THE FUNCTION OF NR3B AND NR4A ORPHAN NUCLEAR RECEPTORS IN OSTEOBLASTS

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ACADEMIC DISSERTATION

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To my dear husband and family

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ABSTRACT

Nuclear receptors comprise a large family of proteins that mediate the effects of small lipophilic molecules such as steroid and thyroid hormones. In addition, there are a group of nuclear receptors that lack identified natural ligands and are referred as orphan nuclear receptors. In this thesis, the function of two such orphan nuclear families has been studied. The NR3B family includes the receptors ERRα (NR3B1), ERRβ (NR3B2) and ERRγ (NR3B3) and the NR4A family includes the receptors NGFI-B (NR4A1), Nurr1 (NR4A2) and Nor1 (NR4A3). NR3B receptors (ERRs) are constitutively active. They are closely related to estrogen receptors but unable to bind natural estrogens as their ligand. However, ERRs bind other ligands, including 4-hydroxytamoxifen and diethylstilbestrol, which function as their inverse agonists inhibiting their activity. NR3B receptors regulate cellular energy balance and carcinogenesis. In addition, it has been suggested that NR3B receptors play a role in bone homeostasis. NR4A receptors are true orphan receptors as their ligand-binding pockets (LBPs) are tightly packed with bulky, hydrophobic side chains, which makes them incapable of binding ligands. NR4A receptors have an important role in the central nervous system in which Nurr1 regulates the differentiation of dopaminergic neurons. However, NR4A receptors are also expressed in peripheral tissues including the bone.

The purpose of this thesis work was to study the signaling and function of NR3B and NR4A orphan nuclear receptors specifically in osteoblasts. The aim was to identify (I) new signaling pathways that regulate the transcriptional activity of NR3B and NR4A receptors, (II) new ligands for the NR3B family, (III) to investigate how NR3B and NR4A orphan nuclear receptors affect the Wnt signaling pathway and finally (IV) to analyze the role of ERRα in osteoblastic differentiation of mesenchymal stem cells (MSCs).

NR4A receptors were found to be regulated by NR3B receptors as ERRα and ERRγ inhibited the transcriptional activity of NR4A receptors in U2-OS cells. Another signaling pathway that was found to repress the activity of NR4A receptors in osteoblasts was the Wnt/β-catenin signaling pathway. β-catenin repressed the transcriptional activities of Nurr1, NGFI-B, and Nor1. On the other hand, NR3B receptors were found to be repressed by NR4A receptors as NGFI-B and Nor1 repressed the transcriptional activity of ERRγ in HeLa cells.

The phytoestrogen equol was identified as a new agonist for ERRγ and ERRβ in PC-3, U2-OS, and SaOS-2 cells. Equol increased the transcriptional activity of ERRγ by increasing the interaction between ERRγ and the co-activator GRIP-1 and by inducing a conformational change in the LBP of ERRγ. We also showed that ERRγ could mediate some of the potentially beneficial health effects of equol as the growth inhibitory effect of equol on PC-3 prostate cancer cells was decreased by blocking ERRγ expression by siRNA.

The Wnt signaling pathway is important for the differentiation and function of osteoblasts. Both the NR3B and also the NR4A receptors were found to repress the transcriptional activity mediated by β-catenin in U2-OS cells. Nurr1 was also able to repress the β-catenin induced expression of Axin2 mRNA in MC3T3-E1 cells.

The MSCs isolated from the bone marrow of 8-12 week old male ERRα knockout (KO) mice showed diminished proliferation, osteoblastic differentiation and expression of the bone marker genes osteocalcin and bone sialoprotein (*BSP*) compared to the cells isolated from their wild-type littermates. The overexpression of ERRα in osteoblastic MC3T3-E1 cell line increased their mineralization and the expression of BSP, Runx2 and alkaline phosphatase mRNAs. *BSP* was shown to be a direct target gene for ERRα and ERRγ as the *BSP* promoter was activated in HeLa cells when transfected with ERRα or ERRγ together with PGC-1α. The adipogenic differentiation of ERRα KO MSCs was also decreased and they expressed less adipogenic markers PPARγ and aP2.

As a conclusion, the studies described in this thesis demonstrated that the transcriptional activity of NR3B and NR4A receptors can be regulated by other orphan nuclear receptors and signaling pathways in osteoblasts. NR3B receptors can also be regulated by ligands and a new agonist, equol, was identified for ERRβ and ERRγ. New roles for NR3B and NR4A were also identified as they were shown to converge with the Wnt signaling pathway in osteoblasts, ERRγ was shown to mediate the growth inhibitory effect of equol in prostate cancer cells, and ERRα was shown to regulate positively MSC proliferation, osteoblastic differentiation and adipogenic differentiation.

ORIGINAL PUBLICATIONS

The thesis is based on following original articles that are referred to in this text by their Roman numerals.

I. Lammi J, Rajalin AM, Huppunen J, Aarnisalo P (2007) Cross-talk between the NR3B and NR4A families of orphan nuclear receptors. Biochem Biophys Res Commun 359: 391-397

II. Hirvonen J*, Rajalin AM*, Wohlfahrt G, Adlercreutz H, Wähälä K, Aarnisalo P (2011) Transcriptional activity of estrogen-related receptor γ (ERRγ) is stimulated by the phytoestrogen equol. J Steroid Biochem Mol Biol 123:46-57 *Equal contribution

III. Rajalin AM, Aarnisalo P (2011) Cross-talk between NR4A orphan nuclear receptors and β-catenin signaling pathway in osteoblasts. Arch Biochem Biophys 509:44-51

IV. Rajalin AM, Pollock H, Aarnisalo P (2010) ERRα regulates osteoblastic and adipogenic differentiation of mouse bone marrow mesenchymal stem cells. Biochem Biophys Res Commun 396: 477-482

In addition to the above studies, some unpublished data are presented.

Original publication I was included in the thesis “Orphan nuclear receptor subfamily NR4A – their interplay with other nuclear receptors and functions in osteoblasts” by Dr. Johanna Lammi. The manuscript of the article II was included in the thesis “Transcriptional regulation by the orphan nuclear receptor ERRγ” by Dr. Johanna Hirvonen.

ABBREVIATIONS

AF	activation function
ALP	alkaline phosphatase
AP-1	activator protein-1
AR	androgen receptor
BALP	bone-specific alkaline phosphatase
BMD	bone mineral density
BMP	bone morphogenetic protein
BSP	bone sialoprotein
CAR	constitutive androstane receptor
Cbfβ	core-binding factor β
C/EBP	CCAAT/enhancer-binding protein
Coll I	collagen type I
CTE	C-terminal extension
DAX-1	dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1
DBD	DNA-binding domain
DES	diethylstilbestrol
Dsh	Dishevelled
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor
ERE	estrogen response element
ERK	extracellular signal-regulated kinase
ERR	estrogen-related receptor
ERRE	estrogen-related receptor response element
ESR	estrogen receptor (gene)
ESRRA	estrogen-related receptor alpha (gene)
ESRRB	estrogen-related receptor beta (gene)
ESRRG	estrogen-related receptor gamma (gene)
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
FXR	farnesoid X receptor
FZD	frizzled
GR	glucocorticoid receptor
GRIP1	glucocorticoid receptor-interacting protein 1
GSK3β	glycogen synthase kinase 3 β
HAT	histone acetyltransferase
HDAC	histone deacetylase
HRE	hormone response element
KO	knockout
LBD	ligand-binding domain
LBP	ligand-binding pocket
Lef	lymphoid enhancer-binding factor
LRP	low density lipoprotein receptor-related protein
LXR	liver X receptor
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor

MEK	MAPK and ERK kinase
MSC	mesenchymal stem cell
NBRE	NGFI-B responsive element
NFATc1	nuclear factor of activated T cell c1
NF-κB	nuclear factor-κB
NGFI-B	nerve growth factor inducible-B
Nor1	neuron-derived orphan receptor 1
NR	nuclear receptor
NTD	amino-terminal domain
Nurr1	Nur-related factor 1
NurRE	Nur-responsive element
OCN	osteocalcin
4-OHT	4-hydroxytamoxifen
OPG	osteoprotegerin
OPN	osteopontin
Osx	osterix
PGC-1	peroxisome proliferator-activated receptor γ coactivator-1
PI3K	phosphatidylinositol 3-kinase
PPAR	peroxisome proliferator-activated receptor
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related peptide
PXR	pregnane X receptor
RANK	receptor activator of nuclear factor-κB
RANKL	receptor activator of nuclear factor-κB ligand
RAR	retinoic acid receptor
RIP140	receptor-interacting protein 140
Runx2	runt-related transcription factor 2
RXR	retinoid x receptor
S.D.	standard deviation
SHP	small heterodimer partner
SIBLING	small integrin-binding ligand N-linked glycoprotein
SMRT	silencing mediator of retinoid and thyroid hormone receptors
Sox	SRY-related HMG-box
SRC	steroid receptor co-activator
Tcf	T cell factor
TGF-β	transforming growth factor β
TNSALP	tissue-nonspecific alkaline phosphatase
TR	thyroid hormone receptor
TRAF6	tumor necrosis factor receptor-associated factor 6
VDR	vitamin D receptor

REVIEW OF THE LITERATURE

1. NUCLEAR RECEPTORS

1.1 The nuclear receptor superfamily

All living organisms, from simple prokaryotic cells such as bacteria to complex eukaryotic systems such as mammals, are composed of cells. These cells have to be able to communicate with each other and with the prevailing environment they are in to be able to function properly. The communication is mediated mainly by different types of molecular signals and cellular receptors that interpret those signals. Four types of receptors are found on the surface of eukaryotic cells: G protein-coupled receptors, ion-channel receptors, tyrosine kinase-linked receptors, and receptors with intrinsic enzymatic activity (Lodish *et al.* 2000). Receptors are embedded in the cell membrane where they convert the extracellular signals that are transmitted in the form of specific ion and protein ligands into one or more intracellular signals that alter the behaviour of the target cell. In addition to the cell surface, receptors can also be located inside a cell. Nuclear receptors (NRs) are transcription factors that are essential for many physiological processes including embryonic development and differentiation, metabolism, and cell proliferation and death. They are located in the cytosol and in the nucleus, and therefore their ligands have to be lipophilic to be able to reach them (Alberts *et al*. 1994, Gronemeyer *et al.* 2004, Mangelsdorf *et al.* 1995).

There are 48 identified NR genes in the human genome. The NR superfamily consists of receptors that bind steroid hormones, such as the estrogen receptors (ERs), the androgen receptor (AR) and the glucocorticoid receptor (GR), and receptors that bind non-steroidal ligands, such as the thyroid hormone receptors (TRs), the vitamin D receptor (VDR), and the retinoic acid receptors (RARs). Some NRs, such as the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), and the peroxisome proliferator-activated receptors (PPARs) bind multiple structurally diverse ligands. Typically, these are naturally occurring metabolites of nutrients (Bain *et al.* 2007, Benoit *et al.* 2004, Gronemeyer *et al.* 2004, Noy 2007).

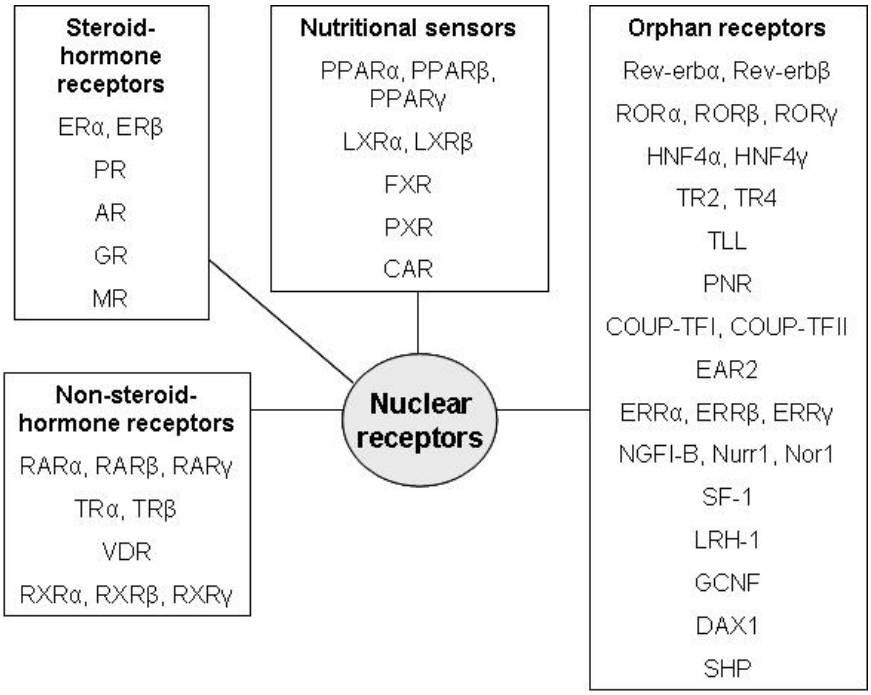

Fig. 1. Human nuclear receptors

In addition to the traditional NRs with known physiological ligands, there are many NRs that were discovered by their structural similarities to the classical NRs and which lacked ligand at the time they were discovered (Giguère 1999). For some of these, physiological ligands have since been found. For retinoid X receptors (RXRs) the recognized ligand was 9-*cis*-retinoic acid (Heyman *et al.* 1992, Levin *et al.* 1992). RXRs participate in a wide range of hormone response systems by associating with other NRs and forming non-permissive or permissive heterodimers. Non-permissive heterodimers are formed, *inter alia*, with RARs, TRs and VDR and can be activated only by the partner's ligand. Permissive heterodimers that can be activated by both RXR's and partner's ligand are formed, *inter alia*, with PPARs and liver X receptors (LXRs) (Germain *et al.* 2006). PPARs, LXRs, farnesoid X receptor (FXR), PXR and CAR are a group of NRs that were described as orphans when first identified but are now known to have large ligand-binding pockets (LBPs) that make them less discriminating and capable of binding several different ligands. As previously mentioned, the ligands that are bound are typically naturally occurring metabolites of nutrients and other compounds. Therefore, it has been suggested that these receptors function as nutritional and metabolic sensors (Benoit *et al.* 2004). PPARs bind for example fatty acids (Göttlicher *et al.* 1992) and eicosanoids (Forman *et al.* 1995, Yu *et al.* 1995), and PPARγ binds selectively thiazolidinediones (Forman *et al.* 1995, Lehmann *et al.* 1995). LXR has been shown to bind different

oxidized derivatives of cholesterol (Janowski *et al.* 1996, Janowski *et al.* 1999). FXR binds bile acids (Makishima *et al.* 1999, Parks *et al.* 1999, Wang *et al.* 1999) and PXR binds C21 steroids pregnanes and several compounds used as drugs, including dexamethasone (Kliewer *et al.* 1998) and hyperforin (Moore *et al.* 2000).

1.2 The structure of nuclear receptors

Nuclear receptors have a characteristic, modular structure, which includes the amino-terminal domain (NTD), a central and highly conserved DNA-binding domain (DBD) and a carboxyl-terminal ligand-binding domain (LBD) which binds the ligand. A schematic representation of the NR structure is shown in figure 2.

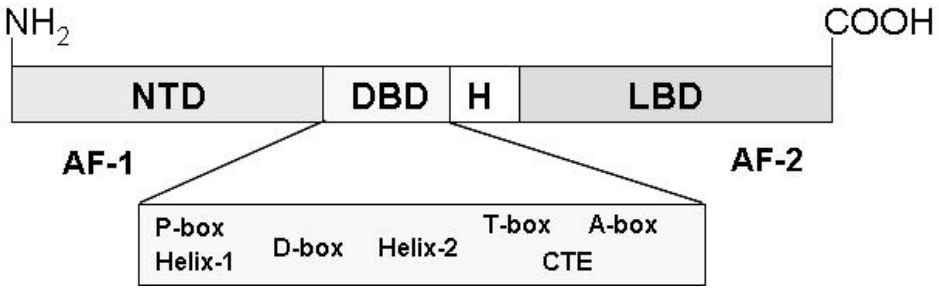

Fig. 2. The schematic layout of the nuclear receptor structure. NRs are composed of an amino-terminal domain (NTD), which contains an activation function 1 (AF-1), DNA-binding domain (DBD), a hinge region (H) and a ligand-binding domain (LBD) that contains an activation function 2 (AF-2). The highly conserved DBD folds into two zinc finger motifs and two α-helices. The residues that are critical for the sequence-specific DNA binding are located in helix 1 and are defined as P-box. The residues of the D-box are located in the C-terminal zinc finger and make up the dimer interface. The C-terminal extension (CTE) contains T-box and A-box that function in receptor DNA binding and heterodimerization (adapted from Bain *et al.* 2007).

1.2.1 The DNA-binding domain

The centrally located DBD docks the NR to specific DNA sequences known as hormone response elements (HREs). The highly conserved DBD folds into two zinc finger motifs, on which each zinc atom is co-ordinated by four cysteine residues. The atoms are necessary to retain stable domain structure (Bain *et al.* 2007, Ribeiro *et al.* 1995). The zinc fingers are common for the whole family, with the exception of SHP (small heterodimer partner) and DAX-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical

region on the X chromosome, gene 1), each of which lack a DBD and do not associate directly with DNA (Gronemeyer *et al.* 2004, Noy *et al.* 2007). Two α-helices extend from the base of the zinc fingers. The first helix (helix 1) contains a region called P-box that interacts with DNA and is responsible for the sequence specificity of the binding. The C-terminal zinc finger contains the D-box, which is involved in dimerization (Aranda and Pascual 2001, Bain *et al.* 2007, Ribeiro *et al.* 1995). The second α-helix (helix 2) contributes to the stabilization of the overall protein structure. After the zinc fingers DBD continues as a C-terminal extension (CTE) that contains the T- and A-boxes, which contribute to the sequence specificity, DNA binding and heterodimerization (Aranda and Pasqual 2001, Bain *et al.* 2007).

1.2.2 The ligand-binding domain

The DBD is linked via a small and flexible hinge region to the C-terminal half of the NR that harbors the LBD. The LBD is a multifunctional domain that mediates ligand binding, homo- and heterodimerization, interaction with heat shock proteins, nuclear localization and ligand-dependent transactivation functions (Aranda and Pasqual 2001, Giguère 1999, Ribeiro *et al.* 1995). The LBD contains 10-13 α-helices (typically 12 numbered H1-H12), several β-turns and connecting loops of varying size arranged into a three-layered "sandwich"-like structure (Benoit *et al.* 2004, Moras and Gronemeyer 1998). The overall architecture of LBD is well conserved among the family members but still different enough to ensure selective ligand recognition. Eleven of the helices form a LBP, whereas C-terminal helix 12 forms a movable lid over the entrance of the pocket (Gronemeyer *et al.* 2004, Moras and Gronemeyer 1998, Noy 2007). Helix 12 also contains residues that are crucial for the function of activation function 2 (AF-2), a highly conserved hydrophobic motif required for co-activator recruitment and ligand-dependent transactivation (Bain *et al.* 2007, Giguère 1999).

1.2.3 The amino-terminal domain

The NTD, which is sometimes referred as an A/B region is the most variable domain both in length and in sequence among the NR family members. The lack of homology may be critical in explaining the different transcriptional responses of closely related NRs binding

to similar response elements. The NTD contains the activation function 1 (AF-1) region that can function as a ligand-independent transcriptional activator or work in synergy with AF-2. The NTD can interact with co-activators and other transcription factors and is involved in both the activation and repression of NR target genes. The NTD can also be post-translationally modified by phosphorylation and sumoylation, which can result in changes in intracellular localization, turnover and protein-protein interactions of the NR (Bain *et al.* 2007, Giguère 1999, Lavery and McEwan 2005, Ribeiro *et al.* 1995).

1.3 The function of nuclear receptors as transcription factors

NRs regulate transcription by binding to specific DNA sequences, HREs, in regulatory segments of their target genes as monomers, homodimers or heterodimers (Giguère 1999, Sonoda *et al.* 2008). HREs can locate on proximal promoter regions upstream of the target gene but also on distal enhancer regions (Deblois and Giguère 2008, Kininis and Kraus 2008). A typical HRE consists of two hexa-nucleotide motifs of AGGTCA or its close variants, separated by a gap of several nucleotides and is possibly preceded by a 5´-flanking A/T-rich sequence. The half-core motifs and 5´-flanking A/T-rich sequence are recognized by the first zinc finger and the CTE. Binding specificity of different NRs is largely achieved by spacing three to five nucleotides in between the elements and by the orientation of the two half sites that can be configured as a direct-repeat, inverted-repeat or everted-repeat (Giguère 1999, Sonoda *et al.* 2008).

The ability of some NRs to bind ligand was acquired during evolution as the ancestral NR was an orphan (Escriva *et al.* 1997). In the absence of a ligand, NRs are either present in the cytoplasm in a complex with heat shock proteins and immunophilin chaperones or in the nucleus constitutively bound to their HREs, where they form a repressive complex with co-repressors and histone deacetylases (HDACs). The HDACs generate a condensed chromatin structure over the target promoter that results in gene repression (Gronemeyer *et al.* 2004, Perissi and Rosenfeld 2005, Sonoda *et al.* 2008). NRs can bind a broad range of different ligands such as glucocorticoids, androgens, mineralocorticoids, progestins, estrogens, thyroid hormones, vitamin D and retinoic acid. The lipophilic ligands travel in the circulation bound to the plasma proteins. After dissociating from the proteins, ligands enter the cell by passive diffusion or by using specific transport processes (Ribeiro *et al.* 1995, Visser *et al.* 2008). NRs can also bind

ligands that are intracellularly originated as metabolic products (Aranda and Pasqual 2001).

When the ligand reaches its receptor it binds to the LBP in the LBD and induces a conformational change. In the case of an activating agonistic ligand, helix 12 is stabilized against the surface of the LBD, which disrupts a repressive hydrophobic groove. The intact repressive hydrophobic groove binds co-repressors which contain a co-repressor nuclear-receptor box, and its disruption causes their release. Instead, a new hydrophobic cleft is formed that allows co-activator recruitment by AF-2. The co-activators, such as the steroid receptor co-activator (SRC) family, typically contain a helical LxxLL motif (where L is leucine and x is any amino acid) and interact with histone acetyltransferases (HATs), chromatin remodeling proteins and the general transcriptional activation machinery to allow initiation of transcription (Bain *et al.* 2007, Gronemeyer *et al.* 2004, Renaud and Moras 2000). However, there are many exceptions to this model. For example, ligand-dependent nuclear-receptor corepressor (LCoR) and receptor-interacting protein 140 (RIP140) can bind to NRs in a ligand-dependent fashion and compete with co-activators by displacing them. Some co-factors, such as the SWI/SNF chromatin remodeling complexes, can function either as repressors or as activators depending on the context. The ability of co-activators and repressors to associate in different complexes allows a temporal- and tissue-specific modulation of target gene transcription (Perissi and Rosenfeld 2005). In contrast to an agonistic ligand, an antagonist inhibits co-activator binding by sterically blocking the ability of helix 12 to approach the core LBD structure or by inducing helix 12 to bind within the hydrophobic cleft thus unproductively mimicking a co-activator (Bain *et al.* 2007). However, helix 12 has more than two positions (on and off). It can adopt several intermediary positions, which enables the design of ligands with different degrees of agonism and antagonism (Gronemeyer *et al.* 2004). With some NRs such as estrogen-related receptors (ERRs), the AF-2 domain is fixed in an active conformation in the absence of ligand, which results in constitutive receptor activation. In these cases, the transcriptional activity of the NR is regulated by nuclear availability of the receptor or its co-activators, by signal-induced receptor modifications such as phosphorylation or acetylation, or by interactions with other transcription factors. However, the transcriptional activity of constitutively active NRs can also be modified by ligands that repress or increase their activity by, for example,

inhibiting or enhancing co-activator binding, respectively (Benoit *et al.* 2004, Sonoda *et al.* 2008).

NRs can also regulate cellular functions via direct interactions with other transcription factors. A classic example of such regulation is mutual transrepression of the GR and NF-κB (nuclear factor-κ) (Gronemeyer *et al.* 2004). In addition, many so-called non-genomic effects have been associated with NRs. These effects are often very rapid and do not need mRNA or protein synthesis to be mediated. An example of such an effect is the regulation of endothelial nitric oxide synthase activity in endothelial cells by estrogen. Steroids have been shown to interact with non-NRs such as ion channels and G protein-coupled receptors at the plasma membrane. Moreover, potentially relevant membrane-bound steroid receptors have been identified for at least ERα, ERβ, progesterone receptor, and AR (Gronemeyer *et al.* 2004, Wierman 2007).

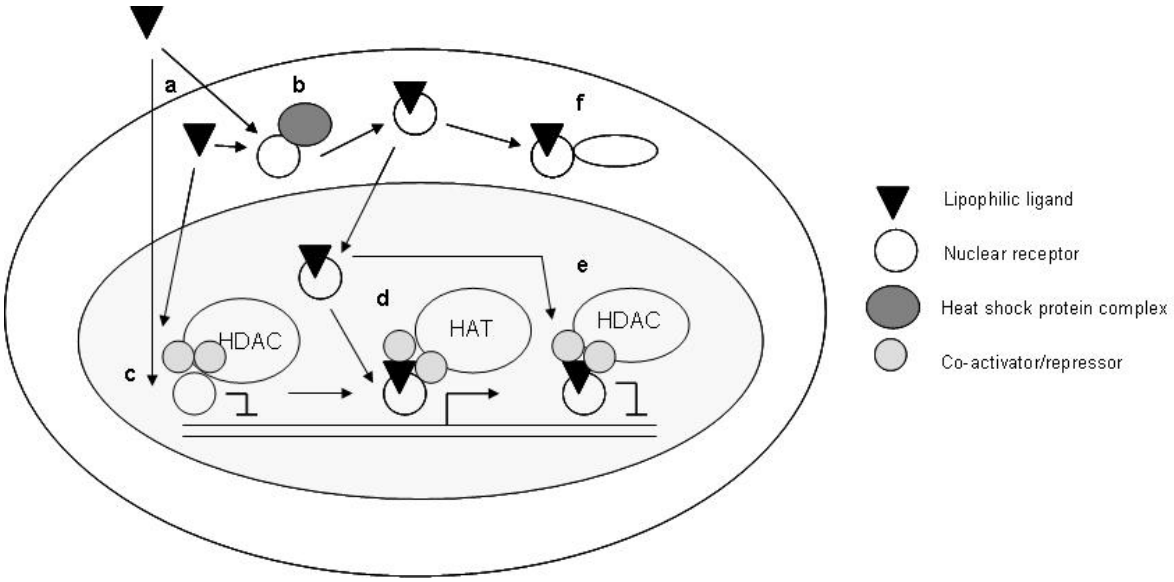

Fig. 3. The function of nuclear receptors. NRs can bind small lipophilic ligands and products of cellular metabolism (a). In the absence of ligand, NRs are present in the cytoplasm in a complex with heat shock proteins (b) or in the nucleus constitutively bound to their HREs, forming a repressive complex with co-repressors and HDACs (c). Ligand binding induces a conformational change that results in either recruitment of co-activators and HATs (agonist) (d) or co-repressors and HDACs (antagonist) (e). NRs can also regulate cellular functions by other mechanisms, for instance, by interacting directly with other transcription factors and repressing or increasing their function (f).

2. ORPHAN NUCLEAR RECEPTORS

Orphan NRs are defined as receptors that lack an identified natural ligand. Some of the NRs, such as PPARs and RXRs, were first identified as orphan receptors but were recategorized after having been shown to bind ligands. However, many receptors still remain in the diverse group of orphan receptors (Benoit *et al.* 2006). ERRs were the first orphan receptors to be discovered (Giguère *et al.* 1988). ERRs are constitutively active in the absence of any ligand (Kallen *et al.* 2004). Their LBDs are still capable of binding ligands, although most of the recognized synthetic ligands inhibit their constitutive activity and therefore function as their inverse agonists. There are also orphan NRs such as hepatocyte nuclear factor 4 (HNF-4) that bind structural ligands, which are unable to leave the receptor once bound and constitute part of the receptor itself (Benoit *et al.* 2006). The members of the NGFI-B receptor family are true orphan NRs incapable of ligand binding because of their small LBP (Wang *et al.* 2003). Although the transcriptional activity of these receptors can not be regulated by ligands, their function is modulated at the level of their expression or by post-translational mechanisms, which will be discussed in more detail in chapter 2.2.1.

2.1 The NR3B orphan nuclear receptor family

The NR3B orphan NR family comprises three members: ERRα (NR3B1), ERRβ (NR3B2) and ERRγ (NR3B3). ERRα was the first orphan NR ever discovered. Its gene (*ESRRA*) was indentified in the search for genes that encode proteins related to ERα and it was accordingly named estrogen-related receptor. The gene that encodes ERRβ (*ESRRB*) was identified by using the ERRα cDNA as the probe (Giguère *et al.* 1988). The last member of the family, ERRγ (*ESRRG*), was finally identified a decade later (Eudy *et al.* 1998, Heard *et al.* 2000, Hong *et al.* 1999).

2.1.1 The structure of the NR3B family

ERR receptors share the typical NR structure with domains necessary for ligand and DNA binding and a nonconserved NTD. The different members of the ERR family show considerable homology in their amino acid sequence. The NTD is the most variable

domain whereas the DBDs of ERRβ and ERRγ show 91% similarity compared to ERRα. The LBD of ERRα is 65% and 63% identical to those of ERRβ and ERRγ, respectively (Fig. 4). In addition to the three main isoforms (ERRα1, ERRβ2, ERRγ2), several splice variants of ERRs have been identified. Human ERRβ has a short-form that is the human ortholog of mouse ERRβ and lacks 67 amino acids from its C-terminus. hERRβ2-Δ10 lacks exon 10 and encodes a different C-terminal end (Zhou *et al.* 2006). ERRγ1 is 23 amino acids smaller than ERRγ2 from the NTD and lacks the functional AF-1 domain that is present in the larger isoform (Heard *et al.* 2000). Although the subject remains poorly known, the different variants could have independent functions in cells (Bombail *et al.* 2010, Tremblay and Giguère 2007). The crystal structures of ERRα (Kallen *et al.* 2004) and ERRγ (Greschik *et al.* 2002) have been solved and they show that the receptors are in their active conformation in the absence of any ligand.

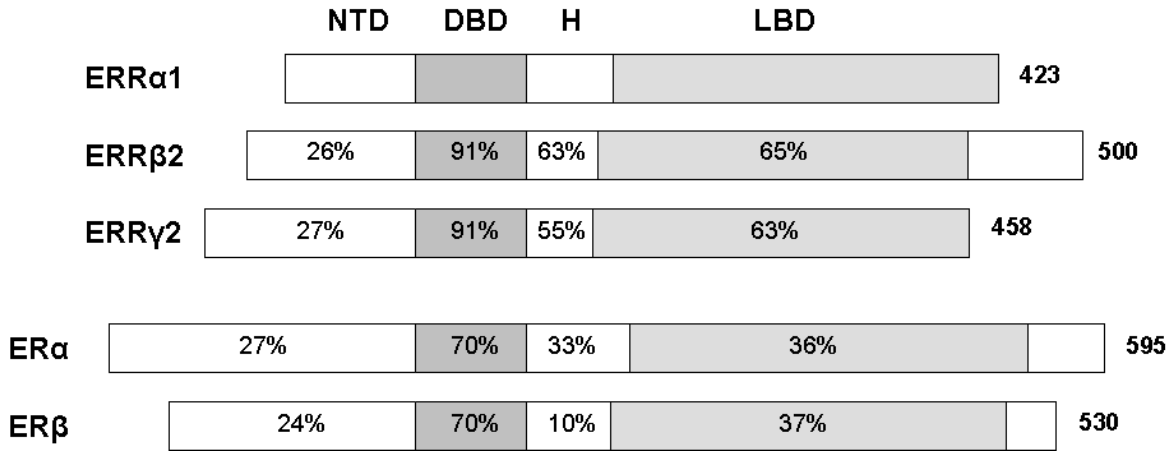

Fig. 4. The amino acid sequence identity between human estrogen-related receptors and estrogen receptors. The percentages represent the similarity of ERRβ and ERRγ proteins and the related ERα and ERβ proteins when compared to ERRα. The length of each protein is also shown. The figure is adapted from Ariazi and Jordan (2006) and Tremblay and Giguère (2007).

2.1.2 The DNA and ligand binding of NR3B receptors

ERRs bind DNA by recognizing an ERR response element (ERRE), which contains the nucleotides 5´-TNAAGGTCA-3´. ERRs can bind to the ERRE as a monomer, a homodimer or a heterodimeric complex that is composed of two distinct ERR isoforms (Barry *et al.* 2006, Johnston *et al.* 1997, Sladek *et al.* 1997). The ERRE sequence can be

recognized and also bound by steroidogenic factor-1 (Wilson *et al.* 1993a). ERRs can bind *in vitro* to the same hormone response element as the ERs (ERE) (Johnston *et al.* 1997, Vanacker *et al.* 1999, Zhang and Teng 2000) but the physiological significance of the ERE binding remains to be determined.

ERRs and ERs share considerable amino acid identity between their respective LBDs (Fig. 4) though none of the ERR family members bind natural estrogens. Although most of the amino acids that line the LBP are identical between ERα and ERRγ, a few differences lead to a smaller ERRγ LBP volume. The most significant of these differences is ERRγ F435, which corresponds to ERα L525. F435 partially fills the ligand binding cavity and prevents ERRγ from binding to the estrogen ligands (Wang *et al.* 2006a). ERRs can still bind several other chemical compounds. The transcriptional activity of all three ERR isoforms is inhibited by the potent synthetic estrogen analog diethylstilbestrol (DES) and its close relatives hexoestrol and dienestrol (Coward *et al.* 2001, Tremblay *et al.* 2001b). A selective estrogen receptor modulator 4-hydroxytamoxifen (4-OHT) acts as an inverse agonist for ERRβ and ERRγ (Coward *et al.* 2001, Tremblay *et al.* 2001a). Due to the small volume of the ERRγ LBP, the receptor has to undergo a large conformational change to be able to bind DES or 4-OHT. Binding of the ligands results in a rotation of F435, which induces the displacement of the AF-2 helix to a position that interferes with the recruitment of co-activators (Greschik *et al.* 2004, Wang *et al.* 2006a). Bisphenol A, which is an ubiquitous environmental contaminant with estrogenic activity, can bind to ERRγ. Bisphenol A does not affect the transcriptional activity of ERRγ but it can prevent 4-OHT from binding to the LBP (Takayanagi *et al.* 2006). ERRα has been found to bind two organochlorine pesticides, toxaphene and chlordane, and a specific synthetic ligand XCT790, which inhibit its activity (Busch *et al.* 2004, Willy *et al.* 2004, Yang and Chen 1999). In addition, there are several synthetic agonists that ERR receptors bind. Phenolic acyl hydrazones GSK4716 and DY131 act as selective ERRβ and ERRγ agonists that increase their transcriptional activity (Yu and Forman 2005, Zuercher *et al.* 2005). The crystal structure of ERRγ with the agonist GSK4716 was solved and it shows that binding of GSK4716 forces a rotation of E275 and R316 that allows access of the ligand to an additional pocket, which was previously shielded. This increases the volume of the combined pocket so that it can accommodate an acyl hydrazone ligand without requiring displacement of the AF-2 helix. GSK4716 induces a small increase in protein stability in an active conformation, which could partly

explain the agonistic effect (Wang *et al.* 2006a). The isoflavones genistein, daidzein, and biochanin A, and the flavone 6,3',4'-trihydroxyflavone can also bind to ERRs and act as their agonists (Suetsugi *et al.* 2003).

Table 1. The specificity of selected ERR ligands. Ligands can either increase (↑) or decrease (↓) the transcriptional activity of the ERRs. Some of the ERR ligands bind specifically to the designated ERR isoforms but have no effect (-) on others.

	ERRα	ERRβ	ERRγ
4-OHT	-	↓	↓
DES	↓	↓	↓
XCT790	↓	-	-
GSK4716	-	↑	↑
daidzein	↑	↑	↑

2.1.3 The function of NR3B family as transcriptional regulators

The LBD of the ERR receptors contains a conserved AF-2 motif that is in an active configuration when in the absence of any ligand (Greschik *et al.* 2002, Kallen *et al.* 2004, Kallen *et al.* 2007, Wang *et al.* 2006a). Therefore, ERRs interact with co-activators in a ligand-independent manner. Because the transcriptional activity of ERRs is mostly dependent on the configuration of co-factor binding, their potencies as transcriptional activators vary according to the cell type and the promoter in question, the overall effect being either negative or positive.

ERRs bind numerous co-regulatory proteins that they share with other NRs. The transcriptional activity of ERRs is greatly enhanced by peroxisome proliferator-activated receptor γ coactivator-1 α and β (PGC-1α and PGC-1β) (Huss *et al.* 2002, Kamei *et al.* 2003, Schreiber *et al.* 2003). Moreover, members of the steroid receptor co-activator family, which include SRC-1 (NcoA1), SRC-2 (NcoA2, GRIP1, TIF2) and SRC-3 (NcoA3, AIB1, ACTR, RAC3, TRAM-1) bind to ERRs and function as their co-activators (Hong *et al.* 1999, Xie *et al.* 1999, Zhang and Teng 2000). RIP140 can either repress or increase the transcriptional activity of ERRs depending on the target sequence on the promoter (Castet *et al.* 2006). Co-repressors of ERRs include PROX1 (prospero-related homeobox 1) (Albers *et al.* 2005, Charest-Marcotte *et al.* 2010) and SMRT (silencing

mediator of retinoid and thyroid hormone receptors) (Wang *et al.* 2006a). The co-regulatory protein interactions can be altered by ligand binding. For example, 4-OHT blocks AF-2 co-activator binding by changing receptor conformation as described in previous chapter. The transcriptional activity of ERRs can also be modulated by other transcription factors, such as SHP which represses their transcriptional activity (Sanyal *et al.* 2002), and by post-translational modifications. The NTDs of ERRα and ERRγ contain a functional phosphorylation-dependent sumoylation motif. In the case of ERRα, the phosphorylation of serine 19 is required for the sumoylation at lysine 14, and both the phosphorylation and the sumoylation decrease the transcriptional activity of ERRα (Tremblay *et al.* 2008, Vu *et al.* 2007). Dimerization can also affect the transcriptional activity of ERRs. For instance, homodimerization increases whereas heterodimerization with ERRα decreases the transcriptional activity of ERRγ (Huppunen and Aarnisalo 2004). ERRα dimers can interact with PGC-1α on DNA whereas monomers can not (Barry and Giguère 2005).

2.1.4 The biological function of the NR3B family

ERRs are ubiquitously expressed in mouse and human. All of the ERR isoforms are expressed in the heart and kidney. ERRα is also highly expressed in many other tissues such as the intestine, skeletal muscle, brain and brown adipose tissue. ERRγ is most highly expressed in the brain and in the spinal cord (Bookout *et al.* 2005). ERRβ is mostly expressed during embryonic development (Luo *et al.* 1997, Mitsunaga *et al.* 2004). After birth it is expressed in the eye with lower concentrations in thyroid, testis and parts of the brain (Bookout *et al.* 2005). NR3B receptors have been shown to regulate many biological processes, including energy metabolism, embryonic development and carcinogenesis.

2.1.4.1 The function of ERRα and ERRγ in energy homeostasis

ERRα (*Esrra*) knockout (KO) mice are viable and fertile and they do not have any gross anatomical alterations. However, they have reduced body weight and peripheral fat deposits and they are resistant to high-fat diet-induced obesity (Luo *et al.* 2003). The lean phenotype of ERRα KO mice can, in part, be explained by lipid malabsorption exhibited by ERRα KO pups (Carrier *et al.* 2004). ERRα KO mice show altered expression and

altered regulation of several enzymes and proteins involved in energy metabolism (Huss *et al.* 2004, Luo *et al.* 2003, Rangwala *et al.* 2007). ERRα KO mice are also unable to maintain body temperature when exposed to cold because of decreased mitochondrial mass and oxidative capacity in their brown adipocytes (Villena *et al.* 2007). ERRα has been shown to have an important role as a regulator of energy metabolism, mitochondrial biogenesis and oxidative phosphorylation in numerous additional studies (Giguère 2008) and therefore the mild phenotype of ERRα KO mice is quite surprising. However, this could be explained by compensation by the other two ERR isoforms. In fact, in the ERRα KO heart, the expressions of ERRγ and PGC-1α are increased, which suggests a compensatory mechanism (Dufour *et al.* 2007). ERRα is a plausible candidate for metabolic diseases due the important role it has in energy metabolism. Nonetheless, in genetic studies no association between the variants of ERRα encoding gene *ESRRA* and type 2 diabetes or obesity was found in caucasian Danish population (Larsen *et al.* 2007). *ESRRA23* is a polymorphic 23-base pair sequence located at position -682 in the 5´-flanking region of the *ESRRA*. It can be found in one to four copies in human chromosomes with higher number of repeats leading to higher expression of ERRα. In Japanese individuals the longer 2.3 genotype of *ESRRA23* was linked to a higher body mass index (BMI) when compared to the shorter 2.2 genotype (Kamei *et al.* 2005). In addition, in mice ERRα was found to be essential for the ATP synthesis and therefore for bioenergetic and functional adaptation of the heart subjected to hemodynamic stressors known to cause heart failure (Huss *et al.* 2007). In humans, the expressions of ERRα and its target genes are decreased in the hearts of cardiomyopathy and end-stage heart failure patients (Karamanlidis *et al.* 2010, Sihag *et al.* 2009).

At birth, the heart undergoes a metabolic switch from a predominant dependence on carbohydrates to a greater dependence on postnatal oxidative metabolism. Disruption of the ERRγ encoding gene *Esrrg* from mice (ERRγ KO mice) blocks this switch which results in lactatemia, electrocardiographic abnormalities and death during the first week of life (Alaynick *et al.* 2007). ERRγ regulates the expression of key ion homeostatic genes, including a voltage-gated potassium channel *Kcne2*, in heart, stomach and kidney, which leads to elevated serum potassium, reductions in gastric acid production markers, and cardiac arrhythmia in ERRγ KO mice. A correlation between specific *ESRRG* single-nucleotide polymorphism genotypes and altered blood pressure has also been reported in humans (Alaynick *et al.* 2010). ERRγ also functions in skeletal

muscle in which it is induced by exercise and it positively regulates mitochondrial activity and oxidative capacity (Rangwala *et al.* 2010).

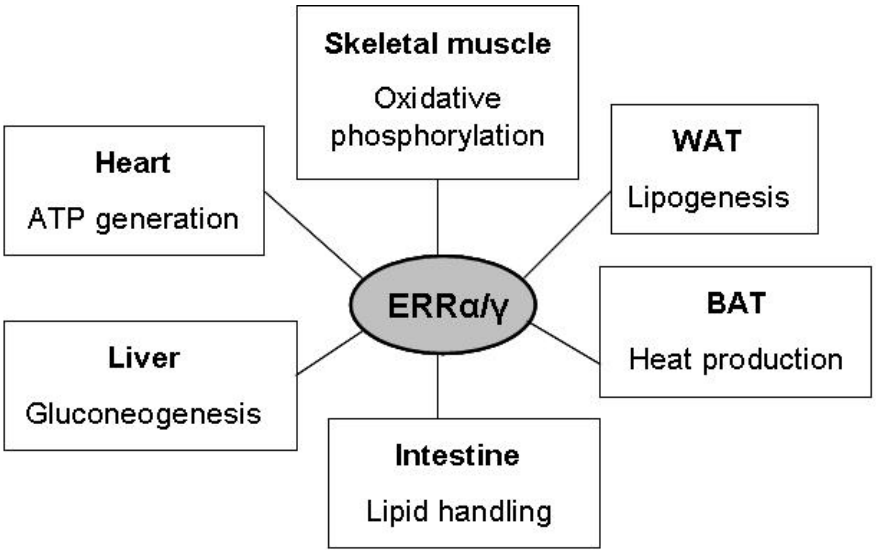

Fig. 5. The metabolic functions of ERRα and/or ERRγ in different tissues.

2.1.4.2 The function of ERRβ in embryonic development

ERRβ has been shown to sustain embryonic stem cell self-renewal and pluripotency by regulating the expression of *Oct4* and *Nanog* genes (van den Berg *et al.* 2008, Zhang *et al.* 2008). ERRβ also functions in concert with Oct4 and Sox2 to mediate reprogramming of mouse embryonic fibroblasts to induced pluripotent stem cells with many of the characteristics of embryonic stem cells (Feng *et al.* 2009). ERRβ is also essential for normal placental formation as ERRβ (*Esrrb*) KO mice die in utero at 10.5 days post coitum due to impaired placental formation (Luo *et al.* 1997). The complement of placental defects with wild-type tetraploid embryos has demonstrated that ERRβ KO embryos have a diminished number of primordial germ cells in their gonads. Despite the defects in primordial germ cell number, the adult ERRβ KO mice are fertile. However, they show behavioural abnormalities with, *inter alia*, circling behaviour and head-tossing (Mitsunaga *et al.* 2004).

2.1.4.3 The function of NR3B receptors in carcinogenesis

ERRs are expressed in many cancer tissues. These include tumors of the breast (Ariazi *et al.* 2002, Deblois *et al.* 2009, Lu *et al.* 2001, Suzuki *et al.* 2004), prostate (Cheung *et al.* 2005, Fujimura *et al.* 2007, Fujimura *et al.* 2010, Yu *et al.* 2008), ovary (Sun *et al.* 2005), and the endometrium (Gao *et al.* 2006, Watanabe *et al.* 2006). The expression of ERRα and ERRγ correlate with unfavourable and favourable biomarkers, respectively, in human breast cancer (Ariazi *et al.* 2002). It has been suggested that ERRα functions as a determinant of heterogeneity in breast cancer (Deblois *et al.* 2009) and ERRγ mediates tamoxifen resistance in invasive lobular breast cancer cells (Riggins *et al.* 2008). The elevated expression of ERRα has been shown to correlate with poor survival in prostate (Fujimura *et al.* 2007) and ovarian (Sun *et al.* 2005) cancers. On the other hand, ERRβ and ERRγ suppress growth of prostate cancer cells (Yu *et al.* 2007, Yu *et al.* 2008). In endometrial carcinoma, the expression of ERRα is positively correlated with cancer stage and myometrial invasion whereas ERRγ negatively correlates with nodal metastasis (Gao *et al.* 2006).

2.2 The NR4A orphan nuclear receptor family

The NR4A subfamily of NRs comprises three members: NGFI-B (NR4A1, Nur77, TR3), Nurr1 (NR4A2, NOT), and Nor1 (NR4A3, MINOR, TEC). *NGFI-B* (nerve growth factor inducible-B) was the first member of the subfamily identified as a gene induced by serum in mouse fibroblast cells (Hazel *et al.* 1988) and by the nerve growth factor in the rat pheochromocytoma cell line PC12 (Milbrandt 1988). *Nor1* (neuron-derived orphan receptor 1) was cloned from forebrain neural cells that were undergoing apoptosis (Ohkura *et al.* 1994) and finally Nurr1 (Nur-related factor 1) was characterized as a brain-specific transcription factor in dopaminergic neurons (Law *et al.* 1992).

2.2.1 The structure and transcriptional regulation by the NR4A family

The structures of NR4A receptors are very similar to each other. Therefore, it has been suggested that the members of the family have evolved from a common ancestral gene (Martínez-González and Badimon 2005, Maxwell and Muscat 2005). The NTD containing

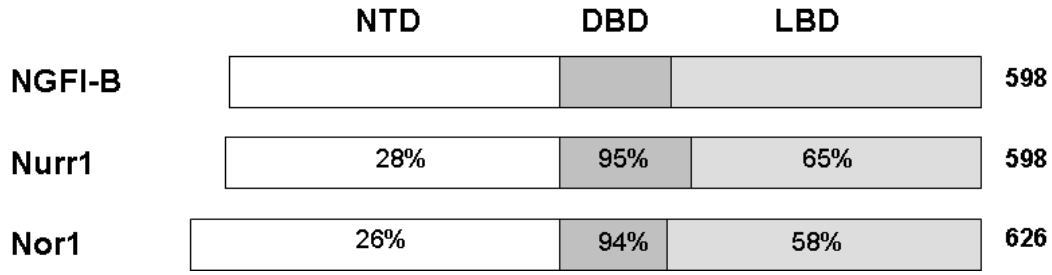

Fig. 6. The schematic representation of the NR4A1 receptor structure. The percentages represent the similarity of Nurr1 and Nor1 amino-terminal domain (NTD), DNA-binding domain (DBD) and ligand-binding domain (LBD) when compared to NGFI-B. The length of each protein is also shown.

the AF-1 is the most variable domain. The NTD of NGFI-B shows 28% amino acid homogeneity with Nurr1 and 26% with Nor1 (Fig. 6) (Martínez-González and Badimon 2005). The AF-1 of NR4A receptors is exceptionally potent and mediates the transactivation, cell specificity and co-factor recruitment of these receptors (Maira *et al.* 2003, Wansa *et al.* 2002, Wansa *et al.* 2003). DBD is well conserved with over 90% homology. It interacts with the consensus response element NGFI-B responsive element (NBRE) AAAGGTCA as monomers and with the palindromic Nur-responsive element (NurRE) TGATATTTX_6AAATGCCA comprising two inverted NBRE sequences spaced by 6 bp as homodimers and heterodimers (Fig. 7) (Maira *et al.* 1999, Philips *et al.* 1997, Wilson *et al.* 1991). NGFI-B and Nurr1 can form heterodimers with retinoic X receptor (RXR) though Nor1 can not. As heterodimers with RXR, Nurr1 and NGFI-B mediate transactivation in response to the RXR ligands through the DR-5 element. The DR-5 element comprises two direct repeats of the consensus NR binding motif separated by five nucleotides (Fig. 7) (Aarnisalo *et al.* 2002, Perlmann and Jansson 1995, Zetterström *et al.* 1996a).

The NR4A receptors are incapable of binding ligands to their LBPs. The structure of the Nurr1 LBD has been determined by X-ray crystallography and it revealed that the LBP is tightly packed with bulky, hydrophobic amino acid side chains (Wang *et al.* 2003) that are conserved throughout the NR4A subfamily (Flaig *et al.* 2005, Wang *et al.* 2003). Moreover, the hydrophobic co-activator binding cleft typical of NRs is filled with polar side chains, which makes it incapable of binding co-regulators (Wang *et al.* 2003). However, there is a hydrophobic patch between helices 11 and 12 that potentially interacts with co-factors and modulates transcriptional activity (Codina *et al.* 2004, Flaig

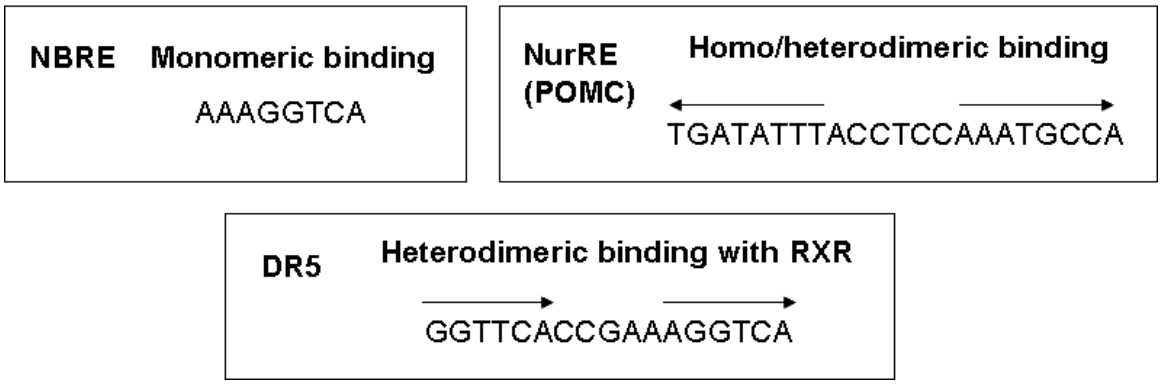

Fig. 7. NR4A receptors can bind DNA as monomers (NBRE), by forming homodimers or heterodimers with other NR4A receptors (NurRE on the pro-opiomelanocortin promoter), or by forming heterodimers with RXR (DR5).

et al. 2005). This patch has been reported to bind, for example, co-repressors SMRT and NCoR (nuclear receptor co-repressor) (Codina *et al.* 2004). In addition, NR4A family has been reported to bind SRC-1, SRC-2, p300 and PCAF (p300/CBP-associated factor) co-regulators through their AF-1 and NTD (Wansa *et al*. 2002). Despite the fact that NR4A receptors do not bind ligands to their LBP, there are compounds that target regions outside of the LBP that regulate receptor/co-factor interactions and receptor functions (Shi 2007). One of these compounds is 6-mercaptopurine, which is a widely used antineoplastic and anti-inflammatory drug that activates Nurr1 (Ordentlich *et al*. 2003) and Nor1 (Wansa *et al.* 2003) through their AF-1 domains. Moreover, other agonists have been described for Nurr1 but their mechanisms of action are still unknown (Dubois *et al.* 2006, Hintermann *et al.* 2007). 1,1-bis(3-indolyl)-1-(*p*-anisyl)methane activates NGFI-B through the LBD (Chintharlapalli *et al.* 2005) and prostaglandin A2 functions as a transactivator of Nor1 by a mechanism that is dependent of both LBD and AF-1 (Kagaya *et al.* 2005).

An important mechanism for regulating the function of NR4A receptors is mediated through the alterations of their protein expression. The NR4A family functions as immediate-early genes and their expression is induced rapidly in response to a range of signals, such as parathyroid hormone (PTH) (Pirih *et al.* 2003, Pirih *et al.* 2005, Tetradis *et al.* 2001a, Tetradis *et al.* 2001b), typical and atypical antipsychotic drugs such as raclopride and olanzapine (Maheux *et al.* 2005), vascular endothelial growth factor (Liu *et al.* 2003) and inflammatory cytokines (Pei *et al.* 2005). Furthermore, physical stimuli such as stress, magnetic fields, mechanical agitation and membrane depolarization can induce their expression (Bandoh *et al.* 1997, Hazel *et al.* 1991, Honkaniemi *et al.* 1994, Katagiri

et al. 1997, Miyakoshi *et al.* 1998). Protein kinase A (Kovalovsky *et al.* 2002, Pirih *et al.* 2003, Song *et al.* 2001, Tetradis *et al.* 2001a, Tetradis *et al.* 2001b), Ca^{2+}/calmodulin dependent kinase II (Kovalovsky *et al.* 2002), protein kinase C, phosphatidylinositol 3-kinase (PI3K) (Lammi and Aarnisalo 2008, Song *et al.* 2001), NF-κB (Pei *et al.* 2005), and mitogen-activated protein kinase pathways (MAPK) (Kovalovsky *et al.* 2002, Lammi and Aarnisalo 2008) have been reported to be involved in the regulation of the NR4A expression.

In addition to the regulation of expression, extracellular stimuli can also influence the NR4A post-translational modifications. For instance NGFI-B can be phosphorylated by several kinases, including the PI3K/Akt and MEK1/2-ERK1/2 MAP kinase pathways. Phosphorylation by PI3K/Akt pathway has been shown to antagonize the DNA binding of NGFI-B and to increase its translocation from the nucleus to the cytoplasm (Cunningham *et al.* 2006, Masuyama *et al.* 2001, Pekarsky *et al.* 2001). Phosphorylation by MEK1/2-ERK1/2 MAP kinase pathway regulates NGFI-B's nuclear export and translocation to mitochondria (Jacobs *et al.* 2004, Wang *et al.* 2009a). Nurr1 has been shown to be sumoylated by PIASγ, which leads to the repression of the transcriptional activity of Nurr1 (Galleguillos *et al.* 2004).

In addition, cross-talk with other signaling pathways has been shown to regulate the transcriptional activity of Nurr1. GR interacts with NR4A receptors and represses their NurRE-dependent transcription (Martens *et al.* 2005). DAX-1 interacts with NGFI-B and thereby represses its transcriptional activity by competing with the co-activator SRC-1 for binding (Song *et al.* 2004).

2.2.2 The biological function of the NR4A family

NR4A receptors play an important role in the central nervous system. Nonetheless, recently, it has become clear that they also function in peripheral tissues and regulate processes such as those of the immune system, energy metabolism, the hypothalamic-pituitary-adrenal axis, and carcinogenesis.

2.2.2.1 The function of NR4A receptors in the central nervous system

Nurr1 is predominantly expressed in the central nervous system with high levels of expression at sites such as the substantia nigra, cerebellum, olfactory bulb, hypothalamus, neocortex and ventral tegmental area (Zetterström *et al.* 1996b). Nurr1 is essential for the development and survival of dopaminergic neurons. Dopamine neurons localize at the substantia nigra and ventral tegmental area where they regulate movement and affective behaviour. Mutations in *Nurr1* gene have been associated with Parkinson's disease, a condition in which dopamine neurons are degenerated (Le *et al.* 2003). *Nurr1* KO mice fail to generate midbrain dopaminergic neurons, are hypoactive and die within two days after birth (Zetterström *et al.* 1997). Furthermore, heterozygous *Nurr1* (*Nurr1* +/-) mice have lower levels of dopamine in the midbrain, prefrontal cortex, and nucleus accumbens, and increased locomotor activity in response to mild stress (Eells *et al.* 2002). *Nurr1* +/- mice also show decreased ethanol preference and wheel running, which associates Nurr1 with excessive reward-seeking behaviour typical for addiction (Werme *et al.* 2003). The other two members of the NR4A family are also expressed in the brain (Zetterström *et al.* 1996a, Zetterström *et al.* 1996b). *NGFI-B* KO mice are hyperactive and have disturbances in both basal and haloperidol-induced dopamine turnover, which suggests a role for NGFI-B in dopamine clearance (Gilbert *et al.* 2006). Nor1 has been associated with the modulation of food intake and energy balance as the underexpression of Nor1 in mice suppresses their food intake and body weight (Nonogaki *et al.* 2009). *Nor1* KO mice also show impaired postnatal axonal growth and region-specific cell death in the hippocampus that are associated with lasting changes in hippocampal excitability and increased susceptibility to chemically induced seizures (Pönniö and Conneely 2004).

2.2.2.2 The function of NR4A receptors in peripheral tissues

NR4A receptors have been shown to play an important role in the cells of the immune system. For instance, they function in T cell receptor-mediated apoptosis during which immature thymocytes and mature T cells that express self-reactive T cell receptors are deleted by apoptosis to eliminate self-reactive and potentially autoimmune lymphocytes. NGFI-B and Nor1 are induced to a high level during T cell receptor-mediated apoptosis in immature thymocytes and T cell hybridomas (Cheng *et al.* 1997, Liu *et al.* 1994,

Woronicz *et al.* 1994) and the blocking of NGFI-B expression or function by antisense or dominant negative NGFI-B inhibits T cell receptor mediated apoptosis (Calnan *et al.* 1995, Liu *et al.* 1994, Woronicz *et al.* 1994, Zhou *et al.* 1996). On the other hand, Nurr1 is strongly expressed in the peripheral blood T cells of multiple sclerosis patients (Satoh *et al.* 2005) in which it regulates the expression of interleukin-17 and interferon-γ, two key cytokines suggested to work in the multiple sclerosis pathogenesis (Doi *et al.* 2008).

In contrast to the apoptotic effects in T cells and thymocytes, the members of the NR4A family are able to mediate prosurvival effects in other tissues. In the endothelium, for example, Nor1 mediates the prosurvival effects of hypoxia-inducible factor 1 (Martorell *et al.* 2009) and in vascular smooth muscle cells Nor1 promotes proliferation (Nomiyama *et al.* 2006).

NR4A receptors regulate many aspects of energy metabolism and expenditure. NR4A receptors are involved in hepatic glucose metabolism. They are induced in the liver by different physiological stimuli, including glucagon stimulation and fasting (Oita *et al.* 2009, Pei *et al.* 2006). Adenovirus-mediated overexpression of NGFI-B in the mouse liver activates multiple genes involved in gluconeogenesis and stimulates hepatic glucose production (Pei *et al.* 2006). In skeletal muscle, the NR4A receptors are induced by β-adrenergic signaling (Pearen *et al.* 2008) and endurance exercise (Mahoney *et al.* 2005). NR4A receptors promote glucose utilization as *NGFI-B* KO mice have reduced expression of genes involved in skeletal muscle glucose and glycogen metabolism (Chao *et al.* 2007). *NGFI-B* KO mice also have an increased susceptibility to diet-induced obesity and insulin resistance in skeletal muscle and liver (Chao *et al.* 2009). NR4A receptors also participate in lipid metabolism. NGFI-B promotes lipolysis in muscle (Maxwell *et al.* 2005), decreases hepatic triglyceride content and modulates plasma lipoprotein profiles by increasing plasma low density lipoprotein cholesterol and by decreasing high density lipoprotein cholesterol (Pols *et al.* 2008). In addition, NR4A receptors may participate in adipogenic differentiation (Chao *et al.* 2008, Fumoto *et al.* 2007) and central regulation of energy homeostasis (Nonogaki *et al.* 2009).

NR4A receptors also function in many other peripheral tissues. They are overexpressed in atherosclerotic lesions obtained from patients with coronary artery disease (Arkenbout *et al.* 2002, Martínez-Gonzaléz *et al.* 2003, Nomiyama *et al.* 2006) and they inhibit macrophage activation and foam-cell formation and differentiation that suggests a protective role in atherogenesis (Bonta *et al.* 2006). The NR4A family has been

linked in the regulation of gene expression in the hypothalamic-pituitary-adrenal axis that is related to inflammation and steroidogenesis. Their expression is rapidly induced by corticotropin releasing hormone (Parkes *et al.* 1993, Philips *et al.* 1997) and adrenocorticotropic hormone (Enyeart *et al.* 1996), and their target genes include pro-opiomelanocortin (Murphy and Conneely 1997, Philips *et al.* 1997), 21-hydroxylase (Wilson *et al.* 1993b), and 20α-hydroxysteroid dehydrogenase (Stocco *et al.* 2000). However, there is normal basal regulation of the hypothalamic-pituitary-adrenal axis and normal steroidogenesis in *NGFI-B* KO mice possibly due to the compensatory effects of related proteins (Crawford *et al.* 1995).

Finally, the NR4A receptor family has been linked to carcinogenesis. The dominating cytoplasmic expression of Nurr1 over nuclear Nurr1 correlates with an advanced pathological stage and an invasive growth pattern in bladder cancer patients. *In vitro*, silencing of endogenous Nurr1 by siRNA reduced the migration of bladder cancer cells (Inamoto *et al.* 2010). Chromosomal translocations that create chimeric fusions of full Nor1 protein fused with the amino-terminal domains of EWS (Ewing sarcoma breakpoint region 1 protein) (Clark *et al.* 1996, Labelle *et al.* 1999), TCF12 (Transcription factor 12) (Sjögren *et al.* 2000), TAF2N (TATA box binding protein (TBP)-associated factor, RNA polymerase II, N) (Attwool *et al.* 1999, Panagopoulos *et al.* 1999, Sjögren *et al.* 1999) or TFG (TRK-fused gene) (Hisaoka *et al.* 2004) proteins have been linked to extraskeletal myxoid chondrosarcoma.

3. THE BONE AND OSTEOBLASTS

In vertebrates, skeletal bone has various physiological roles. Bone functions in locomotion, protection and support of soft tissues but it is also a hematopoietic and endocrine organ and regulates calcium homeostasis (Imai *et al.* 2009, Lee *et al.* 2007, Tortora and Grabowski 2000).

3.1 The structure of bone

Bone is composed mainly of extracellular matrix, which contains both organic and inorganic substances. The inorganic component accounts for 65% of the bone's weight and contains minerals. Calcium and phosphate exist in bone in the form of hydroxyapatite ($3Ca_3(PO_4)_2 \cdot Ca(OH)_2$). The skeleton contains about 99% of the body's calcium, 90% of its phosphate, 50% of its magnesium and 33% of its sodium. The organic matrix consists mainly of collagen (90-95%) whereas the rest is made of proteoglycans, glycoproteins, sialoproteins and a small amount of lipid (Brook and Marshall 2001).

Bones are composed of two different types of bone tissue: cortical (compact) and trabecular (cancellous, spongy) bone. The rigid cortical bone, which constitutes about 80% of the skeleton, forms the external layer of all bones and provides protection and support to resist stress produced by weight and movement. Cortical bone tissue is arranged in units called osteons or Haversian systems. Blood and lymphatic vessels and nerves penetrate the cortical bone through central Haversian canals and perforating Volkmann's canals. The hard, calcified matrix is arranged in ringlike concentric lamellae which are arranged around the central canals. Between the lamellae small spaces called lacunae that contain osteocytes can be found. Radiating in all directions from the lacunae are tiny channels called canaliculi, which are filled with extracellular fluid and which connect lacunae with one another and with the central canals. The areas between osteons contain interstitial lamellae comprising fragments of older osteons, which have been partially destroyed during bone remodeling or growth. In contrast to cortical bone, trabecular bone does not contain true osteons. Instead it contains lamellae that are arranged in an irregular lattice of thin columns of bone called trabeculae. The space between the trabeculae of certain bones is filled with red bone marrow, which produces blood cells. Trabecular bone is light and it tends to be located at sites where bone is not heavily stressed or where stress

is applied from many directions. Trabecular bone constitutes about 20% of bone and makes up most of the bone tissue of short, flat, and irregular shaped bones such as the sternum. Moreover, the epiphyses and a narrow rim around the medullary cavity of the diaphysis of long bones contain trabecular bone (Tortora and Grabowski 2000).

3.2 The different cell types of bone

There are several types of cells in bone. The cells that are specific for bone are preosteoblasts, osteoblasts, osteocytes, and osteoclasts. Preosteoblasts differentiate from the mesenchymal stem cells (MSCs) of the bone marrow. Preosteoblasts in turn can differentiate into osteoblasts, which are bone forming cells that synthesize and secrete collagen fibers and other organic components to the bone matrix. Osteoblasts, preosteoblasts and MSCs are discussed in more detail in next chapter. Osteocytes are mature bone cells that are derived from osteoblasts that have been embedded in the extracellular matrix. Osteocytes do not secrete matrix materials but maintain the metabolic activities of bone tissue such as the exchange of nutrients and waste products with the blood. They make contact with the neighbouring cells and nearby blood supply by means of cytoplasmic processes, which lie in canaliculi. Osteoclasts are large cells that are derived from the fusion of several monocytes, which are concentrated in the endosteum. The side of the cell that makes contact with the bone surface is composed of a ruffled border, by which osteoclast releases lysosomal enzymes and acids that digest the protein and mineral components of the underlying bone (Brook and Marshall 2001, Tortora and Grabowski 2000).

There are two major modes of bone formation, intramembranous and endochondral ossification, which both involve the transformation of a preexisting mesenchymal tissue into bone tissue. Flat bones of the skull are formed by a process of intramembranous ossification by which MSCs first proliferate and condense and then differentiate directly into the bone matrix secreting osteoblasts. In endochondral ossification MSCs first form a hyaline cartilage model by differentiating into chondroblasts and chondrocytes. Cells that surround the cartilage model subsequently start to differentiate into osteoblasts and to form bone matrix and eventually the cartilage model is completely replaced by bone (Gilbert 2000, Tortora and Grabowski 2000).

3.3 The osteoblasts

3.3.1 The osteoblastic differentiation of mesenchymal stem cells

Adult stem cells are undifferentiated cells that include MSCs, which can differentiate into multiple cell lineages such as osteoblasts, adipocytes and chondrocytes. In addition to MSCs, adult stem cells also include hematopoietic stem cells that differentiate into blood cells and osteoclasts, and neural stem cells that differentiate into neural cells (Jeong and Mangelsdorf 2009, Liu *et al.* 2009b).

The differentiation process of MSCs is controlled by specific signals and transcription factors. The key transcription factor for osteoblastic differentiation is Runt related transcription factor 2 (Runx2, Cbfa1). *Runx2* KO mice have a complete lack of ossification due to maturational arrest of osteoblasts (Komori *et al.* 1997, Otto *et al.* 1997). In human, *Runx2* haploinsufficiency causes the autosomal dominant bone disorder cleidocranial dysplasia, which is characterized by defective bone formation (Lee *et al.* 1997, Mundlos *et al.* 1997). Runx2 expression is initiated in the mesenchymal condensations of the developing skeleton (Ducy *et al.* 1997). Runx2 binds to and activates the promoters of several osteoblastic genes, which results in the commitment and establishment of osteoblastic phenotype. These genes include type I collagen (*Coll I*) (Ducy *et al*. 1997, Kern *et al.* 2001), osteopontin (*OPN*) (Ducy *et al.* 1997, Sato *et al.* 1998), and osteocalcin (*OCN*) (Ducy *et al.* 1997). On the other hand, Runx2 inhibits the terminal differentiation of osteoblasts as mice overexpressing Runx2 have an increased number of immature osteoblasts but a decreased number of mature osteoblasts and osteocytes, which results in osteopenia with multiple fractures (Liu *et al.* 2001). In addition, Runx2 regulates the differentiation of hypertrophic chondrocytes as *Runx2* KO mice have delayed chondrocyte maturation (Inada *et al.* 1999).

The expression and function of Runx2 is regulated by several factors, which include growth factors such as bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) and hormones such as PTH. The binding of BMP-2 to its receptor activates Smads, which can interact with Runx2 and enhance its transcriptional activity (Hanai *et al.* 1999, Lee *et al.* 2000). Mice that express dominant negative form of BMP receptor IB in their osteoblasts have reduced bone growth and formation, which indicates an important role for BMPs in osteoblast differentiation (Zhao *et al.* 2002). FGF-2 increases the

phosphorylation of Runx2 improving its transcriptional activity (Xiao *et al.* 2002). Intermittent PTH treatment increases the expression and activity of Runx2 (Krishnan *et al.* 2003). In addition to Smads, numerous other interacting proteins have been reported to regulate the function of Runx2. Runx2 requires the interaction with a co-transcription factor Cbfβ (Core-binding factor β) for the efficient association with DNA and transcriptional activity. Cbfβ is therefore needed for skeletal development (Yoshida *et al.* 2002). Runx2 also binds proteins such as CCAAT/enhancer-binding protein β (C/EBPβ) (Gutierrez *et al.* 2002), and co-activators such as retinoblastoma protein (Thomas *et al.* 2001), TAZ (Transcriptional co-activator with PDZ-binding motif) (Cui *et al.* 2003), and p204 (Liu *et al.* 2005), which all enhance its transcriptional activity. Other transcription factors and co-regulators, including PPARγ (Jeon *et al.* 2003) and Stat1 (signal transducer and activator of transcription 1) (Kim *et al.* 2003), reduce the transcriptional activity of Runx2.

Osterix (Osx) is another osteoblast specific transcription factor that is important for the osteoblastic differentiation. It functions downstream of Runx2 as *Osx* KO mice show a lack of osteoblasts and have defective bone formation in spite of normal Runx2 expression (Nakashima *et al.* 2002). Very little is known about factors that regulate Osx. BMP-2 up-regulates Osx expression during osteoblast differentiation (Nakashima *et al.* 2002). Nuclear factor of activated T cells c1 (NFATc1) binds Osx and activates Osx-dependent *Coll I* promoter activity (Koga *et al.* 2005) and p53 has been shown to negatively regulate Osx expression (Wang *et al.* 2006b).

The canonical Wnt signaling pathway is an important factor in the control of bone formation and bone mass and will be discussed in detail under the heading 3.3.1.1.

In vitro, the bone nodule formation can be divided into three stages: (I) proliferation, (II) extracellular matrix development and maturation, and (III) mineralization. Although an extensive diversity in the expression of osteoblastic marker genes exists, some guidelines have been developed. In general, the expression of alkaline phosphatase (ALP) increases during the extracellular matrix development and decreases again when mineralization is well progressed. OPN peaks prior to bone sialoprotein (BSP) and OCN, which are expressed by differentiated mineralizing osteoblasts (Aubin 2001).

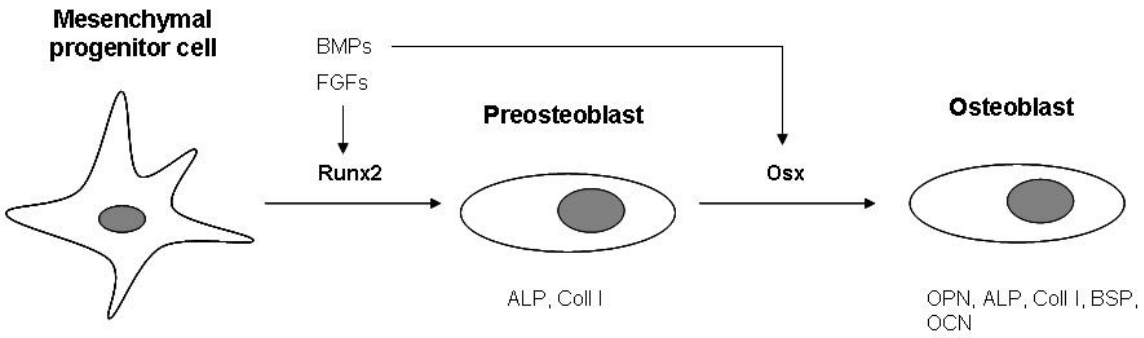

Fig. 8. Osteoblast progenitors in mesenchymal condensations of endochondral and membranous skeletal elements differentiate first into preosteoblasts because of the function of Runx2. Preosteoblasts express low levels of type I collagen (Coll I) and alkaline phosphatase (ALP). To become fully committed osteoblasts, preosteoblasts require the function of Osx. Mature osteoblasts start to secrete osteoblastic markers, including Coll I, ALP, osteopontin (OPN), bone sialoprotein (BSP) and osteocalcin (OCN) into the extracellular matrix and are therefore responsible for the formation of highly mineralized bone matrix.

3.3.1.1 The Wnt signaling pathway

Wnts are a family of 19 secreted glycoproteins that control many important biological processes such as embryogenesis and tumorigenesis. Wnts bind to a membrane receptor complex composed of a Frizzled (FZD) G protein-coupled receptor and a low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) on the cell surface. Wnts can activate three different intracellular signaling cascades: the canonical Wnt/β-catenin pathway, the Wnt/Ca^{2+} pathway and the Wnt/planar polarity pathway. The most comprehensively characterized of these is the canonical Wnt/β-catenin pathway. In the absence of Wnt ligands, a degradation complex of glycogen synthase kinase 3 (GSK3), casein kinase 1α, Axin2 and adenomatous polyposis coli is formed that promotes the phosphorylation of β-catenin, which leads to the ubiquitylation and degradation of β-catenin. When Wnt ligands are present, they bind to their receptors. This activates the cytoplasmic mediators, Dishevelled (Dsh) phophoproteins, which inhibit the β-catenin degradation complex and thereby block the degradation of β-catenin. The stabilized β-catenin protein accumulates within the nucleus, where it binds lymphoid enhancer-binding factor (Lef)/T cell factors (Tcf), displaces co-repressors and recruits transcriptional co-activators to stimulate the expression of specific target genes such as *c-myc* and *cyclin D1*. However, canonical Wnt-signaling can also repress gene expression by mechanisms that are less understood

(Angers and Moon 2009, Bodine 2008, Bodine and Komm 2006, Cadigan and Liu 2006, Kahler and Westendorf 2003, Westendorf *et al.* 2004).

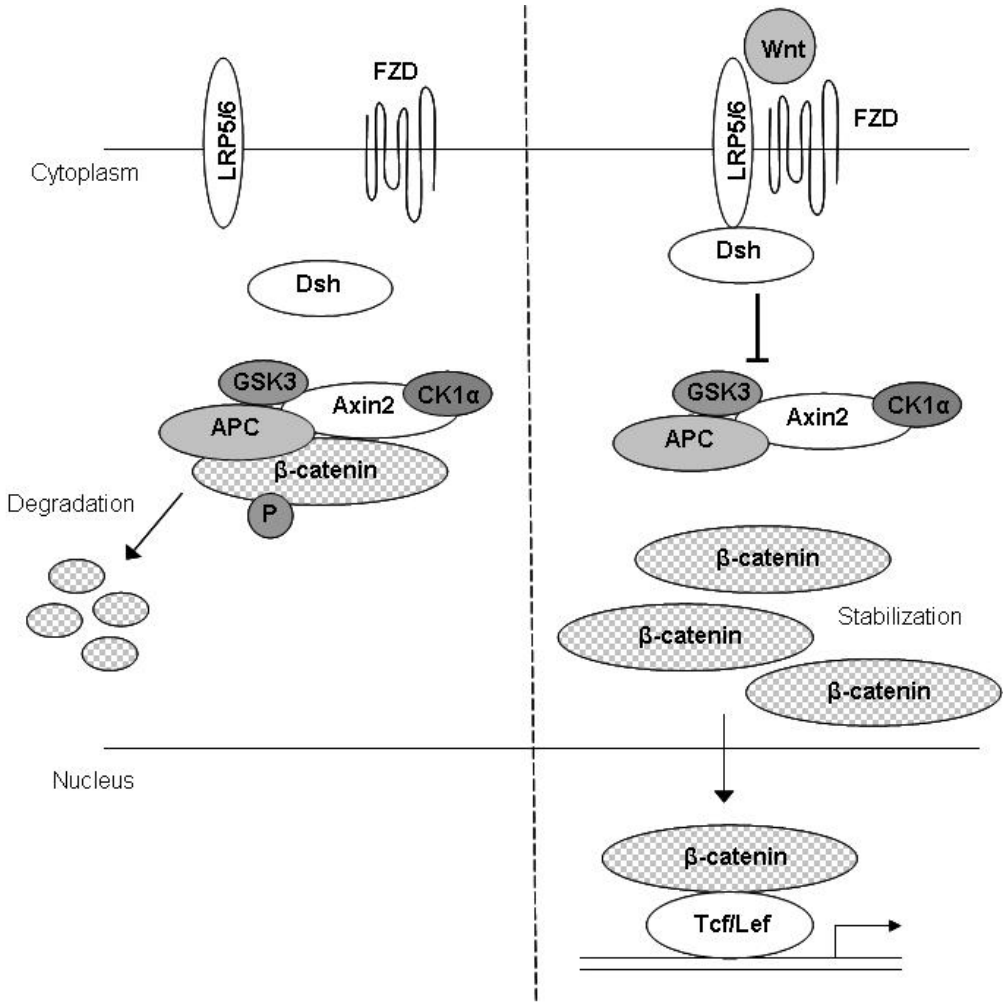

Fig. 9. The function of canonical Wnt signaling pathway. In the absence of Wnt ligands (left), a degradation complex of glycogen synthase kinase 3 (GSK3), casein kinase 1α (CK1α), Axin2 and adenomatous polyposis coli (APC) is formed that promotes the phosphorylation of β-catenin, which leads to the ubiquitylation and degradation of β-catenin. When Wnt ligands are present (right), they bind to their receptors that constitute of Frizzled (FZD) and LRP5/6 proteins, which activates the cytoplasmic mediator Dishevelled (Dsh). Dsh inhibits the β-catenin degradation complex, and thereby blocks the degradation of β-catenin. The stabilized β-catenin protein accumulates and translocates into the nucleus where it binds lymphoid-enhancer binding factor (Lef)/T cell factors (Tcf) and stimulates expression of specific target genes.

Wnt/β-catenin signaling pathway is essential for skeletal development and homeostasis. In human, different LRP5 mutations correlate with high or low bone mass (Boyden *et al.* 2002, Gong *et al.* 2001, Little *et al.* 2002). When β-catenin is deleted early

in the embryonic development in the limb and the head mesenchyme, the loss of β-catenin results in an early osteoblast differentiation that leads to the absence of mature osteoblasts (Hill *et al.* 2005). Canonical Wnt signaling is also required for osteoblast proliferation. *LRP5* KO mice have low bone mass due to decreased osteoblast proliferation and function (Kato *et al.* 2002). The *in vitro* effects of Wnt signaling on osteogenic differentiation are controversial. In mouse, the canonical Wnt signaling pathway seems to promote the osteoblastic differentiation of MSCs (Gaur *et al.* 2005, Gong *et al.* 2001). On the other hand, in the preosteoblastic mouse MC3T3-E1 cell line the Wnt signaling pathway has been found to both increase (Gaur *et al.* 2005) and decrease osteoblastic differentiation and mineralization (Kahler *et al.* 2006, Shi *et al.* 2007). In human MSCs, both stimulatory (Gregory *et al.* 2005) and inhibitory (Boland *et al.* 2004, de Boer *et al.* 2004, Liu *et al.* 2009a) effects have been reported for Wnt signaling pathway.

3.3.1.2 The pluripotency of mesenchymal stem cells

MSCs are pluripotent and can give rise to the cells of different mesodermal cell lineages including osteoblasts, chondrocytes and adipocytes. In addition, they have endodermic and ectodermic differentiation potential although the *in vivo* results are still controversial (Uccelli *et al.* 2008). Of these osteoblastic and adipogenic differentiation especially seem to be in balance as the decrease in bone volume found in osteoporosis, immobilization, or ovariectomy is accompanied by an increase in bone marrow adipose tissue (Ahdjoudj *et al.* 2002, Justesen *et al.* 2001, Martin and Zissimos 1991).

PPARγ is the master regulator of adipogenesis and its forced expression is sufficient to induce adipogenesis in fibroblasts (Tontonoz *et al.* 1994). The temporal expression of C/EBP family also plays a central part in adipogenic differentiation. The cascade starts with early induction of C/EBPβ and δ that leads to the induction of C/EBPα and PPARγ, which activate adipocytic gene transcription (Rosen and MacDougald 2006). In part, the adipocytic differentiation process is controlled by the same factors that control osteoblastic differentiation. For example, Wnt signaling decreases adipogenesis by inhibiting PPARγ and C/EBPα function and by maintaining preadipocytes therefore in an undifferentiated state (Ross *et al.* 2000). BMP-4 commits MSCs to the adipocytic lineage (Tang *et al.* 2004) and BMP-2 can cooperate with PPARγ and C/EBPα to increase adipogenic differentiation (Fux *et al.* 2004, Sottile and Seuwen 2000).

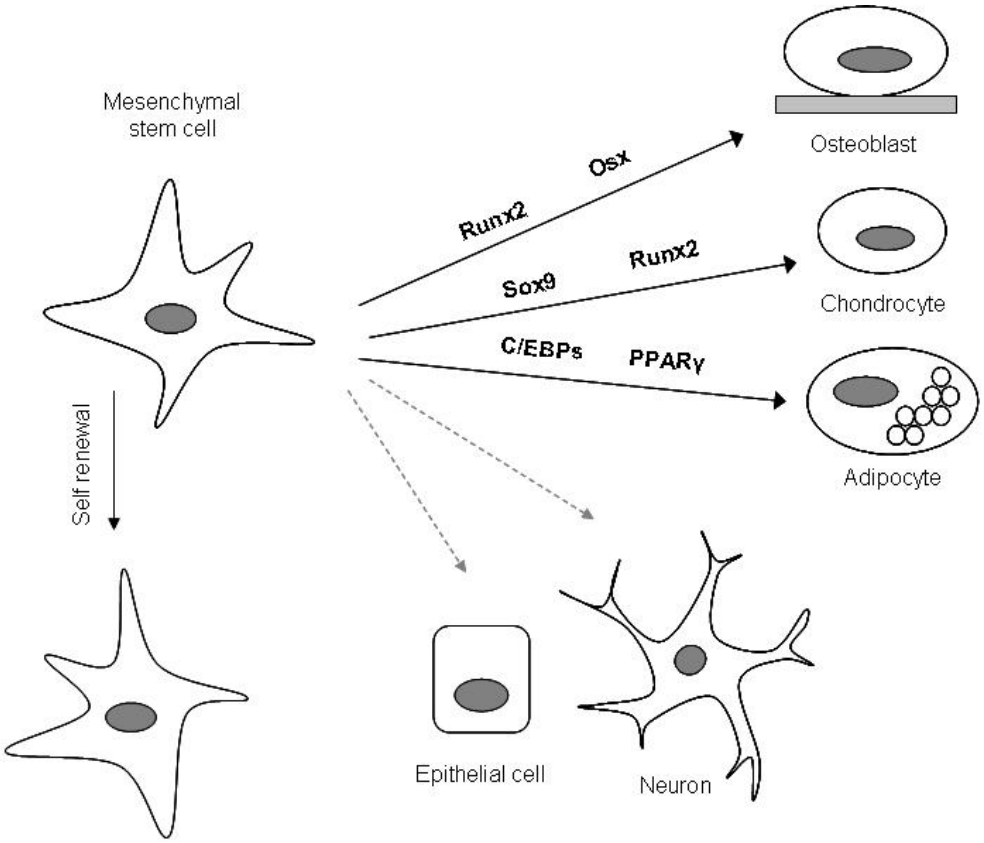

Fig. 10. The mesenchymal stem cells (MSCs) are capable of self renewal. They can also differentiate towards mesodermal lineages, which include osteoblasts, chondrocytes and adipocytes. Osteoblastic differentiation is dependent on the transcription factors Runx2 and Osx. Sox9 and later Runx2 are needed for chondrocyte differentiation, and C/EBPs and PPARγ are obligatory for adipocytic differentiation. The ability of MSCs to differentiate into non-mesodermal lineages *in vivo* is still under debate with controversial results.

MSCs are of interest in regenerative medicine because of their capacity for self-renewal, for their ability to differentiate and because they are relatively easily isolated from a small aspirate of bone marrow (Giordano *et al.* 2007, Mishra *et al.* 2009). MSCs have been used to treat severe osteogenesis imperfecta, which results in increased total bone mineral content associated with an increased growth velocity and fewer fractures (Horwitz *et al.* 1999). MSCs support the growth of hematopoietic progenitors *in vitro* (Robinson *et al.* 2006, Wagner *et al.* 2007) and cancer patients treated with systemic infusion of MSCs with peripheral-blood progenitor cells after high-dose chemotherapy had rapid hematopoietic recovery (Koc *et al.* 2000). MSCs also have immunosuppressive effects and they have been used to effectively prevent graft-versus-host disease (Le Blanc *et al.* 2004, Le Blanc *et al.* 2008). However, in leukemia patients, MSCs might also impair

the therapeutic graft-versus-leukemia effect as a higher incidence of relapses was seen in these patients (Ning *et al.* 2008). The ability of MSCs to migrate to the sites of inflammation, injury and solid tumors, makes them promising as a gene delivery mechanism in gene therapy (Giordano *et al.* 2007, Mishra *et al.* 2009). However, there are several concerns about their safety. It has been proposed that MSCs could be a source of carcinoma-associated fibroblasts (Mishra *et al.* 2009) and could promote tumor growth and metastasis (Djouad *et al.* 2003, Karnoub *et al.* 2007). In other studies, MSCs had suppressing effects on tumor growth (Cousin *et al.* 2009, Khakoo *et al.* 2006). Culturing MSCs *in vitro* makes them susceptible to cytogenic abnormalities. Differentiation of the MSCs into tumor cells after *in vivo* administration has been shown to occur in rodents (Tolar *et al.* 2007). However, it has been demontrated that human MSCs can be safely cultured *in vitro* (Bernardo *et al.* 2007). Hitherto there have been no reports of *in vitro* cultured MSC formed tumors in humans (Tikkanen *et al.* 2010).

3.3.2 The function of osteoblasts

Osteoblasts control most of the functions of bone. They are responsible for bone formation, extracellular matrix mineralization, osteoclast differentiation, and thereby indirectly for bone resorption.

3.3.2.1 Osteoblasts form bone

Osteoblasts synthesize the organic constituents of bone. Most of the organic matrix is highly cross-linked Coll I. Collagen is constituted by three polypeptide α-chains that form a triple-helix structure. Osteoblasts synthesize and secrete collagens in the form of soluble procollagens. During the secretion, propeptides are enzymatically cleaved, which triggers spontaneous self-assembly of collagen molecules into fibrils. Fibrils arrange into complex three-dimensional concentric weaves whose structures are stabilized by several post-translational modifications that allow intermolecular and interfibrillar crosslinks to take place (Blair *et al.* 2002, Viguet-Carrin *et al.* 2006). Collagen plays a substantial role in the toughness of bone and mutations in the genes that encode the α1 or α2 chains of Coll I have been associated with osteogenesis imperfecta (Marini *et al.* 2007).

Mineral is generated within the dense collagen matrix. Osteoblasts regulate the matrix mineralization by releasing small, membrane-bound matrix vesicles that contain concentrated calcium and phosphate which may lead to the first $CaPO_4$ deposits. Initiation of hydroxyapatite crystal formation is facilitated by the activity of phosphatases, such as ALP, which are enriched in the matrix vesicle membranes. The preformed hydroxyapatite crystals are released into the extravesicular fluid, which normally contains homeostatically maintained levels of calcium and PO_4 that are sufficient to support continued nucleation of new hydroxyapatite crystals on preformed hydroxyapatite templates (Anderson 2003).

ALP is ubiquitously found in plants and animals. In human, four ALP isoenzymes are encoded by four genes. One of the genes is expressed in all cells, but especially highly in bone, liver, and kidney, and is designated tissue-nonspecific ALP (*TNSALP*). It is essential for skeletal mineralization as its product hydrolyzes inorganic pyrophosphate, which is an inhibitor of hydroxyapatite formation. In humans, deficiency of the *TNSALP* gene leads to hypophosphatasia with skeletal hypomineralization, that leads to rickets and osteomalacia (Whyte 2010). *TNSALP* KO mice show skeletal hypomineralization that mimicks a severe form of hypophosphatasia (Narisawa *et al.* 1997). Osteoblasts isolated from these mice differentiate normally, but are unable to initiate matrix mineralization *in vitro* (Wennberg *et al.* 2000). Bone-specific ALP (BALP), which is an isoform of the TNSALP isoenzyme, is used as a biochemical marker for bone formation. BALP is a relatively specific marker of osteogenesis and elevated levels occur in conditions such as Paget's disease, bone cancer, and osteomalacia (Coleman *et al.* 2008).

Other matrix proteins that are secreted by osteoblasts include BSP, OPN, and OCN. BSP and OPN are both members of the SIBLING (small integrin-binding ligand N-linked glycoprotein) family (Fisher *et al.* 2001). OPN is expressed in osteoblasts and osteocytes, but also in kidney and epithelial linings, and is secreted in bodily fluids including milk, blood, and urine. *OPN* is upregulated at sites of inflammation and tissue remodeling. The expression of BSP is more restricted as it is mostly produced in cells associated with mineralized tissues and especially highly in osteoblasts. SIBLINGs mediate cell migration, adhesion and survival through interactions with cell surface receptors such as integrins and extracellular matrix constituents including collagen and hydroxyapatite (Ganss *et al.* 1999, Lund *et al.* 2009). BSP enhances osteoblast

differentiation and matrix mineralization *in vitro* (Gordon *et al.* 2007), and the BSP-collagen interaction has been shown to promote hydroxyapatite formation (Baht *et al.* 2008). On the other hand, OPN inhibits hydroxyapatite formation (Boskey *et al.* 1993, Hunter *et al.* 1994). *BSP* KO mouse fetuses and young adults exhibit shorter and hypomineralized bones. Adult *BSP* KO mice display a high trabecular bone mass despite reduced bone formation rate due to impaired bone resorption (Malaval *et al.* 2008). *OPN* KO mice show normal development and bone structure (Rittling *et al.* 1998). The *OPN* deficiency still increases bone fragility possibly due to increased matrix heterogeneity (Thurner *et al.* 2010). In human, elevated serum OPN levels have been shown to be associated with a higher risk of osteoporosis in menopausal women (Chang *et al.* 2010). BSP and OPN are also expressed at pathological sites of mineralization such as microcalcifications in the breast, and they have been suggested to play a role in bone metastasis (Bellahcène and Castronovo 1995, Bellahcène and Castronovo 1997). Indeed, BSP and OPN expressions have been associated with bone metastasis and/or reduced survival in patients with breast (Bellahcène *et al.* 1996, Rudland *et al.* 2002), lung (Donati *et al.* 2005, Papotti *et al.* 2006), and prostate cancer (Forootan *et al.* 2006, Waltregny *et al.* 1998).

OCN is the most abundant noncollagenous protein produced by osteoblasts and it is a terminal marker of osteoblastic differentiation. It has been suggested to OCN has an inhibitory role during bone formation as *OCN* KO mice show increased bone formation (Ducy *et al.* 1996). OCN has a high affinity for mineral ions and hydroxyapatite (Hauschka and Wians 1989) but its exact role in matrix mineralization is not known as there are contradictory results on the subject (Boskey *et al.* 1998, Murshed *et al.* 2004). OCN can also be found in the circulation. Both osteolysis and osteogenesis release OCN into the serum and therefore OCN levels might reflect the overall bone metabolism instead of just osteogenesis (Coleman *et al.* 2008).

After completion of bone formation approximately 50 to 70% of osteoblasts undergo apoptosis whereas the reminder become either osteocytes or inert bone lining cells. Flattened bone-lining cells are thought to be quiescent osteoblasts found in the endosteum. They may regulate the flux of mineral ions into and out of bone extracellular fluid and retain the ability to redifferentiate into functional osteoblasts upon exposure to stimulus such as PTH or to a mechanical force (Clarke 2008, Dobnig and Turner 1995). Osteocytes have been long thought to be rather inactive cells, but new information about

their function has emerged. Osteocytes, for example, regulate bone mass by expressing high levels of sclerostin, which has been shown to be a negative regulator of bone formation in human (Balemans *et al.* 2001, Brunkow *et al.* 2001). Another protein that is produced in large quantities in osteocytes is FGF-23. FGF-23 is a key regulator of phosphorus and vitamin D metabolism, and its excess production can cause hypophosphatemic diseases that are characterized by impaired renal phosphate reabsorption and osteomalacia (Wesseling-Perry 2010).

3.3.2.2 Osteoblasts regulate osteoclastogenesis

Another important function for osteoblasts is the regulation of osteoclastic differentiation. Osteoblasts regulate osteoclastogenesis mainly by expressing macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL). RANKL binds to RANK receptor on osteoclast precursors, which results in the recruitment of TRAF6 and activation of downstream molecules including NF-κB. Ultimately NFATc1 is induced and activated, which initiates the transcription of osteoclastic genes and differentiation. In addition to osteoclastogenesis, RANKL stimulates osteoclast activation and inhibits their apoptosis (Asagiri and Takayanagi 2007, Kearns *et al.* 2008). RANKL mutations have been shown to be associated with osteopetrosis in both mice (Kong *et al.* 1999) and humans (Sobacchi *et al.* 2007). Moreover, *op/op* mice that lack functional M-CSF have osteopetrosis because of a severe deficiency of mature osteoclasts (Yoshida *et al.* 1990). M-CSF expression by osteoblastic stromal cells is required for osteoclastogenesis, but M-CSF *per se* can not complete this process. M-CSF binds to its receptor c-Fms on osteoclast precursor cells and regulates their proliferation and survival (Asagiri and Takayanagi 2007, Boyce and Xing 2008, Kearns *et al.* 2008). Furthermore, osteoprotegerin (OPG) is an important regulator of osteoclastogenesis. It is a decoy receptor that binds to RANKL and therefore prevents its interaction with RANK (Boyce and Xing 2008). *OPG* KO mice develop early onset severe osteoporosis (Bucay *et al.* 1998). In human, the mutations in the *OPG* gene are associated with juvenile Paget's disease (Whyte *et al.* 2002) and idiopathic hyperphosphatasia (Cundy *et al.* 2002). There is a tight regulation of RANKL, OPG and M-CSF expressions in osteoblasts, in which many external factors such as growth factors, hormones, cytokines and drugs take part.

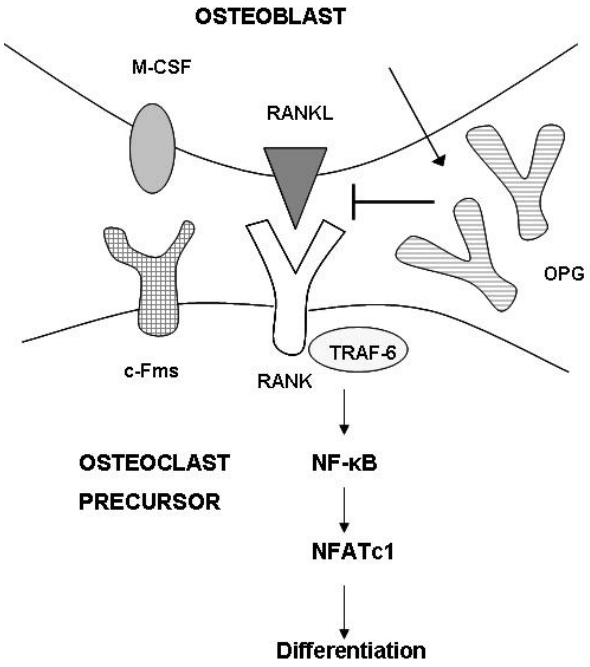

Fig. 11. A simplified model of how osteoblasts regulate osteoclast differentiation. Osteoblasts express RANKL that can bind to its receptor RANK, which is located on the surface of the osteoclast precursor. Binding induces a signaling cascade with TRAF-6, NF-κB, and NFATc1 and leads to the activation of osteoclastic genes and osteoclastogenesis. Osteoblasts also secrete OPG, which is a decoy receptor for RANKL and inhibits RANKL/RANK-binding. In addition, osteoblasts express M-CSF that binds to its receptor c-Fms on osteoclast precursor cells and enhances their proliferation, survival and osteoclastic differentiation.

3.4 Skeletal homeostasis

Bone is a dynamic tissue that is constantly being renewed in a process called bone remodeling, which is necessary to maintain calcium homeostasis and to remove and prevent the accumulation of aged or weakened bone. Bone remodeling occurs in a sequential manner (Fig. 12). Bone surface is activated by unknown signals that attract osteoclast precursor cells from the circulation (activation phase). These cells fuse and form multinucleated cells that adhere to the bone surface, differentiate into mature osteoclasts in response to signals mediated by osteoblasts, and start bone resorption (resorption phase). The degraded bone proteins and matrix minerals enter the osteoclast by endocytosis. They cross the cell in vesicles, and undergo exocytosis on the opposite side of the cell, from where the products diffuse into the nearby blood capillaries. When osteoclasts finish resorbing, they die by apoptosis, which is followed by the recruitment of

mononuclear cells, which prepare the bone surface for bone formation, and preosteoblasts (reversal phase). Preosteoblasts differentiate into functional osteoblasts and fill the cavity with a collagenous matrix that is finally mineralized (formation phase). Bone resorption is a much faster process than bone formation. It takes approximately three months to rebuild the equivalent mass of bone that was resorbed in two to three weeks (Hadjidakis and Androulakis 2006, Henriksen *et al.* 2009, Kearns *et al.* 2008, Tortora and Grabowski 2000).

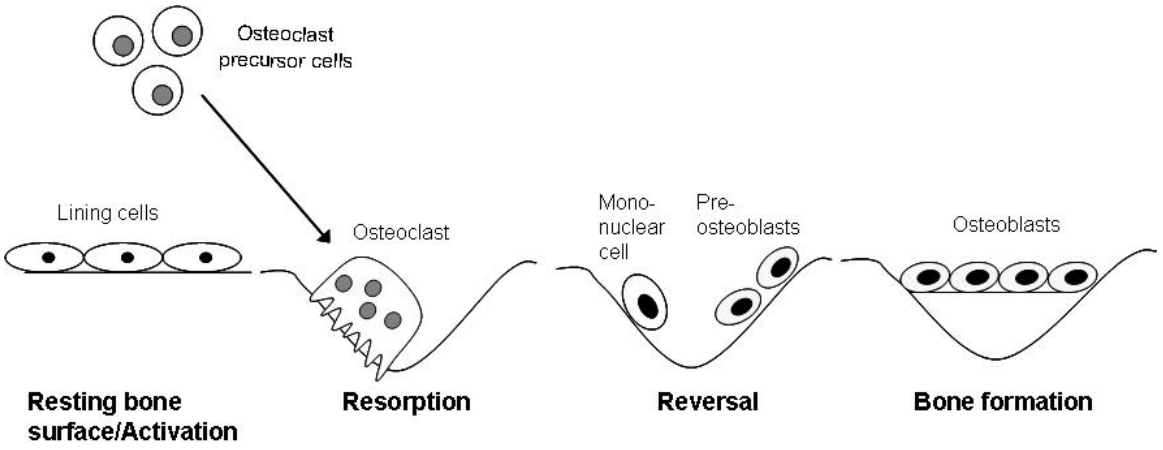

Fig. 12. Bone remodeling occurs in sequential phases. During the activation phase osteoclast precursors are recruited and activated to form multinucleated osteoclasts, which start bone resorption. During the reversal phase mononuclear cells and preosteoblasts are recruited onto the bone surface. In the formation phase preosteoblasts differentiate into functional osteoblasts and fill the cavity with new bone.

The control of bone metabolism is a complex and still largely unknown process. The purpose of having a tight control is to keep the two processes of bone resorption and formation in balance. It is regulated by endocrine, paracrine and autocrine actions of diverse hormones, cytokines and growth factors that in concert with numerous transcription factors act on osteoblast and osteoclast differentiation and function. This regulation will be discussed next emphasizing its effect on bone formation.

3.4.1 The regulation of bone formation and homeostasis

The rate of bone formation is determined by the number and function of osteoblasts. The cell number is regulated partly by those factors that regulate osteoblastic differentiation of

MSCs and pre-osteoblasts already discussed (heading 3.3.1). Many of these factors, including the Wnt signaling pathway (Westendorf *et al.* 2004) and FGFs (Fakhry *et al.* 2005, Valta *et al.* 2006) also enhance the proliferation of preosteoblasts and osteoblasts adding to the increase in osteoblast number.

Estrogens evidently play a critical role in the maintenance of bone homeostasis as exemplified by postmenopausal osteoporosis. The complex effects of estrogen and other nuclear receptors will be discussed in more detail under the next heading. However, decreased bone volume can also be detected in late premenopausal women with normal circulating estrogen levels. Instead, increased bone resorption is associated with elevated follicle-stimulating hormone (FSH) levels (Ebeling *et al.* 1996). FSH has been found to have direct effects on bone as it stimulates osteoclast formation and function via its G protein-coupled receptor FSHR (Sun *et al.* 2006). The early perimenopausal rise in FSH levels in women is attributed to a decrease in inhibin B secretion (Klein *et al.* 1996, Welt *et al.* 1999). Inhibin A and inhibin B are heterodimeric proteins that belong to the transforming growth factor β (TGF-β) superfamily and are able to suppress FSH secretion. The function of inhibins is antagonized by the related homodimeric peptides activin A and activin B that belong to the same TGF-β superfamily (Nicks *et al.* 2010). Both activins and inhibins have been shown to regulate osteoblastogenesis, osteoclastogenesis and bone mass. Activins have been described as being pro-osteoclastogenic but there are reports of both negative and positive effects on osteoblast differentiation (Nicks *et al.* 2009). Inhibins have been shown to repress the differentiation of osteoblasts and osteoclasts (Gaddy-Kurten *et al.* 2002). However, the effect of inhibins seems to be biphasic as continuous Inhibin A exposure *in vivo* has been described to have an anabolic effect on osteoblasts (Perrien *et al.* 2007).

Another biphasic and important regulator of bone mass is PTH. PTH, parathyroid hormone-related peptide (PTHrP) and vitamin D are the main calcium-regulating hormones that regulate bone cell differentiation and mineral transport at multiple points. PTH increases distal tubular calcium re-absorption and increases the production of the active form of vitamin D. Continuous administration of PTH increases osteoclast differentiation, activation and consequently bone resorption by stimulating RANKL and inhibiting OPG expression in osteoblasts. Nonetheless, intermittent PTH treatment has an anabolic effect on bone as it increases osteoblast proliferation and differentiation and inhibits osteoblast apoptosis. PTHrP shares amino acid homology with

PTH and binds the same G protein-coupled receptors (Datta and Abou-Samara 2009, Goltzman 2010). *PTHrP* KO mice show widespread abnormalities in endochondral bone development with decreased chondrocyte proliferation, premature chondrocyte maturation and accelerated bone formation (Karaplis *et al.* 1994). PTHrP has also been reported to increase osteoblast proliferation (Du *et al.* 2000).

Mechanical stimulus is another regulator of bone mass. Increased mechanical loads stimulate bone formation and suppress resorption, whereas unloading has the opposite effect. The exact mechanisms remain unknown but calcium channels, Wnt/β-catenin signaling, integrins, prostaglandin E2, and nitric oxide are some of the proposed mediators (Harada and Rodan 2003, Papachristou *et al.* 2009).

The central nervous system can also regulate bone formation. In mice, factors such as neuropeptides cocaine- and amphetamine-regulated transcript (CART) and neuromedin U (NMU) have been shown to associate with low bone mass with increased bone resorption (Elefteriou *et al.* 2005) and high bone mass with increased bone formation (Sato *et al.* 2007), respectively. The most studied of the centrally affecting factors is leptin. Leptin is a hormone produced by adipose cells that binds to its receptor in the hypothalamus and suppresses appetite and increases energy expenditure (Hamrick and Ferrari 2008, Kawai *et al.* 2009, Takeda 2008). *Ob/ob* mice that lack functional leptin and leptin-receptor deficient *db/db* mice have a high bone mass despite hypogonadism and hypercortisolism (Ducy *et al.* 2000). The suggested mechanism for this phenomenon involves leptin binding to its receptor in the hypothalamus and stimulating the release of noradrenaline from the sympathetic nerve fibers that project into the bone. Noradrenaline in turn is thought to inhibit bone formation by binding to β2-adrenergic receptors on osteoblasts (Ducy *et al.* 2000, Hamrick and Ferrari 2008).

3.4.1.1 The influence of nuclear receptors on bone and osteoblasts

Many NRs regulate bone and osteoblast function. Most important of these are perhaps VDR, GR, AR and ERs, which will be discussed in more detail.

Vitamin D has long been known as a potent stimulant of calcium absorption. Vitamin D deficiency leads to rickets in children and osteomalacia and osteoporosis in adults. VDR mutations that cause complete loss of function of the receptor lead to bone growth abnormalities (Imai *et al.* 2009). *VDR* KO mice have reduced bone mineral density

(BMD) (Yoshizawa *et al.* 1997) but increased bone volume. This phenotype can be corrected by the administration of calcium and phosphate supplements (Amling *et al.* 1999) and therefore vitamin D seems to regulate bone mass mainly by controlling the systemic calcium-phosphate metabolism. Despite this, there is increasing evidence showing that VDR is capable of regulating bone cells directly during bone metabolism (Anderson and Atkins 2008). VDR has been found to inhibit osteoblast differentiation and mineralization *in vitro* (Shi *et al.* 2007, Sooy *et al.* 2005). VDR also induces the expression of RANKL by chondrocytes and osteoblasts, which increases osteoclastogenesis (Kitazawa *et al.* 2003, Masuyama *et al.* 2006, Takeda *et al.* 1999).

Glucocorticoids increase bone resorption and decrease bone formation, which leads to decreased bone mass and osteoporosis as manifested by prolonged steroid therapy and Cushing's syndrome (Imai *et al.* 2009). Glucocorticoids have complex effects on bone and although extensively studied, consensus has yet to be achieved. Glucocorticoids have been shown to induce accelerated apoptosis in osteoblasts (Weinstein *et al.* 1998). However, they increase the ability of osteoprogenitors to form mineralized bone nodules *in vitro* (Jaiswal *et al.* 1997) and dexamethasone, a synthetic glucocorticoid, is commonly used to induce osteoblastic differentiation in MSC cultures.

The bones of males have higher mineral density and a lower risk of osteoporosis and fracture than those of females. This is thought to be due to the anabolic effects of androgenic hormones (Imai *et al.* 2009). Testosterone and other androgens act through AR. Indeed, patients suffering from complete androgen insensitivity syndrome due to a mutation in AR have decreased BMD (Bertelloni *et al.* 1998). *Ar* KO male mice have osteopenia with increased trabecular and cortical bone resorption and formation (Kawano *et al.* 2003). It is still somewhat controversial, which cells in bone are the targets of androgen-AR signaling and whether the effect is systemic or direct (Imai *et al.* 2009).

It has been long known that the decline in circulating estrogen in menopausal women triggers high bone turnover and a decrease in BMD. Ovariectomy induces similar bone defects in experimental animals. This suggests that estrogens and ERs have bone-protective effects (Frenkel *et al.* 2010, Imai *et al.* 2009). Estrogens have both direct and indirect effects on bone. For instance, immune cells participate indirectly in conducting the proskeletal effects of estrogen (Lorenzo *et al.* 2008). In humans and mice, cortical bone expresses mainly ERα but little or no ERβ, whereas trabecular bone contains both receptors (Bord *et al.* 2001, Mödder *et al.* 2004). Despite the clear anti-resorptive and

anabolic effect of estrogen in humans, ERα (*Esr1*) KO female and male mice exhibit increased trabecular bone volume with a decreased number of osteoclasts. This could be explained by the increased testosterone concentration in these mice (Lindberg *et al.* 2001, Parikka *et al.* 2005, Sims *et al.* 2002). Results from studies on cortical bone are conflicting with reports of both increased (Lindberg *et al.* 2001) and decreased (Sims *et al.* 2002) cortical BMD in ERα KO mice. Deletion of *Esr2* (the gene that encodes ERβ) in male mice has no impact on their skeleton. In female mice, ERβ seems to have a repressive effect on bone as a deletion of *Esr2* increases cortical bone mass at the age of 3 months (Windahl *et al.* 1999) and protects them from age-related bone loss as aged animals have an increased trabecular and cortical bone mass at the age of one year (Windahl *et al.* 2001). ERα/ERβ double KO male mice show similar but milder bone phenotype than ERα KO mice but females have decreased trabecular bone mass (Sims *et al.* 2002). Although estrogens have been reported to promote osteoblast commitment (Okazaki *et al.* 2002, Dang *et al.* 2002) and to prevent apoptosis of MSCs and osteoblasts (Almeida *et al.* 2007, Zhou *et al.* 2001), in postmenopausal women, both bone resorption and bone formation have been found to be increased (Garnero *et al.* 1996). However, this can be explained by osteoblast-osteoclast coupling mechanisms. Accordingly, an acute estrogen deficiency of three weeks is associated with a fall in markers of bone formation in both men (Falahati-Nini *et al.* 2000) and women (Charatcharoenwitthaya *et al.* 2007). It also appears that the effects of estrogen on osteoblast differentiation and function may be stage and cell type specific and can vary greatly between individuals (Leskelä *et al.* 2006). Estrogen has been shown to stimulate osteoblastic differentiation in some studies (Qu *et al.* 1998, Waters *et al.* 2001) but not to have any effect or to inhibit differentiation in others (Keeting *et al.* 1991, Robinson *et al.* 1997). Estrogen inhibits osteoclastic differentiation, *inter alia*, by increasing OPG (Hofbauer *et al.* 1999) and decreasing RANKL (Eghbali-Fatourechi *et al.* 2003) production by osteoblastic cells. Estrogen also induces apoptosis in osteoclasts by activating the expression of the Fas ligand. Female mice with osteoclast specific ablation of *Esr1* have low trabecular bone mass, which suggests that the osteoprotective effects of estrogen are partly mediated by osteoclastic ERα in trabecular bone (Nakamura *et al.* 2007). All of these results show that estrogen can function on multiple levels and has both direct and indirect effects on different compartments and cell types of bone, but also reflects the difficulty and complexity of bone research.

Table 2. Summary of the bone phenotypes of different ER KO mice (adapted from Vico and Vanacker 2010). ER KO mice manifest different phenotypes in their trabecular and cortical bone with either increased (↑), decreased (↓), or unaffected (-) bone mass. Some putative effects are still under debate as there are conflicting results (↑/↓).

Genotype	Female		Male	
	Trabecular	Cortical	Trabecular	Cortical
ERα KO	↑	↑/↓	↑	↓
ERβ KO	↑ (one year)	↑ (3 months)	-	-
ERα/ERβ KO	↓	↓	↑	↓

In addition to the classical NRs, many orphan NRs regulate the function of bone and osteoblasts. These include NR3B and NR4A families, which will be discussed next.

3.4.1.1.1 The NR3B family in bone and mesenchymal stem cell differentiation

There are several published studies that address the role of NR3B family in bone and osteoblast differentiation. ERRα is expressed in several human and mouse osteoblastic cell lines that include TE85 and SaOS (Bonnelye *et al.* 1997b). ERRα is also expressed in the ossification zones of the mouse embryo during the onset of bone formation (Bonnelye *et al.* 1997a). In bone the expression of ERRα is not only restricted to osteoblasts as MSCs, chondrocytes, mature osteocytes and osteoclasts all express ERRα (Bonnelye and Aubin 2002a). ERRβ is not expressed in osteoblastic cell lines SaOs, TE85 or primary human osteoblasts (Bonnelye *et al.* 1997b). ERRγ is expressed in osteoblastic cell line MC3T3-E1 and in primary mouse osteoblasts but very modestly (Jeong *et al.* 2009).

ERRs regulate several bone-related genes. One of these is *OPN*, which is regulated by ERRα in a cell context dependent manner. The expression of *OPN* is up-regulated by ERRα in HeLa cells but repressed in ROS17/2.8 cells (Vanacker *et al.* 1998, Zirngibl *et al.* 2008). ERRα also regulates lactofferin (Yang *et al.* 1996), aromatase (Yang *et al.* 1998), and endothelial nitric oxide synthase (Sumi and Ignarro 2003) expression. ERRα is expressed during osteoblast differentiation and it has a positive effect on bone formation *in vitro* in rat calvarial osteoblasts (Bonnelye *et al.* 2001). However, recently there has been some controversy on the subject. Delhon *et al.* showed that ERRα KO mice have slightly increased femoral cancellous BMD and that the absence of ERRα in human

MSCs increases their BMP-2 induced osteoblastic differentiation (Delhon *et al.* 2009). Another independent ERRα KO mouse model was shown to be resistant to bone loss induced by age or estrogen deficiency. However, the younger 14 week old female ERRα KO mice had significantly lower trabecular bone volume and trabecular number, which is in contradiction with that found in the ERRα KO mouse model of Delhon *et al.* (Teyssier *et al.* 2009). In the same study MSCs obtained from the female mice had increased osteoblastic differentiation, which can explain the resistance to estrogen deficiency but not the lower trabecular bone volume in normal state. Wei and colleagues reported osteopetrosis in ERRα KO mice that was mostly explained by decreased bone resorption due to a diminished number of osteoclasts. Osteoblast surface and number were also increased although OCN levels were not significantly altered (Wei *et al.* 2010). Delhon *et al.* and Teyssier *et al.* also studied osteoclast number and function in their ERRα KO models but did not see any significant differences to the wild-type mice (Delhon *et al.* 2009, Teyssier *et al.* 2009). Laflamme and others discovered an association between a frequent regulatory variant of *ESRRA* and BMD. French-Canadian premenopausal women who carried the long *ESRRA* genotype, which increases the expression of ERRα, had a 3.9% higher lumbar spine BMD than those who carried the short *ESRRA* genotype (Laflamme *et al.* 2005). A similar study was performed in another population of premenopausal women and no correlation between *ESRRA* variants and bone density was detected (Giroux *et al.* 2008). All of these studies show that ERRα plays a role in bone metabolism, but the mechanism could be more complex than first anticipated and may involve different cell types of bone.

A silent variant of *ESRRG* has been associated with multiple bone measurements, which could indicate that ERRγ plays a role in bone cell biology (Elfassihi *et al.* 2010). ERRγ expression is increased by BMP-2 in primary osteoblasts, C2C12 and MC3T3-E1 cells and ERRγ physically interacts with Runx2 and represses its transcriptional activity. Intramuscular overexpression of adenoviral ERRγ in mice did not affect intramuscular bone formation by itself *in vivo* but it inhibited BMP-2-induced ectopic bone formation (Jeong *et al.* 2009).

NR3B receptors have been shown to affect other differentiation routes of MSCs. ERRα appears to function in adipogenesis as the underexpression of ERRα inhibits adipogenesis of 3T3-L1 and human MSCs (Delhon *et al.* 2009, Ijichi *et al.* 2007). On the other hand, there is also a study that shows that the underexpression of ERRα increases

adipogenesis in rat calvarial osteoblasts (Bonnelye *et al.* 2002b). The expression of ERRγ increases in mouse mesenchyme-derived cells under the adipogenic differentiation conditions and in the brown and white adipose tissue of mice fed a high-fat diet. ERRγ knockdown by siRNA resulted in down-regulation of adipogenic marker gene expression that implies that ERRγ positively regulates the adipocyte differentiation (Kubo *et al.* 2009). ERRα is also expressed in fetal and adult rat chondrocytes in growth plate and articular cartilage. The overexpression of ERRα in chondrocytic C5.18 cell cultures increases the expression of Sox9 (SRY-related HMG-box 9), which is a master gene in cartilage formation. Reduced expression of ERRα by antisense oligonucleotides led to an inhibition of cartilage formation that was associated with decreased Sox9 and Indian hedgehog expression (Bonnelye *et al.* 2007).

3.4.1.1.2 The NR4A family in bone and mesenchymal stem cell differentiation

The NR4A family members are expressed in bone and osteoblasts. Their expression is induced in osteoblasts by PTH (Pirih *et al.* 2003, Pirih *et al.* 2005, Tetradis *et al.* 2001a, Tetradis *et al.* 2001b) and FGF-8b (Lammi and Aarnisalo 2008). FGF-8b stimulates the proliferation of MC3T3-E1 cells, which is at least partly mediated by Nurr1 and NGFI-B (Lammi and Aarnisalo 2008). NR4A receptors have been shown to regulate some bone related genes. They transactivate the *OPN* promoter directly which increases OPN expression in osteoblastic cells (Lammi *et al.* 2004). Nurr1 also regulates the expression of the *OCN* gene by directly binding to its promoter (Pirih *et al.* 2004). Intermittent PTH administration to mice induces NR4A expression in calvaria, long bones and kidney. Intermittent PTH also increases the expression of OPN and OCN mRNAs in osteoblasts (Pirih *et al.* 2005). Nurr1 is also involved in osteoblast differentiation as Nurr1 siRNA decreases OCN and Coll I A1 expression and ALP activity in MC3T3-E1 cells. The expression of osteoblastic markers is also reduced in calvarial osteoblasts derived from *Nurr1* KO mice (Lee *et al.* 2006).

It has been suggested that NR4A receptors influence adipogenic differentiation. The expression of these receptors is rapidly induced in response to adipogenic cocktail used to induce differentiation in 3T3-L1 preadipocytes (Au *et al.* 2008, Chao *et al.* 2008, Fu *et al.* 2005, Fumoto *et al.* 2007). The role of NR4A receptors in adipogenic differentiation is still controversial with reports of both negative and positive

effects in addition to the absence of effects (Au *et al.* 2008, Chao *et al.* 2008, Fumoto *et al.* 2007). Nurr1 is also thought to have a protective function in cartilage homeostasis as Nurr1 selectively represses the expression of matrix metalloproteinases during inflammation. Matrix metalloproteinases digest components of the extracellular matrix, which leads to the degradation of cartilage, tendon and bone (Mix *et al.* 2007).

3.4.2 Osteoporosis and other bone related diseases

Osteoporosis is a major health problem that affects approximately 400 000 people in Finland. It has been estimated that 30 000 to 40 000 bone fractures are associated with osteoporosis every year (Duodecim 2007). Osteoporosis is characterized by the loss of bone mass and strength due to an imbalance between bone resorption and formation that leads to an increased risk of fractures. Osteoporosis is a heterogenous disease caused by complex interactions among local and systemic regulators of bone cell function (Canalis 2010, Raisz 2005). Many factors, including age, sex, diet, physical activity, medication use, positive family history and menopausal status, influence the risk of osteoporosis. To date, at least 15 genes (*VDR*, *ESR1*, *ESR2*, *LRP5*, *LRP4*, *SOST*, *GRP177*, *OPG*, *RANK*, *RANKL*, *COLL I A1*, *OPN*, *ITGA1*, *OSX*, and *SOX6*) have been assigned as osteoporosis susceptibility genes and over 30 genes have been identified as promising candidates. These susceptibility and candidate genes are clustered in three biological pathways of which the first one is the estrogen pathway (Li *et al.* 2010). As discussed before, estrogen and its receptors obviously play a critical part in the development of osteoporosis in women (Raisz 2005) and also in men (Falahati-Nini *et al.* 2000, Khosla *et al.* 2008). The other two gene clusters include the Wnt/β-catenin and the RANKL/RANK/OPG pathways (Li *et al.* 2010).

The primary treatment for osteoporosis are bisphosphonates. Bisphosphonates include alendronate, risedronate, and etidronate, which are stable analogues of pyrophosphate and they have strong affinity for hydroxyapatite. Bisphosphonates decrease bone turnover and enhance bone mass by inhibiting the function and recruitment of osteoclasts and by increasing their apoptosis. Estrogen replacement therapy prevents bone loss in postmenopausal osteoporosis. However, the hormone replacement therapy is not suitable for all patients as it increases the risk of uterine and breast cancer, stroke, thrombotic events, and cardiovascular diseases. On the

other hand, a selective estrogen receptor modulator raloxifene lacks most of these undesirable side effects and has been approved for the prevention and treatment of postmenopausal osteoporosis. Raloxifene, which acts on bone as estrogen, diminishes the differentiation and activity of osteoclasts while it maintains the function of osteoblasts, which reduces the occurrence of vertebral fracture. Calcitonin is a peptide produced by thyroid, that inhibits osteoclast activity and therefore bone resorption. It is not as efficient as bisphosphonates in inhibiting bone loss but it has an analgesic effect on bone pain not shared by other antiresorptive therapies. Teriparatide (1-34 PTH) is a synthetic PTH analog and is the only accepted anabolic medication available for osteoporosis. The teriparatide treatment is expensive but effective in increasing the BMD, in improving bone microstructure, and in decreasing fracture incidence by stimulating new bone formation by osteoblasts. Teriparatide is approved to treat severe osteoporosis for a maximum of 18 months (Duodecim 2007, Gass and Dawson-Hughes 2006, Migliaccio *et al.* 2007).

Bone is quite a rare place for a primary tumor to occur. In Finland, 50 to 60 cases of malignant primary tumors are found per year, which constitutes only 1/80 of all malignant tumors. The most common primary bone malignancies are myeloma, osteosarcoma, chondrosarcoma, and Ewing sarcoma (Mäkelä 2001). Whereas the incidence of chondrosarcoma and myeloma peaks in adults, osteosarcoma and Ewing sarcoma mainly occur in pediatric patients and young adults and usually develop in the extremities or in the pelvis (Mäkelä 2001, Heare *et al.* 2009).

Instead of primary tumors, cancer metastases commonly develop in the skeleton. For example, breast and prostate cancers preferentially metastase in the bone. The bone microenvironment is highly favourable for tumor invasion and growth. To facilitate the interactions of tumor and bone cells, the bone metastasizing tumor cells often mimic the bone cells. For example, metastatic breast cancer cells have been shown to express BSP and Runx2 (Barnes *et al.* 2003). Physical properties of the bone matrix, such as low oxygen content and acidic pH, promote tumor growth. There are two types of bone metastases, osteoblastic and osteolytic lesions, which result from the imbalance between bone formation and resorption. Osteoblastic lesions are a characteristic of prostate cancer, whereas osteolytic lesions are found in 80% of patients with stage IV breast cancer. In osteolytic lesions, tumor cells secrete factors that stimulate osteoclast function. This, in turn, helps the release of growth factors such as TGF-β and insulin-like growth factors I and II that are immobilized within the bone matrix, which favours tumor growth. In

osteoblastic lesions, tumor cells secrete pro-osteoblastic factors, including Wnt, BMPs and endothelin-1 that stimulate various steps in osteoblast proliferation, differentiation and mineralization. Activated osteoblasts in turn secrete growth factors, such as TGF-β, BMPs and vascular endothelial growth factor, which favor tumor cell survival and proliferation. However, bone lesions are often mixed and show both osteoblastic and osteoclastic elements. Bone metastases lead to many skeletal complications, such as bone pain, hypercalcemia and fractures, which increase morbidity and diminish quality of life (Kingsley *et al.* 2007, Mundy 2002, Virk and Lieberman 2007).

AIMS OF THE STUDY

The members of the orphan NR families NR3B and NR4A are expressed in osteoblasts. However, the regulation of their transcriptional activity and function in osteoblasts is largely unknown. Therefore, the aim of this study was to address the signaling and function of NR3B and NR4A receptors in osteoblasts with the following specific aims:

- To identify new signaling pathways able to regulate the transcriptional activity of NR3B and NR4A receptors in osteoblasts
- To identify possible new ligands for NR3B receptors
- To study how NR3B and NR4A orphan nuclear receptors affect Wnt signaling pathway in osteoblasts
- To analyze the role of ERRα in osteoblastic differentiation of mesenchymal stem cells

MATERIALS AND METHODS

Short descriptions of the materials and methods are presented here. For more detailed descriptions, the reader is referred to the original publications.

Table 3. The methods used in original publications.

Method	Original publication
1. Plasmid construction and recombinant DNA technology	I, II, III, IV
2. Site-directed mutagenesis	I, II, III, IV
3. Cell culture and reporter gene assays	I, II, III, IV
4. Electrophoretic mobility shift assay (EMSA)	I
5. SDS-PAGE and immunoblotting	II, III
6. Partial proteolysis assay	II
7. Cell growth assay	II
8. Molecular modeling	II
9. Primary mesenchymal stem cell isolation and osteoblastic and adipogenic differentiation	IV
10. Primary osteoblast isolation and culture	IV
11. ^{3}H-thymidine incorporation	IV
12. Alizarin red S and Oil Red O stainings	IV
13. RNA extraction and RT-PCR	II, III, IV
14. Statistical analysis	II, III, IV

1. Plasmid construction and recombinant DNA technology

Expression vectors, reporter constructs, and probes were prepared using standard recombinant DNA techniques. The luciferase reporter constructs $NBRE_3$tk-LUC, MH100tk-LUC and $ERRE_3$tk-LUC, and the pCMX-βgal, pCMX-PL1, and pCMX-Gal4 vectors were provided by Dr. Ronald M. Evans (Howard Hughes Medical Institute, The Salk Institute for Biological Studies, La Jolla, CA). The expression vectors for pCMX-Nurr1, pCMX-NGFI-B, pCMX-Nor1, pCMX-Gal4-Nurr1 NTD, pCMX-Gal4-Nurr1 LBD, pCMX-Nurr1Δ1-84, pCMX-Nurr1D589A, pCMX-Nurr1Δ1-84/D589A, and pCMX-Nurr1

DN were gifts from Dr. Thomas Perlmann (Ludwig Institute for Cancer Research, Sweden). pCMX-ERRα, pCMX-ERRβ, and pCMX-ERRγ were provided by Dr. Vincent Giguère (McGill University Health Center, Canada). pM-GRIP1(563-1121) was a gift from Dr. Jorma Palvimo (University of Kuopio, Finland) and pCI-Gal4-RIP140 from Dr. Malcolm Parker (Imperial College London, UK). pCDNA3-hPGC-1α was received from Dr. Anastasia Kralli (Scripps Research Institute, La Jolla, CA) and pCMX-Flag-ERRα from Dr. Toren Finkel (NHLB, NIH, Bethesda, MD). The pGL3-OT reporter and the expression vector for S33Y β-catenin (pCl-neo-β-catenin-S33Y) were gifts from Dr. Bert Vogelstein (The John Hopkins Oncology Center, Baltimore, MD) and the -927 BSP-LUC reporter from Dr. Yorimasa Ogata (Nihon University School of Dentistry at Matsudo, Chiba, Japan). The expression vector for VP16-S33A-β-catenin (pCS2+/βS33A-VP16) was provided by Dr. Edward P. Gelmann (Lombardi Cancer Center, Washington, DC).

2. Site-directed mutagenesis

Mutant plasmids were created by using the QuickChange® Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. The nucleotide sequences of the mutants were confirmed by sequencing.

3. Cell culture and reporter gene assays

All cells were received from the American Type Culture Collection (ATCC). U2-OS, SaOS-2 and 293T cells were maintained in DMEM supplemented with penicillin (25 U/ml), streptomycin (25 U/ml), 10% FBS (v/v) and L-glutamine. HeLa cells were maintained in DMEM supplemented with penicillin (25 U/ml), streptomycin (25 U/ml), 10% FBS (v/v), and non-essential amino acids. PC-3 cells were maintained in F-12 medium supplemented with penicillin (25 U/ml), streptomycin (25 U/ml), and 10% FBS (v/v). MC3T3-E1 subclone 14 cells were maintained in α-MEM supplemented with penicillin (50 U/ml), streptomycin (50 U/ml) and 10% FBS (v/v), and the osteogenic differentiation was induced by adding 50 μg/ml ascorbic acid and 10 mM sodium β-glycerophosphate to the medium.

For reporter assays the cells were seeded on 12-well plates (5 x 10^4 or 6 x 10^4 cells/well) and 24 h later were transfected with FuGENE (Roche Molecular

Biochemicals), FuGENE HD (Roche Molecular Biochemicals) or Lipofectamine 2000 (Invitrogen) according to manufacturer's recommendations with 300 ng of the luciferase reporter plasmid, 100 ng of the pCMX-βgal internal control plasmid, and 50 ng of the expression vectors or empty vector. When indicated, at 20 h after the transfection the cells received fresh medium containing either 2% charcoal-stripped FBS and different hormones, synthetic ligands and phytoestrogens or 10% FBS and LiCl. 24 h later, the cells were harvested, lysed, and assayed for luciferase and β-galactosidase activities. Transfections were performed in triplicate dishes and repeated two to six times.

4. Electrophoretic mobility shift assay (EMSA)

Proteins were produced by coupled *in vitro* transcription and translation in reticulocyte lysates (TNT® Quick Coupled Transcription/Translation Systems, Promega) and incubated with ^{32}P-labeled double-stranded oligonucleotide probes (ERRE, NBRE, OPN S1). The protein-DNA complexes were resolved by electrophoresis on 4% non-denaturing polyacrylamide gel, after which the gel was dried for autoradiography.

5. SDS-PAGE and immunoblotting

PC-3 and U2-OS cells and were transfected on 6-well plates with 2 µg and 1.5 µg of expression or empty vectors, respectively, using FuGENE or Lipofectamine 2000 transfection reagents. When indicated, the cells were treated with vehicle (DMSO), equol, GSK4716 or 4-OHT 20 h after transfection. The cells were harvested 48 h after transfection and protein samples were prepared in Laemmli sample buffer. Proteins were resolved by electrophoresis on a 10% polyacrylamide gel under denaturing conditions. Proteins were then transferred onto Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences). Detections of specific proteins were carried out by using the following antibodies: anti-ERRγ antibody (1 µg/ml, Perseus Proteomics Inc.), Lamin B C-20 antibody (0.2 µg/ml, Santa Cruz Biotechnology), anti-Gal4 DBD RK5C1 antibody (0.28 µg/ml, Santa Cruz Biotechnology), and β-catenin antibody 9562 (1:2000, Cell Signaling). The immunocomplexes were visualized by using horseradish peroxidase-conjugated secondary antibodies and ECL Western blotting detection reagents (Amersham Biosciences).

6. Partial proteolysis assay

Flag-ERRγ was produced by coupled *in vitro* transcription and translation (TNT® Quick Coupled Transcription/Translation Systems, Promega). [^{35}S]methionine-labeled translation mixture was incubated with equol, enterodiol or 4-OHT and digested by trypsin (30 μg/ml or 50 μg/ml). The digestion products were resolved on SDS-PAGE and the radioactive peptides were visualized by autoradiography.

7. Cell growth assay

PC-3 cells were plated on 12-well plates (4.5 x 10^4 cells/well) and 24 h later were transfected with 300 ng of pCMX-FLAG-ERRγ or empty pCMX-FLAG vector using the FuGENE HD reagent. Five hours later, the cells received fresh medium containing 10% charcoal-stripped FBS supplemented with DMSO (vehicle), equol, or GSK4716 when indicated. At 5 h and 72 h post transfection, cells were trypsinized and the cell numbers counted by a Coulter® Particle Counter (Beckman Coulter).

For siRNA experiments, PC-3 cells were plated on 12-well plates (6 x 10^4 cells/well). Subsequently, at 24 h after plating the cells were transfected with 30 pmoles of siRNA duplexes targeted for human ERRγ or non-targeting Luciferase GL2 control siRNA (Qiagen) using the Lipofectamine 2000 reagent. Four hours later, the cells received fresh medium that contained 10% charcoal-stripped FBS supplemented with DMSO or equol. At 4 h and 72 h post transfection, the cells were trypsinized and the cell numbers counted by a Cedex XS semi-automated cell counter (Roche Innovatis AG).

8. Molecular modeling

The binding of equol in the ERRγ ligand-binding pocket was studied by generating a computer model of ERRγ LBD in a complex with equol. Geometric analysis and superposition of ERRγ X-ray structures were performed with Maestro 7.0 (Schrödinger, LLC: Portland, OR).

9. Primary mesenchymal stem cell isolation and osteoblastic and adipogenic differentiation

The ERRα KO mice, in which the *Esrra* gene locus was targeted to delete ERRα amino acids 28-89, were obtained from Amgen Inc. All animals were housed in the animal facility of the University of Helsinki. Male ERRα KO mice (n=3) aged between 8 – 12 weeks and their wild-type littermates (n=3) were euthanized by carbon dioxide followed by cervical dislocation, after which primary mesenchymal stem cells were isolated from their bone marrow. The diaphysis of tibiae and femuri were flushed with α-MEM and the cells were collected by centrifugation. The cells were counted in 2% acetic acid and plated 1 x 10^6 cells/cm^2 in α-MEM containing 10% FCS (v/v), penicillin (50 U/ml) and streptomycin (50 µg/ml). Six days later, the cells were trypsinized and seeded on six-well plates (5 x 10^3 cells/cm^2) and the media was supplemented with 10 mM sodium β-glycerophosphate, 10 nM dexamethasone and 50 µg/ml ascorbic acid to initiate osteoblastic differentiation. To initiate adipogenic differentiation, the cells were seeded on six-well plates (2 x 10^4 cells/cm^2) and the media were supplemented with 5 µg/ml insulin, 1 µM rosiglitazone and 1 µM dexamethasone.

10. Primary osteoblast isolation and culture

Primary mouse osteoblasts were sequentially digested with collagenase from the tibiae and the femuri of 8-12 week old ERRα KO male mice and their wild-type littermates. Cells from the digestions 2-6 were collected and cultured in α-MEM supplemented with 15% FCS (v/v), 10 nM dexamethasone, penicillin (200 U/ml), streptomycin (200 µg/ml), and amphotericin B (2.5 µg/ml) until confluent. For proliferation assays, the cells were replated (2.5 x 10^3 cells/cm^2).

11. ^{3}H-thymidine incorporation

Cell proliferation was studied using mouse mesenchymal stem cells and osteoblasts after 3 and 5 days in culture. After a 6 hour 1 µCi [^{3}H]thymidine pulse the cells were rinsed with ice cold PBS and 5% TCA. The cells were lysed in 0.3 M NaOH and incorporated radioactivity was measured using Wallac 1409 Liquid Scintillation Counter

(PerkinElmer). The proliferation experiments were performed three times in triplicate dishes.

12. Alizarin red S and Oil Red O stainings

Mineralized bone nodules were detected by Alizarin red S staining and lipid accumulation by Oil Red O staining. In Alizarin red S staining, the cultures were washed with PBS, fixed with 3% PFA-PBS, and washed again with PBS and sterile water. The cells were covered with 2% Alizarin red S solution (pH 4.0) for 3 min. The Oil Red O staining was performed by washing the cells with PBS, fixing with 10% formaldehyde, and finally by staining with a filtered 0.3% Oil Red O solution in 60% isopropanol for one hour. After staining the cells were washed extensively and left to dry.

13. RNA extraction and RT-PCR

The PC-3 cells, primary mesenchymal stem cells and osteoblasts, and MC3T3-E1 cells were harvested and RNA samples collected in Trizol (Invitrogen) for RNA extraction. Total RNA was isolated according to the manufacturer's protocol. 1 μg of RNA was used for cDNA synthesis with Superscript II (Invitrogen). Quantitative real-time PCR was performed with LightCycler 480 instrument (Roche Applied Science) and SYBR Green I (Roche Applied Science) according to manufacturer's recommendations. The RT-PCR reactions were performed in duplicate for at least three independent experiments. The results were normalized to G3PDH or TBP and the resulting values were compared with the basal level (=1) and computed as fold inductions. Alternatively, the PCRs were performed with Taq DNA polymerase (GE Healthcare) and the products fractioned on 1% agarose gel.

14. Statistical analysis

All experiments with calculated statistical significances were performed at least three times. The statistical significance of the differences between two groups were assessed by the two-tailed Student's *t* test, unless the reference group was standardized to a constant value (=1) with no variability. In this case, a one-sample *t* test was used. When several *t*

tests were performed, the *p*-values were corrected by the Bonferroni correction. Multiple groups were compared by one-way ANOVA followed by Scheffe's post hoc test to determine the significant differences among groups. A *p*-value of <0.05 was considered to represent a statistically significant difference. All the statistical analyses were performed by using SPSS software (version 15.0).

RESULTS AND DISCUSSION

1. The transcriptional activity of the NR4A family can be regulated by cross-talk with other nuclear receptors and transcription factors in osteoblasts (I, III)

The LBP of NR4A receptors is tightly packed with bulky, hydrophobic amino acid side chains (Wang *et al.* 2003) and thus it is incapable of binding ligands. Therefore it is important to know which other mechanisms are used to regulate the transcriptional activity of NR4A receptors. The activity of NR4A receptors can be regulated by modifying their expression and post-translational status but also by cross-talk with other signaling pathways (Aarnisalo *et al.* 2002, Martens *et al.* 2005, Song *et al.* 2004). Our goal was to identify new signaling pathways that could potentially regulate NR4A receptors in osteoblasts.

1.1 NR3B orphan nuclear receptors repress the transcriptional activity of Nurr1

NR3B and NR4A receptors are co-expressed in many tissues such as the central nervous system and bone (Bonnelye *et al.* 1997a, Bookout *et al.* 2005, Tetradis *et al.* 2001a, Zetterström *et al.* 1996b). To study if ERR receptors can regulate the transcriptional activity of NR4A family, we performed reporter assays in U2-OS osteosarcoma cells. The cells were transfected with the expression plasmids for Nurr1, NGFI-B and Nor1 alone or in combination with ERRγ plasmid along with the $NBRE_3$tk-LUC or OPN-LUC reporters. $NBRE_3$tk-LUC has three NBRE binding sites and *OPN* has been described as a target gene for NR4A receptors (Lammi *et al.* 2004). ERRγ had only a small repressive effect on the transcriptional activity of NGFI-B and Nor1 but it abolished the activity mediated by Nurr1. ERRα was also able to repress Nurr1, whereas ERRβ had no apparent effect.

1.1.1 The DNA-binding domains of ERRγ and Nurr1 are essential for the repressive effect of ERRγ on Nurr1 transcriptional activity

Next we studied the structural requirements needed for the repressive effect of ERRγ on Nurr1. Mutant plasmids were created in which Nurr1 LBD and NTD were fused to the Gal4 DNA-binding domain (Gal4-Nurr1 LBD and Gal4-Nurr1 NTD, respectively). ERRγ

failed to inhibit the transcriptional activity of these mutant receptors lacking DBD. However, ERRγ was capable of inhibiting a Nurr1 deletion mutant that lacked AF-1 but contained DBD and LBD (Nurr1 ΔNTD) and a mutant that contained the DBD and NTD but lacked LBD (Nurr1 ΔLBD). These results suggest that the repressive effect of ERRγ is dependent upon Nurr1 DBD, but not upon either LBD or NTD.

Table 4. Representation of the functional domains present (x) or absent (-) in different Nurr1 and ERRγ expression constructs.

Construct	NTD	DBD	LBD
pCMX-Nurr1	x	x	x
Gal4-Nurr1 LBD	-	-	x
Gal4-Nurr1 NTD	x	-	-
Nurr1 ΔNTD	-	x	x
Nurr1 ΔLBD	x	x	-
ERRγ ΔNTD	-	x	x
ERRγ LBD	-	-	x
Gal4-ERRγLBD	-	-	x

To study which ERR domains were required for the repression of Nurr1, ERRγ mutants were created. ERRγ mutant that lacked most of the NTD (ERRγ ΔNTD) was still able to repress Nurr1, whereas the ERRγ LBD construct that lacked the entire NTD and DBD failed to repress Nurr1. The lack of inhibition was not due to the altered expression or reduced nuclear localization as the expression level was similar to that of the wild-type ERRγ and as Gal4-ERRγLBD that contains a nuclear localization signal failed to repress Nurr1. Therefore, the repressive effect of ERRγ on Nurr1 is dependent on ERRγ DBD. This was confirmed by introducing point mutations in ERRγ DBD. ERRγ C125G and ERRγ R176A in which the mutations are located in the first zinc finger and A-box, respectively, failed to repress Nurr1-mediated OPN-LUC activation.

As DBDs of both Nurr1 and ERRγ were determined to be crucial for the repressive effect, we then studied whether the repression involved competition of DNA binding by EMSA. DNA binding was studied by using the NBRE element and the S1 element of the *OPN* promoter as the probe. *In vitro* translated ERRα and ERRγ proteins bound to these elements very weakly and were unable to prevent Nurr1 protein from binding. This subject

was also studied indirectly in a reporter assay. U2-OS cells were transfected with $NBRE_3$tk-LUC reporter and a dominant negative Nurr1 (Nurr1 DN) variant in which the *Drosophila* repressor protein Engrailed is fused with the DBD of Nurr1. Nurr1 DN had been reported to inhibit transactivation of the NR4A receptors (Castro *et al.* 2001) and accordingly in our study it inhibited the basal $NBRE_3$tk-LUC reporter activity. ERRγ did not diminish the repressive effect of Nurr1 DN, which indicates that it does not directly compete with Nurr1 DN for the DNA binding. This supports the result of the EMSA binding experiments.

1.1.2 The dimerization interface of ERRγ is needed for the repression of Nurr1

Most NRs form homo- or heterodimers. NR4A receptors form heterodimers with RXR (Perlmann and Jansson 1995, Zetterström *et al.* 1996a) that can either inhibit or stimulate the transactivation mediated by NGFI-B and Nurr1 (Aarnisalo *et al.* 2002, Perlmann and Jansson 1995). Therefore, we studied if dimerization could be involved in the repressive effect of ERRγ on Nurr1 transcriptional activity. We introduced mutations to the I-box of ERRγ, a domain mediating receptor dimerization (Aarnisalo *et al.* 2002, Huppunen *et al.* 2004). ERRγ ML(394,395)AA, R390A, and L398A were not able to repress Nurr1 mediated transactivation, which implies that the dimerization interface of ERRγ is important for the repressive effect. The heterodimerization between NR3B and NR4A receptors was studied by using a mammalian two-hybrid assay for which Nurr1 LBD was fused to the yeast Gal4 DNA-binding domain and ERRγ LBD was fused to the herpes simplex virus VP16 activation domain. MH100tk-LUC that is driven by four Gal4-binding sites was used as a reporter. VP16-ERRγ LBD was not able to stimulate transactivation by Gal4-Nurr1 LBD which indicates that the receptors do not interact. We could not detect any dimers between full length Nurr1 and ERRγ proteins in either the EMSA or the co-immunoprecipitation experiments. Therefore, the repression does not involve heterodimerization between Nurr1 and ERRγ. Instead I-boxes might have auxiliary roles in the repression perhaps by stabilizing the receptor conformation or by mediating interaction with other proteins.

Co-activators play an important role in NR transactivation. NR4A and NR3B families have been found to bind to the same co-activators (Hong *et al.* 1999, Wansa *et al.* 2002, Xie *et al.* 1999) and therefore competition for these factors could be one mechanism

by which receptors exert effects on each other's transcriptional activities. Many of the co-activators interact with the AF-2 and to address the potential role of the co-activator competition in repression, the repressive effect of ERRγ AF-2 mutant E429A was studied. ERRγ E429A was able to repress Nurr1 as effectively as the wild-type ERRγ in U2-OS cells and therefore the repression does not involve competition for co-activators that interact with the ERRγ AF-2. However, co-activators can also bind to other NR domains. An example of this is PGC-1α which does not require AF-2 in order to co-activate ERRγ (Huppunen *et al.* 2004). PGC-1α has been reported to enhance both NR3B and NR4A mediated transactivation (Huppunen *et al.* 2004, Nervina *et al.* 2006). In our experimental conditions, PGC-1α was not found to co-activate Nurr1 but rather to repress it. Thus, competition for PGC-1α can not explain the repression mediated by ERRγ on Nurr1.

1.2 Wnt signaling pathway represses the transcriptional activity of NR4A receptors in osteoblasts

Recently, a regulatory circuit between Nurr1 and the Wnt signaling pathway was found. The transcriptional activity of Nurr1 was enhanced by different Wnt signaling cascade activating factors, including β-catenin, lithium chloride (LiCl) and Wnt1 in 293F cells (Kitagawa *et al.* 2007). Both the Wnt/β-catenin signaling pathway and NR4A receptors function in the bone. Therefore, we studied if the proteins influence each other in osteoblasts. U2-OS cells were transfected with $NBRE_3$tk-LUC reporter and the expression vectors for Nurr1, NGFI-B and Nor1 with or without S33Y β-catenin, which is a constitutively active form of β-catenin. Surprisingly, the transcriptional activation induced by all of the NR4A family members was repressed by S33Y β-catenin. This finding was contrary to the results reported by Kitagawa *et al.* (2007) from 293F cells. To confirm the differential regulation of Nurr1 signaling, we transfected 293T cells with $NBRE_3$tk-LUC reporter and the expression vector for Nurr1 and S33Y β-catenin. Indeed, as Kitagawa *et al.* reported, S33Y β-catenin had a small but significant increasing effect on the transcriptional activity of Nurr1 in 293T cells. However, in HeLa cells the transfection of S33Y β-catenin with Nurr1 repressed the transcriptional activity of Nurr1 as in the U2-OS cell line. Therefore, Wnt signaling pathway can have both activating and inhibiting effects on the transcriptional activity of NR4A receptors depending on the cell type. In U2-OS cells, the repressive effect of S33Y β-catenin on Nurr1 induced NBRE-activation was

dose-dependent. Moreover, 20 mM LiCl modestly repressed Nurr1 mediated transactivation. LiCl is evidently quite an inefficient inducer of β-catenin signaling in U2-OS cells as it produced a very small activation of the pGL3-OT reporter compared to the overexpressed β-catenin itself, which could explain the modest repression of Nurr1. S33Y β-catenin also repressed the transcriptional activation induced by Nurr1 on OPN-LUC reporter.

1.2.1 The repressive effect of β-catenin on Nurr1 transactivity involves the ligand-binding domain of Nurr1

We studid which protein domains are important for NR4A family to be able to be repressed by β-catenin. Therefore, U2-OS cells were co-transfected with expression vectors Gal4-Nurr1 LBD and Gal4-Nurr1 NTD with or without S33Y β-catenin. S33Y β-catenin failed to repress the transactivation induced by Gal4-Nurr1 NTD but did repress the transcriptional activity of Gal4-Nurr1 LBD. Nurr1 LBD is therefore needed for the repression. The importance of AF-1 and AF-2 domains was studied on OPN-LUC reporter with Nurr1 AF-1 (Nurr1Δ1-84) and AF-2 (Nurr1D589A) mutants, which lack functional activation functions but are still able to activate OPN-LUC. The Nurr1 AF-1 and AF-2 mutants induced transactivation on the OPN-LUC reporter, which S33Y β-catenin significantly repressed. Therefore AF-1 and AF-2 domains seem to be irrelevant for β-catenin mediated NR4A-repression.

1.2.2 The repressive effect of β-catenin on Nurr1 transactivity is not dependent on direct interaction between the proteins, nor alterations in Nurr1 DNA binding or expression

Kitagawa and colleagues reported that Nurr1 interacts with β-catenin and Lef1 in 293F cells at a site that was mapped to amino acids 363-598 where LBD and AF-2 are situated (Kitagawa *et al.* 2007). In addition, other NRs modulate Wnt signaling pathway by direct interactions and, for example, AR, RAR, VDR, RXR, PPAR, ER, GR and TR interact with β-catenin (Easwaran *et al.* 1999, Guigon *et al.* 2008, Kouzmenko *et al.* 2004, Liu *et al.* 2006, Pálmer *et al.* 2001, Truica *et al.* 2000, Xiao *et al.* 2003). We could not detect an interaction between β-catenin and Nurr1 or NGFI-B in mammalian one- and two-hybrid experiments or in the co-immunoprecipitation experiments in U2-OS cells. In the two-

hybrid experiments VP16-S33A-β-catenin failed to induce the activity of Gal4-Nurr1 LBD, Gal4-Nurr1 NTD or Gal4-NGFI-B LBD expression plasmids on MH100tk-LUC reporter. In one-hybrid experiments full length pCMX-Nurr1 and pCMX-NGFI-B plasmids were transfected in U2-OS cells alone or with VP16-S33A-β-catenin along with the $NBRE_3$tk-LUC reporter. Again, VP16-S33A-β-catenin did not stimulate Nurr1 and NGFI-B activities. In co-immunoprecipitation assays overexpressed Flag-tagged Nurr1 protein was immunoprecipitated from the U2-OS cells but no specific binding between Nurr1 and β-catenin was detected. Co-immunoprecipitation experiments were also conducted by precipitating endogenous or overexpressed β-catenin protein but again no specific binding between β-catenin and Nurr1 was detected. Therefore, we were not able to show that the repressive effect of NR4A family on β-catenin would be dependent on direct interaction between the proteins in U2-OS cells.

We also studied whether β-catenin affects DNA binding of the NR4A receptors. Nurr1 DN and S33Y β-catenin expression plasmids were co-transfected in U2-OS cells. S33Y β-catenin could not abolish the repressive effect of Nurr1 DN on the basal $NBRE_3$tk-LUC reporter activity, which implies that β-catenin does not interfere with the ability of Nurr1 to bind DNA.

Recently, it was shown that the colonic carcinogen deoxycholic acid increases the expression of NGFI-B in colon cancer cells. The mechanism involved stabilization of β-catenin, which formed a complex with the activator protein-1 (AP-1) thas was capable of binding to the AP-1 sites on the *NGFI-B* gene promoter (Wu *et al.* 2011). We did not detect any alteration in the amount of Nurr1 mRNA in U2-OS cells transfected with S33Y β-catenin. Furthermore, the expected result of an increase in protein expression would be its enhanced function rather than the repression observed in our experiments.

In conclusion, we identified two new pathways, NR3B orphan NRs and Wnt signaling pathway, which repress the transcriptional activity of NR4A receptors in osteoblasts. The repressive effect of ERRγ on Nurr1 is dependent on Nurr1 and ERRγ DBDs, but does not involve competition for DNA binding. The repression also involves intact dimerization interfaces but no heterodimerization or competition for co-activator binding. β-catenin repressed transcriptional activation mediated by all of the NR4A receptors by a mechanism dependent on the LBD. As the Wnt signaling pathway has an activating effect on Nurr1 transcriptional activity in 293F (Kitagawa *et al.* 2007) and 293T

cells, it seems that the Wnt signaling pathway can function both as an activator and a repressor of Nurr1 transcriptional activity according to the prevailing cellular circumstances and therefore forms an interesting regulatory mechanism for Nurr1. This is also true for other NRs. For most of the NRs, including AR, RARs, VDR and ERs, Wnt/β-catenin signaling functions as a stimulator of their transcriptional activity (Mulholland *et al.* 2005). However, AR can also be inhibited by the Wnt signaling pathway as AR binds Tcf4 through its DNA-binding domain, which decreases its transcriptional activity (Amir *et al.* 2003). Wnt signaling represses or activates ER activity according to the dominating cellular Tcf factors. When ERs directly interact with Tcf1, the effect on the promoter is synergistic, when ERs interact with Tcf4, the effect is antagonistic (El-Tanani *et al.* 2001). The exact mechanisms of both ERRγ and β-catenin repression on NR4A receptors still remain to be solved. The biological significance of the repression should also be further studied in natural NR4A target genes. Both ERRγ and β-catenin are highly expressed in the central nervous system and Wnt/β-catenin has been shown to regulate dopaminergic differentiation (Cajánek *et al.* 2009) where Nurr1 also has an essential role. Kitagawa and others showed that Nurr1, β-catenin and Lef1 can associate on the same DNA elements and regulate each other's co-factor recruitment in SK-N-MC neuroblastoma cells. In addition, Nurr1 inhibited the nuclear accumulation of β-catenin in these cells (Kitagawa *et al.* 2007). Therefore, it is of potential interest to study further how these pathways converge in neural tissue.

2. The transcriptional activity of NR3B family can be regulated by ligands and cross-talk with other nuclear receptors (I, II)

2.1. Identification of the phytoestrogen equol as an ERRγ and ERRβ ligand

NR3B receptors constitute another family of orphan NRs for which a natural ligand has not been found. However, it was shown that certain isoflavones can bind to ERRs and act as their agonists (Suetsugi *et al.* 2003). Furthermore, phenolic acyl hydrazones GSK4716 and DY131 function as selective ERRβ and ERRγ agonists (Yu and Forman 2005, Zuercher *et al.* 2005). Certain flavonoids were reported to inhibit transcriptional activity of ERRγ by suppressing the interaction between ERRγ and co-activator PGC-1α (Huang *et al.* 2010, Wang *et al.* 2009b). Therefore, we wanted to study how phytoestrogens affect

the transcriptional activity of ERRγ. The subject was studied in U2-OS, SaOS-2 and PC-3 cells, where ERRγ is transcriptionally active (Huppunen *et al.* 2004 and our unpublished results). In accordance with earlier reports, DES and 4-OHT efficiently inhibited the transcriptional activity of ERRγ in PC-3 cells (Coward *et al.* 2001, Tremblay *et al.* 2001b) and GSK4716 increased the activity by a maximum of 3-fold. Estradiol had no effect on the transcriptional activity of ERRγ as previously reported (Giguère *et al.* 1988, Yang *et al.* 1996).

PC-3, U2-OS and SaOS-2 cells were transfected with Gal4-ERRγLBD expression vector along with MH100tk-LUC reporter and treated with genistein, daidzein, equol, enterodiol, and enterolactone to study the effects of phytoestrogens on ERRγ. The only substance that influenced the activity of ERRγ was equol, which had agonistic potential. Equol increased the transcriptional activity of ERRγ in a dose-dependent manner in PC-3 cells transfected with the $ERRE_3$tk-LUC reporter driven by three ERRE binding sites. The maximum stimulation was approximately 2-fold at the concentration of 20 µM.

The effect of equol on ERRγ seemed to be selective as equol did not increase the activity of ERRα or ERRβ above the level of control vector (PL1) in PC-3 or SaOS-2 cells. Equol increased the basal activity of the $ERRE_3$tk-LUC reporter, which is most likely due to the endogenous ERRγ present in PC-3 and SaOS-2 cells. ERRα and ERRβ showed no transcriptional activity in PC-3 or SaOS-2 cells and equol might have a different effect in a situation in which these receptors would be more active. Therefore the ERR expression vectors were transfected in PC-3 cells together with PGC-1α that has been shown to stimulate the activity of these receptors (Huss *et al.* 2002). Equol stimulated the activity induced by ERRβ and ERRγ together with PGC-1α. Equol was not able to significantly increase the activity induced by ERRα and PGC-1α. These results suggest that equol is selective for ERRγ and ERRβ as has been reported for GSK4716 (Zuercher *et al.* 2005).

2.1.1 Equol increases ERRγ co-activator binding

Some phytoestrogens have been shown to inhibit the activity induced by ERRs and PGC-1α by decreasing the interaction between the proteins. The phytoestrogens that have this effect fall into three categories, flavones, flavonols and flavanones. On the other hand, isoflavones genistein and daidzein have either no effect at all or a small stimulating effect

on the transcriptional activity of ERRγ and PGC-1α (Huang *et al.* 2010). Equol falls into the category of isoflavone, which is in accordance with its ability to increase the transcriptional activity of ERRβ/ERRγ and PGC-1α. ERRs have also been shown to bind other co-factors. For example, ERRγ has been reported to interact with GRIP1 (Hong *et al.* 1999), which functions as a co-activator. Moreover, ERRγ interacts with RIP140, which in many cases functions as a transrepressor (Castet *et al.* 2006, Wang *et al.* 2006a). A mammalian two hybrid experiment was performed to study whether equol enhances the interaction between ERRγLBD and GRIP1 or RIP140. PC-3 cells were co-transfected with the expression vectors for Gal4-GRIP1 or Gal4-RIP140 and VP16-ERRγ LBD along with the MH100tk-Luc reporter. Equol enhanced the interaction between ERRγLBD and GRIP1 but had no effect on the interaction between ERRγLBD and RIP140. Therefore, equol was found to increase the transcriptional activity of ERRγ at least partly by increasing the co-activator binding of ERRγ. On the other hand, the effect of equol was not due to an effect on ERRγ protein stability as equol and GSK4716 did not influence the expression level of ERRγ protein in PC-3 cells. In contrast, 4-OHT treatment slightly increased ERRγ expression.

2.1.2 Equol binds to the ERRγ ligand-binding pocket

Next we studied the binding of equol to the ERRγ LBP. Equol decreased the inhibitory effect of 4-OHT on the transcriptional activity of ERRγ in a dose-dependent manner. On the other hand, 4-OHT decreased the stimulatory effect of equol. These results suggest that equol and 4-OHT compete for binding to the LBP. Equol also induced a conformational change in the ERRγ LBD in an assembly assay described by Pissios *et al.* (2000), where helix 1 of the LBD is fused to the yeast Gal4 DNA-binding domain and helices 3-12 are fused to herpes simplex virus VP16 activation domain. The interaction of H1 and H3-12 is measured as the activation of a MH100tk-LUC reporter gene driven by Gal4-binding sites. This interaction has been reported to be influenced by ligand binding, co-repressors, and other signals that modulate the transcriptional activity of LBD (Huppunen *et al.* 2004, Pissios *et al.* 2000, Wang *et al.* 2003). Equol, GSK4716, DES and 4-OHT increased the interaction thus indicating that these ligands induce a conformational change in the ERRγ LBD. In contrast, estradiol and enterodiol had no effect. In partial proteolysis assay, *in vitro* translated ERRγ or ERRγ LBD proteins were treated with

vehicle, equol, enterodiol and 4-OHT and then with trypsin. Trypsin digested vehicle or enterodiol treated proteins completely whereas equol and 4-OHT treated proteins were able to resist digestion. The binding mode of equol to the ERRγ LBP was studied by molecular modeling and compared to the models in which ERRγ was bound to 4-OHT, DES and GSK4716. The *apo*-ERRγ structure could not adopt the equol molecule. Nevertheless, GSK4716 binds to an enlarged cavity at which H12 is in its agonistic conformation. This structure allowed equol binding which correlates well with the agonistic effects of equol observed in experiments. The binding mode was further studied by introducing point mutations in the LBP of ERRγ and by studying the effects of different ligands on these mutants in reporter assays. PC-3 cells were transfected with Gal4-ERRγ LBD expression vector and its mutated variants along with the MH100tk-LUC reporter and subsequently treated with equol, GSK4716, 4-OHT and DES. A272, which is in close proximity with all four ligands, was mutated into a larger phenylalanine with the intention to block the cavity. Indeed, Gal4-ERRγ LBD A272F did not significantly respond to any of the tested ligands. Next F435 was mutated to a smaller leucine to create more space in the binding pocket. Mutating F435 did not affect the inhibitory effect of 4-OHT but changed DES into a weak agonist. This finding is in accordance with an earlier study in which the antagonistic effect of DES on ERRγ was shown to involve a change in the conformation of F435, which then leads to a displacement of H12 (Greschik *et al.* 2002). Equol and GSK4716 remained as agonists and the stimulatory effect of equol was slightly enhanced by the F435L mutation. V313W and Y326W mutations abolished the inhibitory effects of DES and 4-OHT on ERRγ and decreased the agonistic effect of GSK4716. However, equol remained as an agonist which suggests that equol binds to the ERRγ LBP in a slightly different manner than the other ligands studied.

Based on these experiments we identified equol as a new ERRγ ligand. More than that, we showed that in addition to the previously identified synthetic ligands GSK4716 and DY131, there are natural compounds that can function as ERRγ agonists. The equol concentration that was needed to stimulate ERRγ activity was fairly high (5 µM). However, plasma concentrations of phytoestrogens in soya-consuming populations can reach levels as high as 1 µM (Adlercreutz *et al.* 1993, Bloedon *et al.* 2002). Furthermore, phytoestrogens have been shown to concentrate in prostate fluid and tissue where they can reach concentrations up to 10-fold those of serum (Gardner *et al.* 2009,

Hedlund *et al.* 2005). In addition, modulation of the transcriptional activity of NRs in transient transactivation assays often requires ligand concentrations that exceed those required *in vivo*. Therefore, the concentrations needed to stimulate ERRγ in our experiments may well be possible to attain in humans. What should be further studied is the effect of equol on ERRγ target genes. We analyzed the mRNA expression of previously reported ERRγ target genes (*ESRRA*, *PGC-1A*, *PGC-1B*, *p21*, *p27*, *PDK2*, *PDK4*, *GR*, *PLK2*, *MAOB*, and *APOD*) (Park *et al.* 2007, Wang *et al.* 2008, Wang *et al.* 2009b, Wang *et al.* 2010a, Wang *et al.* 2010b, Xie *et al.* 2009, Yu *et al.* 2007, Zhang *et al.* 2006) in GSK4716 or ERRγ siRNA treated PC-3 cells by RT-PCR. None of these genes were induced by GSK4716 or repressed by ERRγ siRNA. We were thus unable to study the role of equol as an ERRγ agonist by analyzing its effect on these previously suggested ERRγ target genes.

2.2 Orphan nuclear receptor NGFI-B represses the transcriptional activity of ERRγ

In addition to ligands, ERR receptors can also be regulated by other NRs. For example, ERs have been shown to inhibit the transcriptional activity of ERRs on monoamine oxidase B promoter (Zhang *et al.* 2006). SHP interacts with ERR receptors and inhibits the transcriptional activity of ERRγ (Sanyal *et al.* 2002). As already discussed, NR3B and NR4A receptors are co-expressed in many tissues and therefore we studied if NR4A receptors can regulate ERR function. HeLa cells were transfected with the expression vector for ERRγ along with the $ERRE_3$tk-LUC reporter. Co-expression of NGFI-B efficiently repressed the activity induced by ERRγ. Nor1 had a modest repressive effect on ERRγ whereas Nurr1 had no effect. The repressive effect of NGFI-B was dose-dependent and NGFI-B totally abolished the activity of ERRγ when NGFI-B and ERRγ were transfected in equal amounts. The repressive effect of NGFI-B was dependent on the DBD, as NGFI-B DBD mutant C252G was not able to repress ERRγ. The repression also involved dimerization interfaces as NGFI-B I-box mutant NGFI-B GKL(522-524)AAA repressed ERRγ less efficiently than the wild-type vector. However, the exact mechanism and the biological relevance of this phenomenon remains to be solved

In conclusion, we identified two new mechanisms capable of regulating the function of ERRγ in osteoblasts. Of these, equol had a positive effect on the transcriptional activity of ERRγ whereas NR4A receptors had a negative effect.

3. NR4A and NR3B families repress the transcriptional activity of Wnt signaling pathway in osteoblasts (III)

3.1 NR4A receptors repress the transcriptional activity mediated by β-catenin

Both β-catenin and the NR4A family are expressed in osteoblasts (Lammi *et al.* 2004, Monaghan *et al.* 2001). β-catenin is essential for skeletal development and maintenance and thus it is important to know which factors regulate the function of the Wnt signaling pathway.

To study if NR4A receptors regulate β-catenin mediated transcriptional activity, U2-OS cells were transfected with pGL3-OT reporter that contains three binding sites for Tcf4, a constitutively active S33Y β-catenin and either Nurr1, NGFI-B or Nor1 expression vectors. S33Y β-catenin induced a very high activation on the pGL3-OT reporter that all members of the NR4A family significantly repressed. LiCl is a known inhibitor of GSK3β (Klein and Melton 1996, Stambolic *et al.* 1996) and it increases the amount of active nuclear β-catenin. 10 mM LiCl treatment was able to induce pGL3-OT reporter and Nurr1 also repressed this induction. The effect of Nurr1 on S33Y β-catenin induced pGL3-OT activity was dose-dependent.

Nurr1 and NGFI-B mutants were used to study the structural requirements of the NR4A receptors needed for β-catenin repression. Nurr1Δ1-84 that lacks AF-1 (AF1 mut), Nurr1 D589A that lacks a functional AF-2 (AF2 mut), and a Nurr1 mutant Δ1-84/D589A in which the function of both activation functions is abolished (AF1/AF2 mut) were transfected to U2-OS cells together with S33Y β-catenin and pGL3-OT reporter. All the mutants were able to repress the S33Y β-catenin induced pGL3-OT activity at the same level as the Nurr1 wild-type. Therefore, AF-1 and AF-2 domains are not needed for the repression of β-catenin. The NGFI-B I-box mutant NGFI-B GKL(522-524)AAA was expressed in U2-OS cells to study if dimerization is involved in the repression. NGFI-B dimerization mutant was still capable of repressing the S33Y β-catenin induced pGL3-OT activation. Therefore homo- or heterodimerization of the NR4A receptors with themselves or with, for example, RXR receptors, does not influence the repressive effect of NR4A receptors on β-catenin. This is in agreement with the notion that Nor1, which does not bind RXR (Zetterström *et al.* 1996a) is also capable of repressing β-catenin. The role of DBD was studied by a NGFI-B DBD mutant C252G. Interestingly, NGFI-B C252G could

not repress the β-catenin mediated pGL3-OT activation, which implies that an intact DNA-binding domain is needed for the repression. Similar results were obtained with Nurr1 DNA-binding mutant C283G.

To investigate if the repression also occurs on the level of β-catenin target genes, the expression of Axin2 mRNA was studied. Axin2 is a negative regulator of the Wnt signaling pathway, as it promotes the phosphorylation and degradation of β-catenin, and a known Wnt/β-catenin/Tcf target gene (Jho *et al.* 2002). The expression of Axin2 is stimulated by canonical Wnt signaling in many different cell types, including the preosteoblastic MC3T3-E1 cell line (Reinhold and Naski 2007). We noted an increase in Axin2 mRNA in S33Y β-catenin transfected MC3T3-E1 cells. When the cells were co-transfected with Nurr1, the expression of Axin2 mRNA decreased by 40%. Therefore Nurr1 is capable of repressing the mRNA expression of β-catenin target genes.

NR4A receptors are immediate early genes whose expression is strongly and rapidly induced by various stimuli that include PTH (Pirih *et al.* 2003, Tetradis *et al.* 2001a, Tetradis *et al.* 2001b) and FGF-8b (Lammi and Aarnisalo 2008). PTH has also been reported to modulate the canonical Wnt signaling pathway (Kulkarni *et al.* 2005). Moreover, FGFs and the Wnt pathway intertwine as FGF signaling antagonizes Wnt-induced transcription (Ambrosetti *et al.* 2008). NR4A receptors thus potentially serve as a link between these signaling pathways in osteoblasts. Wnt signaling is also an important determinant of the osteoblastic and adipogenic differentiation of MSCs (Hill *et al.* 2005, Ross *et al.* 2000). The NR4A family has also been reported to be induced during adipogenic differentiation (Au *et al.* 2008, Chao *et al.* 2008, Fu *et al.* 2005, Fumoto *et al.* 2007) and Nurr1 increases osteoblast differentiation (Lee *et al.* 2006). Therefore, it would be interesting to study if the pathways are linked in these processes.

3.2 NR3B receptors repress the transcriptional activity mediated by β-catenin

In addition to NR4A receptors, NR3B receptors are also expressed in several of the same tissues and cells that express β-catenin. Therefore we studied whether NR3B receptors influence Wnt signaling pathway. U2-OS cells were transfected with expression plasmids for S33Y β-catenin, ERRα and ERRγ along with the pGL3-OT reporter plasmid. ERRγ inhibited S33Y β-catenin mediated activity efficiently, whereas the inhibition elicited by ERRα was more modest (Fig. 13A). The ERRγ DBD mutant ERRγ C125G was used to

study if the DNA binding was needed for the repression. ERRγ C125G was not able to repress S33Y β-catenin mediated transactivation (Fig. 13B). The result is similar to that obtained with NR4A receptors, which implies that orphan NRs could use the same mechanisms to repress β-catenin. As already discussed, PGC-1α is an important co-activator of the ERR receptors. When U2-OS cells were transfected with PGC-1α along with the expression vector for S33Y β-catenin and pGL3-OT reporter, PGC-1α had a repressive effect on S33Y β-catenin mediated activity. PGC-1α was also able to increase the repressive effect of ERRγ (Fig. 13C). On the other hand, the repressive effect of ERRγ on β-catenin was abolished by 4-OHT treatment (Fig. 13D). This finding and also the

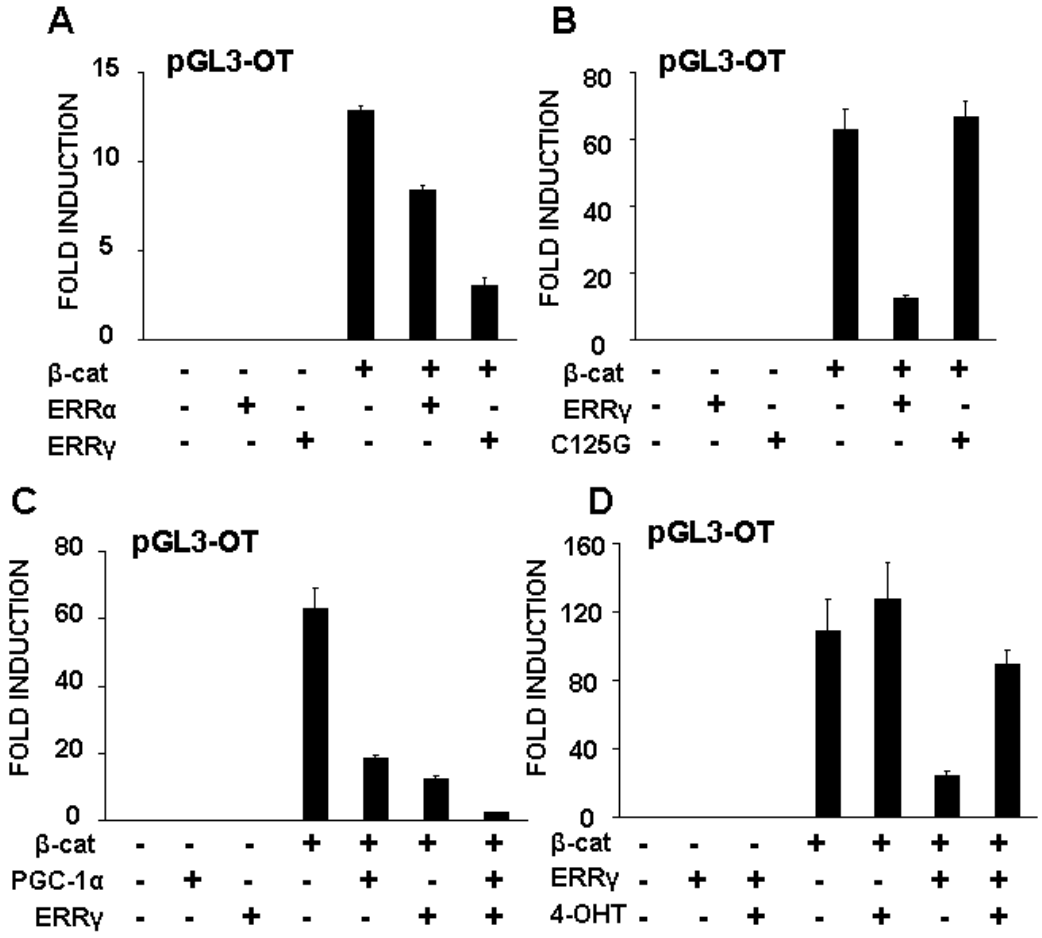

Fig. 13. U2-OS cells were transfected with the pGL3-OT reporter (300 ng) along with the expression plasmids (a total of 50 ng) for β-catenin S33Y, ERRα and ERRγ (A); β-catenin S33Y, ERRγ and ERRγ C125G (B); or β-catenin S33Y, PGC-1α and ERRγ (C) as indicated. (D) U2-OS cells were transfected with the pGL3-OT reporter along with the expression plasmids for β-catenin S33Y and ERRγ and subsequently treated with 1 μM 4-OHT for 24 h as indicated. The experiments were performed in triplicate dishes and repeated at least twice with essentially identical results. The mean ± S.D. of one representative experiment is shown.

dependence of the repressive effect on DBD are in accordance with a mechanism by which ERRγ represses the transcriptional activity of Nurr1 (Lammi *et al.* 2004). Therefore, NR3B receptors could have a repressive mechanism dependent on their DBD and LBD that they use to repress NR4A receptors, Wnt signaling and possibly also other signaling pathways.

Very recently, a study that reported a cross-talk between ERRα and Wnt signaling pathway was published. Wnt signaling pathway and ERRα potentiated each other's transcriptional activity in SKBR3 and MDA-MB-436 breast cancer cells. In addition, both ERRα and β-catenin were involved in the migration of breast cancer cells by inducing the expression of Wnt11 (Dwyer *et al.* 2010). In U2-OS cells, ERRα and ERRγ had an inhibitory effect on Wnt/β-catenin signaling pathway. Therefore, ERRs seem to have both negative and positive effects on Wnt signaling depending on the cell type.

In conclusion, NR4A and NR3B receptors were discovered to repress the function of the Wnt signaling pathway in osteoblasts. As Wnt signaling pathway has an important role in many biological processes and tissues in which NR4A and NR3B receptors also function, this repressive effect is of potential importance. In addition, by regulating the Wnt signaling pathway, NR3B and NR4A receptors may be involved in the pathogenesis of different Wnt signaling pathway related diseases. The biological relevance and mechanisms of the regulation should be further examined by, for example, exploring the repression on β-catenin/Tcf target gene promoters and by studying if the function of Wnt signaling pathway and the processes it regulates are altered in NR3B and NR4A KO mice.

4. NR3B family participates in the growth of osteoblasts and prostate cancer cells and in the differentiation of mesenchymal stem cells (II, IV)

4.1. NR3B receptors regulate the growth of osteoblasts and prostate cancer cells

NR3B receptors have been shown to regulate the proliferation and growth of cells with both positive and negative effects in different cell types (Ijichi et al. 2011, Yu et al. 2008). Therefore, we studied the effect of NR3B receptors on the proliferation and growth of osteoblasts, MSCs and prostate cancer cells.

4.1.1 ERRα increases the proliferation of osteoblasts and mesenchymal stem cells

Blocking the expression of ERRα diminishes the proliferation of rat calvarial osteoblasts dose-dependently (Bonnelye *et al.* 2001). To investigate further the impact of ERRα on proliferation, we studied the proliferation of osteoblasts and MSCs isolated from ERRα KO and wild-type mice by the ^{3}H-thymidine incorporation method on days 3 and 5 of the proliferation phase. ERRα KO MSCs proliferated significantly less than the cells derived from wild-type littermates on both days. ERRα KO osteoblasts also proliferated less but the results did not reach statistical significance. The results were ascertained by cell cycle analysis, which showed that on day 5 there were 14% fewer MSCs and 45% fewer osteoblasts in the S-phase in the ERRα KO cultures. Our results are consistent with the earlier results from rat calvarial osteoblasts and the MSCs derived from another ERRα KO line (Bonnelye *et al.* 2001, Teyssier *et al.* 2009). In contrast, Delhon and colleagues did not detect any difference in the proliferation of ERRα deficient mouse calvarial osteoblasts (Delhon *et al.* 2009). ERRα has also been linked to the regulation of tumor growth and proliferation of breast cancer cells (Deblois *et al.* 2009). The function of ERRα in cell proliferation in different cell types should therefore be further studied.

4.1.2 ERRγ and equol decrease the growth of prostate cancer cells

ERRγ regulates the proliferation of cancer cells in, *inter alia*, breast (Ijichi *et al.* 2011) and prostate cancer (Cheung *et al.* 2005). In prostate cancer cells ERRγ has a negative effect on their proliferation (Cheung *et al.* 2005). Equol has also been shown to decrease the growth of prostate cancer cells *in vitro* (Magee *et al.* 2006, Mitchell *et al.* 2000). Therefore, we examined whether equol was able to enhance further the growth inhibitory effect of ERRγ. PC-3 cells were transfected with the expression vector for ERRγ and treated with equol. Treatment of the mock-transfected cells with equol reduced the cell number 72 h post transfection. The number of cells was further decreased when the cells were transfected with ERRγ. We transfected the cells with siRNA targeted for ERRγ to study if the growth-inhibitory effect of equol in mock-transfected cells was mediated by the endogenous ERRγ. ERRγ siRNA reduced the expression of endogenous ERRγ mRNA to about 50% and abolished the growth-inhibitory effect of equol. GSK4716 also reduced the number of ERRγ transfected PC-3 cells. These results indicate that ERRγ is involved

in mediating the antiproliferative effects of equol. Reciprocally, equol enhances the antiproliferative effects of ERRγ. The ability to produce equol and high serum equol levels have been associated with a reduced prostate cancer risk (Yuan *et al.* 2007) and ERRγ has been indicated as a marker of favourable prognosis in prostate cancer (Fujimura *et al.* 2010). Therefore, the function of ERRγ and equol in prostate cancer cells is potentially of clinical importance and should be further studied by exploring the mechanisms behind the growth inhibition and by investigating if the effect is also obtained *in vivo*. Furthermore, it would be interesting to study if ERRγ also participates in mediating equol effects in ERRγ expressing cells and tissues other than the prostate.

4.2 ERRα regulates the osteoblastic and adipogenic differentiation of mesenchymal stem cells

It has been suggested that ERRα plays a role in bone formation. ERRα has been shown to increase osteoblastic differentiation *in vitro* in rat calvarial osteoblasts (Bonnelye *et al.* 2001). Recently, conflicting results have been obtained. ERRα KO mice were shown to have slightly increased cancellous BMD and primary osteoblasts isolated from these mice showed increased differentiation *in vitro* (Delhon *et al.* 2009). Therefore the effects of ERRα on osteoblastic differentiation and function need more clarification. Osteoblasts and adipocytes differentiate from the same multipotent precursor cells, MSCs. On this account, we also studied the influence of ERRα on adipogenic differentiation.

4.2.1 ERRα increases the osteoblastic differentiation of mesenchymal stem cells

MSCs were isolated from the bone marrow of 8-12 week old ERRα KO male mice and their wild-type littermates. After 6 days in culture the cells were counted, equal numbers were replated and osteogenic differentiation was induced by Na-β-glycerophosphate, dexamethasone and ascorbic acid. ERRα KO mesenchymal cultures had decreased mineralization compared to the wild-type cells on day 20 of differentiation when detected by Alizarin red S staining. The expression of osteoblastic maker genes was determined on days 20 and 25 by RT-PCR to analyze the possible mechanism of ERRα in osteoblast differentiation. The ERRα KO cultures had significantly diminished expression of BSP and OCN mRNA, which might well explain the difference seen in mineralization. The

expression of OPN and ALP was also decreased in every experiment performed but these differences were not statistically significant. The expression of Runx2 was relatively similar in both wild-type and KO cultures. We also studied ERRα mRNA expression and found it to be fairly constant throughout the differentiation process (Rajalin *et al.* unpublished results).

To study how the overexpression of ERRα affects mineralization in preosteoblastic MC3T3-E1 cell line, we transiently transfected cells with empty (pCMX-Flag) or ERRα (pCMX-Flag-ERRα) expression vectors. The overexpression of ERRα increased the mineralization of MC3T3-E1-cells as determined by Alizarin red S staining. Overexpression of ERRα also increased the expression of BSP, Runx2 and ALP mRNAs. The expressions of OPN and OCN were not significantly altered.

The disparity between our results and the results reported by Delhon *et al.* (2009) may be due to the different mouse models, cells used in the experiments and also other differences between the experimental conditions. Delhon and colleagues differentiated mouse calvarial cells in contrast to our MSCs and used BMP-2 in the osteogenic medium. The expression of osteoblastic markers BSP and OCN was also analyzed at a rather early time point (10 d) by Delhon and colleagues compared to our study (20d and 25d). In the study by Teyssier and others MSCs isolated from the ERRα KO female mice had increased osteoblastic differentiation capacity when compared to wild-type cells (Teyssier *et al.* 2009). Again, there were many experimental differences between our studies. Teyssier and others did not use dexamethasone in the osteogenic medium and plated cells isolated from the bone marrow directly in differentiation cultures, whereas we started our differentiation cultures only after expanding MSCs first in an initiation culture. Cell density is a major determinant of the mineralization capacity (Jaiswal *et al.* 1997). Therefore, alterations in factors that affect cell density, such as the number of MSCs in the bone marrow and cell adherence, can affect mineralization in the experimental setting of Teyssier *et al* (2009). Indeed, Wei *et al.* (2010) reported a decrease in the number of bone marrow cells in ERRα KO mice compared to ERRα heterozygous controls. There is also a potential gender-dependent effect of ERRα, which might affect the results. As ERRα has been reported to impinge on the estrogen signaling pathway (Bonnelye *et al.* 2002b), the gender-dependent effects of ERRα are of interest. In addition, the *in vitro* results reported by Teyssier and colleagues do not explain the decreased bone

volume fraction and diminished trabecular number observed in their female mice at the age of 14 weeks (Teyssier *et al.* 2009).

4.2.2 Bone sialoprotein is a target gene for ERRα and ERRγ

As the expression of BSP mRNA was altered in ERRα KO MSCs and also ERRα overexpressing MC3T3-E1 cultures, we next investigated whether the NR3B family can directly control the *BSP* gene promoter. Because MC3T3-E1 cells did not transfect efficiently enough for reporter assays, we decided to use human cervical cancer HeLa cells in which the ERR family is transcriptionally active (Lu *et al.* 2001). HeLa cells were co-transfected with the expression plasmids for ERRα, ERRβ or ERRγ and PGC-1α when indicated along with the human BSP-LUC reporter. ERRα and ERRγ together with PGC-1α activated BSP-LUC reporter efficiently. ERRβ could not activate BSP-LUC reporter. To study the regulation of *BSP* promoter by ERRα further, point mutations were introduced into the ERRα expression vector in order to abolish ERRα DNA-binding (C99G) or to disrupt AF-2 (E413A). Although the expression level of both mutants was comparable to that of the wild-type ERRα, both mutations abolished the transactivation of BSP-LUC. Therefore, the regulation of *BSP* seems to depend upon ERRα DNA-binding and transcriptional activity. The reporter assays together with the mRNA expression analysis show that *BSP* is a target gene for ERRα. In a study by Bonnelye and colleagues the expression of BSP was inhibited in cultures deficient of ERRα and increased in cultures that overexpressed ERRα (Bonnelye *et al.* 2001). In addition to ERRα, ERRγ stimulated BSP-LUC activity when co-transfected with the co-activator PGC-1α. Interestingly, the activation of the BSP-LUC reporter by ERRγ and PGC-1α was not responsive to 1 µM 4-OHT treatment (Fig. 14A) whereas 4-OHT was able to abolish ERRγ driven transactivity on $ERRE_3$tk-luc reporter (Fig. 14B). In contrast to our results, ERRγ was shown to repress Runx2-dependent *BSP* promoter activation in a recent report (Jeong *et al.* 2009). Therefore, ERRs may play different roles in *BSP* regulation depending on the stage of osteoblastic differentiation. BSP is also expressed in breast and prostate cancer cells in which it possibly plays a major role in mineral deposition and preferred bone homing (Ganss *et al.* 1999). ERRα and ERRγ are expressed in many cancer cell lines and ERRα functions as a prognostic marker of breast and endometrial cancer (Ariazi *et al.*

2002, Deblois *et al.* 2009). Therefore the relationship between ERRs and BSP should be further studied in cancer cells.

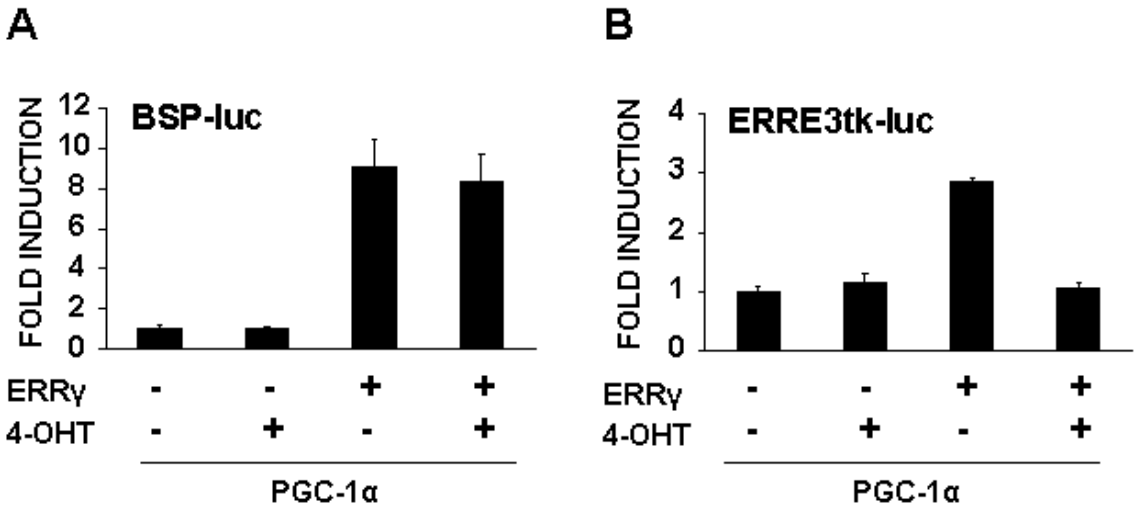

Fig. 14. HeLa cells were transfected with 300 ng of the BSP-LUC (A) or ERRE$_3$tk-luc (B) reporter along with the expression plasmids for PGC-1α (25 ng) and pCMX-ERRγ (25 ng) as indicated. After 24 h the cells were treated with vehicle (EtOH) or 1 µM 4-OHT for another 24 h. The experiments were performed in triplicate dishes and repeated three times with essentially identical results. The mean ± S.D. of one representative experiment is shown.

4.2.3 ERRα increases the adipogenic differentiation of mesenchymal stem cells

To study whether ERRα can affect adipogenic differentiation of MSCs, we induced wild-type and ERRα KO MSCs towards the adipocytic differentiation with a cocktail that contained insulin, dexamethasone and rosiglitazone. After 14 days we stained the cultures with Oil Red O to detect lipids. ERRα KO cultures had diminished numbers of adipocytes compared to the wild-type cultures. In addition, the mRNA expression of adipogenic markers aP2 and PPARγ showed a statistically significant decrease in the ERRα KO cultures on day 7. Therefore, according to our results ERRα has a positive effect on adipogenic differentiation. In line with our result ERRα siRNA was reported to inhibit adipogenesis and overexpression of ERRα by stable transfection up-regulated adipogenic marker genes and promoted triglyceride accumulation during the adipogenic differentiation in 3T3-L1 cells (Ijichi *et al.* 2007). Recently, it was also found that an inverse agonist of ERRα, XCT-790, reduced the expression of PPARγ and aP2, lowered triglyceride content and decreased the size of lipid droplets in a dose-dependent manner in 3T3-L1 cells (Nie and Wong 2009). Silencing ERRα in human MSCs has been shown to

result in decreased adipogenic differentiation and adipocytic marker gene expression (Delhon *et al.* 2009). ERRα KO mice also have reduced fat mass and they are resistant to high-fat diet-induced obesity due to derangements in white adipocyte lipid metabolism (Luo *et al.* 2003) and in lipid absorption from the intestine (Carrier *et al.* 2004). In contrast, silencing ERRα by the antisense method increased the differentiation of rat calvarial osteoblasts into adipocytes (Bonnelye *et al.* 2002b). This potentially stems from the use of cells that are more committed to the osteoblastic lineage.

In conclusion, ERRα controls the differentiation of mouse MSCs by increasing both the adipogenic and osteoblastic differentiation. There are also opposing results about the role of ERRα in osteoblastogenesis, which could indicate that ERRα has cell type-, differentiation stage- and gender-dependent effects that need more clarification. Moreover, the identification of *BSP* as a new target gene for ERRα and ERRγ suggests a new mechanism for their bone related effects. ERRα could thus function in normal bone homeostasis and participate in the pathology of bone related diseases, such as osteoporosis by regulating osteoblast proliferation and differentiation.

CONCLUSIONS

- The activity of NR4A receptors is regulated by NR3B receptors and Wnt signaling pathway in osteoblasts. ERRα, ERRγ and β-catenin repress the transcriptional activity of NR4A receptors in U2-OS cells.

- The phytoestrogen equol was identified as a new agonist for ERRγ in PC-3, U2-OS and SaOS-2 cells. ERRγ increases the growth inhibitory effect of equol on PC-3 cells and could therefore mediate some of the beneficial health effects of equol.

- The activity of NR3B receptor ERRγ is regulated by NR4A receptors as NGFI-B and Nor1 repress its transcriptional activity in HeLa cells.

- NR3B and NR4A receptors regulate the canonical Wnt signaling pathway as they repress the transcriptional activity mediated by β-catenin in U2-OS cells.

- ERRα increases the proliferation of mesenchymal stem cells and osteoblasts. ERRα enhances osteoblastic differentiation and increases the expression of bone sialoprotein in mouse mesenchymal stem cells and MC3T3-E1 preosteoblastic cell line. Bone sialoprotein was identified as a direct target gene for ERRα and ERRγ. ERRα also increases adipogenic differentiation of mesenchymal stem cells

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References

Aarnisalo P, Kim CH, Lee JW, Perlmann T (2002) Defining requirements for heterodimerization between the retinoid X receptor and the orphan nuclear receptor Nurr1. J Biol Chem 277:35118-35123

Adlercreutz H, Markkanen H, Watanabe S (1993) Plasma concentrations of phyto-oestrogens in Japanese men. Lancet 342:1209-1210.

Ahdjoudj S, Lasmoles F, Holy X, Zerath E, Marie PJ (2002) Transforming growth factor β2 inhibits adipocyte differentiation induced by skeletal unloading in rat bone marrow stroma. J Bone Miner Res 17:668-677

Alaynick WA, Kondo RP, Xie W, He W, Dufour CR, Downes M, Jonker JW, Giles W, Naviaux RK, Giguère V, Evans RM (2007) ERRγ directs and maintains the transition to oxidative metabolism in the postnatal heart. Cell Metab 6:13-24

Alaynick WA, Way JM, Wilson SA, Benson WG, Pei L, Downes M, Yu R, Jonker JW, Holt JA, Rajpal DK, Li H, Stuart J, McPherson R, Remlinger KS, Chang CY, McDonnell DP, Evans RM, Billin AN (2010) ERRγ regulates cardiac, gastric, and renal potassium homeostasis. Mol Endocrinol 24:299-309

Albers M, Kranz H, Kober I, Kaiser C, Klink M, Suckow J, Kern R, Koegl M (2005) Automated yeast two-hybrid screening for nuclear receptor-interacting proteins. Mol Cell Proteomics 4:205-213

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1994) Molecular Biology of the cell, 3rd edition, General Principles of Cell Signaling, pp721-734, Garland Publishing, New York

Almeida M, Han L, Martin-Millan M, Plotkin LI, Stewart SA, Roberson PK, Kousteni S, O'Brien CA, Bellido T, Parfitt AM, Weinstein RS, Jilka RL, Manolagas SC (2007) Skeletal involution by age-associated oxidative stress and its acceleration by loss of sex steroids. J Biol Chem 282:27285-27297

Ambrosetti D, Holmes G, Mansukhani A, Basilico C (2008) Fibroblast growth factor signaling uses multiple mechanisms to inhibit Wnt-induced transcription in osteoblasts. Mol Cell Biol 28:4759-4771

Amir AL, Barua M, McKnight NC, Cheng S, Yuan X, Balk SP (2003) A direct β-catenin-independent interaction between androgen receptor and T cell factor 4. J Biol Chem 278:30828-30834

Amling M, Priemel M, Holzmann T, Chapin K, Rueger JM, Baron R, Demay MB (1999) Rescue of the skeletal phenotype of vitamin D receptor-ablated mice in the setting of normal mineral ion homeostasis: formal histomorphometric and biomechanical analyses. Endocrinology 140:4982-4987

Anderson HC (2003) Matrix vesicles and calcification. Curr Rheumatol Rep 5:222-226

Anderson PH, Atkins GJ (2008) The skeleton as an intracrine organ for vitamin D metabolism. Mol Aspects Med 29:397-406

Angers S, Moon RT (2009) Proximal events in Wnt signal transduction. Nat Rev Mol Cell Biol 10:468-477

Aranda A, Pascual A (2001) Nuclear hormone receptors and gene expression. Physiol Rev 81:1269-1304

Ariazi EA, Clark GM, Mertz JE (2002) Estrogen-related receptor α and estrogen-related receptor γ associate with unfavorable and favorable biomarkers, respectively, in human breast cancer. Cancer Res 62:6510-6518

Ariazi EA, Jordan VC (2006) Estrogen-related receptors as emerging targets in cancer and metabolic disorders. Curr Top Med Chem 6:203-215

Arkenbout EK, de Waard V, van Bragt M, van Achterberg TA, Grimbergen JM, Pichon B, Pannekoek H, de Vries CJ (2002) Protective function of transcription factor TR3 orphan receptor in atherogenesis: decreased lesion formation in carotid artery ligation model in TR3 transgenic mice. Circulation 106:1530-1535

Asagiri M, Takayanagi H (2007) The molecular understanding of osteoclast differentiation. Bone 40:251-264

Attwooll C, Tariq M, Harris M, Coyne JD, Telford N, Varley JM (1999) Identification of a novel fusion gene involving $hTAF_{II}68$ and CHN from a t(9;17)(q22;q11.2) translocation in an extraskeletal myxoid chondrosarcoma. Oncogene 18:7599-7601

Au WS, Payne VA, O'Rahilly S, Rochford JJ (2008) The NR4A family of orphan nuclear receptors are not required for adipogenesis. Int J Obes. 32:388-392

Aubin JE (2001) Regulation of osteoblast formation and function. Rev Endocr Metab Disord 2:81-94

Baht GS, Hunter GK, Goldberg HA (2008) Bone sialoprotein-collagen interaction promotes hydroxyapatite nucleation. 27:600-608

Bain DL, Heneghan AF, Connaghan-Jones KD, Miura MT (2007) Nuclear receptor structure: implications for function. Annu Rev Physiol 69:201-220

Balemans W, Ebeling M, Patel N, Van Hul E, Olson P, Dioszegi M, Lacza C, Wuyts W, Van Den Ende J, Willems P, Paes-Alves AF, Hill S, Bueno M, Ramos FJ, Tacconi P, Dikkers FG, Stratakis C, Lindpaintner K, Vickery B, Foernzler D, Van Hul W (2001) Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). Hum Mol Genet 10:537-543

Bandoh S, Tsukada T, Maruyama K, Ohkura N, Yamaguchi K (1997) Mechanical agitation induces gene expression of NOR-1 and its closely related orphan nuclear receptors in leukemic cell lines. Lekemia 11:1453-1458

Barnes GL, Javed A, Waller SM, Kamal MH, Hebert KE, Hassan MQ, Bellahcene A, Van Wijnen AJ, Young MF, Lian JB, Stein GS, Gerstenfeld LC (2003) Osteoblast-related transcription factors Runx2 (Cbfa1/AML3) and MSX2 mediate the expression of bone sialoprotein in human metastatic breast cancer cells. Cancer Res 63:2631-2637

Barry JB, Giguère V (2005) Epidermal growth factor-induced signaling in breast cancer cells results in selective target gene activation by orphan nuclear receptor estrogen-related receptor α. Cancer Res 65:6120-6129

Barry JB, Laganière J, Giguère V (2006) A single nucleotide in an estrogen-related receptor α site can dictate mode of binding and peroxisome proliferator-activated receptor γ coactivator 1α activation of target promoters. Mol Endocrinol 20:302-310

Bellahcène A, Castronovo V (1995) Increased expression of osteonectin and osteopontin, two bone matrix proteins, in human breast cancer. Am J Pathol 146:95-100

Bellahcène A, Castronovo V (1997) Expression of bone matrix proteins in human breast cancer: potential roles in microcalcification formation and in the genesis of bone metastases. Bull Cancer 84:17-24

Bellahcène A, Menard S, Bufalino R, Moreau L, Castronovo V (1996) Expression of bone sialoprotein in primary human breast cancer is associated with poor survival. Int J Cancer 69:350-353

Benoit G, Cooney A, Giguère V, Ingraham H, Lazar M, Muscat G, Perlmann T, Renaud JP, Schwabe J, Sladek F, Tsai MJ, Laudet V (2006) International Union of Pharmacology. LXVI. Orphan nuclear receptors. Pharmacol Rev 58:798-836

Benoit G, Malewicz M, Perlmann T (2004) Digging deep into the pockets of orphan nuclear receptors: insights from structural studies. Trends Cell Biol 14:369-376

Bernardo ME, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A, Montagna D, Maccario R, Villa R, Daidone MG, Zuffardi O, Locatelli F (2007) Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term *in vitro* culture and do not exhibit telomere maintenance mechanisms. Cancer Res 67:9142-9149

Bertelloni S, Baroncelli GI, Federico G, Cappa M, Lala R, Saggese G (1998) Altered bone mineral density in patients with complete androgen insensitivity syndrome. Horm Res 50:309-314

Blair HC, Zaidi M, Schlesinger PH (2002) Mechanisms balancing skeletal matrix synthesis and degradation. Biochem J 364:329-341

Bloedon LT, Jeffcoat AR, Lopaczynski W, Schell MJ, Black TM, Dix KJ, Thomas BF, Albright C, Busby MG, Crowell JA, Zeisel SH (2002) Safety and pharmacokinetics of purified soy isoflavones: single-dose administration to postmenopausal women. Am J Clin Nutr 76:1126-1137

Bodine PVN (2008) Wnt signaling control of bone cell apoptosis. Cell Res 18:248-253

Bodine PVN, Komm BS (2006) Wnt signaling and osteoblastogenesis. Rev Endocr Metab Disord 7:33-39

Boland GM, Perkins G, Hall DJ, Tuan RS (2004) Wnt3a promotes proliferation and suppresses osteogenic differentiation of adult human mesenchymal stem cells. J Cell Biochem 93:1210-1230

Bombail V, Collins F, Brown P, Saunders PT (2010) Modulation of ERα transcriptional activity by the orphan nuclear receptor ERRβ and evidence for differential effects of long- and short-form splice variants. Mol Cell Endocrinol 314:53-61

Bonnelye E, Aubin JE (2002a) Differential expression of estrogen receptor-related receptor α and estrogen receptors α and β in osteoblasts *in vivo* and *in vitro*. J Bone Miner Res 17:1392-1400

Bonnelye E, Kung V, Laplace C, Galson DL, Aubin JE (2002b) Estrogen receptor-related receptor alpha impinges on the estrogen axis in bone: potential function in osteoporosis. Endocrinology 143:3658-3670

Bonnelye E, Merdad L, Kung V, Aubin JE (2001) The orphan nuclear estrogen receptor-related receptor α (ERRα) is expressed throughout osteoblast differentiation and regulates bone formation *in vitro*. J Cell Biol 153:971-984

Bonnelye E, Vanacker JM, Dittmar T, Begue A, Desbiens X, Denhardt DT, Aubin JE, Laudet V, Fournier B (1997a) The ERR-1 orphan receptor is a transcriptional activator expressed during bone development. Mol Endocrinol 11:905-916

Bonnelye E, Vanacker JM, Spruyt N, Alric S, Fournier B, Desbiens X, Laudet V (1997b) Expression of the estrogen-related receptor 1 (ERR-1) orphan receptor during mouse development. Mech dev 65:71-85

Bonnelye E, Zirngibl RA, Jurdic P, Aubin JE (2007) The orphan nuclear estrogen receptor-related receptor-α regulates cartilage formation *in vitro*: implication of Sox9. Endocrinology 148:1195-1205

Bonta PI, van Tiel CM, Vos M, Pols TW, van Thienen JV, Ferreira V, Arkenbout EK, Seppen J, Spek CA, van der Poll T, Pannekoek H, de Vries CJ (2006) Nuclear receptors Nur77, Nurr1, and NOR-1 expressed in atherosclerotic lesion macrophages reduce lipid loading and inflammatory reponses. Aterioscler Thromb Vasc Biol 26:2288-2294

Bookout AL, Jeong Y, Downes M, Yu RT, Evans RM, Mangelsdorf DJ (2005) NURSA Dataset: Tissue-specific expression patterns of nuclear receptors. www.nursa.org/10.1621/datasets.02001

Bord S, Horner A, Beavan S, Compston J (2001) Estrogen receptors α and β are differentially expressed in developing human bone. J Clin Endocrinol Metab 86:2309-2314

Boskey AL, Gadaleta S, Gundberg C, Doty SB, Ducy P, Karsenty G (1998) Fourier transform infrared microspectroscopic analysis of bones of osteocalcin-deficient mice provides insight into the function of osteocalcin. Bone 23:187-196

Boskey AL, Maresca M, Ullrich W, Doty SB, Butler WT, Prince CW (1993) Osteopontin-hydroxyapatite interactions *in vitro*: inhibition of hydroxyapatite formation and growth in a gelatin-gel. Bone Miner 22:147-159

Boyce BF, Xing L (2008) Functions of RANKL/ RANK/OPG in bone modeling and remodeling. Arch Biochem Biophys 473:139-146

Boyden LM, Mao J, Belsky J, Mitzner L, Farhi A, Mitnick MA, Wu D, Insogna K, Lifton RP (2002) High bone density due to a mutation in LDL-receptor-related protein 5. N Engl J Med 346:1513-1521

Brook CGD, Marshall NJ (2001) Essential endocrinology, 4th edition, Calcium regulation, pp 119-136, Blackwell Science Ltd, UK

Brunkow ME, Gardner JC, Van Ness J, Paeper BW, Kovacevich BR, Proll S, Skonier JE, Zhao L, Sabo PJ, Fu Y, Alisch RS, Gillett L, Colbert T, Tacconi P, Galas D, Hamersma H, Beighton P, Mulligan J (2001) Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. Am J Hum Genet 68:577-589

Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL, Boyle WJ, Simonet WS (1998) Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 12:1260-1268

Busch BB, Stevens WC Jr, Martin R, Ordentlich P, Zhou S, Sapp DW, Horlick RA, Mohan R (2004) Identification of a selective inverse agonist for the orphan nuclear receptor estrogen-related receptor α. J Med Chem 47:5593-5596

Cadigan KM, Liu YI (2006) Wnt signaling: complexity at the surface. J Cell Sci 119:395-402

Cajánek L, Ribeiro D, Liste I, Parish CL, Bryja V, Arenas E (2009) Wnt/β-catenin signaling blockade promotes neuronal induction and dopaminergic differentiation in embryonic stem cells. Stem Cells 27:2917-2927

Calnan B, Szychowski S, Chan FKM, Cado D, Winoto A (1995) A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection. Immunity 3:273-282

Canalis E (2010) Update in new anabolic therapies for osteoporosis. J Clin Endocrinol Metab 95:1496-1504

Carrier JC, Deblois G, Champigny C, Levy E, Giguère V (2004) Estrogen-related receptor α (ERRα) is a transcriptional regulator of apolipoprotein A-IV and controls lipid handling in the intestine. J Biol Chem 279:52052-52058

Castet A, Herledan A, Bonnet S, Jalaguier S, Vanacker JM, Cavaillès V (2006) Receptor-interacting protein 140 differentially regulates estrogen receptor-related receptor transactivation depending on target genes. Mol Endocrinol 20:1035-1047

Castro DS, Hermanson E, Joseph B, Wállen A, Aarnisalo P, Heller A, Perlmann T (2001) Induction of cell cycle arrest and morphological differentiation by Nurr1 and retinoids in dopamine MN9D cells. J Biol Chem 276:43277-43284

Chang IC, Chiang TI, Yeh KT, Lee H, Cheng YW (2010) Increased serum osteopontin is a risk factor for osteoporosis in menopausal women. Osteoporosis Int 21:1401-1409

Chao LC, Bensinger SJ, Villanueva CJ, Wroblewski K, Tontonoz P (2008) Inhibition of adipocyte differentiation by Nur77, Nurr1, and Nor1. Mol Endocrinol 22:2596-2608

Chao LC, Wroblewski K, Zhang Z, Pei L, Vergnes L, Ilkayeva OR, Ding SY, Reue K, Watt MJ, Newgard CB, Pilch PF, Hevener AL, Tontonoz P (2009) Insulin resistance and altered systemic glucose metabolism in mice lacking Nur77. Diabetes 58:2788-2796

Chao LC, Zhang Z, Pei L, Saito T, Tontonoz P, Pilch PF (2007) Nur77 coordinately regulates expression of genes linked to glucose metabolism in skeletal muscle. Mol Endocrinol 21:2152-2163

Charatcharoenwitthaya N, Khosla S, Atkinson EJ, McCready LK, Riggs BL (2007) Effect of blockade of TNF-α and interleukin-1 action on bone resorption in early postmenopausal women. J Bone Miner Res 22:724-729

Charest-Marcotte A, Dufour CR, Wilson BJ, Tremblay AM, Eichner LJ, Arlow DH, Mootha VK, Giguère V (2010) The homeobox protein Prox1 is a negative modulator of ERRα/PGC-1α bioenergetic functions. Genes Dev 24:537-542

Cheng LE, Chan FK, Cado D, Winoto A (1997) Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis. EMBO J 16:1865-1875

Cheung CP, Yu S, Wong KB, Chan LW, Lai FM, Wang X, Suetsugi M, Chen S, Chan FL (2005) Expression and functional study of estrogen receptor-related receptors in human prostatic cells and tissues. J Clin Endocrinol Metab 90:1830-1844

Chintharlapalli S, Burghardt R, Papineni S, Ramaiah S, Yoon K, Safe S (2005) Activation of Nur77 by selected 1,1-Bis(3'-indolyl)-1-(*p*-substituted phenyl) methanes induces apoptosis through nuclear pathways. J Biol Chem 280:24903-24914

Clark J, Benjamin H, Gill S, Sidhar S, Goodwin G, Crew J, Gusterson BA, Shipley J, Cooper CS (1996) Fusion of the EWS gene to CHN, a member of the steroid/thyroid receptor gene superfamily, in a human myxoid chondrosarcoma. Oncogene 12:229-235

Clarke B (2008) Normal bone anatomy and physiology. Clin J Am Soc Nephrol 3:S131-S139

Codina A, Benoit G, Gooch JT, Neuhaus D, Perlmann T, Schwabe JW (2004) Idetification of a novel co-regulator interaction surface on the ligand binding domain of Nurr1 using NMR footprinting. J Biol Chem 279:53338-53345

Coleman R, Brown J, Terpos E, Lipton A, Smith MR, Cook R, Major P (2008) Bone markers and their prognostic value in metastatic bone disease: clinical evidence and future directions. Cancer Treat Rev 34:629-639

Cousin B, Ravet E, Poglio S, De Toni F, Bertuzzi M, Lulka H, Touil I, André M, Grolleau JL, Péron JM, Chavoin JP, Bourin P, Pénicaud L, Casteilla L, Buscail L, Cordelier P (2009) Adult stromal cells derived from human adipose tissue provoke pancreatic cancer cell death both in vitro and in vivo. PLoS One 4:e6278

Coward P, Lee D, Hull MV, Lehmann J (2001) 4-Hydroxytamoxifen binds to and deactivates the estrogen-related receptor γ. Proc Natl Acad Sci USA. 98:8880-8884

Crawford PA, Sadovsky Y, Woodson K, Lee SL, Milbrandt J (1995) Adrenocortical function and regulation of the steroid 21-hydroxylase gene in NGFI-B-deficient mice. Mol Cell Biol 15:4331-4336

Cui CB, Cooper LF, Yang X, Karsenty G, Aukhil I (2003) Transcriptional coactivation of bone-specific transcription factor Cbfa1 by TAZ. Mol Cell Biol 23:1004-1013

Cundy T, Hegde N, Naot D, Chong B, King A, Wallace R, Mulley J, Love DR, Seidel J, Fawkner M, Banovic T, Callon KE, Grey AB, Reid IR, Middleton-Hardie CA, Cornish J (2002) A mutation in the gene TNFRSF11B encoding osteoprotegerin causes an idiopathic hyperphosphatasia phenotype. Hum Mol Genet 11:2119-2127

Cunningham NR, Artim SC, Fornadel CM, Sellars MC, Edmonson SG, Scott G, Albino F, Mathur A, Punt JA (2006) Immature CD4+CD8+ thymocytes and mature T cells regulate Nur77 distinctly in response to TCR stimulation. J Immunol 177:6660-6666

Dang ZC, van Bezooijen RL, Karperien M, Papapoulos SE, Löwik CW (2002) Exposure of KS483 cells to estrogen enhances osteogenesis and inhibits adipogenesis. J Bone Miner Res 17:394-405

Datta NS, Abou-Samra AB (2009) PTH and PTHrP signaling in osteoblasts. Cell Signal 21:1245-1254

Deblois G, Giguère V (2008) Nuclear receptor location analyses in mammalian genomes: from gene regulation to regulatory networks. Mol Endocrinol 22:1999-2011

Deblois G, Hall JA, Perry MC, Laganière J, Ghahremani M, Park M, Hallett M, Giguère V (2009) Genome-wide identification of direct target genes implicates estrogen-related receptor α as a determinant of breast cancer heterogeneity. Cancer Res. 69:6149-6157

de Boer J, Siddappa R, Gaspar C, van Apeldoorn A, Fodde R, van Blitterswijk C (2004) Wnt signaling inhibits osteogenic differentiation of human mesenchymal stem cells. Bone 34:818-826

Delhon I, Gutzwiller S, Morvan F, Rangwala S, Wyder L, Evans G, Studer A, Kneissel M, Fournier B (2009) Absence of estrogen receptor-related-α increases osteoblastic differentiation and cancellous bone mineral density. Endocrinology 150:4463-4472

Djouad F, Plence P, Bony C, Tropel P, Apparailly F, Sany J, Noël D, Jorgensen C (2003) Immunosupressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 102:3837-3844

Dobnig H, Turner RT (1995) Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells. Endocrinology 136:3632-3638

Doi Y, Oki S, Ozawa T, Hohjoh H, Miyake S, Yamamura T (2008) Orphan nuclear receptor NR4A2 expressed in T cells from multiple sclerosis mediates production of inflammatory cytokines. Proc Natl Acad Sci USA 105:8381-8386

Donati V, Boldrini L, Dell'Omodarme M, Prati MC, Faviana P, Camacci T, Lucchi M, Mussi A, Santoro M, Basolo F, Fontanini G (2005) Osteopontin expression and prognostic significance in non-small cell lung cancer. Clin Cancer Res 11:6459-6465

Du P, Ye Y, Seitz PK, Bi LG, Li H, Wang C, Simmons DJ, Cooper CW (2000) Endogenous parathyroid hormone-related peptide enhances proliferation and inhibits differentiation in the osteoblast-like cell line ROS 17/2.8. Bone 26:429-436.

Dubois C, Hengerer B, Mattes H (2006) Identification of a potent agonist of the orphan nuclear receptor Nurr1. ChemMedChem 1:955-958

Ducy P, Amling M, Takeda S, Priemel M, Schilling AF, Beil FT, Shen J, Vinson C, Rueger JM, Karsenty G (2000) Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. Cell 100:197-207

Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, Karsenty G (1996) Increased bone formation in osteocalcin-deficient mice. Nature 382:448-452

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89:747-754

Dufour CR, Wilson BJ, Huss JM, Kelly DP, Alaynick WA, Downes M, Evans RM, Blanchette M, Giguère V (2007) Genome-wide orchestration of cardiac functions by the orphan nuclear receptors ERRα and γ. Cell Metab 5:345-356

Duodecim toimitus (2007) Osteoporoosi. Duodecim 123:2691-2696

Dwyer MA, Joseph JD, Wade HE, Eaton ML, Kunder RS, Kazmin D, Chang CY, McDonnell DP (2010) WNT11 expression is induced by estrogen-related receptor α and β-catenin and acts in autocrine manner to increase cancer cell migration. Cancer Res 70:9298-9308

Easwaran V, Pishvaian M, Salimuddin, Byers S (1999) Cross-regulation of β-catenin-LEF/TCF and retinoid signaling pathways. Curr Biol 9:1415-1418

Ebeling PR, Atley LM, Guthier JR, Burger HG, Dennerstein L, Hopper JL, Wark JD (1996) Bone turnover markers and bone density across the menopausal transition. J Clin Endocrinol Metab 81:3366-3371

Eells JB, Lipska BK, Yeung SK, Misler JA, Nikodem VM (2002) Nurr1-null heterozygous mice have reduced mesolimbic and mesocortical dopamine levels and increased stress-induced locomotor activity. Behav Brain Res 136:267-275

Eghbali-Fatourechi G, Khosla S, Sanyal A, Boyle WJ, Lacey DL, Riggs BL (2003) Role of RANK ligand in mediating increased bone resorption in early postmenopausal women. J Clin Invest 111:1221-1230

Elefteriou F, Ahn JD, Takeda S, Starbuck M, Yang X, Liu X, Kondo H, Richards WG, Bannon TW, Noda M, Clement K, Vaisse C, Karsenty G (2005) Leptin regulation of bone resorption by the sympathetic nervous system and CART. Nature 434:514-520

Elfassihi L, Giroux S, Bureau A, Laflamme N, Cole DE, Rousseau F (2010) Association with replication between estrogen-related receptor γ (ESRRγ) polymorphisms and bone phenotypes in women of European ancestry. J Bone Miner Res. 25:901-911

El-Tanani M, Fernig DG, Barraclough R, Green C, Rudland P (2001) Differential modulation of transcriptional activity of estrogen receptors by direct protein-protein interactions with the T cell factor family of transcription factors. J Biol Chem 276:41675-41682

Enyeart JJ, Boyd RT, Enyeart JA (1996) ACTH and AII differentially stimulate steroid hormone orphan receptor mRNAs in adrenal cortical cells. Mol Cell Endocrinol 124:97-110

Escriva H, Safi R, Hänni C, Langlois MC, Saumitou-Laprade P, Stehelin D, Capron D, Pierce R, Laudet V (1997) Ligand binding was acquired during evolution of nuclear receptors. Proc Natl Acad Sci USA 94:6803-6808

Eudy JD, Yao S, Weston MD, Ma-Edmonds M, Talmadge CB, Cheng JJ, Kimberling WJ, Sumegi J (1998) Isolation of a gene encoding a novel member of the nuclear receptor superfamily from the critical region of Usher syndrome type IIa at 1q41. Genomics 50:382-384

Fakhry A, Ratisoontorn C, Vedhachalam C, Salhab I, Koyama E, Leboy P, Pacifici M, Kirschner RE, Nah HD (2005) Effects of FGF-2/-9 in calvarial bone cell cultures: differentiation stage-dependent mitogenic effect, inverse regulation of BMP-2 and noggin, and enhancement of osteogenic potential. Bone 36:254-266

Falahati-Nini A, Riggs BL, Atkinson EJ, O'Fallon WM, Eastell R, Khosla S (2000) Relative contributions of testosterone and estrogen in regulating bone resorption and formation in normal elderly men. J Clin Invest 106:1553-1560

Feng B, Jiang J, Kraus P, Ng JH, Heng JCD, Chan YS, Yaw LP, Zhang W, Loh YH, Han J, Vega VB, Cacheux-Rataboul V, Lim B, Lufkin T, Ng HH (2009) Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. Nat Cell Biol 11:197-203

Fisher LW, Torchia DA, Fohr B, Young MF, Fedarko NS (2001) Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. Biochem Biophys Res Commun 280:460-465

Flaig R, Greschik H, Peluso-Iltis C, Moras D (2005) Structural basis for the cell-specific activities of the NGFI-B and the Nurr1 ligand-binding domain. J Biol Chem 280:19250-19258

Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM (1995) 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 is a ligand for the adipocyte determination factor PPARγ. Cell 83:803-812

Forootan SS, Foster CS, Aachi VR, Adamson J, Smith PH, Lin K, Ke Y (2006) Prognostic significance of osteopontin expression in human prostate cancer. Int J Cancer 118:2255-2261

Frenkel B, Hong A, Baniwal SK, Coetzee GA, Ohlsson C, Khalid O, Gabet Y (2010) Regulation of adult bone turnover by sex steroids. J Cell Physiol 224:305-310

Fu M, Sun T, Bookout AL, Downes M, Yu RT, Evans RM, Mangelsdorf DJ (2005) A Nuclear Receptor Atlas: 3T3-L1 adipogenesis. Mol Endocrinol. 19:2437-2450

Fujimura T, Takahashi S, Urano T, Ijichi N, Ikeda K, Kumagai J, Murata T, Takayama K, Horie-Inoue K, Ouchi Y, Muramatsu M, Homma Y, Inoue S (2010) Differential expression of estrogen-related receptors β and γ (ERRβ and ERRγ) and their clinical significance in human prostate cancer. Cencer Sci 101:646-651

Fujimura T, Takahashi S, Urano T, Kumagai J, Ogushi T, Horie-Inoue K, Ouchi Y, Kitamura T, Muramatsu M, Inoue S (2007) Increased expression of estrogen-related receptor α (ERRα) is a negative prognostic predictor in human prostate cancer. Int J Cancer 120:2325-2330

Fumoto T, Yamaguchi T, Hirose F, Osumi T (2007) Orphan nuclear receptor Nur77 accelerates the initial phase of adipocyte differentiation in 3T3-L1 cells by promoting mitotic clonal expansion. J Biochem. 141:181-192

Fux C, Mitta B, Kramer BP, Fussenegger M (2004) Dual-regulated expression of C/EBP-α and BMP-2 enables differential differentiation of C2C12 cells into adipocytes and osteoblasts. Nucleic Acids Res 32:e1

Gaddy-Kurten D, Coker JK, Abe E, Jilka RL, Manolagas SC (2002) Inhibin supresses and activin stimulates osteoblastogenesis and osteoclastogenesis in murine bone marrow cultures. Endocrinology 143:74-83

Galleguillos D, Vecchiola A, Fuentealba JA, Ojeda V, Alvarez K, Gómez A, Andrés ME (2004) PIASγ represses the transcriptional activation induced by the nuclear receptor Nurr1. J Biol Chem 279:2005-2011

Ganss B, Kim RH, Sodek J (1999) Bone sialoprotein. Crit Rev Oral Biol Med 10:79-98

Gao M, Sun P, Wang J, Zhao D, Wei L (2006) Expression of estrogen receptor-related receptor isoforms and clinical significance in endometrial adenocarcinoma. Int J Gynecol Cancer 16:827-833

Gardner CD, Oelrich B, Liu JP, Feldman D, Franke AA, Brooks JD (2009) Prostatic soy isoflavone concentrations exceed serum levels after dietary supplementation. Prostate 69:719-726

Garnero P, Sornay-Rendu E, Chapuy MC, Delmas PD (1996) Increased bone turnover in late postmenopausal women is a major determinant of osteoporosis. J Bone Miner Res 11:337-349

Gass M, Dawson-Hughes B (2006) Preventing osteoporosis-related fractures: an overview. Am J Med 119:S3-S11

Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS, Javed A, van Wijnen AJ, Stein JL, Stein GS, Lian JB (2005) Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. J Biol Chem 280:33132-33140

Germain P, Chambon P, Eichele G, Evans RM, Lazar MA, Leid M, De Lera AR, Lotan R, Mangelsdorf DJ, Gronemeyer H (2006) International Union of Pharmacology. LXIII. Retinoid X receptors. Pharmacol Rev 58:760-772

Giguère V (1999) Orphan nuclear receptors: from gene to function. Endocr Rev 20:689-725

Giguère V (2008) Transcriptional control of energy homeostasis by the estrogen-related receptors. Endocr Rev 29:677-696

Giguère V, Yang N, Segui P, Evans RM (1988) Identification of a new class of steroid hormone receptors. Nature 331:91-94

Gilbert F, Morissette M, St-Hilaire M, Paquet B, Rouillard C, Di Paolo T, Lévesque D (2006) Nur77 gene knockout alters dopamine neuron biochemical activity and dopamine turnover. Biol Psychiatry 60:538-547

Gilbert SF (2000) Developmental Biology, 6th edition, Osteogenesis: The development of bones. Sinauer Associates, Sunderland (MA)

Giordano A, Galderisi U, Marino IR (2007) From the laboratory bench to the patient's bedside: an update on clinical trials with mesenchymal stem cells. J Cell Physiol 211:27-35

Giroux S, Elfassihi L, Cole DE, Rousseau F (2008) Replication of associations between LRP5 and ESRRA variants and bone density in premenopausal women. Osteoporos Int 19:1769-1775

Goltzman D (2010) Emerging roles for calcium-regulating hormones beyond osteolysis. Trends Endocrinol Metab 21:512-518

Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D, Zacharin M, Oexle K, Marcelino J, Suwairi W, Heeger S, Sabatakos G, Apte S, Adkins WN, Allgrove J, Arslan-Kirchner M, Batch JA, Beighton P, Black GC, Boles RG, Boon LM, Borrone C, Brunner HG, Carle GF, Dallapiccola B, De Paepe A, Floege B, Halfhide ML, Hall B, Hennekam RC, Hirose T, Jans A, Jüppner H, Kim CA, Keppler-Noreuil K, Kohlschuetter A, LaCombe D, Lambert M, Lemyre E, Letteboer T, Peltonen L, Ramesar RS, Romanengo M, Somer H, Steichen-Gersdorf E, Steinmann B, Sullivan B, Superti-Furga A, Swoboda W, van den Boogaard MJ, Van Hul W, Vikkula M, Votruba M, Zabel B, Garcia T, Baron R, Olsen BR, Warman ML (2001) LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell 107:513-523

Gordon JA, Tye CE, Sampaio AV, Underhill TM, Hunter GK, Goldenberg HA (2007) Bone sialoprotein expression enhances osteoblast differentiation and matrix mineralization *in vitro*. Bone 41:462-473

Gregory CA, Gunn WG, Reyes E, Smolarz AJ, Munoz J, Spees JL, Prockop DJ (2005) How Wnt signaling affects bone repair by mesenchymal stem cells from the bone marrow. Ann N Y Acad Sci 1049:97-106

Greschik H, Flaig R, Renaud JP, Moras D (2004) Structural basis for the deactivation of the estrogen-related receptor γ by diethylstilbestrol or 4-hydroxytamoxifen and determinants of selectivity. J Biol Chem 279:33639-33646

Greschik H, Wurtz JM, Sanglier S, Bourguet W, van Dorsselaer A, Moras D, Renaud JP (2002) Structural and functional evidence for ligand-independent transcriptional activation by the estrogen-related receptor 3. Mol Cell 9:303-313

Gronemeyer H, Gustafsson J-Å, Laudet V (2004) Principles for modulation of the nuclear receptor superfamily. Nat Rev Drug Discov 3:950-964

Guigon CJ, Zhao L, Lu C, Willingham MC, Cheng SY (2008) Regulation of β-catenin by a novel nongenomic action of thyroid hormone β receptor. Mol Cell Biol 28:4598-4608

Gutierrez S, Javed A, Tennant DK, van Rees M, Montecino M, Stein GS, Stein JL, Lian JB (2002) CCAAT/enhancer-binding proteins (C/EBP) β and δ activate osteocalcin gene transcription and synergize with Runx2 at the C/EBP element to regulate bone-specific expression. J Biol Chem 277:1316-1323

Göttlicher M, Widmark E, Li Q, Gustafsson JÅ (1992) Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. Proc Natl Acad Sci USA 89:4653-4657

Hadjidakis DJ, Androulakis II (2006) Bone remodeling. Ann N Y Acad Sci 1092:385-396

Hamrick MW, Ferrari SL (2008) Leptin and the sympathetic connection of fat to bone. Osteoporos Int 19:905-912

Hanai J, Chen LF, Kanno T, Ohtani-Fujita N, Kim WY, Guo WH, Imamura T, Ishidou Y, Fukuchi M, Shi MJ, Stavnezer J, Kawabata M, Miyazono K, Ito Y (1999) Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Cα promoter. J Biol Chem 274:31577-31582

Harada S, Rodan GA (2003) Control of osteoblast function and regulation of bone mass. Nature 423:349-355

Hauschka PV, Wians FH Jr (1989) Osteocalcin-hydroxyapatite interaction in the extracellular organic matrix of bone. Anat Rec 224:180-188

Hazel TG, Misra R, Davis IJ, Greenberg ME, Lau LF (1991) Nur77 is differentially modified in PC12 cells upon membrane depolarization and growth factor treatment. Mol Cell Biol 11:3239-3246

Hazel TG, Nathans D, Lau LF (1988) A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. Proc Natl Acad Sci USA 85:8444-8448

Heard DJ, Norby PL, Holloway J, Vissing H (2000) Human ERRγ, a third member of the estrogen receptor-related receptor (ERR) subfamily of orphan nuclear receptors: tissue specific isoforms are expressed during development and in the adult. Mol Endocrinol 14:382-392

Heare T, Hensley MA, Dell'Orfano S (2009) Bone tumors: osteosarcoma and Ewing's sarcoma. Curr Opin Pediatr 21:365-372

Hedlund TE, Maroni PD, Ferucci PG, Dayton R, Barnes S, Jones K, Moore R, Ogden LG, Wähälä K, Sackett HM, Gray KJ (2005) Long-term dietary habits affect soy isoflavone metabolism and accumulation in prostatic fluid in caucasian men. J Nutr 135:1400-1406.

Henriksen K, Neutzky-Wulff AV, Bonewald LF, Karsdal MA (2009) Local communication on and within bone controls bone remodeling. Bone 44:1026-1033

Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C (1992) 9-cis retinoic acid is a high affinity ligand for retinoid X receptor. Cell 68:397-406

Hill TP, Später D, Taketo MM, Birchmeier W, Hartmann C (2005) Canonical Wnt/β-catenin signaling prevents osteoblasts from differentiating into chondrocytes. Dev Cell 8:727-738

Hintermann S, Chiesi M, von Krosigk U, Mathé D, Felber R, Hengerer B (2007) Identification of a series of highly potent activators of the Nurr1 signaling pathway. Bioorg Med Chem Lett 17:193-196

Hisaoka M, Ishida T, Imamura T, Hashimoto H (2004) TFG is a novel fusion partner of NOR1 in extraskeletal myxoid chondrosarcoma. Genes Chromosomes Cancer 40:325-328

Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Spelsberg TC, Riggs BL (1999) Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells. Endocrinology 140:4367-4370

Hong H, Yang L, Stallcup MR (1999) Hormone-independent transcriptional activation and coactivator binding by novel orphan nuclear receptor ERR3. J Biol Chem 274:22618-22626

Honkaniemi J, Kononen J, Kainu T, Pyykkönen I, Pelto-Huikko M (1994) Induction of multiple immediate early genes in rat hypothalamic paraventricular nucleus after stress. Brain Res Mol Brain Res 25:234-241

Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK (1999) Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 5:309-313

Huang Z, Fang F, Wang J, Wong C-W (2010) Structural activity relationship of flavonoids with estrogen-related receptor γ. FEBS Lett 584:22-26

Hunter GK, Kyle CL, Goldberg HA (1994) Modulation of crystal formation by bone phosphoproteins: structural specificity of the osteopontin-mediated inhibition of hydroxyapatite fomation. Biochem J 300:723-728

Huppunen J, Aarnisalo P (2004) Dimerization modulates the activity of the orphan nuclear receptor ERRγ. Biochem Biophys Res Commun 314:964-970

Huppunen J, Wohlfahrt G, Aarnisalo P (2004) Requirements for transcriptional regulation by the orphan nuclear receptor ERRγ. Mol Cell Endocrinol 219:151-160

Huss JM, Imahashi K, Dufour CR, Weinheimer CJ, Courtois M, Kovacs A, Giguère V, Murphy E, Kelly DP (2007) The nuclear receptor ERRα is required for the bioenergetic and functional adaptation to cardiac pressure overload. Cell Metab 6:25-37

Huss JM, Kopp RP, Kelly DP (2002) Peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) coactivates the cardiac-enriched nuclear receptors estrogen-related receptor-α and -γ. Identification of novel leucine-rich interaction motif within PGC-1α. J Biol Chem 277:40265-40274

Huss JM, Torra IP, Staels B, Giguère V, Kelly DP (2004) Estrogen-related receptor α directs peroxisome proliferator-activated receptor α signaling in the transcriptional control of energy metabolism in cardiac and skeletal muscle. Mol Cell Biol 24:9079-9091

Ijichi N, Ikeda K, Horie-Inoue K, Yagi K, Okazaki Y, Inoue S (2007) Estrogen-related receptor α modulates the expression of adipogenesis-related genes during adipocyte differentiation. Biochem Biophys Res Commun 358:813-818

Ijichi N, Shigekawa T, Ikeda K, Horie-Inoue K, Fujimura T, Tsuda H, Osaki A, Saeki T, Inoue S (2011) Estrogen-related receptor γ modulates cell proliferation and estrogen signaling in breast cancer. J Steroid Biochem Mol Biol 123:1-7

Imai Y, Kondoh S, Kouzmenko A, Kato S (2009) Regulation of bone metabolism by nuclear receptors. Mol Cell Endocrinol 310:3-10

Inada M, Yasui T, Nomura S, Miyake S, Deguchi K, Himeno M, Sato M, Yamagiwa H, Kimura T, Yasui N, Ochi T, Endo N, Kitamura Y, Kishimoto T, Komori T (1999) Maturational disturbance of chondrocytes in Cbfa1-deficient mice. Dev Dyn 214:279-290

Inamoto T, Czerniak BA, Dinney CP, Kamat AM (2010) Cytoplasmic mislocalization of the orphan nuclear receptor Nurr1 is a prognostic factor in bladder cancer. Cancer 116:340-346

Jacobs CM, Boldingh KA, Slagsvold HH, Thoresen GH, Paulsen RE (2004) ERK2 prohibits apoptosis-induced subcellular translocation of orphan nuclear receptor NGFI-B/TR3. J Biol Chem 279:50097-50101

Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem 64:295-312

Janowski BA, Grogan MJ, Jones SA, Wisely GB, Kliewer SA, Corey EJ, Mangelsdorf DJ (1999) Structural requirements of ligands for the oxysterol liver X receptors LXRα and LXRβ. Proc Natl Acad Sci USA 96:266-271

Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ (1996) An oxysterol signalling pathway mediated by the nuclear receptor LXRα. Nature 383:728-731

Jeon MJ, Kim JA, Kwon SH, Kim SW, Park KS, Park SW, Kim SY, Shin CS (2003) Activation of peroxisome proliferator-activated receptor-γ inhibits the Runx2-mediated transcription of osteocalcin in osteoblasts. J Biol Chem 278:23270-23277

Jeong BC, Lee YS, Park YY, Bae IH, Kim DK, Koo SH, Choi HR, Kim SH, Franceschi RT, Koh JT, Choi HS (2009) The orphan nuclear receptor estrogen receptor-related receptor γ negatively regulates BMP2-induced osteoblast differentiation and bone formation. J Biol Chem 284:14211-14218

Jeong Y, Mangelsdorf DJ (2009) Nuclear receptor regulation of stemness and stem cell differentiation. Exp Mol Med 41:525-537

Jho EH, Zhang T, Domon C, Joo CK, Freund JN, Costantini F (2002) Wnt/β-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol Cell Biol 22:1172-1183

Johnston SD, Liu X, Zuo F, Eisenbraun TL, Wiley SR, Kraus RJ, Mertz JE (1997) Estrogen-relared receptor α1 functionally binds as a monomer to extended half-site sequences including ones contained within estrogen-response elements. Mol Endocrinol 11:342-352

Justesen J, Stenderup K, Ebbesen EN, Mosekilde L, Steiniche T, Kassem M (2001) Adipocyte tissue volume in bone marrow is increased with aging and in patients with osteoporosis. Biogerontology 2:165-171

Kagaya S, Ohkura N, Tsukada T, Miyagawa M, Sugita Y, Tsujimoto G, Matsumoto K, Saito H, Hashida R (2005) Prostaglandin A2 acts as a transactivator for NOR1 (NR4A3) within the nuclear receptor superfamily. Biol Pharm Bull 28:1603-1607

Kahler RA, Galindo M, Lian J, Stein GS, van Wijnen AJ, Westendorf JJ (2006) Lymphocyte enhancer-binding factor 1 (Lef1) inhibits terminal differentiation of osteoblasts. J Cell Biochem 97:969-983

Kahler RA, Westendorf JJ (2003) Lymphoid enhancer factor-1 and β-catenin inhibit Runx2-dependent transcriptional activation of the osteocalcin promoter. J Biol Chem 278:11937-11944

Kallen J, Lattmann R, Beerli R, Blechschmidt A, Blommers MJ, Geiser M, Ottl J, Schlaeppi JM, Strauss A, Fournier B (2007) Crystal structure of human estrogen-related receptor α in complex with a synthetic inverse agonist reveals its novel molecular mechanism. J Biol Chem 282:23231-23239

Kallen J, Schlaeppi JM, Bitsch J, Filipuzzi I, Schilb A, Riou V, Graham A, Strauss A, Geiser M, Fournier B (2004) Evidence for ligand-independent transcriptional activation of the human estrogen-related receptor α (ERRα): crystal structure of ERRα ligand binding domain in complex with peroxisome proliferator-activated receptor coactivator-1α. J Biol Chem 279:49330-49337

Kamei Y, Lwin H, Saito K, Yokoyama T, Yoshiike N, Ezaki O, Tanaka H (2005) The 2.3 genotype of ESRRA23 of the ERRα gene is associated with a higher BMI than the 2.2 genotype. Obes Res 13:1843-1844

Kamei Y, Ohizumi H, Fujitani Y, Nemoto T, Tanaka T, Takahashi N, Kawada T, Miyoshi M, Ezaki O, Kakizuka A (2003) PPARγ coactivator 1β/ERR ligand 1 is an ERR protein ligand, whose expression induces a high-energy expenditure and antagonizes obesity. Proc Natl Acad Sci USA 100:12378-12383

Karamanlidis G, Nascimben L, Couper GS, Shekar PS, del Monte F, Tian R (2010) Defective DNA replication impairs mitochondrial biogenesis in human failing hearts. Circ Res 106:1541-1548

Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VL, Kronenberg HM, Mulligan RC (1994) Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. Genes Dev 8:277-289

Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R, Weinberg RA (2007) Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 449:557-563

Katagiri Y, Hirata Y, Milbrandt J, Guroff G (1997) Differential regulation of the transcriptional activity of the orphan nuclear receptor NGFI-B by membrane depolarization and nerve growth factor. J Biol Chem 272:31278-31284

Kato M, Patel MS, Levasseur R, Lobov I, Chang BH, Glass DA 2nd, Hartmann C, Li L, Hwang TH, Brayton CF, Lang RA, Karsenty G, Chan L (2002) Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. J Cell Biol 157:303-314

Kawai M, Devlin MJ, Rosen CJ (2009) Fat targets for skeletal health. Nat Rev Rheumatol 5:365-372

Kawano H, Sato T, Yamada T, Matsumoto T, Sekine K, Watanabe T, Nakamura T, Fukuda T, Yoshimura K, Yoshizawa T, Aihara K, Yamamoto Y, Nakamichi Y, Metzger D, Chambon P, Nakamura K, Kawaguchi H, Kato S (2003) Suppressive function of androgen receptor in bone resorption. Proc Natl Acad Sci USA 100:9416-9421

Kearns AE, Khosla S, Kostenuik PJ (2008) Receptor activator of nuclear factor κB ligand and osteoprotegerin regulation of bone remodeling in health and disease. Endocr Rev 29:155-192

Keeting PE, Scott RE, Colvard DS, Han IK, Spelsberg TC, Riggs BL (1991) Lack of a direct effect of estrogen on proliferation and differentiation of normal human osteoblast-like cells. J Bone Miner Res 6:297-304

Kern B, Shen J, Starbuck M, Karsenty G (2001) Cbfa1 contributes to the osteoblast-specific expression of type I collagen genes. J Biol Chem 276:7101-7107

Khakoo AY, Pati S, Anderson SA, Reid W, Elshal MF, Rovira II, Nguyen AT, Malide D, Combs CA, Hall G, Zhang J, Raffeld M, Rogers TB, Stetler-Stevenson W, Frank JA, Reitz M, Finkel T (2006) Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. J Exp Med 203:1235-1247

Khosla S, Amin S, Orwoll E (2008) Osteoporosis in men. Endocr Rev 29:441-464

Kim S, Koga T, Isobe M, Kern BE, Yokochi T, Chin YE, Karsenty G, Taniguchi T, Takayanagi H (2003) Stat1 functions as a cytoplasmic attenuator of Runx2 in the transcriptional program of osteoblast differentiation. Genes Dev 17:1979-1991

Kingsley LA, Fournier PGJ, Chirgwin JM, Guise TA (2007) Molecular biology of bone metastasis. Mol Cancer Ther 6:2609-2617

Kininis M, Kraus WL (2008) A global view of transcriptional regulation by nuclear receptors: gene expression, factor localization, and DNA sequence analysis. Nucl Recept Signal 6:e005

Kitagawa H, Ray WJ, Glantschnig H, Nantermet PV, Yu Y, Leu CT, Harada S, Kato S, Freedman LP (2007) A regulatory circuit mediating convergence between Nurr1 transcriptional regulation and Wnt signaling. Mol Cell Biol 27:7486-7496

Kitazawa S, Kajimoto K, Kondo T, Kitazawa R (2003) Vitamin D3 supports osteoclastogenesis via functional vitamin D response element of human RANKL gene promoter. J Cell Biochem 89:771-777

Klein NA, Illingworth PJ, Groome NP, McNeilly AS, Battaglia DE, Soules MR (1996) Decreased inhibin B secretion is associated with the monotropic FSH rise in older, ovulatory women: a study of serum and follicular fluid levels of dimeric inhibin A and B in spontaneous menstrual cycles. J Clin Endocrinol Metab 81:2742-2745

Klein PS, Melton DA (1996) A molecular mechanism for the effect of lithium on development. Proc Natl Acad Sci USA 93:8455-8459

Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterström RH, Perlmann T, Lehmann JM (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell 92:73-82

Koc ON, Gerson SL, Cooper BW, Dyhouse SM, Haynesworth SE, Caplan AI, Lazarus HM (2000) Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol 18:307-316

Koga T, Matsui Y, Asagiri M, Kodoma T, de Crombrugghe B, Nakashima K, Takayanagi H (2005) NFAT and Osterix cooperatively regulate bone formation. Nat Med 11:880-885

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T (1997) Targeted disruption of Cbfa1 results in complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755-764

Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM (1999) OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 397:315-323

Kouzmenko AP, Takeyama K, Ito S, Furutani T, Sawatsubashi S, Maki A, Suzuki E, Kawasaki Y, Akiyama T, Tabata T, Kato S (2004) Wnt/β-catenin and estrogen signaling converge *in vivo*. J Biol Chem 279:40255-40258

Kovalovsky D, Refojo D, Liberman AC, Hochbaum D, Pereda MP, Coso OA, Stalla GK, Holsboer F, Arzt E (2002) Activation and induction of Nurr77/Nurr1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways. Mol Endocrinol 16:1638-1651

Krishnan V, Moore TL, Ma YL, Helvering LM, Frolik CA, Valasek KM, Ducy P, Geiser AG (2003) Parathyroid hormone bone anabolic action requires Cbfa1/Runx2-dependant signaling. Mol Endocrinol 17:423-435

Kubo M, Ijichi N, Ikeda K, Horie-Inoue K, Takeda S, Inoue S (2009) Modulation of adipogenesis-related gene expression by estrogen-related receptor γ during adipocytic differentiation. Biochim Biophys Acta 1789:71-77

Kulkarni NH, Halladay DL, Miles RR, Gilbert LM, Frolik CA, Galvin RJ, Martin TJ, Gillespie MT, Onyia JE (2005) Effects of parathyroid hormone on Wnt signaling pathway in bone. J Cell Biochem 95:1178-1190

Labelle Y, Bussières J, Courjal F, Goldring MB (1999) The EWS/TEC fusion protein encoded by the t(9;22) chromosomal translocation in human chondrosarcomas is a highly potent transcriptional activator. Oncogene 18:3303-3308

Laflamme N, Giroux S, Loredo-Osti JC, Elfassihi L, Dodin S, Blanchet C, Morgan K, Giguère V, Rousseau F (2005) A frequent regulatory variant of the estrogen-related receptor α gene associated with BMD in French-Canadian premenopausal women. J Bone Miner Res 20:938-944

Lammi J, Aarnisalo P (2008) FGF-8 stimulates the expression of NR4A orphan nuclear receptors in osteoblasts. Mol Cell Endocrinol. 295:87-93

Lammi J, Huppunen J, Aarnisalo P (2004) Regulation of the osteopontin gene by the orphan nuclear receptor NURR1 in osteoblasts. Mol Endocrinol 18:1546-1557

Larsen LH, Rose CS, Sparso T, Overgaard J, Torekov SS, Grarup N, Jensen DP, Albrechtsen A, Andersen G, Ek J, Glümer C, Borch-Johnsen K, Jorgensen T, Hansen T, Pedersen O (2007) Genetic analysis of the estrogen-related receptor α and studies of association with obesity and type 2 diabetes. Int J Obes 31:365-370

Lavery DN, McEwan IJ (2005) Structure and function of steroid receptor AF1 transactivation domains: induction of active conformations. Biochem J 391:449-464

Law SW, Conneely OM, DeMayo FJ, O'Malley BW (1992) Identification of a new brain-specific transcription factor, NURR1. Mol Endocrinol 6:2129-2135

Le WD, Xu P, Jankovic J, Jiang H, Appel SH, Smith RG, Vassilatis DK (2003) Mutations in NR4A2 associated with familial Parkinson disease. Nat Genet 33:85-89

Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringdén O (2008) Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 371:1579-1586

Le Blanc K, Rasmusson I, Sundberg B, Götherström C, Hassan M, Uzunel M, Ringdén O (2004) Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 363:1439-1441

Lee B, Thirunavukkarasu K, Zhou L, Pastore L, Baldini A, Hecht J, Geoffroy V, Ducy P, Karsenty G (1997) Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. Nat Genet 16:307-310

Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, Wozney JM, Kim EG, Choi JY, Ryoo HM, Bae SC (2000) Runx2 is a common target of transforming growth factor β1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol 20:8783-8792

Lee MK, Choi H, Gil M, Nikodem VM (2006) Regulation of osteoblast differentiation by Nurr1 in MC3T3-E1 cell line and mouse calvarial osteoblasts. J Cell Biochem 99:986-994

Lee NK, Sowa H, Hinoi E, Ferron M, Ahn JD, Confavreux C, Dacquin R, Mee PJ, McKee MD, Jung DY, Zhang Z, Kim JK, Mauvais-Jarvis F, Ducy P, Karsenty G (2007) Endocrine regulation of energy metabolism by the skeleton. Cell 130:456-469

Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA (1995) An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). J Biol Chem 270:12953-12956

Leskelä HV, Olkku A, Lehtonen S, Mahonen A, Koivunen J, Turpeinen M, Uusitalo J, Pelkonen O, Kangas L, Selander K, Lehenkari P (2006) Estrogen receptor α genotype confers interindividual variability of response to estrogen and testosterone in mesenchymal-stem-cell-derived osteoblasts. Bone 39:1026-1034

Levin AA, Sturzenbecker LJ, Kazmer S, Bosakowski T, Huselton C, Allenby G, Speck J, Kratzeisen C, Rosenberger M, Lovey A, Grippo JF (1992) 9-cis retinoic acid stereoisomer binds and activates the nuclear receptor RXRα. Nature 355:359-361

Li WF, Hou SX, Yu B, Li MM, Férec C, Chen JM (2010) Genetics of osteoporosis: accelerating pace in gene identification and validation. Hum Genet 127:249-285

Lindberg MK, Alatalo SL, Halleen JM, Mohan S, Gustafsson JÅ, Ohlsson C (2001) Estrogen receptor specificity in the regulation of the skeleton in female mice. J Endocrinol 171:229-236

Little RD, Carulli JP, Del Mastro RG, Dupuis J, Osborne M, Folz C, Manning SP, Swain PM, Zhao SC, Eustace B, Lappe MM, Spitzer L, Zweier S, Braunschweiger K, Benchekroun Y, Hu X, Adair R, Chee L, FitzGerald MG, Tulig C, Caruso A, Tzellas N, Bawa A, Franklin B, McGuire S, Nogues X, Gong G, Allen KM, Anisowicz A, Morales AJ, Lomedico PT, Recker SM, Van Eerdewegh P, Recker RR, Johnson ML (2002) A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. Am J Hum Genet 70:11-19

Liu CJ, Chang E, Yu J, Carlson CS, Prazak L, Yu XP, Ding B, Lengyel P, Di Cesare PE (2005) The interferon-inducible p204 protein acts as a transcriptional coactivator of Cbfa1 and enhances osteoblast differentiation. J Biol Chem 280:2788-2796

Liu D, Jia H, Holmes DI, Stannard A, Zachary I (2003) Vascular endothelial growth factor-regulated gene expression in endothelial cells: KDR-mediated induction of Egr3 and the related nuclear receptors nur77, Nurr1, and Nor1. Aterioscler Thromb Vasc Biol 23:2002-2007

Liu G, Vijayakumar S, Grumolato L, Arroyave R, Qiao H, Akiri G, Aaronson SA (2009a) Canonical Wnts function as potent regulators of osteogenesis by human mesenchymal stem cells. J Cell Biol 185:67-75

Liu J, Wang H, Zuo Y, Farmer SR (2006) Functional interaction between peroxisome proliferator-activated receptor γ and β-catenin. Mol Cell Biol 26:5827-5837

Liu W, Toyosawa S, Furuichi T, Kanatani N, Yoshida C, Liu Y, Himeno M, Narai S, Yamaguchi A, Komori T (2001) Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. J Cell Biol 155:157-166

Liu ZG, Smith SW, McLaughlin KA, Schwartz LM, Osborne BA (1994) Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene nur77. Nature 367:281-284

Liu ZJ, Zhuge Y, Velazquez OC (2009b) Trafficking and differentiation of mesenchymal stem cells. J Cell Biochem 106:984-991

Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J (2000) Molecular cell biology, 4th edition, Overview of extracellular signalling, W.H. Freeman, New York

Lorenzo J, Horowitz M, Choi Y (2008) Osteoimmunology: interactions of the bone and immune system. Endocr Rev 29:403-440

Lu D, Kiriyama Y, Lee KY, Giguère V (2001) Transcriptional regulation of the estrogen-inducible pS2 breast cancer marker gene by the ERR family of orphan nuclear receptors. Cancer Res 61:6755-6761

Lund SA, Giachelli CM, Scatena M (2009) The role of osteopontin in inflammatory processes. J Cell Commun Signal 3:311-322

Luo J, Sladek R, Bader JA, Matthyssen A, Rossant J, Giguère V (1997) Placental abnormalities in mouse embryos lacking orphan nuclear receptor ERRβ. Nature 388:778-782

Luo J, Sladek R, Carrier J, Bader JA, Richard D, Giguère V (2003) Reduced fat mass in mice lacking orphan nuclear receptor estrogen-related receptor α. Mol Cell Biol 23:7947-7956

Magee PJ, Raschke M, Steiner C, Duffin JG, Pool-Zobel BL, Jokela T, Wahala K, Rowland IR (2006) Equol: a comparison of the effects of the racemic compound with that of the purified S-enantiomer on the growth, invasion, and DNA integrity of breast and prostate cells in vitro. Nutr Cancer 54:232-242

Maheux J, Ethier I, Rouillard C, Lévesque D (2005) Induction patterns of transcription factors of the nur family (nurr1, nur77, and nor-1) by typical and atypical antipsychotics in the mouse brain: implication for their mechanism of action. J Pharmacol Exp Ther 313:460-473

Mahoney DJ, Parise G, Melov S, Safdar A, Tarnopolsky MA (2005) Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise. FASEB J 19:1498-1500

Maira M, Martens C, Batsché E, Gauthier Y, Drouin J (2003) Dimer-specific potentiation of NGFI-B (Nur77) transcriptional activity by the protein kinase A pathway and AF-1-dependent coactivator recruitment. Mol Cell Biol 23:763-776

Maira M, Martens C, Philips A, Drouin J (1999) Heterodimerization between members of the Nur subfamily of orphan nuclear receptors as a novel mechanism for gene activation. Mol Cell Biol 19:7549-7557

Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B (1999) Identification of a nuclear receptor for bile acids. Science 284:1362-1365

Malaval L, Wade-Guéye NM, Boudiffa M, Fei J, Zirngibl R, Chen F, Laroche N, Roux JP, Burt-Pichat B, Duboeuf F, Boivin G, Jurdic P, Lafage-Proust MH, Amédée J, Vico L, Rossant J, Aubin JE (2008) Bone sialoprotein plays a functional role in bone formation and osteoclastogenesis. J Exp Med 205:1145-1153

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM (1995) The nuclear receptor superfamily: the second decade. Cell 83:835-839

Marini JC, Forlino A, Cabral WA, Barnes AM, San Antonio JD, Milgrom S, Hyland JC, Körkkö J, Prockop DJ, De Paepe A, Coucke P, Symoens S, Glorieux FH, Roughley PJ, Lund AM, Kuurila-Svahn K, Hartikka H, Cohn DH, Krakow D, Mottes M, Schwarze U, Chen D, Yang K, Kuslich C, Troendle J, Dalgleish R, Byers PH (2007) Consortium for osteogenesis imperfecta mutations in the helical domain of type I collagen: regions rich in lethal mutations align with collagen binding sites for integrins and proteoglycans. Hum Mutat 28:209-221

Martens C, Bilodeau S, Maira M, Gauthier Y, Drouin J (2005) Protein-protein interactions and transcriptional antagonism between the subfamily of NGFI-B/Nur77 orphan nuclear receptors and glucocorticoid receptor. Mol Endocrinol 19:885-897

Martin RB, Zissimos SL (1991) Relationships between marrow fat and bone turnover in ovariectomized and intact rats. Bone 12:123-131

Martínez-González J, Badimon L (2005) The NR4A subfamily of nuclear receptors: new early genes regulated by growth factors in vascular cells. Cardiovasc Res 65:609-618

Martínez-González J, Rius J, Castelló A, Cases-Langhoff C, Badimon L (2003) Neuron-derived orphan receptor-1 (NOR-1) modulates vascular smooth muscle cell proliferation. Circ Res 92:96-103

Martorell L, Gentile M, Rius J, Rodríguez C, Crespo J, Badimon L, Martínez-González J (2009) The hypoxia-inducible factor 1/NOR-1 axis regulates the survival response of endothelial cells to hypoxia. Mol Cell Biol 29:5828-5842

Masuyama N, Oishi K, Mori Y, Ueno T, Takahama Y, Gotoh Y (2001) Akt inhibits the orphan nuclear receptor Nur77 and T-cell apoptosis. J Biol Chem 276:32799-32805

Masuyama R, Stockmans I, Torrekens S, Van Looveren R, Maes C, Carmeliet P, Bouillon R, Carmeliet G (2006) Vitamin D receptor in chondrocytes promotes osteoclastogenesis and regulates FGF23 production in osteoblasts. J Clin Invest 116:3150-3159

Maxwell MA, Cleasby ME, Harding A, Stark A, Cooney GJ, Muscat GE (2005) Nur77 regulates lipolysis in skeletal muscle cells. Evidence for cross-talk between the β-adrenergic and an orphan nuclear hormone receptor pathway. J Biol Chem 280:12573-12584

Maxwell MA, Muscat GEO (2005) The NR4A subgroup: immediate early reponse genes with pleiotropic physiological roles. Nucl Recept Signal 4:e002

Migliaccio S, Brama M, Spera G (2007) The differential effects of bisphosphonates, SERMS (selective estrogen receptor modulators), and parathyroid hormone on bone remodeling in osteoporosis. Clin Interv Aging 2:55-64

Milbrandt J (1988) Nerve growth factor induces a gene homologous to the glucocorticoid gene. Neuron 1:183-188

Mishra PJ, Mishra PJ, Glod JW, Banerjee D (2009) Mesenchymal stem cells: flip side of the coin. Cancer Res 69:1255-1258

Mitchell JH, Duthie SJ, Collins AR (2000) Effects of phytoestrogens on growth and DNA integrity in human prostate tumor cell lines: PC-3 and LNCaP. Nutr Cancer 38:223-228

Mitsunaga K, Araki K, Mizusaki H, Morohashi K, Haruna K, Nakagata N, Giguère V, Yamamura K, Abe K (2004) Loss of PGC-specific expression of the orphan nuclear receptor ERR-β results in reduction of germ cell number in mouse embryos. Mech Dev 121:237-246

Mix KS, Attur MG, Al-Mussawir H, Abramson SB, Brinckerhoff CE, Murphy EP (2007) Transcriptional repression of matrix metalloproteinase gene expression by the orphan nuclear receptor NURR1 in cartilage. J Biol Chem 282:9492-9504

Miyakoshi J, Tsukada T, Tachiiri S, Bandoh S, Yamaguchi K, Takebe H (1998) Enhanced NOR-1 gene expression by exposure of Chinese hamster cells to high-density 50 Hz magnetic fields. Mol Cell Biochem 181:191-195

Monaghan H, Bubb VJ, Sirimujalin R, Millward-Sadler SJ, Salter DM (2001) Adenomatous polyposis coli (APC), β-catenin, and cadherin are expressed in human bone and cartilage. Histopathology 39:611-619

Moore LB, Goodwin B, Jones SA, Wisely GB, Serabjit-Singh CJ, Willson TM, Collins JL, Kliewer SA (2000) St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. Proc Natl Acad Sci USA 97:7500-7502

Moras D, Gronemeyer H (1998) The nuclear receptor ligand-binding domain:structure and function. Curr Opin Cell Biol 10:384-391

Mulholland DJ, Dedhar S, Coetzee GA, Nelson CC (2005) Interaction of nuclear receptors with the Wnt/β-Catenin/Tcf signaling axis: Wnt you like to know? Endocr Rev 26:898-915

Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S, Lindhout D, Cole WG, Henn W, Knoll JH, Owen MJ, Mertelsmann R, Zabel BU, Olsen BR (1997) Mutations involving the transcriptional factor CBFA1 cause cleidocranial dysplasia. Cell 89:773-779

Mundy GR (2002) Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer 2:584-593

Murphy EP, Conneely OM (1997) Neuroendocrine regulation of the hypothalamic pituitary adrenal axis by the nurr1/nur77 subfamily of nuclear receptors. Mol Endocrinol 11:39-47

Murshed M, Schinke T, McKee MD, Karsenty G (2004) Extracellular matrix mineralization is regulated locally; different roles of two gla-containing proteins. J Cell Biol 165:625-630

Mäkelä EA (2001) Luutuumorit. Duodecim 117:2205-2214

Mödder UI, Sanyal A, Kearns AE, Sibonga JD, Nishihara E, Xu J, O'Malley BW, Ritman EL, Riggs BL, Spelsberg TC, Khosla S (2004) Effects of loss of steroid receptor coactivator-1 on the skeletal response to estrogen in mice. Endocrinology 145:913-921

Nakamura T, Imai Y, Matsumoto T, Sato T, Takeuchi K, Igarashi K, Harada Y, Azuma Y, Krust A, Yamamoto Y, Nishina H, Takeda S, Takayanagi H, Metzger D, Kanno J, Takaoka K, Martin TJ, Chambon P, Kato S (2007) Estrogen prevents bone loss via estrogen receptor α and induction of Fas ligand in osteoclasts. Cell 130:811-823

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108:17-29

Narisawa S, Fröhlander N, Millán JL (1997) Inactivation of two mouse alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. Dev Dyn 208:432-446

Nervina JM, Magyar CE, Pirih FQ, Tetradis S (2006) PGC-1α is induced by parathyroid hormone and coactivates Nurr1-mediated promoter activity in osteoblasts. Bone 39:1018-1025

Nicks KM, Fowler TW, Gaddy D (2010) Reproductive hormones and bone. Curr Osteoporos Rep 8:60-67

Nicks KM, Perrien DS, Akel NS, Suva LJ, Gaddy D (2009) Regulation of osteoblastogenesis and osteoclastogenesis by the other reproductive hormones, Activin and Inhibin. Mol Cell Endocrinol 310:11-20

Nie Y, Wong C (2009) Suppressing the activity of ERRα in 3T3-L1 adipocytes reduces mitochondrial biogenesis but enhances glycolysis and basal glucose uptake. J Cell Mol Med 13:3051-3060

Ning H, Yang F, Jiang M, Hu L, Feng K, Zhang J, Yu Z, Li B, Xu C, Li Y, Wang J, Hu J, Lou X, Chen H (2008) The correlation between cotransplantation of mesenchymal stem cells and higher recurrence rate in hematologic malignancy patients: outcome of a pilot clinical study. Leukemia 22:593-599

Nomiyama T, Nakamachi T, Gizard F, Heywood EB, Jones KL, Ohkura N, Kawamori R, Conneely OM, Bruemmer D (2006) The NR4A orphan nuclear receptor NOR1 is induced by platelet-derived growth factor and mediates vascular smooth muscle cell proliferation. J Biol Chem 281:33467-33476

Nonogaki K, Kaji T, Ohba Y, Sumii S, Wakameda M, Tamari T (2009) Serotonin 5-HT2C receptor-independent expression of hypothalamic NOR1, a novel modulator of food intake and energy balance, in mice. Biochem Biophys Res Commun 386:311-315

Noy N (2007) Ligand specificity of nuclear hormone receptors: sifting through promiscuity. Biochemistry 46:13461-13467

Ohkura N, Hijikuro M, Yamamoto A, Miki K (1994) Molecular cloning of a novel thyroid/steroid receptor superfamily gene from cultured rat neuronal cells. Biochem Biophys Res Commun 205:1959-1965

Oita RC, Mazzatti DJ, Lim FL, Powell JR, Merry BJ (2009) Whole-genome microarray analysis identifies up-regulation o Nr4a nuclear receptors in muscle and liver from diet-restricted rats. Mech Ageing Dev 130:240-247

Okazaki R, Inoue D, Shibata M, Saika M, Kido S, Ooka H, Tomiyama H, Sakamoto Y, Matsumoto T (2002) Estrogen promotes early osteoblast differentiation and inhibits adipocyte differentiation in mouse bone marrow stromal cell lines that express estrogen receptor (ER) α or β. Endocrinology 143:2349-2356

Ordentlich P, Yan Y, Zhou S, Heyman RA (2003) Identification of the antineoplastic agent 6-mercaptopurine as an activator of the orphan nuclear receptor Nurr1. J Biol Chem 24791-24799

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89:765-771

Pálmer HG, González-Sancho JM, Espada J, Berciano MT, Puig I, Baulida J, Quintanilla M, Cano A, de Herreros AG, Lafarga M, Munoz A (2001) Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β-catenin signaling. J Cell Biol 154:369-387

Panagopoulos I, Mencinger M, Dietrich CU, Bjerkehagen B, Saeter G, Mertens F, Mandahl N, Heim S (1999) Fusion of the RBP56 and CHN genes in extraskeletal myxoid chondrosarcomas with translocation t(9;17)(q22;q11). Oncogene 18:7594-7598

Papachristou DJ, Papachroni KK, Basdra EK, Papavassiliou AG (2009) Signaling networks and transcription factors regulating mechanotransduction in bone. Bioessays 31:794-804

Papotti M, Kalebic T, Volante M, Chiusa L, Bacillo E, Cappia S, Lausi P, Novello S, Borasio P, Scagliotti GV (2006) Bone sialoprotein is predictive of bone metastases in resectable non-small-cell lung cancer: a retrospective case-control study. J Clin Oncol 24:4818-4824

Parikka V, Peng Z, Hentunen T, Risteli J, Elo T, Väänänen HK, Härkönen P (2005) Estrogen responsiveness of bone formation *in vitro* and altered bone phenotype in aged estrogen receptor-α-deficient male and female mice. Eur J Endocrinol 152:301-314

Park YY, Kim SH, Kim YJ, Kim SY, Lee TH, Lee IK, Park SB, Choi HS (2007) Polo-like kinase 2 gene expression is regulated by the orphan nuclear receptor estrogen receptor-related receptor γ (ERRγ). Biochem Biophys Res Commun 362:107-113

Parkes D, Rivest S, Lee S, Rivier C, Vale W (1993) Corticotropin-releasing factor activates c-fos, NGFI-B, and corticotropin-releasing factor gene expression within the paraventricular nucleus of the rat hypothalamus. Mol Endocrinol 7:1357-1367

Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM (1999) Bile acids: natural ligands for an orphan nuclear receptor. 284:1365-1368

Pearen MA, Myers SA, Raichur S, Ryall JG, Lynch GS, Muscat GE (2008) The orphan nuclear receptor, NOR-1, a target of β-adrenergic signaling, regulates gene expression that controls oxidative metabolism in skeletal muscle. Endocrinology 149:2853-2865

Pei L, Castrillo A, Chen M, Hoffmann A, Tontonoz P (2005) Induction of NR4A orphan nuclear receptor expression in macrophages in response to inflammatory stimuli. J Biol Chem 280:29256-29262

Pei L, Waki H, Vaitheesvaran B, Wilpitz DC, Kurland IJ, Tontonoz P (2006) NR4A orphan nuclear receptors are transcriptional regulators of hepatic glucose metabolism. Nat Med 12:1048-1055

Pekarsky Y, Hallas C, Palamarchuk A, Koval A, Bullrich F, Hirata Y, Bichi R, Letofsky J, Croce CM (2001) Akt phosphorylates and regulates the orphan nuclear receptor Nur77. Proc Acad Sci USA 98:3690-3694

Perissi V, Rosenfeld MG (2005) Controlling nuclear receptors: the circular logic of cofactor cycles. Nat Rev Mol Cell Biol 6:542-554

Perlmann T, Jansson L (1995) A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. Genes Dev 9:769-782

Perrien DS, Akel NS, Edwards PK, Carver AA, Bendre MS, Swain FL, Skinner RA, Hogue WR, Nicks KM, Pierson TM, Suva LJ, Gaddy D (2007) Inhibin A is an endocrine stimulator of bone mass and strength. Endocrinology 148:1654-1665

Philips A, Lesage S, Gingras R, Maira MH, Gauthier Y, Hugo P, Drouin J (1997) Novel dimeric Nur77 signaling mechanism in endocrine and lymphoid cells. Mol Cell Biol 17:5946-5951

Pirih FQ, Aghaloo TL, Bezouglaia O, Nervina JM, Tetradis S (2005) Parathyroid hormone induces the NR4A family of nuclear orphan receptors *in vivo*. Biochem Biophys Res Commun 332:494-503

Pirih FQ, Nervina JM, Pham L, Aghaloo T, Tetradis S (2003) Parathyroid hormone induces the nuclear orphan receptor NOR-1 in osteoblasts. Biochem Biophys Res Commun 306:144-150

Pirih FQ, Tang A, Ozkurt IC, Nervina JM, Tetradis S (2004) Nuclear orphan receptor Nurr1 directly transactivates the osteocalcin gene in osteoblasts. J Biol Chem 279:53167-53174

Pissios P, Tzameli I, Kushner P, Moore DD (2000) Dynamic stabilization of nuclear receptor ligand binding domains by hormone or corepressor binding. Mol Cell 6:245-253

Pols TW, Ottenhoff R, Vos M, Levels JH, Quax PH, Meijers JC, Pannekoek H, Groen AK, de Vries CJ (2008) Nur77 modulates hepatic lipid metabolism through suppression of SREBP1c activity. Biochem Biophys Res Commun 366:910-916

Pönniö T, Conneely OM (2004) Nor-1 regulates hippocampal axon guidance, pyramidal cell survival, and seizure susceptibility. Mol Cell Biol 24:9070-9078

Qu Q, Perälä-Heape M, Kapanen A, Dahllund J, Salo J, Väänänen HK, Härkönen P (1998) Estrogen enhances differentiation of osteoblasts in mouse bone marrow culture. Bone 22:201-209

Raisz LG (2005) Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J Clin Invest 115:3318-3325

Rangwala SM, Li X, Lindsley L, Wang X, Shaughnessy S, Daniels TG, Szustakowski J, Nirmala NR, Wu Z, Stevenson SC (2007) Estrogen-related receptor α is essential for the expression of antioxidant protection genes and mitochondrial function. Biochem Biophys Res Commun 357:231-236

Rangwala SM, Wang X, Calvo JA, Lindsley L, Zhang Y, Deyneko G, Beaulieu V, Gao J, Turner G, Markovits J (2010) Estrogen-related receptor γ is a key regulator of muscle mitochondrial activity and oxidative capacity. J Biol Chem 285:22619-22629

Reinhold MI, Naski MC (2007) Direct interactions of Runx2 and canonical Wnt signaling induce FGF18. J Biol Chem 282:3653-3663

Renaud JP, Moras D (2000) Structural studies on nuclear receptors. Cell Mol Life Sci 57:1748-1769

Ribeiro RCJ, Kushner PJ, Baxter JD (1995) The nuclear hormone receptor gene superfamily. Annu Rev Med 46:443-453

Riggins RB, Lan JP, Zhu Y, Klimach U, Zwart A, Cavalli LR, Haddad BR, Chen L, Gong T, Xuan J, Ethier SP, Clarke R (2008) ERRγ mediates tamoxifen resistance in novel models of invasive lobular breast cancer. Cancer Res 68:8908-8917

Rittling SR, Matsumoto HN, McKee MD, Nanci A, An XR, Novick KE, Kowalski AJ, Noda M, Denhardt DT (1998) Mice lacking osteopontin show normal development and bone structure but display altered osteoclast formation *in vitro*. J Bone Miner Res 13:1101-1111

Robinson JA, Harris SA, Riggs BL, Spelsberg TC (1997) Estrogen regulation of human osteoblastic cell proliferation and differentiation. Endocrinology 138:2919-2927

Robinson SN, Ng J, Niu T, Yang H, McMannis JD, Karandish S, Kaur I, Fu P, Del Angel M, Messinger R, Flagge F, de Lima M, Decker W, Xing D, Champlin R, Shpall EJ (2006) Superior *ex vivo* cord blood expansion following co-culture with bone marrow-derived mesenchymal stem cells. Bone Marrow Transplant 37:359-366

Rosen ED, MacDougald OA (2006) Adipocyte differentiation from the inside out. Nat Rev Mol Cell Biol 7:885-896

Ross SE, Hemati N, Longo KA, Bennett CN, Lucas PC, Erickson RL, MacDougald OA (2000) Inhibition of adipogenesis by Wnt signaling. Science 289:950-953

Rudland PS, Platt-Higgins A, El-Tanani M, De Silva Rudland S, Barraclough R, Winstanley JH, Howitt R, West CR (2002) Prognostic significance of the metastasis-associated protein osteopontin in human breast cancer. Cancer Res 62:3417-3427

Sanyal S, Kim JY, Kim HJ, Takeda J, Lee YK, Moore DD, Choi HS (2002) Differential regulation of the orphan nuclear receptor small heterodimer partner (SHP) gene promoter by orphan nuclear receptor ERR isoforms. J Biol Chem 277:1739-1748

Sato M, Morii E, Komori T, Kawahata H, Sugimoto M, Terai K, Shimizu H, Yasui T, Ogihara H, Yasui N, Ochi T, Kitamura Y, Ito Y, Nomura S (1998) Transcriptional regulation of osteopontin gene *in vivo* by PEBP2α/CBFA1 and ETS1 in the skeletal tissues. Oncogene 17:1517-1525

Sato S, Hanada R, Kimura A, Abe T, Matsumoto T, Iwasaki M, Inose H, Ida T, Mieda M, Takeuschi Y, Fukumoto S, Fujita T, Kato S, Kangawa K, Kojima M, Shinomiya K, Takeda S (2007) Central control of bone remodeling by neuromedin U. Nat Med 13:1234-1240

Satoh J, Nakanishi M, Koike F, Miyake S, Yamamoto T, Kawai M, Kikuchi S, Nomura K, Yokoyama K, Ota K, Kanda T, Fukazawa T, Yamamura T (2005) Microarray analysis identifies an aberrant expression of apoptosis and DNA damage-regulatory genes in multiple sclerosis. Neurobiol Dis 18:537-550

Schreiber SN, Knutti D, Brogli K, Uhlmann T, Kralli A (2003) The transcriptional coactivator PGC-1 regulates the expression and activity of the orphan nuclear receptor estrogen-related receptor α (ERRα). J Biol Chem 278:9013-9018

Shi Y (2007) Orphan nuclear receptors in drug discovery. Drug Discov Today 12:440-445

Shi YC, Worton L, Esteban L, Baldock P, Fong C, Eisman JA, Gardiner EM (2007) Effects of continuous activation of vitamin D and Wnt response pathways on osteoblastic proliferation and differentiation. Bone 41:87-96

Sihag S, Cresci S, Li AY, Sucharov CC, Lehmann JJ (2009) PGC-1α and ERRα target gene downregulation is a signature of the failing human heart. J Moll Cell Cardiol 46:201-212

Sims NA, Dupont S, Krust A, Clement-Lacroix P, Minet D, Resche-Rigon M, Gaillard-Kelly M, Baron R (2002) Deletion of estrogen receptors reveals a regulatory role for estrogen receptors-β in bone remodeling in females but not in males. Bone 30:18-25

Sjögren H, Meis-Kindblom J, Kindblom LG, Aman P, Stenman G (1999) Fusion of the EWS-related gene TAF2N to TEC in extraskeletal myxoid chondrosarcoma. Cancer Res 59:5064-5067

Sjögren H, Wedell B, Meis-Kindblom JM, Kindblom LG, Stenman G (2000) Fusion of the NH2-terminal domain of the basic helix-loop-helix protein TCF12 to TEC in extraskeletal myxoid chondrosarcoma with translocation t(9;15)(q22;q21). Cancer Res 60:6832-6835

Sladek R, Bader JA, Giguère V (1997) The orphan nuclear receptor estrogen-related receptor α is a transcriptional regulator of the human medium-chain acyl coenzyme A dehydrogenase gene. Mol Cell Biol 17:5400-5409

Sobacchi C, Frattini A, Guerrini MM, Abinun M, Pangrazio A, Susani L, Bredius R, Mancini G, Cant A, Bishop N, Grabowski P, Del Fattore A, Messina C, Errigo G, Coxon FP, Scott DI, Teti A, Rogers MJ, Vezzoni P, Villa A, Helfrich MH (2007) Osteoclast-poor human osteopetrosis due to mutations in the gene encoding RANKL. Nat Genet 39:960-962

Song KH, Park JI, Lee MO, Soh J, Lee K, Choi HS (2001) LH induces orphan nuclear receptor Nur77 gene expression in testicular Leydig cells. Endocrinology 142:5116-5123

Song KH, Park YY, Park KC, Hong CY, Park JH, Shong M, Lee K, Choi HS (2004) The atypical orphan nuclear receptor DAX-1 interacts with orphan nuclear receptor Nur77 and represses its transactivation. Mol Endocrinol 18:1929-1940

Sonoda J, Pei L, Evans RM (2008) Nuclear receptors: decoding metabolic disease. FEBS Lett. 582:2-9

Sooy K, Sabbagh Y, Demay MB (2005) Osteoblasts lacking the vitamin D receptor display enhanced osteogenic potential *in vitro*. J Cell Biochem 94:81-87

Sottile V, Seuwen K (2000) Bone morphogenetic protein-2 stimulates adipogenic diferentiation of mesenchymal precursor cells in synergy with BRL 49653 (rosiglitazone). FEBS Lett 475:201-204

Stambolic V, Ruel L, Woodgett JR (1996) Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. Curr Biol 6:1664-1668

Stocco CO, Zhong L, Sugimoto Y, Ichikawa A, Lau LF, Gibori G (2000) Prostaglandin F_2α-induced expression of 20α-hydroxysteroid dehydrogenase involves the transcription factor NUR77. J Biol Chem 275:37202-37211

Suetsugi M, Su L, Karlsberg K, Yuan YC, Chen S (2003) Flavone and isoflavone phytoestrogens are agonists of estrogen-related receptors. Mol Cancer Res 1:981-991

Sumi D, Ignarro LJ (2003) Estrogen-related receptor α1 up-regulates endothelial nitric oxide synthase expression. Proc Natl Acad Sci USA 100:14451-14456

Sun L, Peng Y, Sharrow AC, Iqbal J, Zhang Z, Papachristou DJ, Zaidi S, Zhu LL, Yaroslavskiy BB, Zhou H, Zallone A, Sairam MR, Kumar TR, Bo W, Braun J, Cardoso-Landa L, Schaffler MB, Moonga BS, Blair HC, Zaidi M (2006) FSH directly regulates bone mass. Cell 125:247-260

Sun P, Sehouli J, Denkert C, Mustea A, Könsgen D, Koch I, Wei L, Lichtenegger W (2005) Expression of estrogen receptor-related receptors, a subfamily of orphan nuclear receptors, as new tumor biomarkers in ovarian cancer cells. J Mol Med 83:457-467

Suzuki T, Miki Y, Moriya T, Shimada N, Ishida T, Hirakawa H, Ohuchi N, Sasano H (2004) Estrogen-related receptor α in human breast carcinoma as a potent prognostic factor. Cancer Res 64:4670-4676

Takayanagi S, Tokunaga T, Liu X, Okada H, Matsushima A, Shimohigashi Y (2006) Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor γ (ERRγ) with high constitutive activity. Toxicol Lett 167:95-105

Takeda S (2008) Central control of bone remodeling. J Neuroendocrinol 20:802-807

Takeda S, Yoshizawa T, Nagai Y, Yamato H, Fukumoto S, Sekine K, Kato S, Matsumoto T, Fujita T (1999) Stimulation of osteoclast formation by 1,25-dihydroxyvitamin D requires its binding to vitamin D receptor (VDR) in osteoblastic cells: studies using VDR knockout mice. Endocrinology 140:1005-1008

Tang QQ, Otto TC, Lane MD (2004) Commitment of C3H10T1/2 pluripotent stem cells to the adipocyte lineage. Proc Natl Acad Sci USA 101:9607-9611

Tetradis S, Bezouglaia O, Tsingotjidou A (2001a) Parathyroid hormone induces expression of the nuclear orphan receptor Nurr1 in bone cells. Endocrinology 142:663-670

Tetradis S, Bezouglaia O, Tsingotjidou A, Vila A (2001b) Regulation of the nuclear orphan receptor Nur77 in bone by parathyroid hormone. Biochem Biophys Res Commun 281:913-916

Teyssier C, Gallet M, Rabier B, Monfoulet L, Dine J, Macari C, Espallergues J, Horard B, Giguère V, Cohen-Solal M, Chassande O, Vanacker JM (2009) Absence of ERRα in female mice confers resistance to bone loss induced by age or estrogen-deficiency. PLoS One 4:e7942

Thomas DM, Carty SA, Piscopo DM, Lee JS, Wang WF, Forrester WC, Hinds PW (2001) The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol Cell 8:303-316

Thurner PJ, Chen CG, Ionova-Martin S, Sun L, Harman A, Porter A, Ager JW 3rd, Ritchie RO, Alliston T (2010) Osteopontin deficiency increases bone fragility but preserves bone mass. Bone 46:1564-1573

Tikkanen J, Leskelä HV, Lehtonen ST, Vähäsarja V, Melkko J, Ahvenjärvi L, Pääkkö E, Väänänen K, Lehenkari P (2010) Attempt to treat congenital pseudoarthrosis of the tibia with mesenchymal stromal cell transplantation. Cytotherapy 12:593-604

Tolar J, Nauta AJ, Osborn MJ, Panoskaltsis Mortari A, McElmurry RT, Bell S, Xia L, Zhou N, Riddle M, Schroeder TM, Westendorf JJ, McIvor RS, Hogendoorn PC, Szuhai K, Oseth L, Hirsch B, Yant SR, Kay MA, Peister A, Prockop DJ, Fibbe WE, Blazar BR (2007) Sarcoma derived from cultured mesenchymal stem cells. Stem Cells 25:371-379

Tontonoz P, Hu E, Spiegelman BM (1994) Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. Cell 79:1147-1156

Tortora GJ, Grabowski SR (2000) Principles of anatomy and physiology 9th edition, The skeletal system: Bone tissue, pp. 160-182, John Wiley & Sons, Inc., USA.

Tremblay AM, Giguère V (2007) The NR3B subgroup: an ovERRview. Nucl Recept Signal 5:e009

Tremblay AM, Wilson BJ, Yang XJ, Giguère V (2008) Phosphorylation-dependent sumoylation regulates estrogen-related receptor-α and -γ transcriptional activity through a synergy control motif. Mol Endocrinol 22:570-584

Tremblay GB, Bergeron D, Giguère V (2001a) 4-Hydroxytamoxifen is an isoform-specific inhibitor of orphan estrogen-receptor-related (ERR) nuclear receptors β and γ. Endocrinology 142:4572-4575

Tremblay GB, Kunath T, Bergeron D, Lapointe L, Champigny C, Bader JA, Rossant J, Giguère V (2001b) Diethylstilbestrol regulates trophoblast stem cell differentiation as a ligand of orphan nuclear receptor ERRβ. Genes Dev 15:833-838

Truica CI, Byers S, Gelmann EP (2000) β-catenin affects androgen receptor transcriptional activity and ligand specificity. Cancer Res 60:4709-4713

Uccelli A, Moretta L, Pistoia V (2008) Mesenchymal stem cells in health and disease. Nat Rev Immunol 8:726-736

Valta MP, Hentunen T, Qu Q, Valve EM, Harjula A, Seppänen JA, Väänänen HK, Härkönen PL (2006) Regulation of osteoblast differentiation: a novel function for fibroblast growth factor 8. Endocrinology 147:2171-2182

Vanacker JM, Delmarre C, Guo X, Laudet V (1998) Activation of the osteopontin promoter by the orphan nuclear receptor estrogen receptor related α. Cell Growth Differ. 9 1007-1014

Vanacker JM, Pettersson K, Gustafsson J, Laudet V (1999) Transcriptional targets shared by estrogen receptor-related recetors (ERRs) and estrogen receptor (ER) α, but not by ERβ. EMBO J 18:4270-4279

van den Berg DLC, Zhang W, Yates A, Engelen E, Takacs K, Bezstarosti K, Demmers J, Chambers I, Poot RA (2008) Estrogen-related receptor β interacts with Oct4 to positively regulate Nanog gene expression. Mol Cell Biol 28:5986-5995

Vico L, Vanacker JM (2010) Sex hormones and their receptors in bone homeostasis: insights from genetically modified mouse models. Osteoporos Int 21:365-372

Viguet-Carrin S, Garnero P, Delmas PD (2006) The role of collagen in bone strength. Osteoporos Int 17:319-336

Villena JA, Hock MB, Chang WY, Barcas JE, Giguère V, Kralli A (2007) Orphan nuclear receptor estrogen-related receptor α is essential for adaptive thermogenesis. Proc Natl Acad Sci USA 104:1418-1423

Virk MS, Lieberman JR (2007) Tumor metastasis to bone. Arthritis Res Ther 9:S5

Visser WE, Friesema EC, Jansen J, Visser TJ (2008) Thyroid hormone transport in and out of cells. Trends Endocrinol Metab 19:50-56

Vu EH, Kraus RJ, Mertz JE (2007) Phosphorylation-dependent sumoylation of estrogen-related receptor α1. Biochemistry 46:9795-9804

Wagner W, Roderburg C, Wein F, Diehlmann A, Frankhauser M, Schubert R, Eckstein V, Ho AD (2007) Molecular and secretory profiles of human mesenchymal stromal cells and their abilities to maintain primitive hematopoietic progenitors. Stem Cells 25:2638-2647

Waltregny D, Bellahcène A, Van Riet I, Fisher LW, Young M, Fernandez P, Dewé W, de Leval J, Castronovo V (1998) Prognostic value of bone sialoprotein expression in clinically localized human prostate cancer. J Natl Cancer Inst 90:1000-1008

Wang A, Rud J, Olson CM Jr, Anguita J, Osborne BA (2009a) Phosphorylation of Nur77 by the MEK-ERK-RSK cascade induces mitochondrial translocation and apoptosis in T cells. J Immunol 183:3268-3277

Wang H, Chen J, Hollister K, Sowers LC, Forman BM (1999) Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. Mol Cell 3:543-553

Wang J, Fang F, Huang Z, Wang Y, Wong C (2009b) Kaempferol is an estrogen-related receptor α and γ inverse agonist. FEBS Lett 583:643-647

Wang L, Li Y, Hu P, Teng CT (2008) PGC-1α induces dynamic protein interactions on the ERRα gene multi-hormone response element nucleosome in kidney cells. Biochem J 416:407-419.

Wang L, Zuercher WJ, Consler TG, Lambert MH, Miller AB, Orband-Miller LA, McKee DD, Willson TM, Nolte RT (2006a) X-ray crystal structures of the estrogen-related receptor-γ ligand binding domain in three functional states reveal the molecular basis of small molecule regulation. J Biol Chem 281:37773-37781

Wang SC, Myers S, Dooms C, Capon R, Muscat GE (2010a) An ERRβ/γ agonist modulates GRα expression, and glucocorticoid responsive gene expression in skeletal muscle cells. Mol Cell Endocrinol 315:146-152

Wang X, Kua HY, Hu Y, Guo K, Zeng Q, Wu Q, Ng HH, Karsenty G, de Crombrugghe B, Yeh J, Li B (2006b) p53 functions as a negative regulator of osteoblastogenesis, osteoblast-dependent osteoclastogenesis, and bone remodeling. J Cell Biol 172:115-125

Wang Z, Benoit G, Liu J, Prasad S, Aarnisalo P, Liu X, Xu H, Walker NP, Perlmann T (2003) Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. Nature 423:555-560

Wang Y, Fang F, Wong CW (2010b) Troglitazone is an estrogen-related receptor α and γ inverse agonist. Biochem Pharmacol 80:80-85

Wansa KD, Harris JM, Muscat GE (2002) The activation function-1 domain of Nur77/NR4A1 mediates trans-activation, cell specificity, and co-activator recruitment. J Biol Chem 277:33001-33011

Wansa KD, Harris JM, Yan G, Ordentlich P, Muscat GE (2003) The AF-1 domain of the orphan nuclear receptor NOR-1 mediates trans-activation, coactivator recruitment, and activation by the purine anti-metabolite 6-mercaptopurine. J Biol Chem 278:24776-24790

Watanabe A, Kinoshita Y, Hosokawa K, Mori T, Yamaguchi T, Honjo H (2006) Function of estrogen-related receptor α in human endometrial cancer. J Clin Endocrinol Metab 91:1573-1577

Waters KM, Rickard DJ, Riggs BL, Khosla S, Katzenellenbogen JA, Katzenellenbogen BS, Moore J, Spelsberg TC (2001) Estrogen regulation of human osteoblast function is determined by stage of differentiation and the estrogen receptor isoform. J Cell Biochem 83:448-462

Wei W, Wang X, Yang M, Smith LC, Dechow PC, Sonoda J, Evans RM, Wan Y (2010) PGC1β mediates PPARγ activation of osteoclastogenesis and rosiglitazone-induced bone loss. Cell Metab 11:503-516

Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC (1998) Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Invest 102:274-282

Welt CK, McNicholl DJ, Taylor AE, Hall JE (1999) Female reproductive aging is marked by decreased secretion of dimeric inhibin. J Clin Endocrinol Metab 84:105-111

Wennberg C, Hessle L, Lundberg P, Mauro S, Narisawa S, Lerner UH, Millán JL (2000) Functional characterization of osteoblasts and osteoclasts from alkaline phosphatase knockout mice. J Bone Miner Res 15:1879-1888

Werme H, Hermanson E, Carmine A, Buervenich S, Zetterström RH, Thorén P, Ogren SO, Olson L, Perlmann T, Brené S (2003) Decreased ethanol preference and wheel running in Nurr1-deficient mice. Eur J Neurosci 17:2418-2424

Wesseling-Perry K (2010) FGF-23 in bone biology. Pediatr Nephrol 25:603-608

Westendorf JJ, Kahler RA, Schroeder TM (2004) Wnt signaling in osteoblasts and bone diseases. Gene 341:19-39

Whyte MP (2010) Physiological role of alkaline phosphatase explored in hypophosphatasia. Ann N Y Acad Sci 1192:190-200

Whyte MP, Obrecht SE, Finnegan PM, Jones JL, Podgornik MN, McAlister WH, Mumm S (2002) Osteoprotegerin deficiency and juvenile Paget's disease. N Engl J Med 347:175-184

Wierman ME (2007) Sex steroid effects at target tissues: mechanism of action. Adv Physiol Educ 31:26-33

Willy PJ, Murray IR, Qian J, Busch BB, Stevens WC Jr, Martin R, Mohan R, Zhou S, Ordentlich P, Wei P, Sapp DW, Horlick RA, Heyman RA, Schulman IG (2004) Regulation of PPARγ coactivator 1α (PGC-1α) signaling by an estrogen-related receptor α (ERRα) ligand. Proc Natl Acad Sci USA 101:8912-8917

Wilson TE, Fahrner TJ, Johnston M, Milbrandt J (1991) Identification of the DNA binding site for NGFI-B by genetic selection in yeast. Science 252:1296-1300

Wilson TE, Fahrner TJ, Milbrandt J (1993a) The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. Mol Cell Biol 13:5794-5804

Wilson TE, Mouw AR, Weaver CA, Milbrandt J, Parker KL (1993b) The orphan nuclear receptor NGFI-B regulates expression of the gene encoding steroid 21-hydroxylase. Mol Cell Biol 13:861-868

Windahl SH, Hollberg K, Vidal O, Gustafsson JÅ, Ohlsson C, Andersson G (2001) Female estrogen receptor β (-/-) mice are partially protected against age-related trabecular bone loss. J Bone Miner Res 16:1388-1398

Windahl SH, Vidal O, Andersson G, Gustafsson JÅ, Ohlsson C (1999) Increased cortical bone mineral content but unchanged trabecular bone mineral density in female ERβ(-/-) mice. J Clin Invest 104:895-901

Woronicz JD, Calnan B, Ngo V, Winoto A (1994) Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas. Nature 367:277-281

Wu H, Lin Y, Li W, Sun Z, Gao W, Zhang H, Xie L, Jiang F, Qin B, Yan T, Chen L, Zhao Y, Cao X, Wu Y, Lin B, Zhou H, Wong AS, Zhang XK, Zeng JZ (2011) Regulation of Nur77 expression by β-catenin and its mitogenic effect in colon cancer cells. FASEB J 25:192-205

Xiao G, Jiang D, Gopalakrishnan R, Franceschi RT (2002) Fibroblast growth factor 2 induction of the osteocalcin gene requires MAPK activity and phosphorylation of the osteoblast transcription factor, Cbfa1/Runx2. J Biol Chem 277:36181-36187

Xiao JH, Ghosn C, Hinchman C, Forbes C, Wang J, Snider N, Cordrey A, Zhao Y, Chandraratna RA (2003) Adenomatous polyposis coli (APC)-independent regulation of β-catenin degradation via a retinoid X receptor-mediated pathway. J Biol Chem 278:29954-29962

Xie W, Hong H, Yang NN, Lin RJ, Simon CM, Stallcup MR, Evans RM (1999) Constitutive activation of transcription and binding of coactivator by estrogen-related receptors 1 and 2. Mol Endocrinol 13:2151-2162

Xie YB, Park JH, Kim DK, Hwang JH, Oh S, Park SB, Shong M, Lee IK, Choi HS (2009) Transcriptional corepressor SMILE recruits SIRT1 to inhibit nuclear receptor estrogen receptor-related receptor γ transactivation. J Biol Chem 284:28762-28774

Yang C, Chen S (1999) Two organochlorine pesticides, toxaphene and chlordane, are antagonists for estrogen-related receptor α-1 orphan receptor. Cancer Res 59:4519-4524

Yang C, Zhou D, Chen S (1998) Modulation of aromatase expression in the breast tissue by ERRα-1 orphan rerceptor. Cancer Res 58:5695-5700

Yang N, Shigeta H, Shi H, Teng CT (1996) Estrogen-related receptor, hErr1, modulates estrogen receptor-mediated response of human lactoferrin gene promoter. J Biol Chem 271:5795-5804

Yoshida CA, Furuichi T, Fujita T, Fukuyama R, Kanatani N, Kobayashi S, Satake M, Takada K, Komori T (2002) Core-binding factor β interacts with Runx2 and is required for skeletal development. Nat Genet 32:633-638

Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD (1990) The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature 345:442-444

Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T, Kato S (1997) Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nat Genet 16:391-396

Yu DD, Forman BM (2005) Identification of an agonist ligand for estrogen-related receptors ERRβ/γ. Bioorg Med Chem Lett 15:1311-1313

Yu K, Bayona W, Kallen BC, Harding HP, Ravera CP, McMahon G, Brown M, Lazar MA (1995) Differential activation of peroxisome proliferator-activated receptors by eicosanoids. J Biol Chem 270:23975-23983

Yu S, Wang X, Ng CF, Chen S, Chan FL (2007) ERRγ suppresses cell proliferation and tumor growth of androgen-sensitive and androgen-insensitive prostate cancer cells and its implication as a therapeutic target for prostate cancer. Cancer Res 67:4904-4914

Yu S, Wong YC, Wang XH, Ling MT, Ng CF, Chen S, Chan FL (2008) Orphan nuclear receptor estrogen-related receptor-β suppresses *in vitro* and *in vivo* growth of prostate cancer cells via p21(WAF1/CIP1) induction and as a potential therapeutic target in prostate cancer. Oncogene 27:3313-3328

Yuan JP, Wang JH, Liu X (2007) Metabolism of dietary soy isoflavones to equol by human intestinal microflora – implications for health. Mol Nutr Food Res 51:765-781

Zetterström RH, Solomin L, Jansson L, Hoffer BJ, Olson L, Perlmann T (1997) Dopamine neuron agenesis in Nurr1-deficient mice. Sciene 276:248-250

Zetterström RH, Solomin L, Mitsiadis T, Olson L, Perlmann T (1996a) Retinoid X receptor heterodimerization and developmental expression distinguish the orphan nuclear receptors NGFI-B, Nurr1, and Nor1. Mol Endocrinol 10:1656-1666

Zetterström RH, Williams R, Perlmann T, Olson L (1996b) Cellular expression of the immediate early transcription factors Nurr1 and NGFI-B suggests a gene regulatory role in several brain regions including the nigrostriatal dopamine system. Brain Res Mol Brain Res 41:111-120

Zhang X, Zhang Y, Wang T, Esteban MA, Pei D (2008) Esrrb activates Oct4 transcription and sustains self-renewal and pluripotency in embryonic stem cells. J Biol Chem 283:35825-35833

Zhang Z, Chen K, Shih JC, Teng CT (2006) Estrogen-related receptors-stimulated monoamine oxidase B promoter activity is down-regulated by estrogen receptors. Mol Endocrinol 20:1547-1561

Zhang Z, Teng CT (2000) Estrogen receptor-related receptor α1 interacts with coactivator and constitutively activates the estrogen response elements of the human lactoferrin gene. J Biol Chem 275:20837-20846

Zhao M, Harris SE, Horn D, Geng Z, Nishimura R, Mundy GR, Chen D (2002) Bone morphogenetic protein receptor signaling is necessary for normal murine postnatal bone formation. J Cell Biol 157:1049-1060

Zhou S, Zilberman Y, Wassermann K, Bain SD, Sadovsky Y, Gazit D (2001) Estrogen modulates estrogen receptor α and β expression, osteogenic activity, and apoptosis in mesenchymal stem cells (MSCs) of osteoporotic mice. J Cell Biochem Suppl 36:144-155

Zhou T, Cheng J, Yang P, Wang Z, Liu C, Su X, Bluethmann H, Mountz JD (1996) Inhibition of Nur77/Nurr1 leads to inefficient clonal deletion of self-reactive T cells. J Exp Med 183:1879-1892

Zhou W, Liu Z, Wu J, Liu JH, Hyder SM, Antoniou E, Lubahn DB (2006) Identification and characterization of two novel splicing isoforms of human estrogen-related receptor β. J Clin Endocrinol Metab 91:569-579

Zirngibl RA, Chan JSM, Aubin JE (2008) Estrogen receptor-related receptor α (ERRα) regulates osteopontin expression through a non-canonical ERRα response element in a cell context-dependent manner. J Mol Endocrinol 40:61-73

Zuercher WJ, Gaillard S, Orband-Miller LA, Chao EY, Shearer BG, Jones DG, Miller AB, Collins JL, McDonnell DP, Willson TM (2005) Identification and structure-activity relationship of phenolic acyl hydrazones as selective agonists for the estrogen-related orphan nuclear receptors ERRβ and ERRγ. J Med Chem 48:3107-3109