Orphan nuclear receptor subfamily NR4A – their interplay with other nuclear receptors and functions in osteoblasts

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SUMMARY

Nurr1, NGFI-B and Nor1 (NR4A2, NR4A1 and NR4A3, respectively) belong to the NR4A subfamily of nuclear receptors. The NR4A receptors are orphan nuclear receptors, which means that activating or repressing ligands for these receptors have not been found. NR4A receptors are immediate early genes; their expression is rapidly induced in response to various stimuli including growth factors and the parathyroid hormone (PTH). The studies concerning the NR4A receptors in the central nervous system have demonstrated that they have a major role in the development and function of the dopaminergic neurons of the midbrain and in regulating hypothalamus-pituitary-adrenal-axis. However, the peripheral functions of the NR4A family are largely unknown.

Cultured mouse primary osteoblasts, a preosteoblastic cell line and several osteoblastic cell lines were used to investigate the role of NR4A receptors in osteoblasts. NR4A receptors were shown to directly bind to and activate the promoter of the osteopontin gene (OPN) in osteoblastic cells, thus regulating its expression. OPN is a major bone matrix protein expressed throughout the differentiation of preosteoblastic cells into osteoblasts. The activation of the OPN promoter was shown to be dependent on the activation function-1 located in the N-terminal part of Nurr1 and to occur in both monomeric and RXR heterodimeric forms of NR4A receptors. Furthermore, PTH was shown to upregulate OPN expression through the NR4A family. It was also demonstrated that the fibroblast growth factor-8b (FGF-8b) induces the expression of NR4A receptors in osteoblasts as immediate early genes. This induction involved phosphatidylinositol-3 kinase, protein kinase C, and mitogen activated protein kinase, which are all major pathways of FGF signalling. Nurr1 and NGFI-B were shown to induce the proliferation of preosteoblastic cells and to reduce their apoptosis. FGF-8b was shown to stimulate the proliferation of osteoblastic cells through the NR4A receptors. These results suggest that NR4A receptors have a role both in the differentiation of osteoblasts and in the proliferation and apoptosis of preosteoblast.

The NR4A receptors were found to bind to the same response element on OPN as the members of the NR3B family of orphan receptors do. Experiments focussing on their joint functions on OPN promoter were conducted. NR4A receptors and NR3B receptors were overexpressed in several cell lines to study their activity in reporter assays. Mutual repression was observed between the NR4A receptors and the NR3B receptors. This repression was shown to be dependent on the DNA-binding domains of both receptor families, but to result neither from the competition of DNA binding nor from the competition for coactivators. As

the repression was dependent on the relative expression levels of the NR4As and NR3Bs, it seems likely that the ratio of the receptors mediates their activity on their response elements. Rapid induction of the NR4As in response to various stimuli and differential expression of the NR3Bs can effectively control the gene activation by the NR4A receptors.

NR4A receptors can bind DNA as monomers, and Nurr1 and NGFI-B can form permissive heterodimers with the retinoid X receptor (RXR). Permissive heterodimers can be activated with RXR agonists, unlike non-permissive heterodimers, which are formed by RXR and retinoic acid receptor or thyroid hormone receptor (RAR and TR, respectively). Non-permissive heterodimers can only be activated by the agonists of the heterodimerizing partner. The mechanisms behind differential response to RXR agonists have remained unresolved. As there are no activating or repressing ligands for the NR4A receptors, it would be important to find out, how they are regulated. This study aimed at revealing the mechanisms regulating the expression and activity of NR4A receptors and their RXR heterodimers. Chimeras created between the non-permissive RAR and the permissive Nurr1 were used in reporter assays. Permissiviness of Nurr1/RXR heterodimers was linked to the N-terminal part of Nurr1 ligand-binding domain. This region has previously been shown to mediate the interaction between NRs and corepressors. Non-permissive RAR and TR, permissive Nurr1 and NGFI-B, and RXR were overexpressed with corepressors silencing mediator for retinoic acid and thyroid hormone receptors (SMRT), and with nuclear receptor corepressor in several cell lines. Nurr1 and NGFI-B were found to be repressed by SMRT. The interaction of RXR heterodimers with corepressors was weak in permissive heterodimers and much stronger in non-permissive heterodimers. Non-permissive heterodimers also released corepressors only in response to the agonist of the heterodimeric partner of RXR. In the permissive Nurr1/RXR heterodimer, however, SMRT was released following the treatment with RXR agonists. Corepressor release in response to ligands was found to differentiate permissive heterodimers from non-permissive ones. Corepressors were thus connected to the regulation of NR4A functions.

In summary, the studies presented here linked the NR4A family of orphan nuclear receptors to the regulation of osteoblasts. Nurr1 and NGFI-B were found to control the proliferation and apoptosis of preosteoblasts. The studies also demonstrated that cross-talk with the NR3B receptors controls the activity of these orphan receptors. The results clarified the mechanism of permissiviness of RXR-heterodimers. New information was obtained on the regulation and functions of NR4A receptors, for which the ligands are unknown.

ORIGINAL PUBLICATIONS

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

II Lammi J, Rajalin AM, Huppunen J, Aarnisalo P. Cross-talk between the NR3B and NR4A families of orphan nuclear receptors (2007). *Biochemical and Biophysical Research Communications* 359:391-397.

III Lammi J, Perlmann T, Aarnisalo P. Corepressor interaction differentiates the permissive and non-permissive retinoid X receptor heterodimers (2008). *Archives of Biochemistry and Biophysics* 472:105-114.

IV Lammi J, Aarnisalo P. FGF-8 Stimulates the Expression of NR4A Orphan Nuclear Receptors in Osteoblasts (2008). *Molecular and Cellular Endocrinology.* In press.

Publication I also appears in the thesis of Johanna Hirvonen (2008).

ABBREVIATIONS

ACTH	adrenocorticotropic hormone
AF-1	activation function 1
AF-2	activation function 2
ALP	alkaline phosphatase
AR	androgen receptor
CBP	CREB-binding protein
CNS	central nervous system
Col1A1	collagen type I alpha 1
CTE	c-terminal extension
DBD	DNA-binding domain
DR	direct repeat
ER	estrogen receptor
ERE	estrogen response element
ERR	estrogen-related receptor
ERRE	ERR response element
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FXR	farnesoid X receptor
GR	glucocorticoid receptor
GST	glutathione S-transferase
HAT	histone acetyl transferase
HDAC	histone deacetylase
HPA	hypothalamus-pituitary-adrenal axis
HRE	hormone response element
LBD	ligand-binding domain
LXR	liver X receptor
MAPK	mitogen activated protein kinase
NBRE	NGFI-B response element
NCoR	nuclear receptor corepressor
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
OPN	osteopontin
PGC-1	peroxisome proliferator activated receptor γ coactivator-1
PI-3K	phosphatidylinositol-3 kinase
PKA	protein kinase A
PKC	protein kinase C
Pol II	RNA polymerase II
POMC	pro-opiomelanocortin
PPAR	peroxisome proliferators-activated receptor
PR	progesterone receptor
PTH	parathyroid hormone
PTHR1	parathyroid hormone receptor 1
RANK	receptor activator of nuclear factor-κB
RANKL	receptor activator of nuclear factor-κB ligand
RAR	retinoic adic receptor
Runx2	runt-related transcription factor-2
RXR	retinoid X receptor
SMRT	silencing mediator for retinoic adic and thyroid hormone receptors
SRC	steroid receptor coactivator
SRM	selective receptor modulator
TH	tyrosine hydroxylase
TR	thyroid hormone receptor
VDR	vitamin D3 receptor

REVIEW OF THE LITERATURE

1. Nuclear receptor signalling

1.1 Superfamily of nuclear receptors

A living organism is capable of responding to signals that it receives from the surrounding environment. In a multicellular organism, the signalling molecules are secreted by endocrine organs, the surrounding cells, or by the target cell itself. There are several types of signalling molecules, such as small peptides, polypeptides and lipids. Some molecules, e.g. fatty acids and plant derived phytochemicals, are obtained via food intake. The signalling molecules, ligands, are bound by several specific receptors. These receptors are located either on the cell surface or inside the cells. The binding of a ligand to a cell surface receptor usually leads to the activation of kinases. Kinases activate a cascade of phosphorylations, which leads to activation or repression of transcription factors and to upregulation or downregulation in the transcription of target genes, respectively. Nuclear receptor (NR) ligands are lipid-soluble compounds that can penetrate through the cell membrane and bind to their compatible intracellular receptors (Gronemeyer et al. 2004). NRs are devided into families and subfamilies based on their structure and evolution (Escriva et al. 2004). Steroid receptors belong to the superfamily of nuclear receptors, and include the receptors for androgens, estrogens, progestins, mineralocorticoids and glucocorticoids (Mangelsdorf et al. 1995, Aranda and Pascual 2001). Non-steroidal NRs include the receptors for thyroid hormones, retinoids and vitamin D (Aranda and Pascual 2001). In addition, several other NRs are controlled with ligands of varied structure (Hummasti and Tontonoz 2008). The nuclear receptor superfamily also includes a group of structurally similar proteins with no identified or natural ligands. These receptors are called orphan receptors (Laudet 1997, Giguere 1999).

The family of nuclear receptors regulates multiple and diverse functions connected to development, growth, reproduction, and homeostasis (Novac and Heinzel 2004). The NR family consists of receptors that bind specific sequences on DNA to regulate transcription. In addition to NRs themselves, many other proteins are involved in the NR-mediated regulation of transcription. Some coregulators, namely coactivators and

corepressors, bind to the receptor containing complexes and enhance or inhibit the transcriptional effects by the receptor, respectively (Aranda and Pascual 2001).

1.2 Structure of nuclear receptors

The majority of the NR superfamily members have the same structure; they are formed by successive domains with different functions (fig. 1, Mangelsdorf and Evans 1995). The amino-terminal domain (NTD) is the most variable domain among different NRs, both in length and sequence. It includes a ligand-independent activation function-1 (AF-1) region (Evans and Hollenberg 1988, Tora et al. 1989, Simental et al. 1991) and can interact with coregulators (Ikonen et al. 1997, Hittelman et al. 1999, Aranda and Pascual 2001).

The DNA-binding domain (DBD) is the most conserved domain in NRs. The DBD interacts with DNA and is involved in receptor dimerization and interactions with coregulatory proteins (Baumann et al. 1993, Lee et al. 1993, Berglund et al. 1997). It is also required for cross-talk with other transcription factors such as NF-κB and AP-1 (Aarnisalo et al. 1999, Björnström and Sjöberg 2002).

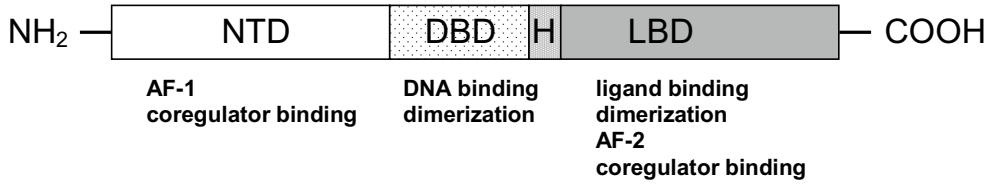

Fig. 1. The modular structure of nuclear receptors. NTD, the amino-terminal domain; DBD, the DNA-binding domain; H, the hinge; LBD, the ligand-binding domain. The main functions of each domain are shown. AF-1, the activation function-1; AF-2, the activation function-2.

The ligand-binding domain (LBD) is formed of 12 helices (H1-H12). It contains a pocket-like structure between the folded helices, the ligand-binding pocket, needed for ligand binding. LBD also contains a dimerization surface and a second activation function region, AF-2 (Moras and Gronemeyer 1998).

The hinge domain serves as a hinge between the DBD and LBD and allows the rotation of the LBD with respect to DBD. In many cases, the hinge also includes a nuclear localization signal and residues that are involved in corepressor interaction (Hörlein et al. 1995, Daury et al. 2001, Nascimento et al. 2006, Haelens et al. 2007).

The conserved structure and amino acid content of NRs has led to theory that all NRs have evolved from one or very few ancestral NRs (Owen and Zelent 2000, Escriva et al. 2004). All NRs also share the basic mechanisms of action, and all multicellular organisms possess some kind of NR activity (Owen and Zelent 2000). The NR superfamily is further divided into subfamilies consisting of closely related NRs (see fig. 2). Mutations during the evolution of organisms have also changed the sequence and functions of NRs. This has enabled more complex regulation of a more complex organism.

Subfamily	Group	Trivial name	Gene
Thyroid hormone receptor-like	Thyroid hormone receptor	TRα	NR1A1
		TRβ	NR1A2
	Retinoic acid receptor	RARα	NR1B1
		RARβ	NR1B2
		RARγ	NR1B3
Retinoid X receptor-like	Retinoid X receptor	RXRα	NR2B1
		RXRβ	NR2B2
		RXRγ	NR2B3
Estrogen receptor-like	Estrogen receptor	ERα	NR3A1
		ERβ	NR3A2
	Glucocorticoid receptor	GR	NR3C1
	Estrogen receptor-related receptor	ERRα	NR3B1
		ERRβ	NR3B2
		ERRγ	NR3B3
Nerve growth factor IB-like	NGF-induced factor B	NGFI-B	NR4A1
	Nur related factor 1	Nurr1	NR4A2
	Neuron-derived orphan receptor 1	Nor1	NR4A3

Fig. 2. Nuclear receptors are divided into subfamilies based on the structural similarities of DBD and LBD. Subfamilies are further divided into groups. Group members are usually named using Greek symbols. This figure shows the division of selected NRs. Modified from Nuclear receptor nomenclature committee 1999.

1.3 Nuclear receptor ligands

The classical NRs are regulated by ligands. The regulatory system composed of hormones, receptors and their functions is complex, and it has taken time to understand the basic events. At the beginning of the 20th century, hormones were isolated based on their abilities to control development, cell differentiation and organ physiology (Mangelsdorf et al. 1995). Radiolabeled ligands helped to recognize the proteins that were affected by the ligands and translocated to the nucleus (Jensen et al. 1966). Later, these proteins were found to be nuclear receptors (Yamamoto 1985). After the cDNAs of the glucocorticoid receptor and estrogen receptor (GR and ER, respectively) were solved in 1985, more nuclear receptors have been found based on the similarities between the sequences and, in particular, those of the DBD (Mangelsdorf et al. 1995). Some of these receptors have been identified as the targets for known hormones, but many were classified as orphan receptors, because their hormones/ligands were unidentified at the time (Giguere et al. 1988). Since NRs have major roles in controlling the metabolism, reproduction, and well-being of a person, pharmaceutical studies targeting NR functions have been extensive. Studies concerning drug design have also advanced the knowledge of NR functions and helped in the search for natural ligands. Several ligands have been identified by analyzing receptor activity after treating cells with tissue extracts, pharmacological compounds or natural chemicals (Heyman et al. 1992, Bocos et al. 1995, Willy et al. 1995, Lambe and Tugwood 1996, Tremblay et al. 2001). Also other methods, such as scintillation proximity assay and fluorescence resonance energy transfer, have been used to identify NR ligands (Nichols et al. 1998, Zhou et al. 1998). Over 10 % of the medically usefull drugs act by binding the LBDs of NRs (Goodwin and Moore 2004).

NR ligands can be natural or synthetic. Examples of natural and synthetic NR ligands are given in fig. 3. Steroid hormones are synthesized from cholesterol and iodothyronine hormones from tyrosine. Steroid and thyroid hormones are sythesized in specialized cells, for example testosterone in the Leydig cells of the testis, and triiodothyronine by the follicular cells of the thyroid gland. Inactive forms of vitamin A and D are obtained via food intake, vitamin D prescursor can also be synthesized in the skin. Retinoic acids are formed by oxidations from dietary vitamin A by non-specialized cells (Hardikar and Suchy 2005 p. 1001), and the active form of vitamin D, calcitriol, is formed in the liver and kidneys by successive hydroxylations of the inactive vitamin D (Barret and Barret 2005 p. 1095).

	receptor	ligand(s)	natural/synthetic	agonist (+)/ antagonist(-)
steroid receptors	ER	estradiol	N	+
		4-OHT	S	SERM
	AR	testosterone	N	+
retinoic acid receptors	RAR	9-trans retinoic acid	N	+
	RXR	9-cis retinoic acid	N	+
		SR11237	S	+
orphan receptors	PPARα	GW6471	S	–
	PPARγ	Roziglitazone	S	+
		15-Deoxy-Δ12,14-prostaglandin	N	+
	FXR	bile acids	N	+/ –

Fig. 3. Examples of NR ligands. N, natural; S, synthetic; SERM, selective estrogen receptor modulator. Additional abbreviations and references are in the text.

Nuclear receptors bind agonists and antagonists, i.e. activators and repressors, respectively. Inverse agonists are compounds that bind to the receptors and stabilize the inactive conformation of the receptor, thus having repressing effect on the basal transcriptional activity (Gronemeyer et al. 2004). Selective receptor modulators (SRMs) are ligands that have an agonistic or antagonistic character in a cell and tissue context dependent manner. It is suggested that the agonistic or antagonistic character is due to different coregulator pools present in different cells (Smith and O'Malley 2004).

The theory that all NRs are evolved from one ancestral receptor is supported by the evidence indicating that ligands are partially conserved. For instance, the same ligand, 9-cis retinoic acid, binds its receptor with same the specificity in jellyfish as in humans (Kostrouch et al. 1998). Biosynthesis of steroid hormones is composed of successive enzymatic steps. Steroid receptors and converting enzymes are to some degree found in primitive water vertebrates, but more complete system is present in land vertebrates (Baker 2004). ER has been reported to be the most ancient steroid receptor and other steroid receptors have evolved from it by series of duplications (Baker 2004). The volume of the natural ligands is also suggested to be conserved. For example, the volumes of estradiol,

testosterone, cortisol, thyroid hormone, and retinoic acids are very much alike, even though these ligands have varying molecular weights (Bogan et al. 1998).

1.4 Ligand-binding domain and ligand-dependent activation of nuclear receptors

The studies on the crystal structures of the LBDs of various nuclear receptors have demonstrated that the LBDs have a common structure. The LBD consists of 12 α-helices (H1 to H12) and one β-turn arranged in an antiparallel α-helical sandwich forming a structure that buries the ligand-binding pocket within the core of the LBD (Wurtz et al. 1996, Wang et al. 2003). Ligand binding alters the conformation of the LBD and results in altered localization of NR and changes in the associated proteins (Togashi et al. 2005).

To activate gene transcription, nuclear receptors have to be in the nucleus. The cellular localization prior to ligand binding varies among different steroid receptors. GR is mostly located in the cytoplasm, and the ligand induces the dissociation of the heat shock proteins and GR is transported to nucleus (Picard and Yamamoto 1987, Sackey et al. 1996). In contrast to GR, unliganded ER and the progesterone receptor (PR) seem to be mostly nuclear (Htun et al. 1999, Lim et al. 1999). Non-steroidal receptors, such as the retinoic acid receptor, retinoid X receptor, and vitamin D receptor (RAR, RXR, and VDR, respectively), bind DNA but do not activate transcription in the absence of ligands, they rather repress it (Aranda and Pascual 2001).

NR LBDs without a ligand are in an open *apo* conformation, and ligand binding induces a change into a closed *holo* conformation (Egea et al. 2000). Upon binding of an agonistic ligand, H10, H11 and H5 are repositioned. This leads to “closing” of the ligand-binding pocket formed by the LBD with H12 (Renaud et al. 1995). The ligand-induced repositioning of H12 provides the surface(s) for coactivator interactions and generates the active AF-2. In cases of constitutively active receptors, such as Nurr1, NGFI-B and ERRγ (estrogen-related receptor γ), the H12 is constantly in an active position (Greschik et al. 2002, Wang et al. 2003, Sablin et al. 2003, Suino et al. 2004). The binding of an antagonistic ligand induces a conformation distinct from the agonist-induced conformation; in the antagonist-induced conformation, H12 is placed in an "antagonist position", which means that the receptor adopts an inactive conformation. In this conformation, coactivators cannot interact with the receptor (Egea et al. 2000). The crystal structure of the peroxisome proliferators-

activated receptor α (PPARα) LBD bound to an antagonist and a corepressor SMRT (silencing mediator for retinoic adic and thyroid hormone receptors) shows that the antagonist-bound receptor adopts a conformation which favours the binding of corepressors (Xu et al. 2002). The crystal structure of ERα LBD complexed with the partial antagonist raloxifen shows that ERα has the antagonistic conformation (Brzozowski et al. 1997). In contrast, in the ERα LBD bound to a pure ER antagonist ICI 164,384 the H12 is highly mobile and the AF-2 structure is inactivated (Pike et al. 2001). It has been suggested that a pure estrogen antagonist also flips H12 to a position that disturbs the activity of AF-1 (Pike et al. 2001). In conclusion, the binding of ligands induces conformational changes in the LBDs, affecting the H12 in particular, thus regulating the AF-2 and the binding of coregulators.

1.5 The DNA-binding domain

Nuclear receptors activate transcription by binding to hormone response elements (HREs) usually located upstream of the transcription initiation site (Chandler et al. 1983, Ham et al. 1988, Pascussi et al. 2003, Wulf et al. 2008). NRs generally interact with the major groove of DNA, as it is wider and thus exposes more functional groups than the minor groove (Khorasanizadeh and Rastinejad 2001). The DBDs of NRs consist of three helices that form two zinc-finger modules, each with four cysteines arranged around zinc2+ ions (Kellenbach et al. 1991). The first helix extends from the C-terminal part of the first zinc-finger to the region between the two zinc-fingers and contains the amino acids responsible for the sequence-specific recognition of the DNA (Claessens and Gewirth 2004). Five amino acids in the base of the first zinc finger termed the “P-box” are are especially important for the recognition of the HRE sequence (Carson-Jurica et al. 1990). The amino acids in the first helix, responsible for the sequence-specific DNA binding (CGSCKVF), are 100% conserved among all but one of the steroid hormone receptors (Tsai and O’Malley 1994). The “D-box” (amino acids CRGSKD) of the base of the second zinc finger partly mediates the DNA binding-dependent dimerization of certain receptors, like GR and ER (Freedman 1992). The third helix stretches from the C-terminal end of the second zinc-finger into the C-terminal extension (CTE) of the DBD. CTE contains an A-box which is responsible for recognizing the sequence preceding the classical response element (Wilson et al. 1992). CTE also contains a T-box which is important for HRE recognition of nuclear receptor dimers (Zechel et al. 1994).

1.6 Response elements on DNA

HREs consist of consensus sequences. When NRs bind DNA as dimers, usually two closely situated HREs, half-sites, are needed. The identity of an HRE resides in three features: the sequence of the base pairs in the half-sites, the number of base pairs between the half-sites, and the relative orientation of the two half-sites (Glass 1994, Zechel et al. 1994, Umesono et al. 1991). Each receptor dimer that binds DNA has to recognize the sequences, spacing and orientation of the half-sites within their HREs (Claessens and Gewirth 2004). In a widely accepted model, HREs typically contain two consensus hexameric half-sites. Steroid receptors recognize the half-site consensus sequence AGAACA, while ERs and other NRs bind to the half-site consensus sequence AGGTCA (Glass 1994, Umesono et al. 1991). RXR seems to recognize specifically only 3 of the 6 bases of the half-site, and it has been suggested that RXR is more relaxed in HRE specificity (Zhao et al. 2000). Monomeric (single) NRs bind to a single half-site (Glass 1994). Most receptors bind HREs as homodimers (a dimer formed between two similar NRs, eg. steroid receptors) or heterodimers (a dimer formed between two different NRs, eg. non-steroidal receptors). Interfaces formed by the D-box are responsible for the spacing distance between the two half-sites of the palindromic HRE (Freedman 1992). Dimeric receptors bind to response elements containing two half-sites that can be arranged as direct repeats (DRs) with zero to five spacing bases (Glass 1994). DRs separated by 3, 4, or 5 bases mediate preferentially regulation by vitamin D, thyroid hormone, and retinoic acid, respectively (Umesono et al. 1991), but other DRs are also acceptable (see fig. 4). Dimerization and flexibility in HRE binding add complexity to gene regulation.

1.7 Dimerization of nuclear receptors

Dimerization of nuclear receptors is mostly mediated by the LBD (Perlmann et al. 1996), but also to some extent by DBD. The dimerization process of ER has been studied in detail (Brzozowski et al. 1997). In ER, dimerization is mediated by the H11 helices of each receptor that interact via a stretch of conserved residues at their aminoterminal ends. Additional dimer interactions are provided by regions of H8, the loop between helices H9 and H10, and H10 (Brzozowski et al. 1997). This way of forming dimers appears to be common for NRs, as

similar arrangements are observed for the unliganded RXR and for both the unliganded and liganded PPARγ (Bourguet et al. 1995, Nolte et al. 1998).

Steroid receptors form homodimers, with the exception of ER. ER has two isoforms, ERα and ERβ, which can form heterodimers with each other (Cowley et al. 1997, Whitfield et al. 1999). Many non-steroidal nuclear receptors, such as the thyroid hormone receptor (TR) bind DNA as monomers, homodimers or as heterodimers with RXR (Rastinejad 2001). TR can form heterodimers also with RAR (Glass et al. 1989, Lee and Privalsky 2005).

Formation of different homodimers or heterodimers alters the functions and transcriptional activity of the receptors. ERα/ERβ heterodimers activate transcription more efficiently than ERβ homodimers but less efficiently than ERα homodimers (Cowley et al. 1997). In the case of TR, the favoured response element of the DNA changes when it binds as a monomer, homodimer or heterodimer. As a monomer, TRα barely binds response elements, but as a homodimer the binding is rather strong. The binding is enhanced even further upon heterodimerization with RXR or RAR (Lee and Privalsky 2005, Velasco et al. 2007). TR/RXR and TR/RAR heterodimers activate response elements efficiently (Lee and Privalsky 2005, Velasco et al. 2007). In addition, binding of triiodothyronine changes the dimerization of TRs and affects DNA binding (Andersson et al. 1992).

Some orphan nuclear receptors (e.g. Nor1 and the retinoid-related orphan receptors) regulate transcription only as monomers, whereas other orphan receptors can also form homodimers or heterodimers (Giguere 1999). Orphan nuclear receptors can form heterodimers either with RXR (Giguere 1999) or among their own subfamily or group members (Maira et al. 1999, Huppunen and Aarnisalo 2004). Some orphan NRs form heterodimers with both RXR and subfamily members (Maira et al. 1999). In conclusion, monomeric, homodimeric and heterodimeric receptors bind DNA differently, bind to response elements, and regulate gene expression.

1.7.1. RXR as a dimerization partner

RXR was first identified due to its similarity with RAR (Mangelsdorf et al. 1990). Shortly after its identification it was recognized that this protein was forming heterodimers with VDR, TR and RAR on their HREs and was required for high affinity DNA binding of these receptors (Yu et al. 1991, Kliewer et al. 1992a, Kliewer et al. 1992b). Since then, these and

several other non-steroidal nuclear receptors have been shown to heterodimerize with RXR, including Nurr1, NGFI-B, PPARs, LXR (liver X receptor) and FXR (farnesoid X receptor; Rastinejad 2001). Non-steroidal receptors heterodimerize with RXR more often than form homodimers. Heterodimers formed between TR and RXR are activated by low TR agonist concentrations more efficiently than by TR alone, and their activity is greatly enhanced by low concentrations of retinoic acid (Zhang et al. 1992). In general, RXR heterodimers are more potent activators than monomers or homodimers (Umesono et al. 1991, Zhang et al. 1992, Forman et al. 1995). As mentioned earlier, the RXR-heterodimeric partners are separated from RXR on DNA by bases according to the 1-to-5 rule (see fig. 4, Glass 1994). This means that the number of bases between the half-sites can vary from one to five and that the preferred dimerization partner changes as the number of bases changes. Each base between the half sites changes the distance and binding angle between the partners. The dimerization surfaces that RXR uses for heterodimerizing with different partners are partly selective (Lee et al. 2000, Aarnisalo et al. 2002). In addition, the binding of RXR to DNA is flexible (Holmbeck et al. 1998, Zhao et al. 2000). The heterodimerizing surface of RXR is different from that of any other mammalian NR, as it has unique structures in H9 and H10 (Gampe et al. 2000), and, indeed, RXR is the only mammalian NR capable of heterodimerizing with multiple partners.

RXR heterodimers are classified into two major groups, non-permissive and permissive heterodimers (see fig. 4, Mangelsdorf and Evans 1995, Forman et al. 1995). Non-permissive heterodimers are only activated by the ligands of the heterodimerizing partner receptor. Permissive heterodimers are activated by the ligand of the partner, but they allow effective activation by RXR ligands as well. On the basis of the crystal structures of non-permissive RAR/RXR and permissive PPARγ/RXR heterodimers it has been concluded that PPARγ H12 interacts with helix 7 of RXR LBD. This interaction is lacking in RAR/RXR heterodimer (Gampe et al. 2000, Bourguet et al. 2000). The interaction could stabilize the PPARγ H12 in a position that permits interactions with coactivators even in the absence of a PPARγ agonist and may provide a structural basis for the permissivity of PPARγ/RXR heterodimers (Gampe et al. 2000, Bourguet et al. 2000). The binding of corepressors, the conformation of RXR, the specific ligand itself, and the precise nature of the HRE are also likely to play a role in the regulation of permissiveness (Niessen et al. 1996, DiRenzo et al. 1997, Ahuja et al. 2003). The spacing between the half-sites does not seem to influence to permissiviness (fig. 4).

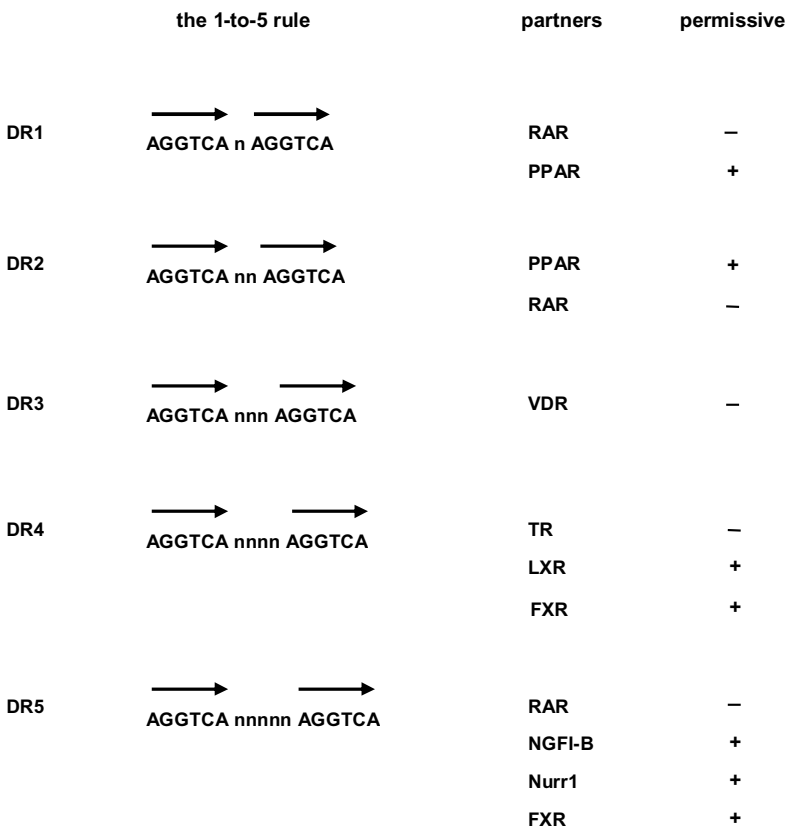

Fig. 4. Schematic illustration of the direct repeat (DR) binding sites of RXR heterodimers, the specific dimerization partners on each DR, and the permissiviness (+, permissive; -, non-permissive) of the heterodimers. n represents any base in between the half-sites.

Coregulators seem to function differently in permissive and non-permissive RXR heterodimers. Permissive partner receptors are bound to the corepressors in the absence of their own agonist (Direnzo et al. 1997). In the absence of RXR agonist, RXR does not bind coactivators nor corepressors (Zhang et al. 1999). It has been suggested that in permissive RXR heterodimers coactivators are bound to RXR or the partner receptor in response to either RXR or partner agonist. Agonist binding to both receptors in the permissive dimer can result in synergistic effect and further enhance the binding of coactivators (Ahuja et al. 2003). Furthermore, it seems that the binding of an RAR ligand to RAR/RXR heterodimers blocks the coactivator binding triggered by RXR agonist (Germain et al. 2002). There are also non-classical RXR heterodimers, since the heterodimers formed by RXR and the constitutive androstane receptor seem to be either permissive or non-permissive depending on the HRE

(Tzameli et al. 2003). The role of corepressors in regulating the permissiviness of RXR heterodimers has remained unclear.

1.8 Transcriptional regulation by nuclear receptors

The DNA of a cell is packaged into a protein/DNA structure, chromatin. Chromatin consists of DNA bound to histones, and DNA can be either inaccessible or accessible to transcription factors depending on the state of the chromatin. Heterochromatin means tightly packed, inaccessible chromatin, euchromatin is loose and accessible to transcription. Chromatin can be made accessible in different ways, e.g. by ATP-dependent remodelling, ubiquitination, and, most importantly, the the actions of histone acetyl transferases (HATs) which acetylate the lysines in histones' tails (see fig. 5). Acetylation decreases the interaction between histones and DNA, and results in loosened euchromatin (Struhl 1998, Verdone et al. 2005, Kinyamu et al. 2005, Choundary and Varga-Weisz, 2007).

NRs can bind specific DNA elements both in the absence (non-steroidal NRs) and presence (steroid receptors) of ligands. Upon ligand binding, NRs are released from corepressors or heat shock protein-complexes, and coactivators can bind to the receptor and activate the AF-2 (Greshik and Moras 2003). When activating transcription, NRs loosen the chromatin to euchromatin with the help of coactivators with HAT activity (see below). Once the chromatin is loosened and the DNA is "open" and accessible to transcription, the basal machinery of transcription forms the preinitiation complex (Kumar et al. 2004). Genes are transcribed to mRNA and to new proteins. After transcription, chromatin is packed to heterochromatin again by histone deacetylases (HDACs, Struhl 1998). HDACs are recruited to NR by corepressors. NRs therefore control gene expression by loosening and unloosening the chromatin. NRs do this by HATs and HDACs present in the coregulatory proteins.

1.9 Corepressors and coactivators

NR activity is modulated by interactions with corepressors and coactivators, and these interactions inhibit or enhance the transcriptional activity of NRs, respectively. Antagonists recruit corepressors to the LBD, resulting in a complex that repsesses transcription, whereas agonists enable the binding of coactivators and activate transcription (Dotzlaw et al. 2003).

SMRT and NCoR are related corepressors that interact with HDAC-3, a member of the histone deacetylace family. The complexes formed by NRs, corepressors, and the HDAC-3 tighten the chromatin by deacetylations, thus repressing transcription (fig. 5, Heinzel et al. 1997, Guenther et al. 2000, Wen et al. 2000, Li et al. 2000).

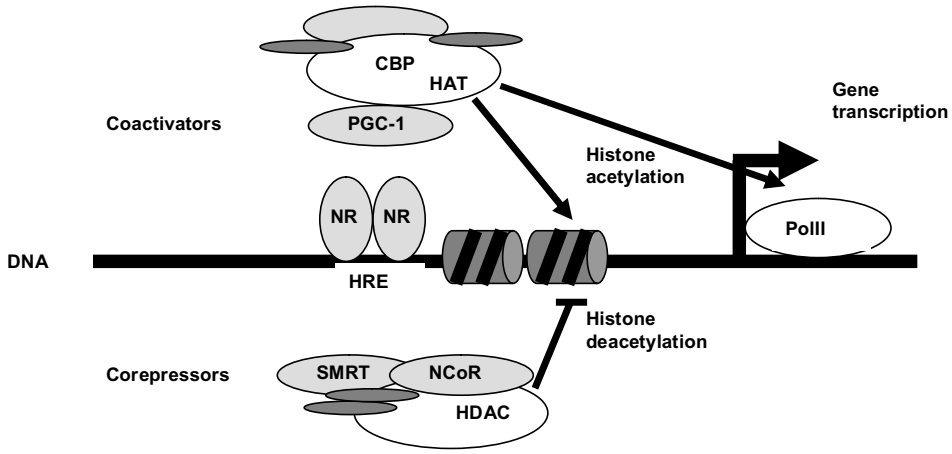

Fig. 5. Corepressor and coactivator complexes are bound to NR and tighten or loosen chromatin with their HDAC and HAT activities, respectively. Pol II is recruited to loosened DNA by NR or coactivator complex, and the gene is transcribed to mRNA (adapted from Dilworth and Chambon 2001, Lonard and O'Malley 2007)

SMRT and NCoR seem to be responsible for the repressing effect of unliganded, DNA bound RAR and TR (Hörlein et al. 1995, Glass and Rosenfeld 2000). NCoR and SMRT are also recruited by antagonist-bound steroid receptors (Lavinsky et al. 1998).

Coactivators, such as CREB-binding protein (CBP) and steroid receptor coactivator (SRC) -1, -2, and -3, are suggested to mediate transcription in two major modes. They modify the chromatin structure with their HAT activity. Additionally, they may interact with basal transcription factors and with polymerase II (Pol II; Wärnmark et al. 2003). Pol II is responsible for the transcription of genes to mRNA. Coactivators thus bridge the NRs to the transcriptional machinery (Kwok et al. 1994, Nakajima et al. 1997, Chen et al. 1999, Dilworth

and Chambon 2001). CBP is also capable of interacting with SRCs through its C-terminal domain (Yao et al. 1996). PGC-1 (PPARγ coactivator-1) is a coactivator that interacts with CBP and SRCs to obtain HAT activity to the NR complex. It also interacts with the Mediator, a large activating complex formed by numerous proteins that enhances transcription by further interacting with Pol II (Malik and Roeder 2000, Finck and Kelly 2006).

1.10 Non-classical functions of nuclear receptors

1.10.1 Ligand-independent activation of nuclear receptors

The functions of nuclear receptors are also regulated in a ligand-independent manner. This means that NRs are activated, bind to their HRE and initiate transcription without ligand binding (Weigel and Zhang 1998). In this respect, post-translational modifications play a major role. Most of the members of the nuclear receptor family are phosphoproteins (Weigel 1996) and they can be activated by phosphorylation, regardless of ligand binding. PR was found to be activated by phosphorylation in a ligand-independent manner in 1990. This was found to derive from the activation of the protein kinase A (PKA) pathway (Denner et al. 1990). Since then, several nuclear receptors including ER, androgen receptor (AR) and PPARγ have been shown to be activated in a ligand-independent manner by e.g. phosphorylations, sumoylations, or methylations (Darne et al. 1998, Patrone et al. 1998, Poukka et al. 2000, Sadar et al. 2000, Schreihofer et al. 2001, Al-Rasheed et al. 2004, Pierson-Mullany and Lange 2004, Al-Ghnaniem et al. 2007). Phosphorylation can occur on distinct parts of the receptor (Gioeli et al. 2002), but it has been suggested that it mainly functions to regulate, either activate or repress, the AF-1 (Miller et al. 2005, Thomas et al. 2008). In addition, it has been suggested that phosphorylations and other post-translational modifications also regulate the functions of HSPs and coactivators thus regulating transcriptional activity of NRs (Weigel and Zhang 1998, Ueda et al. 2002, Saporita et al. 2007).

Orphan nuclear receptors have also been shown to be modulated by post-translational modifications in ligand-independent manner (Hammer et al. 1999, Khan et al. 2005, Lee et al. 2005). For example, the orphan nuclear receptor TR2 was found to be

phosphorylated at two different serines by the PKC pathway. Phosphorylation of the more C-terminal serine was found to be critical for the activity of TR2 (Khan et al. 2005). The orphan nuclear receptor COUP-TFI (chicken ovalbumin upstream promoter transcription factor I) has been shown to be phosphorylated by two different pathways, resulting in differential outcomes on its activity (Gay et al. 2002). Post-translational modifications could thus provide an important ways of controlling the activity of orphan nuclear receptors.

1.10.2 Non-genomic functions of nuclear receptors

In the classical model of NR action, ligand binding leads to a conformational change in the NR structure and results in binding of coactivators and/or releasing of corepressors. The ligand-receptor complex binds the HREs of the target genes and this leads to the regulation of gene transcription (Aranda and Pascual 2001). The genomic effects of NRs usually have response times from hours to days. There have been implications of a much more rapid, seconds to minutes, response for ligands. The rapid response can be seen even in the presence of specific inhibitors of transcription or translation. These responses are termed non-genomic effects (Lösel and Wehling 2003). Non-genomic effects are regulated by binding of a ligand to a receptor, which leads to activation of signalling cascades and to changes in cell function, but does not involve DNA-binding of the receptor (Lösel and Wehling 2003).

Steroids have a wide range of non-genomic functions in different cell types (Schwartz et al. 1996, Baldi et al. 1998, Whiting et al. 2000). Non-genomic effects can be regulated in two ways. Firstly, steroid receptors present on the plasma membrane can transmit the signal into the cell. Secondly, large concentrations of steroids produce non-genomic effects by altering membrane fluidity without receptor binding (Wehling 1997, Rauch and Flint 2008). All the major signalling pathways (PKA, protein kinase C, PKC; phosphatidylinositol-3 kinase, IP-3k; mitogen activated protein kinase, MAPK) and transport systems seem to have a role in mediating the non-genomic effects of different ligands (Migliaggio et al. 1996, Kousteni et al. 2003). It has been suggested that at least ERs may be present at the cell surface (Norfleet et al. 1999, Taleghany et al. 1999, Powell et al. 2001). Small amounts of ER are located at the cell membrane (Zhang et al. 2002) and these receptors can mediate the effects of different growth factors (Levin 2003). It seems that non-genomic effects of steroids are more prevalent than originally assumed.

2. Orphan nuclear receptors

In addition to ligand-regulated NRs, there is a large group of proteins termed orphan nuclear receptors. These receptors were first recognized based on their resemblance to classical NRs and their ligands were unknown (Giguère 1999). Since their identifications, some orphan NRs have been found to respond to synthetic or natural ligands (see fig. 3). For example, PPARγ has been shown to bind several natural (e.g. fatty acids and eicosanoids) and synthetic ligands (e.g. thiazolidinediones and 5-aminosalicylic acid, Desvergne and Wahli 1999, Toth et al. 2007). Some of the orphan nuclear receptors are still devoid of known ligands.

2.1 NR4A subfamily of orphan nuclear receptors

The NR4A subfamily consists of three members, the nerve growth factor inducible B, (NGFI-B, also known as NR4A1, Nur77, TR3), nur-related factor 1, (Nurr1, NR4A2, TINUR, NOT) and neuron derived orphan receptor 1 (Nor1, NR4A3, MINOR, Milbrandt 1988, Law et al. 1992, Ohkura et al. 1996). NGFI-B was found to be induced when cells were treated with the nerve growth factor (Milbrandt 1988), and Nurr1 and Nor1 were cloned due to their resemblance to other nuclear receptors (Law et al. 1992, Ohkura et al. 1996). Based on their sequence homology, NGFI-B, Nurr1 and Nor1 were suggested to form a subfamily (Law et al. 1992, Ohkura et al. 1996, Giguere 1999). A Drosophila homolog DHR38 and a C. Elegans homolog CeNHR-6 also belong to this group (Sutherland et al. 1995, Maglich et al. 2001). Some reviews have set the NR4A receptors as an evolutionary lineage of their own among all NRs (Escriva-Garcia et al. 2003). Some identify the ERRs as close relatives and the other orphan receptors as more distinct ones (Benoit et al. 2006).

2.1.1 Structure of NR4A receptors

The structure of the NR4As is similar to that of ligand-regulated NRs. They share high homology in the DBD, moderate in the LBD, and the N-terminus has more differencies (fig.

6, Maruyama et al. 1997). The crystal structures of Nurr1 and NGFI-B LBDs have been resolved and show that they both consist of 12 helices and that the ligand-binding pocket is filled with bulky sidechains from residues that are conserved in the family. This suggests that these receptors do not bind lipophilic ligands in the classical way (Wang et al. 2003, Flaig et al. 2005).

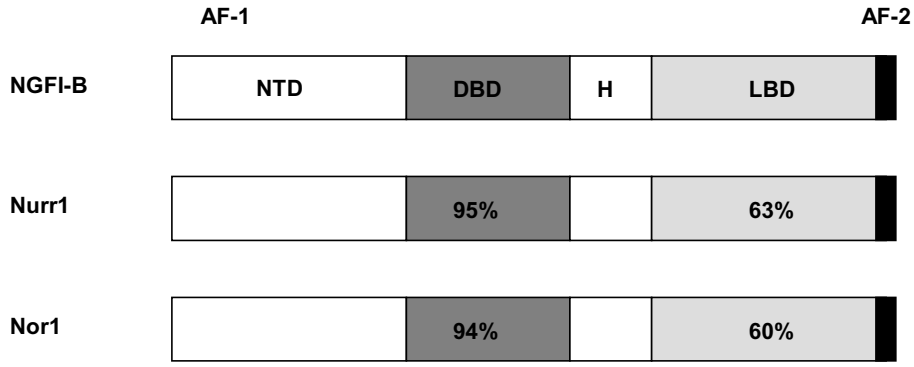

Fig. 6. The structure and sequence homology of NR4A receptors. The similarity between different domains compared to those of NGFI-B are given in prosentage. AF-1, activation function-1; AF-2, activation function-2; NTD, N-terminal domain; DBD, DNA-binding domain; H, hinge domain; LBD, ligand-binding domain.

2.1.2 NR4A receptor expression

Nurr1 is expressed at high levels in the central nervous system (CNS), especially in the dopaminergic neurons of the midbrain (Saucedo-Cardenas and Conneely 1996, Zetterström et al. 1996a, 1996b). It is also detected in peripheral tissues such as adrenals, liver, and bone (Scearce et al. 1993, Honkaniemi et al. 2000, Tetradis et al. 2001a). NGFI-B is widely expressed in peripheral tissues including lung, liver, kidney, adrenals, and bone (Davis and Lau 1994, Ohkubo et al. 2002, Shin et al. 2004, Pirih et al. 2005). Nor1 expression is high in the pituitary gland and lower in kidney, skeletal muscle and adrenals (Ohkura et al. 1996, Marayama et al. 1997). During development, NGFI-B expression starts at E16,5 (Milbrandt 1988), Nurr1 and Nor1 expression can be seen already at E7 (Cheng et al. 1997, DeYoung et al. 2003).

The expression of the receptors of the NR4A subfamily is strongly induced by external stimuli. NR4As are expressed as immediate early genes (stress response genes) with maximum induction after only 30 to 60 minutes after stimulation in tissues such as adrenals and bone (Honkaniemi et al. 2000, Tetradis et al. 2001a, 2001b). The range of physiological signals that induce the expression of NR4A receptors is wide, from fatty acids and peptide hormones to stress and magnetic field (Maxwell and Muscat 2006).

The induction of the NR4A receptors has been shown to involve several pathways, including MAPK, PKC and PKA pathways (Kovalovsky et al. 2002, Darragh et al. 2005, Bourhis et al. 2008). Phosphorylation of many transcriptional activators, such as NF-κB, Sp1, AP-1, and CREB has been implicated in the transcriptional regulation of NR4A expression (Yoon and Lau 1994, Saucedo-Cardenas et al. 1997, Chen et al. 1998, Ichinose et al. 1999, Fass et al. 2003). Nurr1 promoter also contains the binding sites for these transcription factors (Saucedo-Cardenas et al. 1997).

2.1.3 Gene regulation by NR4A receptors

NR4A family members bind as monomers to the consensus DNA sequence AAAGGTCA. This element is termed NBRE (NGFI-B response element, see fig. 7). The A-box of the DBD is responsible for recognizing the AA sequence preceding the classical response element of non-steroidal nuclear receptors (AGGTCA, Wilson et al. 1992, Meinke and Sigler 1999). NGFI-B, Nurr1 and Nor1 have been shown to bind DNA as homodimers and as heterodimers with each other on the NurRE (nur-response element, AAAT(G/A)(C/T)CA), which is an inverted repeat of two slightly converted NBRE half-sites (Philips et a. 1997a, fig. 7). NGFI-B activates NurRE much more potently than NBRE (Philips et al. 1997a). The Nurr1/NGFI-B and Nor1/NGFI-B heterodimers activate NurRE much more potently than Nurr1/Nor1 heterodimers (Maira et al. 1999). Heterodimers formed within the NR4A family also bind NurRE much more efficiently than monomeric or homodimeric NR4As (Philips et al. 1997a, Maira et al. 1999). It has been shown that NGFI-B bound to a NurRE recruits coactivators of the SRC family to the AF-1 (Maira et al. 2003). NGFI-B bound to NBRE does not recruit SRCs. Maybe differential expression of the NR4A receptors can influence the abundance in which the highly active and less active NR4A heterodimers are present in the cell. In addition, the amounts of homodimers and monomers may be affected. The promoters of different genes

can be activated depending on the expression levels of Nurr1, NGFI-B and Nor1. Gene activation is also dependent on the differential activity of NurRE and NBRE on target genes.

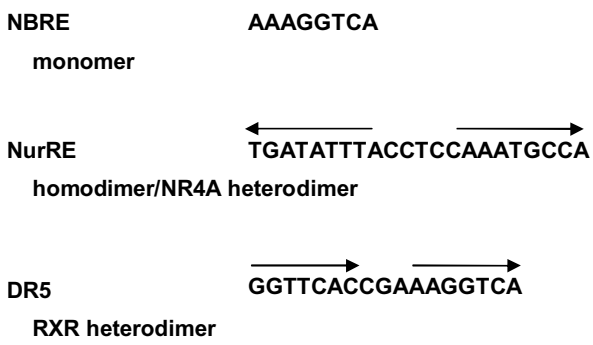

Fig. 7. Response elements of the NR4A family. NBRE, NGFI-B response element; NurRE, nur-response element; DR5, direct repeat separated by 5 nucleotides.

Nurr1 and NGFI-B bind DNA as heterodimers with the RXR on DR5 elements (fig. 7). Nurr1/RXR heterodimers are also formed on NBREs without RXR binding to DNA (Forman et al. 1995, Aarnisalo et al. 2002, Sacchetti et al. 2002). Heterodimerization with unliganded RXR represses Nurr1 transactivation on the NBRE-reporter (Forman et al. 1995). Nurr1/RXR and NGFI-B/RXR heterodimers are permissive and therefore efficiently activated by RXR agonists, such as natural 9cis-RA and synthetic LG69 and SR11247 (Forman et al. 1995, Aarnisalo et al. 2002). RXR agonists have been shown to promote the survival of neurons by affecting Nurr1/RXR heterodimers (Wallén-McKenzie et al. 2003). Heterodimerization with RXR and agonistic regulation of these heterodimers thus seem to have physiological importance, although an endogenous target promoter for Nurr1/RXR or NGFI-B/RXR heterodimers has not been reported. The role of RXR and heterodimerization in the functions of Nurr1 and NGFI-B need clarification.

Nurr1 transactivates target genes constitutively and ligand-independently. AF-1 and AF-2 both have a role in transactivation, and their activity is also dependent on the cell type (Castro et al. 1999, Wansa et al. 2002, Nordzell et al. 2004). The LBDs of Nurr1, NGFI-B, and the Drosophila homologue DHR38 are constantly in a conformation that is similar to that of other NRs in the ligand-bound state with the AF-2 in active conformation (Wang et al. 2003, Baker et al. 2003, Flaig et al. 2005). In Nurr1, the active AF-2 conformation is

stabilized by intramolecular interactions in the absence of ligands. The transcriptional activities of Nurr1 and NGFI-B have been shown to differ from each other (Castro et al. 1999, Flaig et al. 2005). For example, in the human embryonic kidney derived 293 cells, Nurr1 efficiently activates the NBRE-reporter, whereas NGFI-B does not. Mutation analysis showed that the differences may be due to the H12s of Nurr1 and NGFI-B being slightly shifted in relation to one another. Exchange between the H12s of Nurr1 and NGFI-B or smaller mutations of the H12 caused exchanging of the transcriptional properties (Flaig et al. 2005).

The structural and mutagenesis studies of the Nurr1 LBD show that the classical coactivator binding cleft seen in other NRs is missing in Nurr1 (Castro 1999, Wang et al. 2003). This may be the reason, why interactions between classical coactivators, for example SRC-1, and Nurr1 AF-2 located in the LBD, have not been seen (Castro et al. 1999, Wansa et al. 2002, Wang et al. 2003). The recruitment of coactivators to the AF-1 region has been shown to be important in the ligand-independent transcriptional activity of NRs (Hammer et al. 1999, Tremblay et al. 1999). NGFI-B and Nurr1 have been shown to recruit coactivators AF-1-dependently (Wansa et al. 2002, Maira et al. 2003).

Corepressors SMRT and NCoR bind Nurr1 very weakly compared to their binding to other NRs (Codina et al. 2004). SMRT has also been shown to interact with NGFI-B (Sohn et al. 2001). SMRT binds Nurr1 LBD to a site near the AF-2 (Codina et al. 2004), and same area has been suggested to function as a coregulator surface in NGFI-B (Flaig et al. 2005). This binding site has been suggested to bind both corepressors and coactivators (Sohn et al. 2001, Codina et al. 2004) by a mechanism similar to that of other NRs. However, the differencies in coactivator and corepressor binding compared to other nuclear receptors are likely to explain partly the constitutive activity of the NR4A receptors and the permissive nature of Nurr1/RXR and NGFI-B/RXR heterodimers. The precise mechanism of permissiviness has remained partly unclear.

In addition to dimerization, several mechanisms have been described to control the transcriptional activity of the NR4A receptors (Pekarsky et al. 2001, Kovalovsky et al. 2002, Galleguillos et al. 2004, Chintharlapalli et al. 2005). NR4As are regulated by phosphorylations. Phosphorylation can either enhance or repress the transcriptional activities of the NR4A receptors. For example, the MAPK pathway phosphorylates NGFI-B and enhances its transactivation on POMC (pro-opiomelanocortin) promoter (Kovalovsky et al. 2002). Nurr1 is phosphorylated by MAPK at sites near the AF-1. These phosphorylations upregulate its activity on tyrosine hydroxylase (TH) promoter (Zhang et al. 2007). Phosphorylation of serine 350 in the NGFI-B DBD has been shown to decrease its binding to

NBRE (Hirata et al. 1993), thus offering another possible target for regulation by phosphorylation, in addition to the modulation of AFs. Nurr1 has also been shown to possess two putative sumoylation sites at lysines 91 and 577. Mutational analyses have shown that sumoylation at position 91 can repress the transcriptional activity of Nurr1, and sumoylation at position 577 can enhance it (Galleguillos et al. 2004). Ubiquitination or methylation of the receptors of the NR4A family have not been reported.

Although Nurr1 and NGFI-B lack the classical ligand-binding pocket found in the other NRs, putative ligands have been identified. 6-mercaptopurine has been shown to activate Nurr1, NGFI-B, and Nor1. Activation of Nor1 by 6-mercaptopurine was found to be regulated by the first 150 amino acids of its N-terminal and linked to the AF-1 region. Phosphorylation was thought to be the mechanism of action, but no direct phosphorylation site was found (Wansa et al. 2003). Prostaglandin A2 was found to activate Nor1 and to bind directly to the LBD of Nor1. However, this work did not present any mechanism of activation connected to coregulatory proteins or phosphorylations (Kagaya et al. 2005). Selected 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methanes increased DNA binding by NGFI-B. These compounds are studied for their anticarcinogenic activities. Coactivators SRC-1 and PGC-1 were shown to interact with NGFI-B LBD more efficiently when cancer cells were treated with different 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methanes (Chintharlapalli et al. 2005). In addition, benzamidizoles, compounds used as parasiticides and fungicides, have been reported to be ligands for Nurr1 (Dubois et al. 2006). It seems that these compounds regulate the activity of Nurr1 and NGFI-B in a manner similar to NRs regulated with ligands (ie. by recruiting coactivators or by phosphorylations), although the ligand-binding pocket is unavailable for ligand binding. However, the H12 of Nurr1 and NGFI-B is in active position in the absence of any ligand. The crystal structure of Nurr1 or NGFI-B bound to one of these compounds should be resolved to obtain more information. The biological functions of the ligands should also be studied. As Nurr1 and NGFI-B can be controlled by synthetic ligands, it is possible that an endogeneous ligand also exists.

2.1.4 Localization of NR4A receptors

NR4A receptors are usually located in the nucleus (Davis et al. 1993). Katagiri et al. (2000) have studied the pathways mediating the localization of NGFI-B. They have demonstrated

that in NGFI-B, there are two nuclear localization signal sequences in the DBD and three nuclear export signal sequences in the LBD. They have shown that the nerve growth factor can induce NGFI-B relocalization to the cytoplasm by phosphorylating serine 105 in the A-box. This phosphorylation was shown to be regulated by the MAPK pathway. In their studies, the nerve growth factor also induced relocalization of RXR as a heterodimer with NGFI-B (Katagiri et al. 2000). Relocalization of NGFI-B to cytoplasm has also been observed in response to apoptotic factors (Li et al. 2000). Cytoplasmic NGFI-B was found to associate with mitochondria which led to depolarization of the mitochondrial membrane and release of cytochrome c (Li et al. 2000). It was further demonstrated that these events were the result of interaction with an anti-apoptotic protein Bcl-2 and that the interaction changed the nature of Bcl-2 from anti- to pro-apoptotic (Lin et al. 2004). It seems that the cytoplasmic NGFI-B has functions that are distinct from the nuclear NGFI-B.

2.1.5 Biological functions of NR4A receptors

NR4A receptors have been shown to be involved in inflammation, steroidogenesis, and energy metabolism (Maxwell and Muscat 2006). NR4A receptors are also linked to functions of smooth muscle cells and atherogenesis and to the regulation of apoptosis in certain tumour types (Maxwell and Muscat 2006). NR4A receptors therefore have varied functions in controlling multiple physiological events.

The fact that the NR4A receptors regulate genes involved in the dopamine synthesis and dopamine transporter links them to dopamine system. Overexpression of Nurr1 facilitates pluripotent embryonic stem cells to differentiate to dopamine cells (Chung et al. 2002). Mice with targeted deletion of the Nurr1 gene die after birth, propably due to problems with the control of respiratory functions (Zetterström et al. 1997, Castillo et al. 1998, Nsegbe et al. 2004). The homozygous mice lack TH, L-aromatic amino decarboxylase, and other dopamine markers from the midbrain area, as the dopamine cells of the midbrain are not developed and properly migrated (Zetterström et al. 1997, Castillo et al. 1998). These results prove that NR4A receptors control the development and functions of the dopaminergic cells of the midbrain. In the study by Chu and others (2002) it was demonstrated that the Nurr1 expressing neurons of the midbrain were reduced with aging. In this study, the expression of TH was similarly reduced. The quantity of TH is the limiting step of the synthesis of

dopamine (Bear et al. 2001 p. 143). Chu and others suggest that the reduction of the Nurr1 expressing neurons leads to reduced expression of TH and therefore reduced dopamine synthesis. Reduction in dopamine synthesis has been linked to disturbance of motorical functions and loss of cognitive abilities, and to many age-related diseases, such as Parkinson's, Huntington's, and Alzheimer's diseases (Bäckman et al. 2006). Disturbed function of Nurr1 has also been linked to Parkinson's disease (Olson et al. 1998, Grimes et al. 2006) and to Alzheimer's disease (Chu et al. 2006). Reduced levels of Nurr1 and NGFI-B proteins have been linked to severe disorders of the CNS (Buervenich et al. 2000, Xing et al. 2006). The expression levels of the NR4As in CNS have been shown to be incfluenced by antipsychotic drugs (Maheux et al. 2005). It seems that Nurr1 is involved in several age-related and other pathological conditions of the midbrain area.

NR4A receptors are key players in the regulation of the HPA (hypothalamic-pituitary-adrenal) axis (Okabe et al. 1998, Philips et al. 1997a, Philips et al. 1997b, Fernandez et al. 2000). Nurr1 and NGFI-B induce the expression of the corticotrophin releasing hormone secreted by the hypothalamus of the brain (Murphy and Conneely 1997, Murphy et al. 2001). Nurr1 also activates the POMC promoter, resulting in the production of adrenocorticotropic hormone (ACTH) from the anterior pituitary (Murphy and Conneely 1997). ACTH stimulates the secretion of additional hormones, glucocorticoids such as cortisol, from the adrenals. NR4A receptors thus participate in the regulation of the secretion of different hormones. Glucocorticoids are involved in many functions, e.g. in the regulation of blood glucose levels and in anti-inflammatory reactions. Nurr1 expression is high in inflammatory arthritis and can be downregulated with glucocorticoids (Murphy et al. 2001). Mouse lines overexpressing NGFI-B show improper maturation of the thymocytes and almost complete lack of T cells (Kuang et al. 1999). Nor1 overexpressing mice are small and their body weight is only half of that of wild type mice, their thymus and spleen are also atrophic (Kagaya et al. 2005). Based on this evidence, it seems that NGFI-B and Nor1 are essential in controlling the tissues connected with the immunological system. There have also been reports of cross-talk between GR and NR4A receptors (Philips et al. 1997b, Martens et al. 2005). Cross-talk with other NRs have not been reported.

Two different lines of Nor1-deficient mice have been generated and studied in separate laboratories. In the first line, Nor1 knock outs are viable, but have dysfunctions in the inner ear and in the CNS (Pönniö et al. 2002, Pönniö and Conneely 2004). In the other line, knock outs die during development due to improper development of the anterior mesoderm and defects in gastrulation (DeYoung et al. 2003).

Crawford et al. (1995) studied NGFI-B knock-out mice. They seem to have normal overall physiology which is surprising, since NGFI-B has been linked with major regulatory pathways (Maxwell and Muscat 2006). NGFI-B knock-out mice are hyperactive and their dopamine neurons function abnormally (Gilbert et al. 2006). It has been shown that the amount of induced Nurr1 in lipopolysaccharide treated NGFI-B knock out mice is three times higher than in normal mice (Crawford et al. 1995). It has also been shown that the basal expression levels of Nurr1 in NGFI-B knock-outs is higher in certain areas of the brain of these mice compared to normal mice (Gilbert et al. 2006). Nurr1 could compensate for the loss of NGFI-B, since they both have been linked with similar functions and their expression is partially overlapping.

NR4A receptors have been shown to have an inhibiting role in the formation of atherosclerotic lesions (Bonta et al. 2006, 2007). They inhibit the functions of macrophages, and NGFI-B inhibits the proliferation of smooth muscle cells of the vascular wall, simultaneously promoting differentiation (Bonta et al. 2007). The physiological importance of NR4A receptors as protective mediators of vascular functions needs more clarification.

2.2 NR3B subfamily of orphan nuclear receptors

Estrogen-related receptors – the NR3B subfamily of orphan nuclear receptors, see fig. 1 – include three members: estrogen-related receptor α (ERRα), estrogen-related receptor β (ERRβ), and ERRγ (NR3A1, NR3A2 and NR3A3, respectively, Giguere 1999). ERRα and ERRβ were identified based on their similarity with ERα (Giguère et al. 1988), as they show high similarity in their DNA binding domain (68 % for ERα compared with ERRα). ERRγ was found by Hong et al. (1999) when studying proteins that interacted with the coactivator glucocorticoid receptor interacting protein 1, and also by Eudy et al. (1998) while they studied a specific locus of chromosome 1.

Crystal structure studies have shown that the ligand-binding pockets of ERRα and ERRγ are very small (Greschik et al. 2002, Kallen et al. 2004), but that synthetic ligands of ERRα and ERRγ can force amino acids to rearrange so that a larger ligand will fit into the pocket (Wang et al. 2006, Kallen et al. 2007). Endogeneous ligands for ERRs have not been found, but synthetic ligands, such as inverse ER agonists diethylstilbestrol (Coward et al. 2001), 4-hydroxytamoxifen (4-OHT, Coward et al. 2001), and 4-hydroxytoremifene

(Huppunen et al. 2004) have been reported. XCT790 has been specifically developed for ERRα (Busch et al. 2004, Willy et al. 2004). Synthetic agonists have also been reported for ERRβ and ERRγ (Zuercher et al. 2005).

The unliganded ERRγ is constanly in the active conformation and can interact with coactivator proteins (Greschik et al. 2002). Diethylstilbestrol and 4-OHT displace H12 of ERRγ, which leads to reduced binding of coactivators (Greschik et al. 2004, Wang et al. 2006). Thus, in contrast to NR4A receptors, NR3B receptors can be regulated with synthetic ligands, perhaps also with natural ones.

ERRs bind to a half-site consensus sequence TnAAGGTCA referred to as ERRE (ERR element, n being any base) and to estrogen response elements (ERE, AGGTCAnnnTGACCT, Yang et al. 1996, Johnston et al. 1997). ERRs bind DNA as monomers or homodimers and can also form heterodimers with each other as well as with ERα (Yang et al. 1996, Johnston et al. 1997, Huppunen and Aarnisalo 2004). Dimerization has been shown to modulate the transcriptional activity of ERRγ. Homodimerization increases the activity of ERRγ, whereas heterodimerization with ERRα inhibits its activity (Huppunen and Aarnisalo 2004). The meaning of the formation of ER-ERR heterodimers is still unclear.

2.2.1 Expression and biological functions of NR3B receptors

The ERR isoforms are expressed ubiquitously (Tremblay and Giguère 2007). All ERRs are expressed at elevated levels in tissues that need a lot of energy, such as the heart and the kidneys, and their functions have been strongly linked to the regulation of energy metabolism (Luo et al. 2003, Carrier et al. 2004, Tremblay and Giguère 2007). Recent studies have revealed more than 500 possible target genes for ERRs, many connected with the functions of the heart and regulatory networks (Tremblay and Giguere 2007). ERR target genes include TRα, PPARα, monoamine oxidase B, and apolipoprotein A4 (Vanacker et al. 1998, Carrier et al. 2004, Huss et al. 2004, Willy et al. 2004, Zhang et al. 2006).

ERRα expression is especially high in the intestine, brown adipose tissue and skeletal muscles, and its expression levels in the liver, uterus and bone have been shown to vary according to circadian rhythm (Horard et al. 2004, Tremblay and Giguère 2007). Mice with targeted disruption of the ERRα gene have reduced body weight and peripheral fat deposits (Luo et al. 2003) as well as altered expression of several genes involved in lipid

metabolism (Luo et al. 2003, Carrier et al. 2004). ERRβ is mainly expressed during embryogenesis, and ERRβ knock-out mice die at E10,5 (Luo et al. 1997). ERRβ is also expressed in the adult heart and kidneys (Bookout et al. 2005). ERRγ expression can be seen particularly in the brain stem and spinal cord (Tremblay and Giguère 2007). ERRγ knock-outs die shortly after birth due to problems in the mitochondrial oxidative metabolism (Alaynick et al. 2007).

Interestingly, many NRs are up or downregulated in the cardiac ventricles of the E18,5 ERRγ null mice (Alaynick et al. 2007). These NRs include LXR (up), NGFI-B (down), and ERRα and ERRβ (down). This links ERRγ to biological mechanisms controlled by other NRs.

ERRs are also linked to cancer. In breast and prostate cancers, ERRα has been shown to be a negative prognosis indicator, as ERRγ is considered as a better prognosis indicator (Tremblay and Giguère, 2007). ERRs thus seem to be involved in the regulation of many biological functions.

3. Orphan nuclear receptors and fibroblast growth factors in bone tissue

3.1 Bone tissue

Bone is a complex and constantly changing tissue. Bone tissue server many bodily functions. The skeleton and muscles together allow vertebrates to move and maintain posture. Bone tissue serves as a reserve of calcium and phosphate (Saladin 2001 p. 231). Bone has also haematological functions, as bone marrow inside the bone cavity produces erythrocytes, leukocytes and thrombocytes (Saladin 2001 p. 686). Specialized populations of bone cells form, maintain and remodel bone tissue. Bone is remodelled constantly in response to hormonal stimuli and mechanical loading (Hadjidakis and Androulakis 2006).

3.1.1 Bone structure and cell types

Bones are composed of cortical and trabecular bone. Cortical bone consists of cylinder-shaped, tightly packed units called osteons, while trabecular bone is more porous and does not contain osteons. The bone matrix has an organic component, primarily type I collagen, which gives it tensile strength. In addition, the bone matrix has an inorganic component, primarily hydroxyapatite (Ca10(PO4)6(OH)2), which gives it stiffness to compression (Saladin 2001 p. 231).

There are four types of bone cells based on their locations, morphology and functions: preosteoblasts, osteoblasts, osteocytes and osteoclasts. Preosteoblasts are formed from mesenchymal stem cells located in the bone marrow. Preosteoblasts differentiate further into bone-forming osteoblasts. Osteocytes are formed from osteoblasts trapped inside newly formed bone. Osteoclasts have a separate stem cell line, blood-borne monocytes, and they resorb bone (Hadjidakis and Androulakis 2006, Saladin 2001 p. 231).

3.1.2 Differentiation of osteoblasts

Bone marrow stroma contains mesenchymal stem cells, which have both significant proliferation capacity and ability to differentiate into chondrocytes, myoblasts, neurons, adipocytes, or osteoblasts (Porada et al. 2006). Mesenchymal stem cells are committed to osteoblast lineage by induction of the runt-related transcription factor-2 (Runx2, Banerjee et al. 1997, Ducy et al. 1998). Stem cells proceed to mature osteoblasts in three stages: proliferation, extracellular matrix development and maturation, and mineralization (Aubin and Triffith 2002). Other transcription factors, such as Wnt, osterix and Indian hedgehog, control Runx2 function or act downstream to regulate the expression of several genes (Karsenty and Wagner 2002, Yoshida et al. 2004). The coordinate expression of these transcription factors results in the expression of several genes that are important for the differentiation and function of preosteoblasts and mature osteoblasts, such as alkaline phosphatase (ALP), type I collagen, osteopontin (OPN), osteonectin, bone sialoprotein, and osteocalcin (OCN). These proteins are expressed sequentially during the process of osteogenesis (Aubin and Triffitt 2002). OPN is expressed highly in early preosteoblasts and remains highly expressed in mature osteoblasts, while OCN is expressed only in mature osteoblasts (Liu et al. 2003).

3.1.3 Bone remodelling

Osteoclasts are large multinucleated cells that are formed by fusions of mononuclear monocytes from the bone marrow (Väänänen et al. 2000). The receptor activator of nuclear factor-κB (RANK) and RANK ligand (RANKL) play important roles in the differentiation and activation of osteoclasts (see fig. 8). Osteotropic factors, such as parathyroid hormone (PTH), 1,25(OH)2D3 (vitamin D), and fibroblast growth factor-2, stimulate osteoblasts to produce RANKL. It has been suggested that the initiation of RANKL production is the key step in bone remodelling (Hsu et al. 1999, Hadjidakis and Androulakis 2006). RANKL binds to its receptor RANK on the cell surface of the osteoclast precursor. This activates the osteoclasts to resorp bone material (Lacey et al. 1998, Hadjidakis and Androulakis 2006).

Osteoclasts resorp bone by dissolving hydroxyapatite from bone tissue and breaking down the organic matrix enzymatically (Silver et al. 1988, Väänänen et al. 2000). After degradation by osteoclasts, osteoblasts fill the formed cylindrical canal, and new osteon is formed (Hadjidakis and Androulakis 2006).

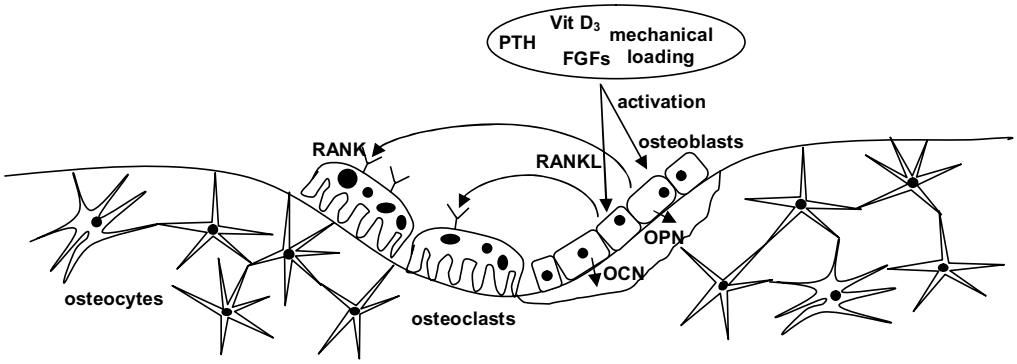

Fig. 8. Bone remodelling. Osteoblasts are stimulated by PTH, mechanical loading, and additional factors. Osteoclasts degrade bone in response to RANKL secreted by active osteoblasts.

3.1.4 Parathyroid hormone in bone

Parathyroid hormone (PTH) is secreted from the parathyroid. Most traditional actions of PTH are mediated by binding to the G-protein linked receptor, PTHR1 (PTH receptor 1). In bone,

PTHR1 is present mostly in osteoblasts (Gardella and Jüppner 2001, Potts 2005) and also detected in osteoclasts in certain pathological conditions (Langub et al. 2001). PTH controls the levels of calcium and phosphate in the blood. In hypocalcemia, the secretion of PTH is increased. This increases calcium absorption from the intestine by stimulating the synthesis of active vitamin D, and increases the reabsorption of calcium in the kidneys. PTH also mobilizes calcium from bone. PTH stimulates osteoblasts to activate osteoclasts with RANKL. Osteoclasts, by bone resorption, degrade bone and release calcium to blood (Potts 2005).

Continuous administration of PTH decreases bone mass (Tam et al. 1982). Intermittent administration of PTH, however, increases bone mass (Hock et al. 1988). This type of PTH administration also reduces the incidence of fractures in postmenopausal women and elderly men (Neer et al. 2001), and is therefore used to treat osteoporosis. Intermittent PTH has been shown to reduce the apoptosis of osteoblasts and to stimulate their proliferation by activating the PKA-pathway (Jilka 2007). Intermittent PTH increases the expression of RUNX2 protein (Krishnan et al. 2003). In addition, PTH treatment has been connected to increased osteoblastogenesis (Bellido et al. 2005). This suggests PTH a role in differentiation of osteoblasts. PTH also stimulates bone growth and improves fracture healing (Krishnan et al. 2003, Tsiridis et al. 2007, Barnes et al. 2008). PTH thus has a major role in controlling the formation and maintenance of bone tissue.

The differences in the outcome of intermittent and continuous administration of PTH have remained poorly understood. Intermittent and continuous PTH treatments have been shown to regulate differentially gene expression in rat femurs after 7 days of administration (Onyia et al. 2005). Jilka (2003) suggests that intermittent PTH could activate short bursts of survival signalling by mediating factors like Bad and BCL-2. According to his theory, during continuous administration of PTH the negative feedback loop remains active, thus preventing subsequent anti-apoptotic signals. It has also been suggested that in intermittent administration the PTH pulse is long enough to stimulate bone growth, but too short to activate bone resorption (Potts 2005).

3.2 Fibroblast growth factors and their receptors in bone

3.2.1 Fibroblast growth factors and their receptors

Fibroblast growth factors (FGFs) are a family of small polypeptide growth factors that control the proliferation and differentiation of various cell types (Powers et al. 2000, Xu et al. 2005, Monfils et al. 2006, Kim et al. 2007, Araki et al. 2007, Ng et al. 2008). Up to date, 23 members of the family have been identified in humans and mice (Fukumoto 2008).

FGFs produce their effects by signalling through transmembrane tyrosine kinase receptors, FGF receptors (FGFRs). There are four known FGF receptors (FGFR-1 through FGFR-4), and in humans seven principal receptor isoforms (Powers et al. 2000). Alternative splicing of FGFR1, FGFR2, and FGFR3 results in two functional receptor isoforms, the –b and –c receptors, which have different ligand-binding properties (Powers et al 2000, Eswarakumar et al. 2005). FGFs activate FGF receptors by inducing receptor dimerization (Ornitz et al. 1992, Spivak-Kroizman et al. 1994). This dimerization leads to transphosphorylation of the receptors and activation of several signalling pathways leading to a variety of biological functions including angiogenesis and development (Powers et al. 2000).

3.2.2 Fibroblast growth factors and their receptors –functions in bone

FGFs and FGFRs play important roles in skeletal development. In mice, the mutations or altered expression levels of FGF-2, -3, -4, -9, and -18 result in many skeletal disorders (Ornitz and Marie 2002). FGFR-2 is needed for osteoblast proliferation and mice lacking FGFR-2 have dwarfism phenotype and reduced bone density (Yu et al. 2003). FGFR-3 is considered as negative regulator of bone growth (Deng et al. 1996). In humans, different mutations in FGFR genes result in many skeletal dysplasias (Ornitz and Marie 2002, Marie et al. 2005). FGFs mediate limb formation and control osteogenesis (Cohn et al. 1995, Lewandoski et al. 2000, Moore et al. 2002, Mina et al. 2007, Zhou and Armstrong 2007). At least FGF-2, -4, -6, -8, -9, -18, and -23 have been linked to the regulation of osteoblastic cells (Marie 2003, Fakhry et al. 2005, Bosetti et al. 2007, Kawata et al. 2007). FGF-2, -4, and -8 have been shown to stimulate Runx2 expression in bone marrow cells and/or in osteoblasts (Zhou et al. 2000, Zhang et al.

2002, Kim et al. 2003), thus promoting preosteoblast formation. FGF-23 suppresses osteoblast differentiation and mineralization in calvarial osteoblasts and parietal bone cultures (Wang et al. 2008). FGF-2 has been shown to stimulate osteoclast formation and late differentiation and bone resorption (Zuo et al. 2004). The FGF family therefore seems to control the differentiation and mineralization of osteoblasts as well as bone turnover.

FGFRs have been shown to have important roles in bone. FGFR-mediated signaling in osteoblasts involves MAPK, PI-3K, PKC, phospholipase Cγ, and SRC-kinase pathways (Debiais et al. 2001). FGFR-1 is needed when undifferentiated bone marrow cells differentiate to osteogenic lineage (Ng et al. 2008). FGFR-2 is required for osteoblast proliferation and for the maintenance of osteogenesis (Yu et al. 2003). Targeted knock-out of FGFR-2c resulted in delayed bone ossification, dwarfism, and misshapen skull. FGFR-2c is also required for normal expression of Runx2 (Eswarakumar et al. 2002). Targeted knock-out of FGFR-3, on the other hand, caused bone over-growth, as the long bones and tails grew more and for a longer time than those of the wild type littermates (Deng et al. 1996). Thus, multiple FGFRs mediate the signalling by the FGFs in bone.

3.2.3 Fibroblast growth factor-8

FGF-8 was originally identified as a gene encoding two secreted androgen-induced growth factors that were responsible for the androgen dependent growth of the SC-3 mammary carcinoma cell line (Tanaka et al. 1992). Alternative splicing of the FGF-8 gene can result in eight different isoforms, FGF-8a through -8h, and FGF-8a, -8b, -8e and -8f are expressed in humans (Gemel et al. 1996, Valve et al. 2001). Of these, FGF-8b has the greatest ability to activate FGFRs in mitogenic assays as well as transform NIH-3T3 cells (MacArthur et al. 1995, Blunt 1997). FGF-8 can bind to and activate several of the FGFRs, namely FGFR-2c, FGFR-3c and FGFR-4 (Ornitz et al. 1996, Blunt et al. 1997).

Mutagenesis studies have shown that mice lacking FGF-8 die at E8,5 due to defective gastrulation (Meyers et al. 1998). During development, FGF-8 has important roles in the development of both the limb bud and the dopaminergic cells of the midbrain (Crossley and Martin 1995, Zhou et al. 2007). Nurr1 has a key role in the development of the same dopaminergic cells (Zetterström et al. 1997), and is expressed in the developing limb buds (Zetterström et al. 1996a). In addition, Nor1-deficient mice show similar defects in gastrulation as those seen in the FGF-8-deficient mice (Sun et al. 1999, DeYoung et al. 2003).

It is possible that NR4A receptors and FGF-8 regulate common processes during development.

In the normal adult, FGF-8b is only expressed in certain cells involved in spermatogenesis and oogenesis (Valve et al. 1997). However, it is expressed in human breast, ovarian and prostate cancer (Tanaka et al. 1998, Valve et al. 2000, Ruohola et al. 2001). FGF-8 overexpression in prostate cancer is associated with decreased survival (Dorkin et a. 1999), and the expression of FGF-8b has been shown to correlate with the tumor stage and grade (Gnanapragasam et al. 2003). Furthermore, proliferation of the S115 mammary carcinoma cell line is increased in cells stably overexpressing FGF-8b (Mattila et al. 2001). FGF-8b is therefore linked to tumour malignancy and proliferation of tumour cells.

FGF-8b treatment increases proliferation of mesenchymal stem cells and stimulates their differentiation to osteoblasts when cultured in the presence of osteogenic compounds (Valta et al. 2006). Thus, FGF-8 seems to have a role in the regulation of bone cell proliferation and differentiation.

3.3 The NR4A and NR3B orphan nuclear receptors in bone

During development, Nurr1 is expressed in hind limbs at E11,5 (Zetterström et al. 1996). In the adult, Nurr1, NGFI-B and Nor1 are expressed in long bones and in calvaria (Pirih et al. 2006). Their basal expression in bone is relatively low compared to that in e.g. brain tissue and adrenals (Bookout et al. 2005). However, PTH treatment induces the expression of the members of the NR4A-subfamily as immediate early genes in primary mouse osteoblasts through the PKA pathway in vitro (Tetradis et al. 2001a, 2001b, Pirih et al. 2003). The NR4A family has also been shown to be induced in vivo in calvariae and long bones when PTH is injected to mice daily (Pirih et al. 2005). In addition to PTH, also other activators of PKA and PKC upregulate the expression of Nurr1 in osteoblasts (Pirih et al. 2004).

The role of NR4A receptors in bone has not been studied until recently. In a study by Lee and others (2006) Nurr1 siRNA was used to downregulate Nurr1 expression in osteoblasts. Reduced expression of Nurr1 resulted in downregulation of the osteoblastic differentiation markers OCN and collagen type I alpha 1 (Col1A1) and in reduced ALP activity. OCN and Col1A1 expression and ALP activity are also downregulated in cultured primary osteoblasts from Nurr1 null mice. Nurr1 has been shown to bind to and activate an

NBRE element in the promoter of the OCN gene (Pirih et al. 2004). These findings suggest the NR4A receptors an important role in bone metabolism and in differentiation of osteoblasts. Other possible target genes and the biological functions of NR4A receptors in bone tissue remain to be solved.

Mouse ERRα has been shown to be expressed in developing long bones in the ossification centers at E15,5 and in bones formed by intramembranous ossification. In addition, ERRα is expressed in osteoblasts and osteoblastic cell lines (Bonnelye et al. 1997, Bonnelye et al. 2001). ERRα is also present in proliferating and differentiating osteoblasts and regulates bone formation (Bonnelye et al. 2001, Bonnelye and Aubin 2002). Downregulation of ERRα inhibits the formation of bone and cartilage (Bonnelye et al. 2001, Bonnelye et al. 2007). ERRα target genes in bone tissue include lactoferrin, TR, and aromatase (Yang et al. 1996, Vanacker et al. 1998, Yang et al. 1998). The role of ERRα in mediating OPN promoter activity is still somewhat unclear, but it seems that the OPN promoter contains several possible binding sites and that these functions are cell type specific (Vanacker et al. 1998, Zirngibl et al. 2008).

AIMS OF THE STUDY

The NR4A subfamily of orphan nuclear receptors consists of three constitutively active ligand independent members. This study addressed the functions of NR4A receptors in osteoblasts, and how the activity of NR4A receptors could be regulated.

The aims of the study were the following:

- To identify osteoblastic genes that are regulated by the NR4A receptors

- To find out if NR4A receptors participate in regulating the proliferation of osteoblastic cells

- To study how PTH and FGF-8b regulate the expression and functions of the NR4A receptors in osteoblasts

- To examine how the transcriptional activity of NR4A receptors can be controlled in the absence of ligands

MATERIALS AND METHODS

Detailed descriptions of the materials and methods used in this study are found in the original publications according to Table 1.

Table 1. Methods used in this study.

detection of apoptosis	IV
electrophoretic mobility shift assay	I, II
GST-pull down assay	III
immunocytochemistry	I, II, III
in vitro transcription and translation	I, II, III
mammalian cell culture	I, II, III, IV
plasmid construction and recombinant DNA technology	I, II, III
proliferation assay	IV
quantitative real-time PCR	IV
reporter gene assay	I, II, III
rt PCR	I
SDS-PAGE and immunoblotting	III
transfection of mammalian cells	I, II, III, IV

RESULTS AND DISCUSSION

1. Regulation of the expression of NR4A receptors by FGF-8b (Paper IV)

FGF-8b has functions in proliferating and differentiating osteoblastic cells and in developing dopaminergic cells (Ye et al. 1998, Valta et al. 2006). NR4A receptors have previously been shown to be induced by different growth factors (Lau and Nathans 1985, Liu et al. 2003, Roussa et al. 2006). As Nurr1 has been shown to be involved in the development and differentiation of dopaminergic neurons (Zetterström et al. 1997, Castillo et al. 1998, Chung et al. 2002), and there are observations that NR4A receptors have functions also in osteoblast (Pirih et al. 2004, Lee et al. 2006), we asked if FGF-8b could be one of the factors that induce the NR4As as immediate early genes and hypothesized that there is a connection between them in osteoblast regulation.

We studied whether NR4A expression could be stimulated by FGF-8b in osteoblastic cells. We used preosteoblastic MC3T3-E1 (hereafter referred to as MC3T3) cells from a subclone that is capable of bone nodule formation. The cells were treated with FGF-8b for different time periods, and subsequently total the RNA was isolated and studied for NR4A family expression by quantitative PCR. FGF-8b was found to stimulate the expression of Nurr1, NGFI-B and Nor1 as immediate early genes. The expression was detectable after 30 min and the expression of Nurr1 and NGFI-B peaked at 1h and then rapidly declined to and below the basal level at 2h and 4 h, respectively. The expression of Nor1 remained elevated until 2h and then declined to the basal level by 4h. The expression of Nor1 was stimulated more (16 fold) than that of Nurr1 (6) and NGFI-B (8). Regarding Nurr1 and NGFI-B, the mRNA expression was markedly increased already at a dose of 2,5 ng/ml and 5 ng/ml of FGF-8b, respectively, and further stimulated with higher concentrations. Nor1 mRNA expression was markedly increased with a higher concentration, 25 ng/ml of FGF-8b. 25 ng/ml of FGF-8b resulted in maximal induction of all NR4A receptors. At higher concentrations the induction was slightly diminished.

FGF-8b binds to FGFRs on the cell surface and regulates gene expression through several signalling pathways. We used different inhibitors to investigate which signalling pathways were responsible for the induction of the expression of NR4A receptors.

Inhibitors of the MAPK, PI-3K and PKC pathways blocked the stimulating effect of FGF-8b efficiently. Our results suggest that all major FGFR signalling pathways are involved in mediating the effects of FGF-8b on the NR4A family of orphan nuclear receptors (fig. 9). The expression levels of NR4A receptors have previously been shown to be regulated via PKA, PKC, calmodulin kinase II, MAPK, and PI-3K pathways (Song et al. 2001, Tetradis et al. 2001a, 2001b, Kovalovsky et al. 2002, Pirih et al. 2003). Our observation that several signaling pathways are involved in mediating the induction of the NR4A receptor expression by FGF-8b is thus in line with the previous reports on the induction of NR4A receptors.

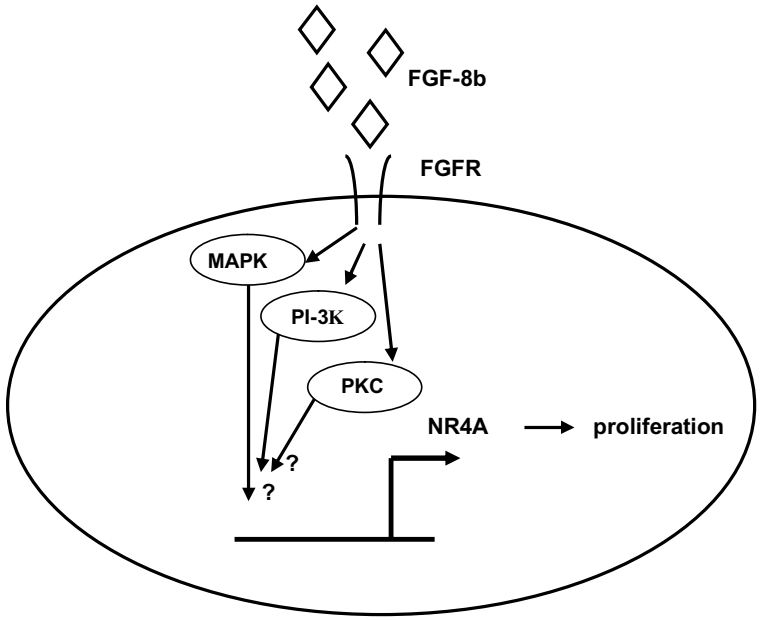

Fig. 9. Proposed model for signalling triggered by FGF-8b in preosteoblastic cells. FGF-8b binds to FGFR and activates several signalling pathways. Yet unidentified mechanism induces the expression of NR4A receptors that in turn affect the expression of downstream proteins. This eventually leads to increased proliferation of preosteoblasts.

We did not study whether the blockage of one or several signalling pathways affects the biological outcome of FGF-8b treatment (i.e. increased proliferation) or which binding site(s) on NR4A promoters is/are utilized for the induction. These questions remain to be solved.

Several pathways induce the NR4A receptors in osteoblasts. This could be an explanation to the rapid and high induction of the mRNAs of these proteins in response to FGF-8b and other stimuli, as many pathways simultaneously stimulate the NR4A mRNA

production. The tumour necrosis factor α has been linked to Nurr1 function and it can block the PKC pathway (Lee et al. 2000, O'Kane et al. 2008). Our results suggest that in situations in which one or more of the NR4A induction mediating pathways are blocked, the induction of NR4A receptors is not blocked completely.

Nor1 cannot heterodimerize with RXR (Perlmann and Jansson 1995, Zetterström et al. 1996b), and it also functions differently also in cross-talk with ERRs (see below). Nor1 is expressed more potently, but also more slowly as compared to the expression of Nurr1 and NGFI-B. The Nor1 expression also requires higher concentrations of FGF-8b. The NR4A receptors induced by PTH also show differential responses with respect to time and concentration (discussed in Pirih et al. 2003). These differences could be of importance when regulating different biological functions mediated by NR4A receptors.

Following PTH treatment of osteoblasts, Nurr1 is induced primarily through the PKA pathway (Tetradis et al. 2001a). Nervina and coworkers have shown (2006) that the Nurr1 coactivator PGC-1 is rapidly induced after PTH treatment, and that this induction is also mediated by PKA. They also demonstrated that PGC-1 can be induced by PKC activation, although this does not result from PTH treatment. Rapid induction of relevant coactivators further potentiates the functions of induced NR4A receptors (Nervina et al. 2006). Whether FGF-8b also induces PGC-1 or other potential coactivators in preosteoblastic cells remains to be clarified.

2. New biological functions for NR4A receptors (Papers I, IV)

2.1 Osteopontin, new target gene for NR4A receptors

To clarify the role of NR4A receptors in bone, we studied the genes that NR4As regulate in osteoblastic cells. We screened promoter regions of several osteoblastic genes for potential NBREs. Two were found in the promoter area of the OPN gene. When U2-OS and MC3T3 osteoblastic cells were transiently transfected with Nurr1, the expression of OPN was increased, suggesting that OPN could be a target gene for the NR4As.

OPN is important for bone homeostasis. OPN is present in non-mineralized bone and it is suggested to act as a binding agent between the collagen proteins and to

regulate the growth and directions of hydroxyapatite crystals (Sodek et al. 2000). Overexpression of OPN in bone marrow-derived osteoblasts resulted in higher a expression of other differentiation markers and also in increased bone nodule formation (Kojima et al. 2004). Thus OPN seems to be involved in triggering the differentiation of osteoblasts. OPN knock-out mice seem to have normal skeleton. However, in these mice postmenopausal bone resorption was inhibited (Yoshitake et al. 1999). These mice were also resistant to PTH-induced bone resorption, possibly due to impaired RANKL functions (Ihara et al. 2001). In OPN deficient mice, intermittent PTH treatment also enhanced the improvement of bone mineral density and cortical bone mass compared to wild type mice (Kitahara et al. 2003). It seems that OPN acts as a suppressor of PTH signalling-induced increase of bone mass (Ono et al. 2008). These results link PTH signalling to OPN functions.

PTH has both anabolic and catabolic effects in bone. As PTH induces the expression of NR4A receptors (Tetradis et al. 2001a, 2001b, Pirih et al. 2003), and as it seems that OPN might be a target gene for the NR4As, we studied whether PTH regulated OPN expression through the NR4A receptors. Treatment of the cells with 100 nM PTH induced Nurr1 mRNA expression as an immediate early gene in osteoblastic cells. Eight hours later, OPN mRNA expression was also increased. The dominant-negative Nurr1 variant (Nurr1 DN, Castro et al. 1999) binds to DNA as the wild type Nurr1 and represses promoter activity. While binding DNA, the dominant negative Nurr1 also diminishes the DNA binding of all members of NR4A receptors, thus blocking their activating functions. An increase in the expression of OPN after PTH treatment could be inhibited by transfecting the cells with Nurr1 DN. These results suggested that NR4A receptors had a role in mediating PTH-induced OPN expression.

The OPN promoter was found to contain two putative NBRE elements. Therefore, the -857/+191 region of the mouse OPN promoter was cloned upstream of the luciferase reporter gene. To see whether the NR4As regulated OPN promoter activity directly, we performed reporter gene assays in transiently transfected osteoblastic cells (U2-OS, SaOS2, and MG63 cells). Nurr1, NGFI-B and Nor1 transactivated the OPN promoter in several osteoblastic cell lines but not in kidney-derived cells. This demonstrates that OPN promoter activation was osteoblast specific. To find out if this transactivation was due to direct binding of NR4A receptors to the OPN promoter, we used mutated Nurr1 constructs. The mutations were introduced in the first zinc finger (Nurr1 C283G) and in the A-box (Nurr1 R334A, Castro et al. 1999), which prevent the DNA binding of Nurr1. These mutants were not able to transactivate the OPN promoter, suggesting that Nurr1 binds directly to the

OPN promoter. The OPN promoter contains two putative response elements for NR4A receptors that have previously been named as the S1 and S2 elements (Vanacker et al. 1998). We used wild type and mutated S1 and S2 elements in electromobility shift assay (EMSA) to examine, whether Nurr1 directly bound to these sites. Nurr1 was found to bind directly to the S1 but not to the S2 site. In reporter assay, Nurr1 was found to activate the OPN reporter when S2 was mutated. Nurr1 did not activate the OPN promoter that was mutated at the S1 site. These results confirm that Nurr1 binds directly to the S1 element of the OPN gene promoter and transactivates it. This result together with our transfection data confirm that OPN is a target gene for the NR4A subfamily of orphan nuclear receptors (see fig. 10).

Since Nurr1 and NGFI-B can heterodimerize with RXR, we studied whether they transactivated the OPN promoter as monomers or as heterodimers with RXR. We used a mutated Nurr1 that lacks the ability to heterodimerize with RXR (Nurr1 KLL554-556AAA, Aarnisalo et al. 2002). This mutant was able to transactivate the OPN promoter as effectively as the wild type Nurr1. This suggests that Nurr1 does not have to heterodimerize in order to activate the OPN promoter. Our result is in line with the findings that Nor1, which does not heterodimerize with RXR, was also able to transactivate the OPN promoter. However, when the cells were transfected with the wild type Nurr1 and treated with the RXR agonist SR11237, the treatment efficiently increased the transactivation of the OPN promoter. This suggests that Nurr1 can also bind to the OPN promoter and transactivate it as a heterodimer with RXR (fig. 10).

As Nurr1 has been shown to regulate genes in an AF-1 or AF-2-dependent manner depending on the cell type and the promoter (Castro et a. 1999, Sohn et al. 2001, Wansa et al. 2002, Maira et al. 2003), we tested whether the AF-1 or AF-2 was responsible for the Nurr1-mediated transactivation of the OPN promoter in U2-OS cells. We used a Nurr1 variant mutated at both AFs (Nurr1Δ1-84/D589A, Castro et al. 1999). This mutant lacks the N-terminal region shown to contain the AF-1. In addition, the AF-2 is mutated. The transactivation by Nurr1 was abolished completely. We then used Nurr1 variants with separately mutated AF-1 and AF-2 (Nurr1 Δ1-84 and Nurr1D589A, respectively, Castro et al. 1999). The OPN promoter was shown to be activated by the AF-2 mutated Nurr1 as efficiently as by the wild type Nurr1. In contrast, the Nurr1 mutant lacking the AF-1 was less effective in activating the OPN promoter.

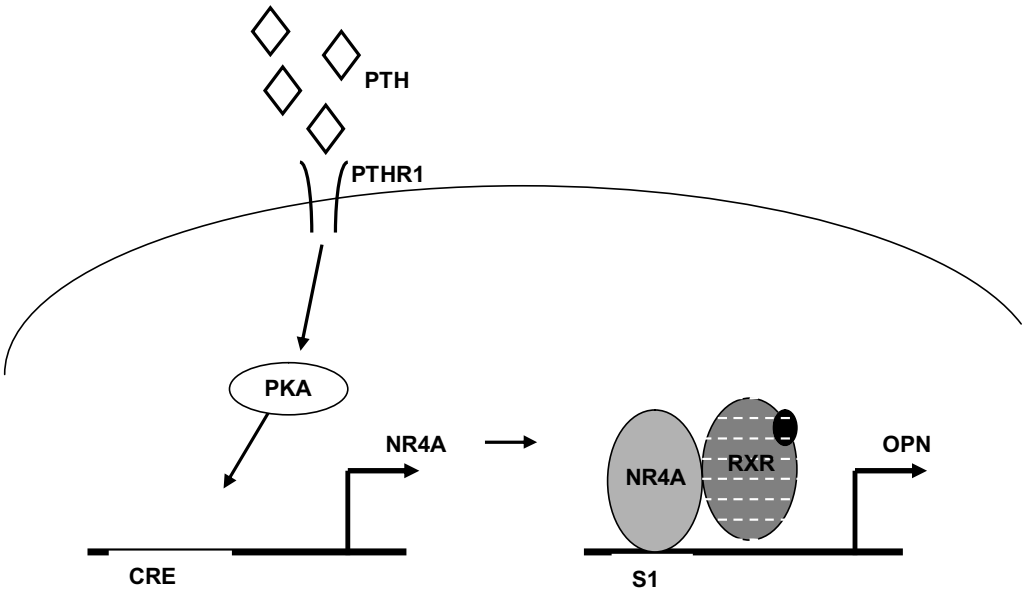

Fig. 10. PTH regulates OPN synthesis *via* NR4A receptors. PTH signaling induces NR4A receptors primarily through PKA pathway and propably by affecting the cAMP responsive element (CRE) on Nurr1 promoter (Tetradis et al. 2001a, 2001b). NR4A receptors then bind S1 element on OPN promoter and stimulate OPN production. Heterodimerization with RXR is not required, but treatment with RXR agonist enhances stimulation.

These results suggest that the transactivation by Nurr1 is mostly AF-1 dependent on this promoter and in this cell line. Previously it has been suggested that a character between the amino acids 1 and 160 of Nurr1, which includes the AF-1, is responsible for the specific activation of different promoters and also for binding of coactivator SRC-2 (Wansa et al. 2002).A coactivator is probably linked to AF-1-dependent, NR4A mediated regulation of the OPN-promoter. SRC-2 and PGC-1 have been linked to the functions of the NR4A receptors (Wansa et al. 2002, Nervina et al. 2006). The role of these coactivators in the OPN gene activation by NR4A receptors should be further investigated.

Vitamin D is involved in bone homeostasis, and VDR can be found in osteoblasts, osteoclast precursors and mature osteoclasts (Mee et al. 1996, Johnson et al. 1996, Langub et al. 2000). Addition of 1,25(OH)2D3 to a primary culture of human bone marrow stromal cells results in increased expression of ALP and OPN suggesting that 1,25(OH)2D3 has a role in regulating the differentiation of osteoblasts (Beresford et al. 1994). VDR has been shown to bind the OPN promoter at a vitamin D responsive element at 761

bases upstream of the OPN start codon (Noda et al. 1990). To study whether Nurr1 and VDR co-operated in OPN promoter regulation, we transfected cells with Nurr1 and treated them with vitamin D. The combined effect of Nurr1 and vitamin D was synergistic. When the S1 site was mutated, vitamin D was still able to increase the activity of the OPN promoter. This suggests that Nurr1 and vitamin D bind and activate the OPN promoter at distinct sites.

In vivo, PTH is cleared from circulation in 2-3 hours (Jilka 2007). In cell culture conditions, PTH is degraded slowly, as it takes more than 24 hours (Ishizuya et al. 1997). In the study by Ishizuya and others (1997), it was demonstrated that the length of the PTH exposure is crucial for bone nodule formation. They treated primary osteoblasts with PTH for 3 and 6 hours during every 48 hour cycle. In one experimental group, the PTH-containing medium was left on cells for the whole 48 hours, but no PTH was added to cells during this 48 hour period. Differences in the bone nodule formation were dramatic. No bone nodules were formed in the dishes where the PTH-containing medium was not changed. Nodule formation was most efficient on dishes treated with PTH for 3 or 6 hours. In addition, short term (4 hours *vs* 12 or 24 hours) PTH treatment has also been shown to upregulate unique genes (Partridge et al. 2006). Our results on OPN induction were obtained in conditions between intermittent and continuous PTH treatment, as the PTH containing medium was not removed after exposure. On the other hand, the maximum exposure time was eight hours. If we had replaced the PTH containing medium after 1-3 hours of exposure, the response in the induction of OPN could have been different.

In bone, PTH has been shown to upregulate the expression of many genes, such as type 1 collagen and bone sialoprotein (Thiébaud et al. 1994, Yang and Gerstenfeld 1996). PTH has also been shown to activate the OCN reporter in osteoblastic cells (Yu and Chandasekhar 1997). The evidence indicates that both PTH and NR4A receptors are linked to many important proteins of bone besides OPN, suggests NR4As a major role in the regulation of bone homeostasis. Since the NR4A receptors are immediate early genes, their expression is rapidly increased by different stimuli, and their expression is rapidly decreased after the offset of the original stimulation. The suggestion that that short, intermittent PTH treatment activates bone formation but does not have time to activate bone resorption (Potts 2005), implies that NR4A receptors could be mediators of the anabolic effects of short term PTH treatment.

After our results were published, another group reported that also the OCN promoter is regulated by PTH via Nurr1 (Pirih et al. 2004). Their work demonstrated that PTH induces Nurr1 mRNA and results in increased binding of Nurr1 on the OCN promoter in

mouse primary osteoblasts. In addition, Nurr1 was shown to bind the OCN promoter alone, not as a heterodimer with RXR. Later, the same group published an observation that intermittent administration of PTH for 1 week increased the expression of OPN and OCN mRNA in vivo in calvariae and long bones (Pirih et al. 2005). PTH also induces the expression of coactivator PGC-1α as an immediate early gene (Nervina et al. 2006). PGC-1α in turn enhances the activity of Nurr1 on the OCN promoter in mouse primary osteoblasts. In our studies, PGC-1 did not stimulate Nurr1 activity. We used U2-OS cells in our experiments with PGC-1. Later it was revealed that U2-OS cells do not contain endogenous PGC-1 (Rajalin A, personal communication). The fact that we did not observe PGC-1 stimulation of Nurr1 is probably due to different regulatory systems present in different cell lines. Nurr1 has also been linked to the differentiation of osteoblasts in studies using the siRNA technique to knock down Nurr1 expression. In these studies, Nurr1 siRNA prevented the expression of OCN and Col1A1, inhibited ALP activity, and decreased the number of bone nodules in differentiating MC3T3 cells (Lee et al. 2006).

These results together demonstrate a new role for the NR4A family in mediating bone-related genes. More studies need to be carried out in order to find out the extent of the NR4A regulated events in bone development and remodelling.

2.2 Nurr1 and NGFI-B stimulate the proliferation of preosteoblastic cells

The members of the NR4A subfamily have been shown to act both in an apoptotic and anti-apoptotic manner depending on the cell type, and also to affect the proliferation of different cell types (Cheng et al. 1997, Li et al. 1998, Kolluri et al. 2003, Nomiyama et al. 2006). FGF-8b has been shown to stimulate the proliferation of bone marrow cells (Valta et al. 2006). As we had demonstrated that FGF-8b induces the NR4A receptors in preosteoblasts, we decided to study whether the NR4A receptors are involved in FGF-8b stimulated proliferation.

In our experiments, overexpression of Nurr1 and NGFI-B increased the proliferation of preosteoblastic MC3T3 cells. Nor1 did not stimulate proliferation. Also FGF-8b treatment for 20 hours stimulated proliferation. When the cells were transfected with Nurr1 DN, FGF-8b treatment had no effect on proliferation. This suggests that FGF-8b induces the NR4A family members and then Nurr1 and NGFI-B act to stimulate proliferation (fig. 9). Nor1 is not capable of forming heterodimers with RXR. As overexpression of Nor1 did not

stimulate proliferation, we tested whether RXR heterodimerization was involved. We used 1 μM RXR agonist SR11237 to activate the endogeneous RXR in Nurr1, NGFI-B and Nor1 overexpressing cells. However, this had no effect on proliferation, suggesting that RXR heterodimerization is not involved. It therefore remains unclear which properties of Nurr1 and NGFI-B are responsible of the stimulation of proliferation.

To study the effect of NR4A receptors and FGF-8b on apoptosis of preosteoblasts, we overexpressed Nurr1, NGFI-B and Nor1 in MC3T3 cells. The overexpression of Nurr1 and NGFI-B decreased apoptosis significantly, whereas Nor1 had no effect. However, the treatment of cells with FGF-8b affected the apoptosis of neither the non-transfected nor NR4A overexpressing cells. It therefore seems that Nurr1 and NGFI-B reduce apoptosis independently of FGF-8b. Our results suggest that Nurr1 and NGFI-B increase cell growth by both increasing proliferation and reducing apoptosis simultaneously.

Our results demonstrate that overexpression of Nor1 does not regulate the proliferation or apoptosis of preosteoblastic cells. However, a study using Nor1 knock-out animals showed that Nor1 is required for the proliferation of aortic smooth muscle cells (Nomiyama et al. 2006). The Study by Pönniö et al. (2002) with a Nor1 knock-out model showed that Nor1 is essential for the proliferation of nonsensory epithelial cells of the inner ear. Disruption of Nor1 did not affect the apoptosis of these cells. It has also been shown that constitutive overexpression of Nor1 results in massive apoptosis in thymocytes (Cheng et al. 1997). Nor1 therefore controls proliferation and apoptosis differently in different cell types and in different organs.

FGF-8b is expressed in breast and prostate tumours (Tanaka et al. 1998, Ruohola et al. 2001). Breast and prostate tumours also frequently metastasize to bone. These metastases can be either osteolytic (bone degrading), osteoblastic (bone forming), or have characters from both osteolytic and osteoblastic forms (Mundy 2002). It has been suggested that osteolytic metastases are caused by osteoclast-activating factors that are released from the tumour cells. Osteoblastic metastases could be caused by factors produced by the cancer cells that stimulate osteoblast proliferation and differentiation (Mundy 2002). The FGF-8b that is produced and secreted by breast and prostate tumour cells may induce the expression of the NR4A receptors in the neighbouring osteoblasts. This signalling could have a major role in the regulation of the proliferation of osteoblastic metastases in bone.

Nurr1 expression can be seen in developing hind limbs at E11,5 (Zetterström et al. 1996a). As FGF-8 signalling is also linked with limb development (Vogel et al. 1996), the co-operation of the NR4A family and FGF-8b should be further studied in developing bone.

OPN is expressed through proliferational and differentiational stages of osteoblasts and is considered a protein associated with active proliferation, as it is thought to regulate and prevent mineralization (Aubin et al. 1995, Sodek et al. 2000). The NR4A family could also activate other genes that stimulate proliferation. OCN is mainly expressed by osteoblasts in the late differentiational stages (Aubin et al. 1995). Coll1a1 is a marker for advanced differentiation and has been shown to harbour several potential Nurr1 binding sites (Lee et al. 2006). In the study by Lee and others (2006), Nurr1 promoted the differentiation of MC3T3-E1 cells. It is possible that FGF-8b regulates osteoblast proliferation and differentiation by inducing the NR4A subfamily, thus triggering the expression of OPN and OCN. Further studies are needed to investigate whether the other osteoblast differentiation markers could also be target genes for the NR4A family.

In bone, OPN has been shown to suppress the proliferation and differentiation of a MC3T3 cell subclone capable to bone nodule formation (Huang et al. 2004). It has also been demonstrated that in these subclones, the expression of OPN is increased at higher passage numbers and correlates with weakened nodule formation (Huang 2001). PTH is capable of inducing the proliferation of osteoblasts (Datta et al. 2007, Pettway et al. 2008). This proliferation was linked to the non-confluent stage of culture, and the phenomenom was reported in MC3T3 cells capable of bone nodule formation (Datta et al. 2007). It seems that the differentiational status and the level of maturation affect the outcome of different treatments of osteoblastic cells. In our FGF-8b experiments, we used mesenchymal stem cells capable of forming nodules, preosteoblastic MC3T3 cells capable of forming nodules, MC3T3 cells not capable of forming nodules, and osteoblastic U2-OS cells not capable of forming nodules. Of these, FGF-8b induced NR4A receptors only in cells capable of forming nodules. This suggests that NR4A receptors are not induced by FGF-8b in more mature osteoblastic cells. It is possible that the FGF-8b-induced NR4A receptors are linked to increased OPN expression in maturing osteoblasts. FGF-8b, NR4A receptors, and OPN could successively regulate the proliferation and differentiation of osteoblastic cells. This hypothesis needs further experiments.

Our studies have linked FGF-8b to the regulation of NR4A expression. In addition to osteoblasts, NR4A receptors have also been linked to the functions of dopaminergic neurons (Zetterström et al. 1997, Castro et al. 2001, Chung et al. 2002). In a study by Chung and others (2002), Nurr1 was overexpressed in pluripotent embryonic stem cells, and this overexpression was shown to potentiate the formation of dopaminergic neurons. Combined FGF-8 treatment was found to further increase differentiation to

dopaminergic neurons, potentiating the stimulating effect that Nurr1 had alone. Dopaminergic neurons of the midbrain are involved, among others, in learning, reward, and in the regulation of movement, (Robbins and Everitt 1996, Hollerman and Schultz 1998, Jankovic et al. 2005). These cells are lost in patients with Parkinson's disease (Stoessl 2008). The connection among FGF-8b signalling, the NR4A family, and the development and regulation of dopamine neurons should be further addressed.

3. Cross-talk with the receptors of the NR3B group represses the activity of NR4A orphan receptors (Papers I, II)

NR4A receptors and ERRα have been proposed to bind to the same S1 response element on the OPN promoter and to activate it (Vanacker et al. 1998, Vanacker et al. 1999). ERRα has also been shown to bind the S2 element. Our results in paper I showed that NR4A receptors bind to the S1 element on the OPN promoter. We examined if the NR4A receptors and NR3Bs co-operate in controlling OPN promoter activation.

When ERRα and ERRγ were transfected to SaOS-2 cells, the OPN-luc reporter was not activated in contrast to previous results (Vanacker et al. 1998). However, when overexpressed together with Nurr1, ERRα and ERRγ were able to repress the transactivation of OPN caused by Nurr1. This repression was dependent on the ratio of transfected plasmids, as increasing amounts of ERRγ resulted in more efficient repression. ERRα and ERRγ also repressed the activating effects of Nurr1 on the NBRE element. ERRβ did not have the same effect, not even with increasing amounts of transfected ERRβ plasmid. When the NR4A receptors were overexpressed in osteoblastic cells with the ERRγ and OPN-luc reporter, only the activity of Nurr1 was efficiently repressed, whereas the activities of NGFI-B and Nor1 were only slightly repressed.

We then transfected Nurr1, NGFI-B and Nor1 to the osteoblastic cells with the ERRγ and ERRE-LUC element. NGFI-B repressed efficiently the activating effects of ERRγ on ERRE, whereas Nor1 repressed ERRγ slightly and Nurr1 had no effect. Again, increased amounts of NGFI-B resulted in a more efficient repression. NR4A receptors and NR3Bs were found to mutually repress each other's ability to activate target elements.

It is of interest that not all, but one or two members of the NR4A and NR3B families take part in the observed mutual repression (i.e. only Nurr1 acitivity is repressed by ERRγ, ERRβ does not repress Nurr1 activity, and only NGFI-B represses effectively ERRγ activity). These differential repressions could have an important role in the tissues expressing different sets of NR4A receptors and ERRs. Furthermore, when the NR4A and NR3B receptors were cotransfected in osteoblastic cells, ERRα and ERRγ repressed the ability of Nurr1 to activate both NBRE and OPN reporters. ERRβ failed to repress the activity of NR4A receptors. This could originate from ERRβ is propably not being expressed in the adult bone. In humans, ERRβ has been shown to be expressed in adults in tissues such as breast, prostate, testis and kidney, but its most important functions take place during development (Luo et al. 1997, Zhou et al. 2006). In mice, ERRβ has not been shown to be expressed in bone tissue (Bookout et al. 2005). However, NR4A receptors and ERRβ could have important cross-talk during development.

To investigate which part of the receptors contained the property needed for the mutual repression, we used mutated NGFI-B and ERRγ constructs in reporter assays. The mutations abolishing the DNA binding ability of ERRγ (C125G; first zinc finger, and R176A; A-box, Huppunen et al. 2004) lacked the ability to repress the activation of OPN by Nurr1. Similarly, mutation in the NGFI-B DBD (C252G; first zinc finger) did not repress the binding of ERR to ERRE. Our results thus suggest that intact DBD is needed for the repression. In order to study the involvement of DNA binding in repression, we used EMSA to investigate the binding of DNA by NR4A receptors and NR3Bs. ^{32}P-labelled OPN S1 element, NBRE, and ERRE were used as probes. Both ERRα and ERRγ bound to the OPN S1 and S2 elements and NBRE, although much less efficiently than Nurr1. In a competition assay, ERRα and ERRγ were found not to compete for DNA binding with Nurr1 on the NBRE or OPN S1 elements. ERRs could still be binding the S2, therefore repressing the activating effect of Nurr1 simultaneously bound to S1. When the OPN S2 element of the OPN reporter was mutated and used in transfections with Nurr1 and ERRγ, repression was not blocked. This indicates that ERRγ does not inhibit OPN activity by binding to S2 element.

Nurr1 and NGFI-B were shown not to bind ERRE in EMSA, and increasing amounts of NGFI-B did not have effect on the binding of ERRγ to the ERRE element. In addition, the repressive effect that Nurr1 DN had on the basal level of the NBRE reporter was not abolished by ERRγ. This implies that ERRγ does not interfere with the DNA binding of Nurr1. We also conducted an experiment in which transfected cells were treated with 4-OHT. 4-OHT is an inverse agonist of ERRγ that does not reduce ERRγ DNA binding but has been

shown to release coactivator SRC-1 (Coward et al. 2001). 4-OHT treatment of the cells abolished the repressive effect of ERRγ on Nurr1. These results together suggest that alterations in the DNA binding of Nurr1 are not involved in the repression of its ability to activate target elements.

In addition to DNA binding properties, DBD also contains sequences important for the subcellular localisation of the receptors (Cadepond et al. 1992, Black et al. 2001), and localization has been shown to be connected to NR4A functions. We observed no altered localization when Nurr1 and ERRγ were cotransfected into U2-OS cells. This suggests that the repression of NR4As and NR3Bs abilities to activate target elements is not due to changes in the localization.

NRs can form heterodimers that can inhibit or stimulate their transactivation (Zhang et al. 1992, Cowley et al. 1997, Maira et al. 1999, Huppunen and Aarnisalo 2004). The formation of heterodimers often involves the I-boxes of the LBD (Perlmann et al. 1996b), and Nurr1 uses this interface when heterodimerizing with RXR (Aarnisalo et al. 2002). We used ERRγ constructs mutated at the I-boxes (ML-AA, R390A, L398A, Huppunen et al. 2004), and these mutants failed to repress the ability of Nurr1 to activate the OPN promoter. Also, an I-box mutant of NGFI-B (GKL522-524AAA) repressed the ability of ERRγ to activate the ERRE reporter only modestly. This implies that the dimerization of NR4As and NR3Bs could be involved in the mutual repression. In mammalian two-hybrid assay, however, no interaction between the LBDs of Nurr1 and ERRγ was observed. Furthermore, no dimeric bands were detected in EMSA, and dimer formation was not observed in co-immunoprecipitated samples. This suggests that the mutual repression is probably not due to the formation of inactive heterodimers.

Competition for coactivators could be the cause for mutual repression between NR4As and NR3B. The involvement of coactivators was assessed using ERRγ mutated at the AF-2 (E429A, Huppunen et al. 2004). This mutation had no effect on the repression of Nurr1. PGC-1 is a coactivator shown to modulate the activity of both NR4A and NR3B receptors (Huppunen et al. 2004, Nervina et al. 2006), in addition, PGC-1 has also been shown not to require AF-2 to activate ERRγ (Huppunen et al. 2004). In our experiments, PGC-1 was not able to coactivate Nurr1 in U2-OS cells. Thus it seems that at least PGC-1 was not the coactivator the NR4As and NR3Bs are competing for in osteoblastic cells.

Based on our results, it seems that the mutual repression between NR3B and NR4A receptors is dependent of three factors: 1) The properties present at the I-box. 2) The properties present in the DBD. 3) The relative expression levels of these receptors. Further

analyses are needed to investigate whether mutual repression is caused by competition for other coactivators present in different cell lines. Also PGC-1 could be involved in different cell lines or in primary osteoblasts. As both PGC-1 and NR4A receptors are induced by PTH treatment (Nervina et al. 2006), it is possible that the upregulation of PGC-1 takes place to secure the amount of correct coactivator in gene regulation process by NR4A receptors.

There have not been many reports of ligands controlling the activity of NR4A receptors. However, our results show that 4-OHT can be used to control Nurr1 activity through ERRγ. 4-OHT treatment of osteoblastic cells overexpressing Nurr1 resulted in a slightly enhanced activation of the OPN-promoter. When Nurr1 and ERRγ were co-expressed in cells and treated with 4-OHT, the repression caused by ERRγ was blocked effectively. In situations in which Nurr1 activity is decreased, 4-OHT could induce activity partly by blocking the repressing ability of ERRγ.

In their recent study Zirngibl and others (2008) showed that ERRα repressed the activity of the OPN promoter in osteoblastic ROS17/2.8 cells. Vanacker and others (1998) have shown that ERRα activates the OPN promoter in ROS17/2.8 cells. In our studies, in osteoblastic SaOS2 cells, ERRα did not stimulate nor inhibit the OPN promoter. The repression of OPN by ERR α seen in the study by Zirngibl and others, did not require DNA binding of ERRα as in the cross-talk with NR4A. Furthermore, Zirngibl and others showed that ERRα does not regulate the OPN promoter by binding to any of the suggested S1 to S6 binding elements. There can be another binding site for ERRα, or ERRα could in some other way regulate the OPN promoter and this mechanism may be the explanation for the repression we observed. The role of ERRα in different osteoblastic cells needs more clarifying.

Mutual repression has also been reported between the receptors of the NR4A family and GR in pituitary cells (Philips et al. 1997b, Martens et al. 2005). This repression is mediated in a manner similar to what we have seen between NR4A receptors and the NR3Bs, i.e. by a feature present in the DBD of Nurr1 and NGFI-B. Furthermore, the repression reported between NR4As and GR it does not require formiation of NR4A/GR dimers (Martens et al. 2005). As in the case of the NR4As and NR3Bs, this mutual repression has also been shown to vary depending on the relative expression levels of the receptors (Philips et al. 1997b). In adrenal cells, however, Nurr1 has been shown to be stimulated by a direct interaction with GR (Carpentier et al. 2008). It has been suggested that there are cell specific cofactors that control the activating and repressing qualities of the NR4A-GR interaction. Transcriptional activity of NR4A receptors may thus be controlled by the number of receptors in relation to other factors present in the cells at a certain moment.

ERRγ overexpression has been shown to suppress the proliferation of prostate cancer cell lines and to decrease the colony formation capability of these cells (Yu et al. 2007). This phenomenon was shown to be linked to the DBD, as the deletion of the first zinc finger of ERRγ abolished the suppression. Tumour formation was shown to be reduced significantly when ERRγ was overexpressed. In addition, it was shown that ERRγ agonist decreased the proliferation of prostate cancer cells. Nurr1 and NGFI-B have also been linked to proliferation by us in paper IV and by others (Castro et al. 2001, Kolluri et al. 2003, Mullican et al. 2007). This suggests that ERRγ has a common pathway with Nurr1 and NGFI-B in the regulation of proliferation. A higher relative expression of ERRγ could repress the stimulating effect of Nurr1 and NGFI-B on proliferation and thus suppress proliferation. This suggestion requires further studies.

NR4As and NR3Bs could jointly mediate several biological functions by regulating common, known or yet unknown, target promoters in a manner dependent on their relative expressions. Several tissues, such as the brain, express both NR4As and NR3Bs. It is possible that the transcriptional activities of NR4A receptors are regulated in a complex manner by regulating the levels of expressions of NR4As, NR3Bs, and other cofactors. This cross-talk should be investigated with respect to other cell types, such as the dopaminergic cells, and other promoter areas besides OPN, such as OCN.

4. The mechanism of permissiviness of Nurr1-RXR heterodimers (Paper III)

Permissiviness of NRs in RXR heterodimers has been linked to the LBDs (Gampe et al. 2000, Germain et al. 2002). We investigated how permissiviness is determined. We started by studying which part of the LBD is mediating the permissiviness of Nurr1/RXR heterodimers. We created chimeric constructs that contain parts of the permissive Nurr1 and non-permissive RAR LBDs (fig. 11, constructs A, B, C and D). We then examined their activity on a reporter gene driven by βRE, a DR5 type of HRE, and treated the cells with RXR agonist SR11237. RAR chimera containing the Nurr1 LBD (construct A, fig. 11) was found to form permissive heterodimers with RXR in reporter assays, thus confirming the previous results of permissiviness being linked to the LBD.

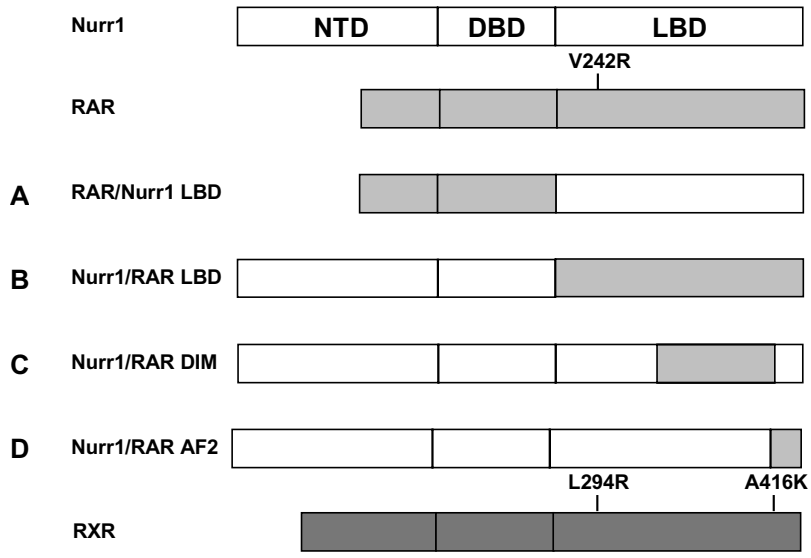

Fig. 11. Schematic presentation of the Nurr1/RAR chimeras and locations of RAR and RXR mutations.

Chimeras containing the dimerization area of RAR LBD (C) as well as the AF-2 area of RAR LBD (D) were found in reporter assays to form permissive RXR heterodimers. However, when the whole LBD of Nurr1 was replaced with RAR LBD (B), permissiviness was lost. These results suggest that the N-terminal part of the LBD is crucial for permissiviness. This part of the LBD has been shown to be important for the binding of corepressors (Hu and Lazar 1999). Using SMRT and NcoR, we to investigated, whether corepressors are involved in permissiviness.

In reporter gene assays, the ability of Nurr1 to induce NBRE was repressed when cotransfected with SMRT, suggesting that Nurr1 may interact with SMRT. The interaction of Nurr1 and SMRT was demonstrated in mammalian two hybrid assay. Mammalian two hybrid assays also showed that this interaction was very weak compared to the interactions between RXR, RAR, TR and SMRT. Similar results were obtained with NCoR. SMRT interacted also with NGFI-B. This interaction was more efficient than with Nurr1. This is in line with previous results showing that SMRT interacts more potently with NGFI-B than with Nurr1 (Sohn et al. 2001). Permissiviness has been previously suggested to be linked to weak binding of corepressors (Germain et al. 2002). Our results show that

permissive RXR heterodimerization partners bind corepressors more weakly than non-permissive partners.

To find out how Nurr1/RXR heterodimers interacted with SMRT, we performed GST pull-down (glutathione S-transferase pull-down) assays. Both RXR and SMRT alone interacted with Nurr1 LBD. When Nurr1 LBD, RXR and SMRT were incubated together, Nurr1 interacted with both RXR and SMRT. Nurr1/RXR heterodimers also seemed to bind SMRT more efficiently than the Nurr1 monomer. To further study the effect of heterodimerization on the interaction between Nurr1 and SMRT, we performed reporter gene assays. Interaction between SMRT and Nurr1/RXR heterodimer was stronger than the interaction between SMRT and Nurr1 monomer. This increase was abolished when using the RXR mutant unable to heterodimerize with Nurr1 (A416K, Lee et al. 2000). Thus the Nurr1/RXR heterodimer seemed to interact more efficiently with SMRT than the Nurr1 monomer did. The finding that the RXR mutant unable to bind corepressors (L294R, Aarnisalo et al. 2002) did not, as the wild type, evoke an increase in Nurr1-SMRT interaction implies that RXR recruits SMRT to the Nurr1/RXR heterodimer.

Ligand binding can release corepressors from NRs (Xu et al. 1999, White et al. 2004). To study whether there is a difference as to how ligands control the activity of permissive and non-permissive RXR heterodimers with recpect to corepressors, we treated transiently transfected cells with RXR ligands. In the cells transfected with Nurr1 and RXR, RXR agonists (synthetic SR11237 and natural 9-cis-retinoic acid (9-cis-RA)) inhibited the repression of the reporter gene caused by SMRT. However, these ligands failed to inhibit the repression of the reporter gene by SMRT in the cells transfected with RAR and RXR or TR and RXR. Only the agonists for RAR and TR (TTNPB and T3, respectively) were able to inhibit the repression by SMRT in these cells (see fig. 12). These results suggest that the interaction between NR dimers and SMRT is regulated differently by RXR agonists in permissive and non-permissive heterodimers. Reporter gene assays performed with chimeric Nurr1/RAR constructs supported these results.

We studied the release of SMRT from RXR heterodimers in more detail. Direct interaction between RXR and SMRT was abolished by SR11237 even when cotransfected with Nurr1. We then transfected cells with RXR, SMRT, and RAR. In mammalian two hybrid assay, RXR/RAR interacted with SMRT. This interaction was even increased when the cells were treated with SR11237. This increase of interaction was blocked when RAR was mutated at the site important for corepressor binding (V242R, Hu and Lazar, 1999). This suggests that the increased interaction between RAR/RXR heterodimers and SMRT is due to a stronger

interaction between RAR and SMRT. Taken together, it seems that in the permissive Nurr1/RXR heterodimer, SMRT interacts with RXR. In the non-permissive RAR/RXR heterodimer, SMRT interacts mostly with RAR.

In addition to corepressor binding, it seems that also coactivators mediate the permissive characteristics of RXR heterodimers (DiRenzo et al. 1997, Yang et al. 2000, Germain et al. 2002, Kassam et al. 2003). In permissive PPAR/RXR heterodimers, both PPAR and RXR agonist Induce the binding of a coactivator to the heterodimer. In non-permissive RAR/RXR heterodimers, RAR agonist induces binding of a coactivator, whereas RXR agonist has no effect on coactivator binding (DiRenzo et al. 1997, Yang et al. 2000). RXR agonist can not release SMRT from the RAR/RXR heterodimer (Germain et al. 2002), which is in line with our results. It has also been shown that the ratio of corepressors and coactivators can affect the activity caused by a ligand (Smith et al. 1997, Liu et al. 2002). Differential release of corepressors from permissive and non-permissive heterodimers could affect the cofactor pools in a cell and have a role in mediating the effects of NRs and their ligands.

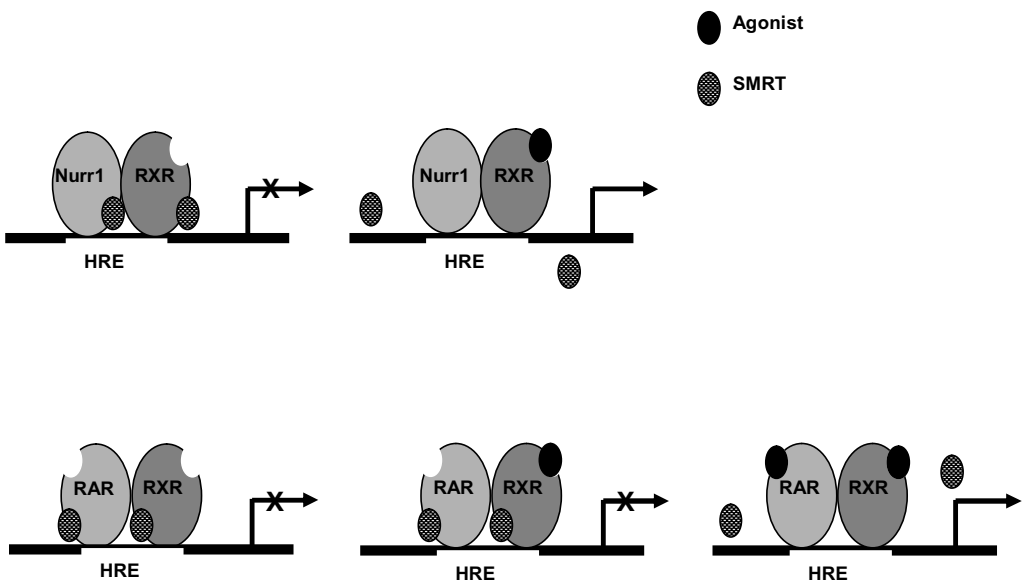

Fig. 12. Corepressor release is differentially regulated in permissive and non-permissive RXR heterodimers. In the permissive Nurr1/RXR heterodimer, the corepressor SMRT is recruited to the dimer by RXR, and released in response to RXR agonist. In the non-permissive RAR/RXR heterodimer, SMRT is recruited by RAR, and released only in response to the agonist of RAR.

It has been shown that the expression levels of RAR and PPAR determine which partner, RAR or PPAR, will heterodimerize with RXR (DiRenzo et al. 1997). This implies that there is competition for the RXR molecules present in a cell at a particular time. The amount of the Nurr1 and NGFI-B and, on the other hand, of RXR and its other heterodimerization partners could affect the number of different heterodimers formed in the cell. Nurr1 and NGFI-B could have an advantage in the formation of heterodimers when stress or growth factors induce their expression as immediate early genes. Heterodimerization with unliganded RXR could repress the transcriptional activities of NGFI-B and Nurr1 when no activity is needed. Heterodimerization with RXR could also repress the excessive activity of sudden a induction of Nurr1 and NGFI-B. However, Nor1 can perform its functions regardless of RXR, other heterodimerization partners, or RXR ligands, as it does not form RXR heterodimers. Nor1 can therefore perform the functions of the NR4A subfamily alone if Nurr1 and NGFI-B are blocked in RXR heterodimers. In addition, the monomeric and heterodimeric functions can be separately controlled.

Our studies show that SMRT interacts with Nurr1 and represses its activating functions on the NBRE element. SMRT interacts with monomeric Nurr1, as proved by the GST pull-down assay. However, SMRT interacts with Nurr1/RXR heterodimer more efficiently. Based on our results, the OPN promoter is activated by both monomeric and RXR-heterodimeric Nurr1. How SMRT interacts with Nurr1 and regulates its activity on the OPN promoter remain to be solved.

5. Future prospects

This study has provided insight to how the expression or activity of the NR4A subfamily of orphan nuclear receptors is regulated.

We have shown that FGF-8b treatment of osteoblasts induces NR4A expression in preosteoblasts (IV). In addition, we have demonstrated that Nurr1 and NGFI-B stimulate the proliferation of preosteoblasts. They also mediate the proliferative effects of FGF-8b (IV). We have presented evidence on how NR4A receptors take part in mediating the downstream effects of PTH treatment by targeting the OPN gene (I). The transcriptional activity of NR4A receptors is controlled by inhibiting cross-talk with NR3B receptors (I, II). The inhibiting

effect of NR4A-NR3B cross-talk can be blocked by 4-OHT (I). We have also presented data on how corepressor release separates permissive heterodimers (formed by Nurr1 and NGFI-B with RXR) from non-permissive RXR heterodimers (III).

Our studies and other recent investigations have demonstrated that NR4A receptors have a role in bone metabolism. These studies have revealed definite, such as OPN and OCN, and putative, such as Col1a1 and ALP, target genes for the NR4As (I, Pirih et al. 2004, Lee et al. 2006). The downregulation of Nurr1 has already been demonstrated to inhibit the formation of bone nodules (Lee et al. 2006). The role of NR4A receptors in osteoblastic differentiation and bone remodelling needs more clarification. The expression of NR4A receptors in differentiating osteoblasts should be examined. In addition, the phenotypes of the osteoblasts in NR4A deficient mice should be studied in detail. We and others have demonstrated that NR4A receptors are quickly and transiently induced in response to PTH treatment (I, Tetradis et al. 2001a, 2001b, Pirih et al. 2003). As intermittent administration of PTH is used for the treatment of osteoporosis, the expression and activity of NR4As in osteoporotic bone should be determined both before and after the PTH treatment.

It is of importance to clarify the role of Nurr1 and NGFI-B in proliferation of osteosarcoma cells or other cancerous cell lines. Since also ERRγ have been linked to proliferation of prostate cancer cells (Yu et al. 2007), the combined influences of NR4A expression and ERRγ should be examined. In addition, the roles of ERRγ agonists should be studied in this respect. The expression and activity of NR4A receptors should be studied in ERR null mice and vice versa.

We have demonstrated that the activity of Nurr1 on the NBRE element can be repressed by corepressor SMRT (III). Whether SMRT also interacts with and represses the activity of NR4A receptors on HREs, as it does on the NBRE element, needs more clarification. Further studies should be conducted to determine whether there are more mechanisms and perhaps other NRs and their ligands regulating NR4A receptors.

CONCLUSIONS

The NR4A subfamily of nuclear receptors consists of orphan nuclear receptors with no known activating or repressing ligands. The functions of these receptors in bone tissue have not been studied in detail. This work characterizes their functions in osteoblasts and evaluates the mechanisms regulating their transcriptional activity.

- Nurr1 and NGFI-B control preosteoblastic cells both by increasing proliferation and decreasing apoptosis. In addition, they mediate the proliferative stimulation by FGF-8b (see fig. 13). It is noteworthy that Nor1 does not have these effects. The biological functions of the closely related NR4A receptors are therefore different.

- The increased expression of OPN in osteoblasts in response to PTH is partly mediated by NR4A receptors. NR4A receptors are thus linked to the regulation of a major bone matrix protein.

- The transcriptional activity of NR4A receptors can be regulated by cross-talk with NR3B receptors. The activity of NR4A receptors is increasingly repressed when cells express increasing amounts of ERRα and ERRγ. The transcriptional activity of NR4A receptors can be enhanced by blocking ERRγ activity with 4-OHT, a synthetic inhibitor of ERRγ. Cross-talk with other nuclear receptors may serve as an important regulator of constitutively active NR4A receptors.

- The expression of NR4A receptors is rapidly induced by FGF-8b. The NR4A receptors being rapidly induced by many external stimuli suggests that their expression level is, in fact, an important mechanism in regulating the transcriptional activity they relay. When strongly and briefly induced, the expression of NR4A receptors can overcome the repressing effect of NR3Bs in the cell.

- In non-permissive RXR heterodimers, the corepressors are strongly bound to the dimer, whereas in permissive Nurr1/RXR and NGFI-B/RXR heterodimers the binding of corepressors is very weak. In permissive heterodimers, the corepressors are released

in response to the binding of RXR agonists. In non-permissive heterodimers, in contrast, the corepressors are released in response to the partner's agonist.

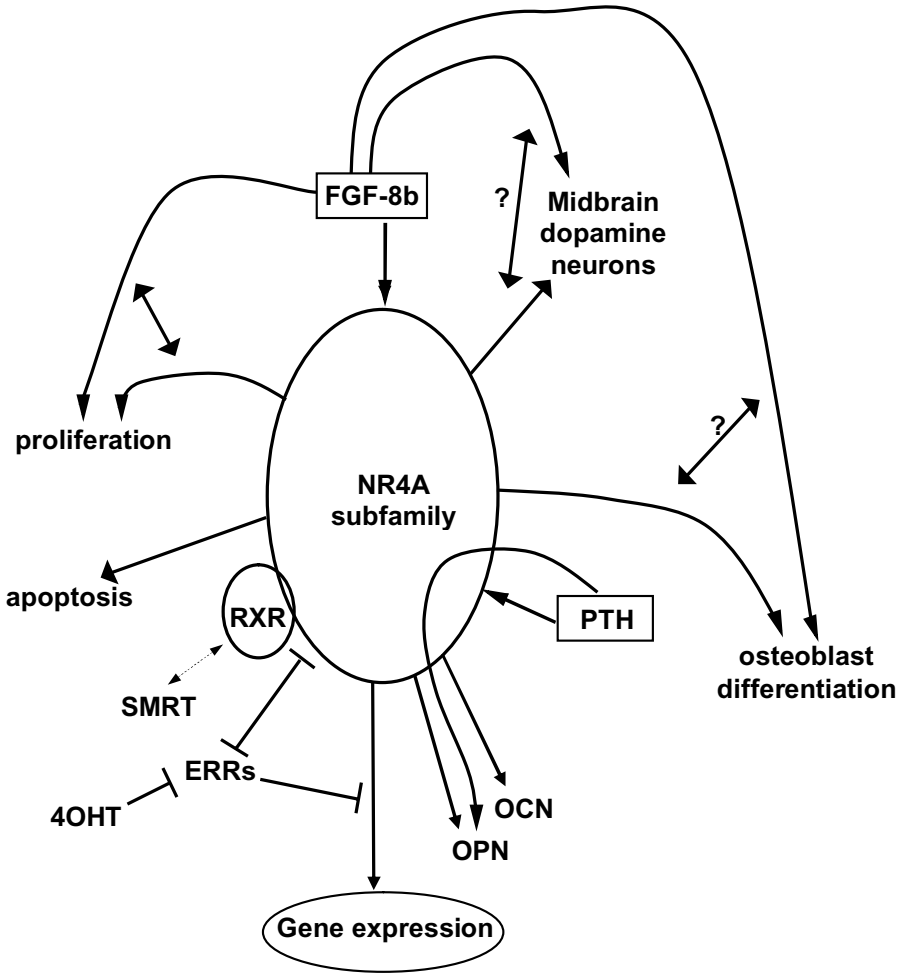

Fig. 13. Summary of results obtained in this study combined with previous findings by others. In this summary the question marks point at matters that require further studies. The results concerning proliferation, apoptosis, and heterodimerization with RXR are only valid for Nurr1 and NGFI-B. For abbreviations see page 7. References are in the text.

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Johanna Lammi

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