Estrogen receptor-related receptor α (ERR α) regulates osteopontin expression through a non-canonical ERR α response element in a cell context-dependent manner

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Abstract

We previously demonstrated that the orphan nuclear receptor, estrogen receptor-related receptor α (ERR α) is highly expressed in osteoblasts and osteoclasts, regulates osteogenesis and expression of osteoblast-associated markers in the rat calvaria cell differentiation system, and is dysregulated in the rat ovariectomy model of postmenopausal osteoporosis. There are conflicting published data on the transcriptional regulation by ERR α of the gene for osteopontin (OPN), an extracellular matrix protein required in bone remodeling, and a potential direct target mediating ERR α effects in bone. We therefore readdressed OPN gene regulation by ERR α in both osteoblastic (rat osteosarcoma ROS17/2.8 cells) and non-osteoblastic (HeLa) cell lines using a mouse proximal 2 kb OPN promoter fragment. A minimal OPN promoter fragment spanning from -56 to $+9$ bp is activated in HeLa cells but repressed it in ROS17/2.8 cells. Adenine scanning mutagenesis revealed the presence of a non-canonical ERR α response element in this minimal promoter. Surprisingly, prototypical inactivating mutations in the activation function 2 (AF2) domain or a naturally occurring allelic variant of ERR α (ERR α H408) were all better activators than wild-type ERR α in HeLa cells, activities that were generally paralleled by repression in ROS17/2.8 cells. Finally, we found that the N-terminus of ERR α harbors a repressor domain that acts in a cell context-dependent manner. We conclude that OPN is an ERR α target gene whose promoter is regulated by ERR α in a cell context-dependent manner and that a predicted silencing mutation in AF2 or a more flexible helix 12 increases ERR α transcriptional activity, effects with implications for ERR α as a therapeutic target in bone.

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Introduction

Nuclear receptors (NRs) are structurally and functionally modular with the N-terminal A/B domain regulating transcriptional activity in a ligand-independent fashion (activation function 1 (AF1)) and a conserved centrally located DNA-binding domain (DBD) composed of two zinc fingers. The DBD allows the NR to control transcription directly by binding to cognate response elements found in a subset of promoters or indirectly by tethering to other transcription factors (Kushner *et al.* 2000, Safe *et al.* 2004). The C-terminal ligand/hormone-binding domain (HBD) is composed of 12 α -helices that form a centrally located ligand-binding pocket, with helix 12 harboring the ligand-dependent AF2 (Bourguet *et al.* 2000). NRs are controlled by a large and increasing number of co-repressors and co-activators, the balance of which determines transcriptional activity (Privalsky 2004, Spiegelman & Heinrich 2004). Generally, co-repressors bind to a hydrophobic surface in the HBD and are released due to a conformational shift involving helices

3, 5, and 12 upon ligand binding, which repositions the AF2 domain, closing the ligand-binding pocket to form a new hydrophobic binding surface allowing co-activators to bind (Nettles & Greene 2005). The binding of the co-activator to the NR is greatly stabilized by a charge clamp involving a conserved lysine (K) in helix 3 and either a glutamic acid (E) or aspartic acid (D) found in the AF2 domain (Li *et al.* 2003). Recent evidence has pointed to an important role for the A/B domain in binding the co-activators and interactions with the C-terminus of the NR (Schaufele *et al.* 2005).

The estrogen receptor-related receptor (ERR) subfamily of NRs comprises three distinct genes (ERR α (nuclear receptor 3b; NR3B1), ERR β (NR3B2), and ERR γ (NR3B3)) most closely related to the estrogen receptor (ER) subfamily (ER α (NR3A1) and ER β (NR3A2)) with 68% identity in their DBD (Nuclear Receptors Nomenclature Committee 1999, Giguere 2002). ERRs bind to a response element (ERRE) with the consensus sequence 5'-TCAAGGTCA-3', while the ER response element (ERE) comprises two inverted repeats of 5'-AGGTCA-3' separated by three nucleotides

(Giguere 2002). The similarity in their respective response elements and the high conservation of their DBD underlie the ability of ERR and ER to regulate some genes via either response element (Giguere 2002, Bonnelye & Aubin 2005). The solved crystal structure of the ERR α HBD showed that it is too small to accommodate estrogen or similar ligands (Kallen *et al.* 2004), explaining why ERR α does not bind and is not activated by estrogen directly. However, estrogen binding to ER α upregulates ERR α expression (Shigeta *et al.* 1997, Bonnelye *et al.* 2002) and ER α can physically interact with ERR α (Yang *et al.* 1996), strengthening the idea that these two receptor families impinge on each others signaling (Vanacker *et al.* 1999a, Kraus *et al.* 2002). Intriguingly, the crystal structure of ERR α also suggests that the AF2 domain is in the traditional activated conformation, supporting the view that ERR α acts as a constitutive weak activator (Kallen *et al.* 2004). It must be recognized, however, that the crystal structure was solved for ERR α bound with the co-activator peroxisome proliferator activated receptor gamma co-activator 1 alpha (PGC1 α), suggesting that helix 12 may not always be in the active conformation. In any case, while maximal activation by ERR α requires co-activators, including PGC1 α (Huss *et al.* 2002, Schreiber *et al.* 2003, Gaillard *et al.* 2007), less is known about ERR and co-repressor interactions, although ERR α is known to interact with the co-repressors DSS-AHC critical region on the X chromosome, gene 1 (DAX1), small heterodimer partner (SHP), and receptor interacting protein (RIP140) (Sanyal *et al.* 2002, Park *et al.* 2005, Castet *et al.* 2006).

We reported previously that ERR α expression is much higher than that of ER α or ER β in bone and cartilage (Bonnelye & Aubin 2002) and that upregulation of ERR α increases, while antisense knockdown reduces, bone and cartilage formation in cell culture models (Bonnelye *et al.* 2001, 2007). Concomitantly, we observed regulation of a number of genes necessary for, and/or associated with, the differentiation programs in both osteoblast and chondrocyte lineages (Aubin 2001, Lefebvre & Smits 2005). Among those regulated in osteoblasts were osteopontin (OPN; Bonnelye *et al.* 2001), a secreted phosphorylated multifunctional glycoprotein whose regulation depends on a number of NRs with roles in bone biology (Noda *et al.* 1990, Craig & Denhardt 1991, Vanacker *et al.* 1998, Lee *et al.* 2000, Bonnelye *et al.* 2001, Denhardt *et al.* 2001, Ogawa *et al.* 2005, Shen & Christakos 2005). However, there are conflicting reports on the regulation of OPN by ERR α in osteoblastic cells, with some reporting upregulation (Vanacker *et al.* 1998, Bonnelye *et al.* 2001) and others reporting downregulation, the latter via interference of ERR α with the orphan NR nuclear receptor related 1 (Nurr1) (Lammi *et al.* 2004). We therefore readdressed OPN regulation by ERR α and report that mOPN

regulation by ERR α is cell context dependent and does not involve the previously identified ERRE, but rather a non-canonical site overlapping the inverted CAAT box. We also provide evidence that mutation or complete removal of the AF2 domain renders mERR α a more potent activator dependent on the presence of the A/B domain. Our data suggest that interactions between the N- and C-termini influence the alignment of the AF2 domain and subtle changes in this interaction dictate the transcriptional response and presumably the interactions with co-regulatory molecules.

Materials and methods

Plasmids

The ERR α open reading frame was amplified using RT-PCR from mouse muscle cDNA and cloned into a modified pcDNA3.1 vector (Invitrogen) that had the SV40 promoter replaced with an internal ribosome entry site to make pcDINmERR α . Point mutations and deletions of mERR α were made using standard PCR approaches with pcDINmERR α as the starting plasmid and oligonucleotides that incorporated restriction sites for cloning and mutation identification (Ausubel *et al.* 1987). The OPN promoter plasmids pGL2mOPN-1981+78 and pGL2mOPN-624+78 were a kind gift from D A Towler (Bidder *et al.* 2002). Additional OPN promoter deletions and mutations were made using standard techniques and cloned into pGL2B (Promega). The mammalian Gal4 DNA-binding domain expression vectors pM1-3 and pM1VP16 were a kind gift from Sadowski & Ptashne (1989) and the pFRluc reporter plasmid was obtained from Stratagene (La Jolla, CA, USA). The pM1 plasmid was modified to allow construction of both N- and C-terminal Gal4 fusion proteins. The pGFP-C1 plasmid was from Clontech. The pSilencer 3.1-H1 hygro and pSilencer negative control (pSi neg) plasmids were obtained from Ambion, Inc. The oligonucleotide 5'-GATC-CAGGGTTCCTCAGAGACTGATTCAAGAGATCAGT-CTCTGAGGAACCCCTTTTTTGGAAA-3' targeting the 5'-end of mouse and rat ERR α was designed with the program at the Ambion web site (www.ambion.com) and cloned into pSilencer 3.1 to make pSi 5' α . Other oligonucleotide sequences used in making the plasmids are available upon request. All plasmids were sequence verified.

Cell culture

All cells were kept in a humidified incubator at 37 °C in a 95% air–5% CO₂ atmosphere. HeLa cells (gift from L Attisano, University of Toronto) were maintained in

Dulbecco's modified Eagle's medium (DMEM) (D5796, Sigma) + 10% fetal bovine serum (FBS), MC3T3-E1 clone 26 cells (Wang *et al.* 1999) were maintained in α MEM + 10% FBS, and ROS17/2.8 cells (Majeska *et al.* 1985) were maintained in α MEM + 15% FBS. Cells were routinely passaged at 80–90% confluence and were plated onto 24-well plates at the cell density described in the figure legends the day before transfection. On the day of transfection, the medium was changed and cells were transfected using Lipofectamine 2000 (Invitrogen) with 10 ng pRLtkluc (Promega) as internal control for transfection efficiency. Luciferase activities were measured 48 h later on an EG&G Berthold Microplate Luminometer LB96V (EG&G Berthold GMBH & Co., Bad Wildbad, Germany) using the Dual-Luciferase reporter assay system (Promega). Transfections were repeated at least thrice and a representative experiment is shown. To confirm that changes in endogenous ERR α expression level altered OPN gene expression, ROS 17/2.8 cells at 70% confluence in 6-well plates were transiently transfected using Lipofectamine 2000 with 5 μ g of either pSi neg or pSi 5' α , and RNA was extracted 48 h later using TRI reagent (Sigma) following the manufacturer's instructions. After conversion of the RNA to cDNA with Superscript II (Invitrogen), real-time PCR was performed (Bio-Rad MyiQ cyclor) and relative expression levels of rOPN were determined using rL32 as internal control.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed essentially as described (Ausubel *et al.* 1987). Briefly, either the wild-type mOPN promoter oligonucleotides 5'-TCGAGCAGCTGATTGGTGGA-GACTGTCTGGACCAGCATTT-3' and 5'-AAATGCTGGTCCAGACAGTCTCCACCAATCAGCTGC-3' or the mutant 6A2 oligonucleotides 5'-TCGAGCCATGGCTGATTaaaaaGACTGTCTGGACCAGCATTT-3' and 5'-AAATGCTGGTCCAGACAGTCTtttttAATCAGCCATGGC-3' were annealed and the resulting overhang was filled in with [α -³²P]dCTP and Klenow fragment. The probe was purified using a spun column and 30 000 c.p.m. of probe were used in the binding reaction. HeLa cells were transfected with either control vector (pcDIN), full length ERR α (pcDINmERR α), or ERR α DNA-binding mutant (pcDINmERR α C99G) and nuclear extracts were prepared 48 h after transfection. The probe and nuclear extracts were combined and incubated on ice for 20 min before being loaded on a pre-run 0.5% Tris-Borate-Ethylenediaminetetraacetic acid (TBE) non-denaturing gel at 4 °C for 2 h, dried, and exposed to film. For competition experiments, either 10- or 50-fold excess unlabeled oligonucleotide was included in the incubation step.

Statistical analysis

All data are plotted as means $\pm$ s.d. of triplicate determinations; results of representative experiments are shown and experiments were repeated a minimum of thrice. Data were analyzed using Student's *t*-test.

Results

ERR α does not regulate mOPN via the S1 element in osteoblasts or HeLa cells

We made sequential deletions of a 2 kb fragment of the mOPN promoter so as to progressively delete previously identified putative ERREs S1–S6 (Vanacker *et al.* 1998; Fig. 1A). Based on basal activity of the 2 kb or truncated promoter constructs in HeLa cells, we identified a positive regulatory element between –789 and –629 and a negative regulatory element between –629 and –419 (Fig. 1B). Deletion to –24 resulted in a further dramatic decrease, consistent with removal of the CAAT and TATA boxes that have been shown to be required for basal promoter activity (Kabe *et al.* 2005, Hummelshoj *et al.* 2006). Co-transfection of the mOPN promoter constructs with mERR α caused a two- to threefold enhancement over basal promoter activity in HeLa cells until sequences between –253 and –24 were deleted (Fig. 1B). Transfection of the same constructs into the osteoblastic ROS 17/2.8 cell line showed similar trends in basal promoter activity (compare Fig. 1B and C); however, co-transfection of the promoter constructs with mERR α repressed (30–50%) promoter activity until sequences between –253 and –24 were deleted (Fig. 1C). Real-time PCR analysis of ROS17/2.8 cells overexpressing ERR α (data not shown) or underexpressing ERR α (shRNA-transfected cells) confirmed that ERR α represses OPN in ROS17/2.8 cells (Fig. 1D). We conclude that mERR α regulates the mOPN promoter in a cell context-dependent manner independently of the previously identified ERREs (S1 element) and that the regulatory element lies between –253 and –24.

mERR α regulates the mOPN promoter through a non-canonical ERRE

A transcription element search system (TESS) database (Schug 2003) search of the proximal –253 to +78 bps of the mOPN promoter for transcription factor binding sites revealed an abundance of predicted specificity protein 1 (SP1) as well as two AP1 sites. The upstream AP1 site is thought to be used by a variety of signaling pathways (Bidder *et al.* 2002, Ogawa *et al.* 2005) and the downstream AP1 site overlaps the CAAT box. Visual inspection of the sequence identified a weak consensus ERRE (GGGAGGTCT) between –17 and –9 which

also overlaps a predicted SP1 site. To determine whether mERR α utilized any of these predicted sites, we made a series of promoter deletions and mutated the newly predicted ERRE1 from GGGAGGTCT to GGGgaaTCT (m1), which would abolish ERR binding and destroy the SP1 site (Fig. 2A). Deletion of

sequences between +10 and +78 had no effect on basal promoter activity in HeLa cells nor did it diminish the ability of mERR α to activate the -253 to +9 mOPN promoter (Fig. 2B). Mutation of the putative ERRE1 slightly increased the basal promoter activity, but did not abolish the ability of mERR α to activate the -253 to +9 m1 construct (Fig. 2B). Since mERR α was shown previously to act through SP1 sites (Castet *et al.* 2006) that are still present in the -253 to +9 construct, we tested a minimal mOPN promoter from -56 to +9 that retains the CAAT and TATA boxes, as well as a previously identified binding site for Smad proteins (Hullinger *et al.* 2001; Fig. 2D). Removal of the upstream SP1 sites reduced activity of the -56 to +9 basal promoter while mutating the ERRE1/SP1 site in -56 to +9 m1 had no further effect (Fig. 2B). Importantly, mERR α still activated or repressed these two minimal promoters in HeLa or ROS17/2.8 cells respectively, indicating that the regulation was not due to the predicted ERRE1 or SP1 sites in either case.

To further delineate the site used by mERR α to regulate mOPN, we made adenine scanning mutations (Fig. 2D). The wild-type -56 to +9 promoter and adenine scan mutants 6A3 and 6A4 gave robust activation in the presence of mERR α (Fig. 2E), whereas 6A1 and 6A2 had minimal effects on basal promoter activity while completely abolishing the ability of mERR α to activate the mOPN promoter in HeLa cells (Fig. 2E). In ROS17/2.8 cells, on the other hand, 6A1 and more so 6A2 abolished mERR α 's ability to repress the promoter while the wild-type or the 6A3 and 6A4 promoter mutant constructs still repressed (Fig. 2F). Notably, basal promoter activity is much more affected by the 6A1 and 6A2 mutations in ROS17/2.8 cells than in HeLa cells (compare Fig. 2E and F). These results suggest that regulation of mOPN is through a non-canonical binding site or through interaction with proteins bound near the CAAT box but not the Smad site.

Figure 1 ERR α regulates mOPN in a cell context-dependent manner. (A) Schematic of the mOPN promoter constructs used, with the location of the previously proposed ERR binding sites (S1 to S6) indicated (Vanacker *et al.* 1998). ERR α activates the mOPN promoter in (B) HeLa cells and represses it in (C) ROS17/2.8 osteosarcoma cells through a site located between -253 and -24 bp. HeLa cells (45 000 cells/well) were transfected with 150 ng of reporter and 150 ng empty (control) or full length mERR α expression plasmid. ROS17/2.8 cells (20 000 cells/well) were transfected with 50 ng reporter and 400 ng empty (control) or full length mERR α expression plasmid. The mean $\pm$ s.d. of triplicate wells from a representative experiment is shown; consistent activation (HeLa) or repression (ROS17/2.8) was significant ($P < 0.05$) with all promoter fragments other than the -24 to +78. (D) Knockdown of ERR α expression with a shRNA plasmid relieves repression and activates the endogenous OPN promoter.

Figure 2 *ERRα* regulates mOPN through a non-canonical site. (A) Schematic of the mOPN promoter region from -253 to $+78$ and the various deletion constructs used to define the site mERR α uses to regulate mOPN expression. The relative position of known and putative transcription factors binding sites that mERR α could potentially interact with are indicated. Activation in (B) HeLa cells or repression in (C) ROS17/2.8 cells does not require the Sp1 or the low homology ERRE sites. HeLa cells (40 000 cells/well) were transfected with 150 ng reporter and 100 ng empty (control) or full length mERR α expression plasmid. ROS17/2.8 cells (30 000 cells/well) were transfected with 75 ng reporter and 400 ng empty (control) or full length mERR α expression plasmid. (D) Schematic and sequence of the mOPN promoter from -56 to $+9$ as well as the adenine scan mutations used to identify the non-canonical site used by ERR α . Mutating the sequence between the CAAT and Smad boxes abolishes ERR α regulation of mOPN in both (E) HeLa and (F) ROS17/2.8 cells. HeLa cells (40 000 cells/well) were transfected with 200 ng reporter and 200 ng empty (control) or full length mERR α expression plasmid. ROS17/2.8 cells (20 000 cells/well) were transfected with 50 ng reporter and 400 ng empty (control) or full length mERR α expression plasmid. The mean $\pm$ s.d. of triplicate wells from a representative experiment is shown; consistent activation (HeLa) or repression (ROS 17/2.8) was significant ($P < 0.05$) with all promoter fragments other than the 6A1 and 6A2 mutants.

Activation of mOPN in HeLa cells requires an intact DBD while repression in ROS17/2-8 cells is independent of direct DNA binding

Mutating cysteine to glycine in the zinc finger of NRs abolishes DNA binding and has been used to discriminate between the requirement for direct response element binding versus binding via a second protein to regulate transcription (Liu *et al.* 1996, Jakacka *et al.* 2001, Huppunen *et al.* 2004). A DBD mutant, mERRαC99G, increased promoter activity (30%) over basal levels in HeLa cells (Fig. 3A), indicating that on the 2 kb mOPN promoter, mERRα activates transcription by both DBD-dependent and DBD-independent modes. However, an intact DBD was required for activating the -629 and smaller mOPN promoter deletions (Fig. 3A). In ROS17/2.8 cells, repression was observed for both the wild-type and the DBD mutant mERRα (Fig. 3B), indicating that repression in the osteoblastic cells most likely involves protein-protein interactions; this interaction appears to be mediated by protein(s) that binds between the CAAT box and the Smad site as repression is no longer observed in the 6A2 promoter mutant (Fig. 3B). Repression is observed in the 6A3 promoter mutant indicating that mERRα does not require binding to Smads to repress in ROS17/2.8 cells.

In an EMSA using a -56 to -24 probe, we detected four complexes (I to IV), all of which were competed away with unlabeled oligonucleotide (Fig. 3C, compare lanes 2 and 4). Transfecting HeLa cells with control vector diminished complex IV (Fig. 3C, compare lanes 5 and 6), however, we should point out that complex IV showed variable intensity between different nuclear protein extracts (data not shown). Transfection of mERRα reduced the intensity of complexes I and II (Fig. 3C, lanes 6 and 7), while mERRαC99G did not (Fig. 3C, lanes 6 and 8) indicating potential ERRα binding to complexes I and II. Incubation of the 6A2 mutant oligonucleotide with any of the HeLa cell extracts caused loss of complexes I and II only (Fig. 3C, compare lanes 5-8 with 9-12), while use of the 6A2 mutant oligonucleotide as competitor of labeled wt probe caused loss of III and IV only (data not shown).

Role of the AF2 domain of ERRα in transcriptional regulation of mOPN

The AF2 domain is required for maximal transcriptional activation by most NRs (Bourguet *et al.* 2000). However, deletion of amino acids 413-419 of mERRα to create mERRαmAF2 (Fig. 4A), which lacks helix 12 including the important E415 that forms part of the

charge clamp and should render mERRα transcriptionally inactive, surprisingly led to greater activation of the 2 kb mOPN promoter than seen with wt mERRα (Fig. 4B, compare wt with mAF2). This novel observation suggested that either E415 is less important in co-activator binding or that the smaller C-terminus allowed for better binding of co-activators. Most ERRα isoforms reported have a proline at position 408 (P408) preceding helix 12, but the originally described mERRα (Giguere *et al.* 1988) has a histidine (H) at this position (Fig. 4A). When we tested the H408 allele (mERRαH408) on the 2 kb mOPN promoter in HeLa cells, we found that it is more active than the wt mERRα allele (Fig. 4B), supporting the notion that conformational flexibility in helix12 affects the transcriptional outcome. Similarly to what we observed with mERRα-mAF2, an AF2 domain deletion of the H408 allele (mERRαH408mAF2) is also an even better activator of the 2 kb promoter than mERRαH408 (Fig. 4B, compare H408 and H408mAF2). The mERRαH408 was also more active than mERRα on all of the promoter deletions that we tested in HeLa cells (data not shown). We tested the same series of mERRα constructs with the -253 to +9 mOPN promoter and observed the same trends as with the 2 kb mOPN promoter (Fig. 4B). The mERRαmAF2 deletion places the terminal aspartic acid (D422) residue in the same relative position as would be occupied by E415 (Fig. 4A), potentially leaving the charge clamp intact which could explain why mERRαmAF2 is more active. If this were the case, then a C-terminal deletion to E515 should mimic the result we obtained with mERRαmAF2, but the mERRαΔC415 mutant was totally inactive on the -253 to +9 mOPN promoter (Fig. 4C, compare ΔC415 with mAF2) and the -629+78 and the -56+9 promoters (data not shown). A mutant with the charge reversed, mERRαΔCE415N, as well as a deletion mutant to amino acid 414 (mERRαΔC414), mimicked mERRαmAF2 activity (Fig. 4C and data not shown) indicating that the charge clamp residue in the AF2 domain is dispensable for activation. A larger C-terminal deletion that removes both helices 11 and 12 (mERRαΔC392) retained the ability to activate mOPN (Fig. 4C), and the degree of activation depended on the amount of mOPN promoter that was present in the luciferase constructs, but was always less than that seen with mERRαmAF2 (data not shown). When we mutated the other charge clamp residue (K243) to alanine (mERRαK243A), both the -629+78 and the -56+9 mOPN promoters were activated two- to threefold (data not shown), while the -253+9 promoter was unresponsive (Fig. 4C). Taken together, these data suggest that one charge clamp residue is sufficient to recruit co-activators to mERRα depending on the promoter context, while the positioning of helix 12 determines the strength of the transcriptional response.

Figure 3 Regulation of *mOPN* promoter activity requires an intact DNA-binding domain in HeLa but not ROS17/2.8 cells. (A) HeLa cells (40 000 cells/well) were transfected with 200 ng of indicated *mOPN* promoter constructs together with 250 ng empty (control) or wild-type *mERRα* or an *ERRα* DNA-binding mutant (*mERRαC99G*) expression vector. Activation by *mERRαC99G* was significant ($P < 0.05$) only with the -1981+78 construct. (B) ROS17/2.8 cells (30 000 cells/well) were transfected with 50 ng of indicated *mOPN* promoter constructs together with 400 ng empty (control) or wild-type *mERRα* or an *ERRα* DNA-binding mutant (*mERRαC99G*) expression vector. The mean $\pm$ s.d. of triplicate wells from a representative experiment is shown: consistent repression was significant ($P < 0.05$) with all promoter fragments other than 6A2. (C) EMSA of HeLa cell nuclear extracts showing binding of complexes (I to IV) to the -56 to -24 region of the *mOPN* promoter (lane 2 compared with lane 1) that can be specifically competed off using 10- or 50-fold unlabeled competitor oligonucleotide (lanes 3 and 4 respectively). EMSA of HeLa cell nuclear extracts (lane 5) or HeLa cell nuclear extracts from cells transfected with empty vector (+control, lane 6) or an *ERRα* DNA-binding mutant (+*mERRαC99G*, lane 8) show no differences in the complexes formed, while nuclear extracts from HeLa cells overexpressing wild-type *mERRα* (lane 7) shows reduction in complexes I and II. Complexes I and II do not form in the presence of the mutant 6A2 oligonucleotide indicating that complexes I and II either interact with or contain *ERRα*.

Figure 4 C-terminal mutants uncover a non-canonical role for the AF2 domain. (A) Amino acid sequence of the carboxy terminus of mERRα with helix 12 containing the AF2 domain underlined as well as the various mutants tested. (B) Hyperactivity of mERRα (wt) is observed by mutating the AF2 (mAF2) domain or by increasing the rotational freedom of helix 12 by a proline 408 to histidine mutation (H408 and H408mAF2). HeLa cells (40 000 cells/well) were transfected with 50 ng of the mOPN-1981 + 78 promoter construct together with 400 ng empty expression vector (control) or indicated mERRα expression plasmids. (C) Charge and size of side chain in the ultimate position are important in order to hyperactivate mERRα. Hyperactivity is observed for all C-terminal mutants tested, except when it is negatively charged glutamic acid at position 415 (ΔC415). Both positive and negative charges can be hyperactive at position 415 as long as it is one carbon shorter than the glutamic acid as demonstrated by the ΔCE415N and mAF2 mutants. HeLa cells (40 000 cells/well) were transfected with 200 ng of the mOPN-235 + 9 promoter construct together with 150 ng empty expression vector (control) or indicated mERRα expression plasmids. The mean ± s.d. of triplicate wells from a representative experiment is shown: consistent activation was significant ($P < 0.05$) with all mERRα isoforms tested except ΔC415 and K243A.

The A/B and HBD domains of mERRα have opposing transcriptional activities

To determine whether the different domains of mERRα behave independently, we next tested several Gal4DBD fusion proteins (Fig. 5A) using a luciferase reporter containing 5× Gal4 binding sites in both HeLa (Fig. 5B) and ROS17/2.8 (Fig. 5C) cells. Fusing Gal4DBD (Gal4) to the N-terminus of full length mERRα or the previously hyperactive mERRαmAF2 or mERRαΔC392 mutants repressed the Gal4 reporter in HeLa cells (Fig. 5B). This, together with the fact that the wt HBD fused to Gal4 activated the reporter fourfold while the mAF2HBD and ΔC392HBD were incapable of activating the reporter (Fig. 5B), supports the notion that the N-terminus plays a role in controlling the activity of mERRα. Similar trends were seen in ROS17/2.8 cells where, intriguingly, the wt HBD elicited a very robust 28-fold activation compared with the Gal4 control alone while the mAF2HBD and ΔC392HBD fusions were inactive (Fig. 5C), suggesting that the N-terminus acts as a repressor of mERRα activity. We tested this further by fusing the A/B domain of mERRα to either the N-terminus of Gal4, which preserves the relative domain orientation in the native protein, or the C-terminus of Gal4. Irrespective of how the A/B domain was fused or the cell line used, the Gal4 reporter luciferase was repressed (Fig. 5B and C), indicating that the A/B domain of mERRα harbors a repressor function and that there is communication between the A/B and AF2 domains in native mERRα.

To further address the hypothesis that fusing protein domains to the N-terminus affects the ability of mERRα to regulate the mOPN promoter by disrupting N-/C-terminal communication, we fused GFP (244 amino acids), Gal4 (148 amino acids), or VP16 (48 amino acids) to the N-terminus of mERRα and tested them on the 2 kb, -253 and -56 mOPN promoters. Consistent with our previous results, native mERRα activated all three of the mOPN promoters two- to threefold in HeLa cells, the GFP and the VP16 fusions abolished activation of all three promoters, and the Gal4 fusion activated both the -253 and -56 mOPN reporters but not the 2 kb promoter (Fig. 5D). In ROS17/2.8 cells, mERRα represses all three mOPN reporters (Fig. 5E) as we had demonstrated before (Fig. 1C). GFPmERRα repressed only the -253 mOPN promoter, while GAL4mERRα repression was indistinguishable from that of the native mERRα on all three of the reporters (Fig. 5E). VP16mERRα activated the 2 kb mOPN promoter about twofold (Fig. 5E) and activated the endogenous rOPN promoter as shown by real-time PCR (data not shown) indicating that VP16 can turn mERRα into a constitutive activator. However, VP16mERRα repressed the -253 and -56 promoters (Fig. 5E).

Figure 5 *ERRα* protein domain fusions reveal the importance of different domains for transcriptional regulation. (A) Schematic of the Gal 4 DBD fusions made to address which domains of *ERRα* are needed for transcriptional activation/repression. (B) HeLa cells (45 000 cells/well) or (C) ROS17/2.8 cells (30 000 cells/well) were transfected with 150 ng pFRLuc reporter and 500 ng of the Gal4 fusion proteins depicted in (A). Activation/repression was significant ($P < 0.05$) for all constructs tested with the exception of mAF2HBD and Δ C392HBD in both HeLa and ROS17/2.8 cells. (D) HeLa cells (40 000 cells/well) were transfected with 200 ng of the indicated pGL2mOPN promoter reporter plasmid and 150 ng non-tagged mERR α (–) or different sized N-terminally tagged *ERRα* fusion proteins. (E) ROS17/2.8 cells (30 000 cells/well) were transfected with 50 ng of the indicated pGL2mOPN promoter reporter plasmid and 400 ng non-tagged mERR α (–) or different sized N-terminally tagged *ERRα* fusion proteins. Empty expression vector or the appropriate fusion protein vector was used as the control for the transfections. The GFP, Gal4, or VP16 fusion proteins add 244, 148, or 48 amino acids to the N-terminus of mERR α respectively. The mean $\pm$ s.d. of triplicate wells from a representative experiment is shown: significant ($P < 0.05$) activation or repression is described in the text.

The data indicate that proper conformation of the N-terminus and promoter context are important in regulating the transcriptional activity of mERRα.

Discussion

We report here that mERRα is a cell context-dependent regulator of the mOPN promoter through a non-canonical binding site, activating in HeLa cells but repressing in the osteoblastic ROS17/2.8 cells. We also found that the AF2 domain was dispensable for this activation while the A/B domain functions as a repressor, a repressor function that can be masked by the carboxy terminus of mERRα in a cell context-dependent manner.

mERRα and cell context-dependent regulation of the mOPN promoter

Previous studies were discrepant in showing either that mERRα positively regulates mOPN mediated by two ERRE sites (S1 and S2) around 700 bps upstream from the transcription start site in HEK293 and ROS17/2.8 cells (Vanacker *et al.* 1998) or that it has no effect on its own but blocks Nurr1-mediated transactivation of OPN in the osteoblastic SaOS cell line (Lammi *et al.* 2004). These latter authors demonstrated that Nurr1 bound to the S1 site in a manner not competable by ERRα, suggesting that ERRα acted through a different site (Lammi *et al.* 2004). Our data support the view that mOPN repression by mERRα in the osteoblastic cell lines is mediated through a novel site. The basis of the discrepancy in activation versus repression in ROS17/2.8 cells is at present unclear. However, a serum factor that modulates mERRα activity (Vanacker *et al.* 1999b) seems an unlikely explanation, given that three different batches of serum gave us comparable results (data not shown). Sequence polymorphisms in the mOPN promoters may contribute to the differences in regulation observed, as they are known to do in human OPN (Giacopelli *et al.* 2004, Hummelshoj *et al.* 2006). We also cannot discount the possibility that the ROS17/2.8 cells have diverged between laboratories (Grigoriadis *et al.* 1985) and are expressing different subsets/levels of co-activators or co-repressors; such a possibility is supported by the ability of PGC1α to turn mERRα into an activator when it is cotransfected into unresponsive HeLa cells (Huppunen *et al.* 2004). In any case, our observation that endogenous OPN expression was repressed in ERRα-transfected ROS17/2.8 cells and increased in ROS17/2.8 cells transfected with shRNA against ERRα (Fig. 1D) is consistent with ERRα being a negative regulator of OPN transcription in the osteoblastic cells, a result we confirmed in the mouse pre-osteoblast cell line MC3T3-E1 (clone26; data not shown). It is also worth noting that neither we (NIH

3T3 fibroblasts; data not shown) nor others (NB-E fibroblasts; Vanacker *et al.* 1998) have been able to detect either repression or activation of the OPN promoter in fibroblastic cells; we also have not detected regulation of OPN by ERRα in the monkey kidney (*Cercopithecus aethiops* 1 (COS1)) or rat chondrocyte (C5·18) cell lines (data not shown).

Requirement for an intact DBD is cell context dependent

Activation of mOPN in HeLa cells depends on a non-canonical site at −56 to −45 and requires a functional DBD, as disruption of the second zinc finger in the mERRαC99G construct abolished activation. The residual activity observed with the −2 kb mOPN promoter construct suggests that in this context mERRαC99G is tethered to other transcription factors. Although we demonstrated that mERRα alters the binding of protein complexes at this site in a DBD-dependent manner, we were not able to show definitive direct binding of mERRα to this site by EMSA, nor were we able to supershift the complexes using a commercially available antibody (data not shown). This suggests that mERRα binds to the site only weakly/transiently or via protein–protein interactions. It is known that the −56 to −45 region is important for basal transcriptional activity and is capable of binding the CAAT box factor NF-Y (Kabe *et al.* 2005), but whether mERRα binds to the −56 to −45 region directly or uses NF-Y as a bridging factor is at present not known. Nevertheless, it is notable that repression of mOPN by mERRα in ROS17/2.8 cells is mediated by the same non-canonical ERRE element, but does not require an intact DBD, suggesting that mERRα binds to different factors or adopts a different conformation when interacting with the promoter in ROS17/2.8 versus HeLa cells.

mERRα domain requirements and interactions in the regulation of the mOPN promoter

Our data suggest the novel conclusion that helix 12 of mERRα is not only dispensable for activation, but also its absence renders mERRα hyperactive. In most NRs, helix 12 contributes several residues that form part of the hydrophobic surface and the charge clamp, the latter formed by two conserved amino acids, a lysine (K) residue in helix 3 (K243 in mERRα) and in most NRs a glutamic acid (E) in helix 12 (E415 in mERRα). A few NRs have an aspartic acid (D) in helix 12 as part of their charge clamp. Co-activators bind to the AF2 domain via a LXXLL motif (L is leucine and X is any amino acid) termed the NR interaction domain, with amino acids surrounding this core sequence imparting NR

selectivity and binding affinity (Gaillard *et al.* 2006). Our mERRαmAF2 mutant deletes amino acids 413–419, removing all of helix 12, including the charge clamp E415 and several hydrophobic residues that form part of the co-activator binding surface (Kallen *et al.* 2004). The fact that this made mERRα hyperactive not only in HeLa cells but also in other cell lines and with other promoters (data not shown), suggests that mERRαmAF2 is either able to recruit co-activators better or bind them in a different conformation such that a more active complex results. In support of this idea is the observation that reducing the hydrophobicity by mutating leucines to alanines in helix 12 of ERRα has been shown to reduce but not abolish activity, suggesting that such a mutated ERRα still binds co-activators (Kraus *et al.* 2002, Zhang *et al.* 2006), including PGC1α or RIP140 (Huss *et al.* 2002, Castet *et al.* 2006). Neutralizing the charge in helix 12 has also been shown to activate ERRα depending on the promoter/co-activator used (Gaillard *et al.* 2007). Our data with mERRαmAF2, which carries a change predicted to keep the charge clamp intact, suggest that charge *per se* is less important than having one less carbon in the side chain as demonstrated by the mERRαΔCE415N mutant. The charge near the C-terminus is totally dispensable as a deletion mutant to residue 392 (mERRαΔC392) activates transcription of the *mOPN* promoter with an activity between that of wild-type and mERRαmAF2 levels depending on the promoter complexity. Mutation of the other charge clamp residue K243 to alanine retains activity, which varied with the size of the promoter fragment used. Taken together, the data suggest that in ERRα only one of the charge clamp residues is sufficient to allow binding of co-activator(s). This is consistent with recent data from Gaillard *et al.* (2007) who demonstrated that in order to abolish ERRα activation of a 3× ERE-TATA-luc reporter by PGC1α, both the charge clamp residues had to be neutralized. The fact that activation via another co-activator, SRC2, was abolished by neutralizing the charge clamp in either helix 3 (K243A) or AF2 domain (triple mutant K411N, E415Q, E418Q) suggests that these interactions are co-activator, and most likely, promoter context dependent.

We also found that more subtle changes in the conformation of helix 12, i.e., H408 versus P408, increase the activity of mERRα. We predict that the H408 compared with the P408 allele imparts more degrees of rotational freedom to helix 12, perhaps better accommodating co-activator binding or conversely reducing co-repressor binding. There is precedent to suggest that subtle changes in the conformation of the AF2 domain alter co-activator binding (Paige *et al.* 1999, Nettles & Greene 2005). Such allelic changes could have important biological and clinical consequences for carriers. For example,

increased ERRα expression, due to an autoregulatory ERRα promoter polymorphism comprising amplification of ERRE elements, is correlated with higher bone mineral density (BMD) and body mass index (BMI) in certain populations (Kamei *et al.* 2005, Laflamme *et al.* 2005). Our results suggest that the H408 allele, which also exists in the rat (Accession # NP_001008511), may also influence BMD and BMI by being more active.

A second novel conclusion, based on our results with heterologous protein domains fused to mERRα, is that the N-terminus of mERRα contains a repressor domain whose activity is influenced by cell and promoter context and it is the interplay between the N- and C-termini that affect the transcriptional outcome. We uncovered a repressor domain within the A/B domain of mERRα (Fig. 5 and (Zhang & Teng 2001)) that, somewhat surprisingly, can shut down the strong activation activity of VP16 (Sladek *et al.* 1997). This was not due to a defective VP16 fusion protein since we detected an appropriately sized protein by western blotting and the VP16mERRα activates transcription of a 3× ERRE-tk-luc plasmid (data not shown). Further support for a regulatory interaction between the N- and C-termini of ERRα comes from our HBD mutants, and the data again suggest that repression is dominant over the ability to activate. This is not because a repressor is bound to the HBD, as the wt HBD fused to the Gal4 DBD gave a robust 28-fold activation, suggesting that ROS17/2.8 cells are not lacking an activator molecule, but rather that the N-terminal part of mERRα is able to suppress the activation. Proximity of the N- and C-termini for NRs has been described and, in the case of the androgen receptor (AR), it plays an important role in activating transcription: the N-terminus of AR contains a sequence FXXLF that binds to its own AF2 domain with the net result being longer ligand retention (He *et al.* 1999). The N- and C-termini of AR have also been shown to interact in a yeast two hybrid test and FRET (He *et al.* 1999, Schaufele *et al.* 2005). The A/B domain of mERRα is quite short and does not possess a sequence resembling either FXXLF or LXXLL, however, that does not preclude the possibility that the N- and C-termini are in close spatial proximity. In other NRs, it has also been demonstrated that while co-activator(s) bind to the AF2 domain with its LXXLL motif, other parts of the co-activator(s) bind sequences in the N-terminus. Our data are consistent with a model in which at least two out of the three regions (A/B, K243, or AF2) of mERRα cooperate to regulate transcription. Thus, the structure of the N-terminus of mERRα appears to be more important than previously appreciated and is dependent on the cell context to determine whether mERRα acts as a repressor or an activator.

We conclude that OPN is an ERR α target gene whose promoter is regulated by ERR α through a non-canonical ERR α response element in a cell context-dependent manner and that a predicted silencing mutation in AF2 or a more flexible helix 12 increases ERR α transcriptional activity, effects with implications for ERR α as a therapeutic target in bone.

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Disclosure

The authors have nothing to disclose.

References

- Aubin JE 2001 Regulation of osteoblast formation and function. *Reviews in Endocrine and Metabolic Disorders* **2** 81–94.
- Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA & Struhl K 1987 In *Current Protocols in Molecular Biology in Current Protocols*, Ed VB Chanda. New York: Wiley.
- Bidder M, Shao JS, Charlton-Kachigian N, Loewy AP, Semenkovich CF & Towler DA 2002 Osteopontin transcription in aortic vascular smooth muscle cells is controlled by glucose-regulated upstream stimulatory factor and activator protein-1 activities. *Journal of Biological Chemistry* **277** 44485–44496.
- Bonnelye E & Aubin JE 2002 Differential expression of estrogen receptor-related receptor α and estrogen receptors α and β in osteoblasts *in vivo* and *in vitro*. *Journal of Bone and Mineral Research* **17** 1392–1400.
- Bonnelye E & Aubin JE 2005 Estrogen receptor-related receptor α : a mediator of estrogen response in bone. *Journal of Clinical Endocrinology and Metabolism* **90** 3115–3121.
- Bonnelye E, Merdad L, Kung V & Aubin JE 2001 The orphan nuclear estrogen receptor-related receptor α (ERR α) is expressed throughout osteoblast differentiation and regulates bone formation *in vitro*. *Journal of Cell Biology* **153** 971–984.
- Bonnelye E, Kung V, Laplace C, Galson DL & Aubin JE 2002 Estrogen receptor-related receptor α impinges on the estrogen axis in bone: potential function in osteoporosis. *Endocrinology* **143** 3658–3670.
- Bonnelye E, Zirngibl RA, Jurdic P & Aubin JE 2007 The orphan nuclear estrogen receptor-related receptor- α regulates cartilage formation *in vitro*: implication of Sox9. *Endocrinology* **148** 1195–1205.
- Bourguet W, Germain P & Gronemeyer H 2000 Nuclear receptor ligand-binding domains: three-dimensional structures, molecular interactions and pharmacological implications. *Trends in Pharmacological Sciences* **21** 381–388.
- Castet A, Herledan A, Bonnet S, Jalaguier S, Vanacker JM & Cavaillès V 2006 Receptor-interacting protein 140 differentially regulates estrogen receptor-related receptor transactivation depending on target genes. *Molecular Endocrinology* **20** 1035–1047.
- Craig AM & Denhardt DT 1991 The murine gene encoding secreted phosphoprotein 1 (osteopontin): promoter structure, activity, and induction *in vivo* by estrogen and progesterone. *Gene* **100** 163–171.
- Denhardt DT, Giachelli CM & Rittling SR 2001 Role of osteopontin in cellular signaling and toxicant injury. *Annual Review of Pharmacology and Toxicology* **41** 723–749.
- Gaillard S, Gräsfeder LL, Haeffle CL, Lobenhofer EK, Chu TM, Wolfinger R, Kazmin D, Kovacs TR, Muoio DM, Chang CY *et al.* 2006 Receptor-selective coactivators as tools to define the biology of specific receptor-coactivator pairs. *Molecular Cell* **24** 797–803.
- Gaillard S, Dwyer MA & McDonnell DP 2007 Definition of the molecular basis for estrogen receptor-related receptor- α -cofactor interactions. *Molecular Endocrinology* **21** 62–76.
- Giacopelli F, Marciano R, Pistorio A, Catarsi P, Canini S, Karsenty G & Ravazzolo R 2004 Polymorphisms in the osteopontin promoter affect its transcriptional activity. *Physiological Genomics* **20** 87–96.
- Giguere V 2002 To ERR in the estrogen pathway. *Trends in Endocrinology and Metabolism* **13** 220–225.
- Giguere V, Yang N, Segui P & Evans RM 1988 Identification of a new class of steroid hormone receptors. *Nature* **331** 91–94.
- Grigoriadis AE, Petkovich PM, Ber R, Aubin JE & Heersche JN 1985 Subclone heterogeneity in a clonally-derived osteoblast-like cell line. *Bone* **6** 249–256.
- He B, Kemppainen JA, Voegel JJ, Gronemeyer H & Wilson EM 1999 Activation function 2 in the human androgen receptor ligand binding domain mediates interdomain communication with the NH2-terminal domain. *Journal of Biological Chemistry* **274** 37219–37225.
- Hullinger TG, Pan Q, Viswanathan HL & Somerman MJ 2001 TGF β and BMP-2 activation of the OPN promoter: roles of smad- and hox-binding elements. *Experimental Cell Research* **262** 69–74.
- Hummelshøj T, Ryder LP, Madsen HO, Odum N & Svejgaard A 2006 A functional polymorphism in the Eta-1 promoter is associated with allele specific binding to the transcription factor Sp1 and elevated gene expression. *Molecular Immunology* **43** 980–986.
- Huupponen J, Wohlfahrt G & Aarnisalo P 2004 Requirements for transcriptional regulation by the orphan nuclear receptor ERR γ . *Molecular and Cellular Endocrinology* **219** 151–160.
- Huss JM, Kopp RP & Kelly DP 2002 Peroxisome proliferator-activated receptor coactivator-1 α (PGC-1 α) coactivates the cardiac-enriched nuclear receptors estrogen-related receptor- α and - γ . Identification of novel leucine-rich interaction motif within PGC-1 α . *Journal of Biological Chemistry* **277** 40265–40274.
- Jakacka M, Ito M, Weiss J, Chien P-Y, Gehm BD & Jameson JL 2001 Estrogen receptor binding to DNA is not required for its activity through the nonclassical AP1 pathway. *Journal of Biological Chemistry* **276** 13615–13621.
- Kabe Y, Yamada J, Uga H, Yamaguchi Y, Wada T & Handa H 2005 NF-Y is essential for the recruitment of RNA polymerase II and inducible transcription of several CCAAT box-containing genes. *Molecular and Cellular Biology* **25** 512–522.
- Kallen J, Schlaeppli JM, Bitsch F, Filipuzzi I, Schilb A, Riou V, Graham A, Strauss A, Geiser M & Fournier B 2004 Evidence for ligand-independent transcriptional activation of the human estrogen-related receptor α (ERR α): crystal structure of ERR α ligand binding domain in complex with peroxisome proliferator-activated receptor coactivator-1 α . *Journal of Biological Chemistry* **279** 49330–49337.

- Kamei Y, Lwin H, Saito K, Yokoyama T, Yoshiike N, Ezaki O & Tanaka H 2005 The 2.3 genotype of ESRR23 of the ERR α gene is associated with a higher BMI than the 2.2 genotype. *Obesity Research* **13** 1843–1844.
- Kraus RJ, Ariazi EA, Farrell ML & Mertz JE 2002 Estrogen-related receptor α 1 actively antagonizes estrogen receptor-regulated transcription in MCF-7 mammary cells. *Journal of Biological Chemistry* **277** 24826–24834.
- Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM & Webb P 2000 Estrogen receptor pathways to AP-1. *Journal of Steroid Biochemistry and Molecular Biology* **74** 311–317.
- Laflamme N, Giroux S, Loredó-Osti JC, Elfassihi L, Dodin S, Blanchet C, Morgan K, Giguere V & Rousseau F 2005 A frequent regulatory variant of the estrogen-related receptor α gene associated with BMD in French–Canadian premenopausal women. *Journal of Bone and Mineral Research* **20** 938–944.
- Lammi J, Hupponen J & Aarnisalo P 2004 Regulation of the osteopontin gene by the orphan nuclear receptor NURR1 in osteoblasts. *Molecular Endocrinology* **18** 1546–1557.
- Lee M, Na S, Jeon D, Kim H, Choi Y & Baik M 2000 Induction of osteopontin gene expression during mammary gland involution and effects of glucocorticoid on its expression in mammary epithelial cells. *Bioscience, Biotechnology, and Biochemistry* **64** 2225–2228.
- Lefebvre V & Smits P 2005 Transcriptional control of chondrocyte fate and differentiation. *Birth Defects Research. Part C, Embryo Today: Reviews* **75** 200–212.
- Li Y, Lambert MH & Xu HE 2003 Activation of nuclear receptors: a perspective from structural genomics. *Structure* **11** 741–746.
- Liu R-T, Suzuki S, Takeda T & DeGroot LJ 1996 An artificial thyroid hormone receptor mutant without DNA binding can have dominant negative effect. *Molecular and Cellular Endocrinology* **120** 85–93.
- Majeska RJ, Nair BC & Rodan GA 1985 Glucocorticoid regulation of alkaline phosphatase in the osteoblastic osteosarcoma cell line ROS 17/2.8. *Endocrinology* **116** 170–179.
- Nettles KW & Greene GL 2005 Ligand control of coregulator recruitment to nuclear receptors. *Annual Review of Physiology* **67** 309–333.
- Noda M, Vogel RL, Craig AM, Prah J, DeLuca HF & Denhardt DT 1990 Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D3 receptor and 1,25-dihydroxyvitamin D3 enhancement of mouse secreted phosphoprotein 1 (SPP-1 or osteopontin) gene expression. *PNAS* **87** 9995–9999.
- Nuclear Receptors Nomenclature Committee 1999 A unified nomenclature system for the nuclear receptor superfamily. *Cell* **97** 161–163.
- Ogawa D, Stone JF, Takata Y, Blaschke F, Chu VH, Towler DA, Law RE, Hsueh WA & Brummer D 2005 Liver x receptor agonists inhibit cytokine-induced osteopontin expression in macrophages through interference with activator protein-1 signaling pathways. *Circulation Research* **96** e59–e67.
- Paige LA, Christensen DJ, Gron H, Norris JD, Gottlin EB, Padilla KM, Chang C-Y, Ballas LM, Hamilton PT, McDonnell DP *et al.* 1999 Estrogen receptor (ER) modulators each induce distinct conformational changes in ER α and ER β . *PNAS* **96** 3999–4004.
- Park YY, Ahn SW, Kim HJ, Kim JM, Lee IK, Kang H & Choi HS 2005 An autoregulatory loop controlling orphan nuclear receptor DAX-1 gene expression by orphan nuclear receptor ERR γ . *Nucleic Acids Research* **33** 6756–6768.
- Privalsky ML 2004 The role of corepressors in transcriptional regulation by nuclear hormone receptors. *Annual Review of Physiology* **66** 315–360.
- Sadowski I & Ptashne M 1989 A vector for expressing GAL4(1-147) fusions in mammalian cells. *Nucleic Acids Research* **17** 7539.
- Safe S, Kim K & Kivie M 2004 Nuclear receptor-mediated transactivation through interaction with Sp proteins. In *Progress in Nucleic Acid Research and Molecular Biology*, vol 77, pp 1–36. Academic Press.
- Sanyal S, Kim JY, Kim HJ, Takeda J, Lee YK, Moore DD & Choi HS 2002 Differential regulation of the orphan nuclear receptor small heterodimer partner (SHP) gene promoter by orphan nuclear receptor ERR isoforms. *Journal of Biological Chemistry* **277** 1739–1748.
- Schafele F, Carbonell X, Guerbadot M, Borngraeber S, Chapman MS, Ma AA, Miner JN & Diamond MI 2005 The structural basis of androgen receptor activation: intramolecular and intermolecular amino-carboxy interactions. *PNAS* **102** 9802–9807.
- Schreiber SN, Knutti D, Brogli K, Uhlmann T & Kralli A 2003 The transcriptional coactivator PGC-1 regulates the expression and activity of the orphan nuclear receptor estrogen-related receptor α (ERR α). *Journal of Biological Chemistry* **278** 9013–9018.
- Schug J 2003 In *Using TESS to Predict Transcription Factor Binding Sites in DNA Sequence*, Ed AD Baxevanis. New York: Wiley (ch 5).
- Shen Q & Christakos S 2005 The vitamin D receptor, Runx2, and the Notch signaling pathway cooperate in the transcriptional regulation of osteopontin. *Journal of Biological Chemistry* **280** 40589–40598.
- Shigeta H, Zuo W, Yang N, DiAugustine R & Teng CT 1997 The mouse estrogen receptor-related orphan receptor α 1: molecular cloning and estrogen responsiveness. *Journal of Molecular Endocrinology* **19** 299–309.
- Sladek R, Bader JA & Giguere V 1997 The orphan nuclear receptor estrogen-related receptor α is a transcriptional regulator of the human medium-chain acyl coenzyme A dehydrogenase gene. *Molecular and Cellular Biology* **17** 5400–5409.
- Spiegelman BM & Heinrich R 2004 Biological control through regulated transcriptional coactivators. *Cell* **119** 157–167.
- Vanacker JM, Delmarre C, Guo X & Laudet V 1998 Activation of the osteopontin promoter by the orphan nuclear receptor estrogen receptor related α . *Cell Growth and Differentiation* **9** 1007–1014.
- Vanacker JM, Pettersson K, Gustafsson JA & Laudet V 1999a Transcriptional targets shared by estrogen receptor – related receptors (ERRs) and estrogen receptor (ER) α , but not by ER β . *EMBO Journal* **18** 4270–4279.
- Vanacker JM, Bonnelye E, Chopin-Delannoy S, Delmarre C, Cavaillès V & Laudet V 1999b Transcriptional activities of the orphan nuclear receptor ERR α (estrogen receptor-related receptor- α). *Molecular Endocrinology* **13** 764–773.
- Wang D, Christensen K, Chawla K, Xiao G, Krebsbach P & Franceschi R 1999 Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct *in vitro* and *in vivo* differentiation/mineralization potential. *Journal of Bone and Mineral Research* **14** 893–903.
- Yang N, Shigeta H, Shi H & Teng CT 1996 Estrogen-related receptor, hERR1, modulates estrogen receptor-mediated response of human lactoferrin gene promoter. *Journal of Biological Chemistry* **271** 5795–5804.
- Zhang Z & Teng CT 2001 Estrogen receptor α and estrogen receptor-related receptor α 1 compete for binding and coactivator. *Molecular and Cellular Endocrinology* **172** 223–233.
- Zhang Z, Chen K, Shih JC & Teng CT 2006 Estrogen-related receptors-stimulated monoamine oxidase B promoter activity is down-regulated by estrogen receptors. *Molecular Endocrinology* **20** 1547–1561.

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