Estrogen-related receptor α regulates osteoblast differentiation via Wnt/β-catenin signaling

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Abstract

Based on its homology to the estrogen receptor and its roles in osteoblast and chondrocyte differentiation, the orphan nuclear receptor estrogen-related receptor α (ERRα (ESRRA)) is an intriguing therapeutic target for osteoporosis and other bone diseases. The objective of this study was to better characterize the molecular mechanisms by which ERRα modulates osteoblastogenesis. Experiments from multiple systems demonstrated that ERRα modulates Wnt signaling, a crucial pathway for proper regulation of bone development. This was validated using a Wnt-luciferase reporter, where ERRα showed co-activator-dependent (peroxisome proliferator-activated receptor gamma co-activator 1α, PGC-1α) stimulatory effects. Interestingly, knockdown of ERRα expression also enhanced WNT signaling. In combination, these data indicated that ERRα could serve to either activate or repress Wnt signaling depending on the presence or absence of its co-activator PGC-1α. The observed Wnt pathway modulation was cell intrinsic and did not alter β-catenin nuclear translocation but was dependent on DNA binding of ERRα. We also found that expression of active ERRα correlated with Wnt pathway effects on osteoblastic differentiation in two cell types, consistent with a role for ERRα in modulating the Wnt pathway. In conclusion, this work identifies ERRα, in conjunction with co-activators such as PGC-1α, as a new regulator of the Wnt-signaling pathway during osteoblast differentiation, through a cell-intrinsic mechanism not affecting β-catenin nuclear translocation.

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Introduction

Estrogen-related receptor α (ERRα and NR3B1 (ESRRA)) is an orphan nuclear hormone receptor capable of regulating the transcription of genes involved in multiple cellular and physiological processes (Bonnelye & Aubin 2005, Ariazi & Jordan 2006, Huss et al. 2007). The best characterized of these is its role in energy metabolism, in which ERRα is co-activated by the peroxisome proliferator-activated receptor γ (PPARγ) co-activator 1-α (PGC-1α) to regulate many genes required for mitochondrial function (Vega & Kelly 1997, Luo et al. 2003, Carrier et al. 2004, Schreiber et al. 2004). Although ERRα was identified based on sequence similarity to the estrogen receptor α (ERα; Giguere et al. 1988), ERRα does not bind estrogen as a ligand. It is, however, co-expressed with ERα in estrogen-responsive cell types including osteoblasts (Bonnelye & Aubin 2002).

Studies have shown ERRα to be an important regulatory factor in several facets of bone biology. First, ERRα is expressed during the formation of ossification zones during mouse development in vivo as well as in vitro in primary rat calvarial (RC) cells (Bonnelye et al. 1997, 2001). Inhibiting ERRα blocks differentiation of RC cells into mature osteoblasts, while over-expression enhances differentiation, affecting multiple stages of osteoblast development. In addition, several known transcriptional targets of ERRα have roles in bone development, most importantly osteopontin.
ERRα has also been implicated in the formation and maintenance of cartilage by regulating the transcription of Sox9 (Bonmyle et al. 2007). It is unclear whether co-activation by PGC-1α is required for these activities of ERRα, although PGC-1α has been implicated in chondrogenesis through co-activating NURR1 (NR4A2) (another nuclear hormone receptor) and SOX9 (Kawakami et al. 2005, Nervina et al. 2006). Two recent studies have demonstrated bone phenotypes in ERRα knockout (KO) mice. One shows moderately increased femoral cancellous bone volume and density in female ERRα KO mice and suggests a role in osteoblastogenesis (Delhon et al. 2009). Another shows resistance to bone loss in female KO mice and further suggests that ERRα acts as an inhibitor of osteoblast differentiation in vitro (Teyssier et al. 2009).

The Wnt pathway is another important player in bone development (reviewed in Bodine & Komm (2006)). During canonical Wnt/β-catenin signaling, Wnt ligands such as Wnt3a activate LDL receptor-related protein (LRP (LRP1)) receptors, resulting in translocation of β-catenin to the nucleus where it activates transcription of target genes. Other Wnt ligands, such as Wnt5a and Wnt11, can specify the activation of non-canonical pathways including the Wnt/calcium, Wnt/cAMP, and JNK pathways. Wnt/β-catenin signaling is required for osteoblast differentiation and plays a key role in deciding the fate of mesenchymal stem cells both during development and in mature bone (Day et al. 2005, Hill et al. 2005). Although comparatively little is known about the effects of noncanonical Wnt signaling, one study has implicated it in skeletal development (Tu et al. 2007).

Functional cross talk between ERRα and the Wnt pathway has recently been observed to be important in cancer cell migration (Dwyer et al. 2010). ERRα expression enhanced β-catenin-dependent transcription from a TCF/LEF reporter, and ERRα was shown to physically interact with β-catenin and LEF1 in several breast cancer cell lines. Further, Wnt11 was shown to be a target of co-regulation by ERRα and β-catenin at two sites in the Wnt11 promoter, either via binding adjacent sites or via protein–protein interactions. Our work suggests that cross talk between ERRα and Wnt signaling also occurs and plays a functional role in osteoblast differentiation.

Here, we investigate the molecular mechanism by which ERRα affects osteoblast differentiation, using multiple model systems and approaches. We identified WNT pathway modulation by ERRα as one such mechanism. We found that ERRα regulates transcription of genes involved in Wnt signaling in mouse calvarial explants. Further, ERRα can negatively or positively regulate a Wnt-luciferase reporter in vitro, depending on the presence or absence of the co-activator PGC-1α. This activation requires ERRα DNA-binding capability, is independent of secreted factors, and does not involve β-catenin nuclear translocation.

Materials and methods

Ethics statement

All procedures using animals were performed in accordance with institutional regulations and ethics guidelines under IACUC-reviewed and approved protocols.

Tissue culture and other materials

Anti-FLAG M2 and monoclonal anti-β-actin antibodies were purchased from Sigma. Tissue culture reagents were purchased from Gibco/Invitrogen, except McCoy’s 5A, which was made in-house. U2OS and CSH10T1/2 cells were purchased from ATCC (Manassas, VA, USA). U2OS cells were cultured as described (Bhat et al. 2007). CSH10T1/2 cells were cultured in DMEM with high glucose and Glutamax (Invitrogen) with 10% FBS. The hMSCs were purchased from Cambrex, Inc. (East Rutherford, NJ, USA) and cultured as described (Liu et al. 2007b).

Constructs and adenovirus

All adenoviruses were generated by ViraQuest, Inc. (North Liberty, IA, USA), with the exception of 16×TCF-luc and Wnt3a. ERRα and PGC-1α cDNAs purchased from Open BioSystems (Lafayette, CO, USA) were N-terminally tagged and cloned into pAdORI-Flag-ERRα and pAdORI-Flag-PGC-1α. CMV-GFP was purchased from Promega. 16×TCF-luciferase has been previously described (Bhat et al. 2007). Point mutants in pAdORI-FLAG-ERRα were generated using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions. The oligonucleotides used were C99G: 5’-GCATCCTGTGA GCCCTGGACCGCGTC-3’ and R150A: 5’-CAA GGAGGGAGTG GCCCTGGACCGCGTC-3’.

Calvarial organ culture

Neonatal mouse calvaria were prepared from 4-day-old pups as described previously (Traianedes et al. 1998, Mundy et al. 1999) with slight modifications (Liu et al. 2007a). Briefly, calvaria were excised and cut into half along the sagittal suture. Each half calvarium was placed with the concave surface downward on a stainless steel grid (Small Parts, Inc., Miami, Fl, USA) in a 12-well tissue culture dish (Becton Dickinson, Oxnard, CA, 2007).
USA). Each well contained 1 ml BGJ medium with 0.1% BSA. Calvaria in 12-well plates were incubated in a humidified atmosphere of 5% CO₂ and the medium was changed after 1, 3, and 5 days. Calvaria prepared from littermates were used in each experiment. For adenovirus-mediated gene transfer, calvaria were infected with GFP (control) or with appropriate adenoviruses (2×10⁹ particles/well) and incubated for 7 days. Fresh adenoviruses were added when medium was changed on day 4. After organ culture, calvaria were fixed in 10% neutral phosphate-buffered formaldehyde at room temperature for 48 h and then decalcified for 3 h in 10% EDTA in PBS. Calvaria in each group were embedded in paraffin in the same paraffin block, and 4 μm sections were stained with hematoxylin–eosin.

For histomorphometrical analysis, consistent bone areas (200 μm away from frontal sutures) were selected. In brief, a 200 μm square grid was placed on each calvaria and the number of osteoblasts and total bone area were determined with the Osteomeasure System (Osteometrics, Inc., Atlanta, GA, USA). All cells on the bone surface were counted as osteoblasts. All results are expressed as the mean ± s.e.m. for four to six cultures. Statistical analyses between groups were performed by one-way ANOVA.

**RNA isolation and transcriptional profiling**

Mouse calvarial explants were prepared and infected as described earlier and cultured for 4 days before RNA isolation. Briefly, calvarval extracts were homogenized in cold denaturing buffer (Promega, RNAagents) containing β-mercaptoethanol. Aqueous phase was collected following phenol/chloroform/isoamyl alcohol extraction and RNA precipitated with isopropanol. RNA was then purified with the RNeasy Micro kit (Qiagen). Gene expression profiling was performed using Mouse Genome 430 2.0 arrays, as described earlier and cultured for 4 days before RNA isolation. For analysis, five endogenous control genes (18S, GAPDH, B2M, HPRT1, and PPIA) were pooled together as reference using an in-house-developed algorithm. The relative amount of each target gene mRNA to reference was calculated as the average 2−ΔCt, where ΔCt = Ct − C(reference). The threshold cycle Ct was calculated automatically by the SDS2.2 software (Applied Biosystems). Genes with Ct > 35 were set to 35, and genes with a Welch t-test P-value of <0.05 between ΔCt in treatment vs GFP were considered to be significantly changed.

**Reporter assays**

In general, cells were seeded at confluence in 96-well plates and infected the following day with 16×TCF and treatment virus. Cells were lysed and luciferase was monitored using Luciferase Reporter Assay reagents and protocols (Promega) in a Victor 3 luminometer (Perkin Elmer, Waltham, MA, USA). Results are represented as the mean and s.d. and are representative of at least two separate experiments.

Infection and differentiation of C3H10T1/2 cells were carried out as described for the alkaline phosphatase assay, with infection on day 13 of differentiation. For C3H10T1/2 epistasis experiments with Wnt3a, MOI1000 of adenovirus was pre-incubated in serum-free media with poly-L-lysine to improve infection efficiency (Bereziat et al. 2005). For the DKK1 experiment, C3H10T1/2 cells were seeded previously (Berasi et al. 2006).

**TaqMan low-density arrays**

C3H10T1/2 cells were infected with GFP, Wnt3a, or ERRz+PGC-1α with a total MOI of 1000 essentially as described (Orlicky & Schack 2001). Twenty-four hours after infection, cells were lysed using QIAshredder, and RNA was isolated by RNeasy Mini kits according to the manufacturer’s instructions (Qiagen). For U2OS, RNA was collected 12 h after infection. Total RNA (400 ng) was reverse transcribed using the High-Capacity cDNA Archive Kit as per manufacturer’s instructions (Applied Biosystems, Foster City, CA, USA). RNA was applied to the organism-specific custom-designed TLDA containing 80 (mouse) or 87 (human) genes from Applied Biosystems Assays-on-Demand Gene Expression Products. Endogenous 18S, GAPDH, B2M, GUSB, HPRT1, and PPIA probes were included as control and reference genes for normalization. A total of 100 μl reaction mixture with 5 μl cDNA template (100 ng mRNA) and 50 μl 2× TaqMan Universal PCR Master Mix (Applied Biosystems) was added to each line of the LDA so that each reaction cell contained 1 μl reaction mixture with 1 ng mRNA. The PCR amplifications were performed in an ABI Prism 7900HT sequence detection system (Applied Biosystems) using the following amplification protocol: 2 min at 50 °C, 10 min at 94.5 °C, 40 cycles of 97 °C for 30 s, and 59.7 °C for 1 min.

For analysis, five endogenous control genes (18S, B2M, GUSB, HPRT1, and PPIA) were pooled together as reference using an in-house-developed algorithm. The relative amount of each target gene mRNA to reference was calculated as the average 2−ΔCt, where ΔCt = Ct − C(reference). The threshold cycle Ct was calculated automatically by the SDS2.2 software (Applied Biosystems). Genes with Ct > 35 were set to 35, and genes with a Welch t-test P-value of <0.05 between ΔCt in treatment vs GFP were considered to be significantly changed.
were infected with 16×TCF for 1 h, media changed, and then infected with ERRz+PGC-1α or GFP at time points before luciferase was measured. Virus mixes were replaced with fresh media 4 h after each infection.

For conditioned media transfer, U2OS cells seeded at 2 million cells/10 cm dish were infected with GFP, ERRz+PGC-1α, or Wnt3a for 4 h and then changed to media containing 0.1 mg/ml heparin. After 3 days, conditioned media was collected and sterile filtered. Reporter assays were performed in U2OS cells treated with 50% conditioned media overnight. For coculture experiments, U2OS cells were seeded to T25 flask at 1 million cells and infected with each adenoviral treatment separately (16×TCF MOI50, others MOI250). Four hours after infections, cells were trypsinized and counted and then combined as follows: cells expressing 16×TCF were kept constant at 5K/well, and Wnt3a- or ERRz+PGC-1α-expressing cells were added at 5K, 10K, and 15K/well and supplemented with GFP to maintain a total of 20K cells/well of a 96-well plate. Luciferase was measured 2 days later.

**C3H10T1/2 alkaline phosphatase assays**

C3H10T1/2 cells were seeded in black/clear bottom 96-well tissue culture plates at 4×10³ cells/cm² 1 day before infection. For four to six replicate wells were performed per condition, and blank wells were included. Cells in each well were infected with the appropriate adenovirus at an MOI of 400 in 50 μl reduced serum media (2%). Infection was carried out for more than 1 h at 37 °C, then viral inoculum was replaced with growth media (GM), and cells were allowed to recover for 4 h. GM was then replaced with osteogenic differentiation medium: GM containing 50 μg/ml l-ascorbic acid (AA), 10 mM β-glycerol phosphate, and 100 nM menadione sodium bisulfite (vitamin K3). Osteogenic differentiation medium was replaced every 2–3 days throughout differentiation.

At the desired time point, alkaline phosphatase was measured as follows: wells were washed twice with PBS, then 50 μl 4-methylumbellifferyl phosphate (4-MUP; Sigma) was added, and the plate was incubated at 37 °C for 15 min. Fluorescence was measured on a Victor luminometer using the umbrelliferone settings (excitation 355 nM, emission 460 nM, and CW lamp energy 1120) for 1 s/well. Counts were normalized to those from blank wells with only 4-MUP. After reading, 50 μl of 2× protein lysis buffer (200 mM Tris–HCl, pH 9.8; 0-4% Triton X-100) was added to each well and the plates were frozen at −80 °C. After all the time points were complete, protein concentration was determined by BCA, and the alkaline phosphatase measurements were normalized to the protein concentration.

For osteocalcin transcript measurements, cells were lysed at day 10 and RNA isolated using the SV96 RNA Isolation System (Promega). TaqMan was performed on 5 μl RNA and data were analyzed by the comparative C_Δ Δt method according to the manufacturer’s instructions (Applied Biosystems). The primers and probes used were mOC F: CGGCCCTGAGTCTGCACAA; mOC R: AAGGTAGTGAACAGACTCGG; mOC probe: CTT CATGTCACAGCAGGGA.

**Human MSC viral infection and histochemical staining**

Human MSCs were seeded at 6000/cm² in 24-well plates and allowed to adhere and proliferate overnight. Cells were infected for 24 h in 0-4 ml/cm² MSCGM using GFP, ERRz, and/or PGC-1α adenoviruses at MOI750 in the presence of hCAR (MOI=750) to improve infection efficiency. After 24 h, cells were washed once in PBS, and MSCGM supplemented with 0-05 mM AA and 10 mM β-GP was added. For ERRz knockdown in hMSC, cells were infected with adenoviruses containing ERRz-specific or scramble control shRNA for 72 h in 0-4 ml/cm² MSCGM at MOI750 in the presence of hCAR (MOI750). After 72 h, cells were washed once in PBS and placed in MSCGM supplemented with 0-05 mM AA, 10 mM β-GP, and 100 nM dexamethasone. Medium was changed every 5 days. Formation of mineralized nodules was determined by alizarin red-S histochemical staining. To quantify the level of alizarin red-S staining, the dye was eluted with 1 ml/well of 10% (w/v) cetylpyridinium chloride. Alizarin red-S in the eluted samples was quantified (vs a standard curve of 0–800 μM dye) at 562 nm with a microplate reader.

**High content analysis of β-catenin nuclear translocation**

C3H10T1/2 cells were seeded at 5K/well into 96-well plates (Perkin Elmer Viewplate-96F TC). After treatment, cells were washed once with PBS, fixed with 4% PFA for 10 min, washed twice with PBS, permeabilized by incubation in 1% Triton X-100 in PBS for 3 min, and washed thrice with PBS. Antibody staining was performed in PBST (0-05% Tween-20) with 1% goat serum (Invitrogen). Mouse anti-β-catenin (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) was added at a 1:200 dilution, incubated 1 h with shaking at room temperature and washed three times with PBST. Alexa-labeled goat antimouse IgG (Invitrogen/Molecular Probe) was added at a 1:200 dilution and incubated for 1 h at room temperature with shaking.

After washing three times with PBST, DAPI was added at final concentration of 300 nM to stain nuclei (Invitrogen/Molecular Probe). Samples were stored in PBS and scanned by Array Scan with the Nuclear Translocation Bioapplication. In short, the intensity of β-catenin in the nucleus and cytoplasm was normalized.
to the number of pixels identified in each region. The total ratio of nuclear to cytoplasmic β-catenin was then determined per well and compared across treatments. Data were downloaded into Spotfire for analysis.

Results

**ERRα and PGC-1α modulate transcription of Wnt/β-catenin genes**

ERRα has been demonstrated to have effects in several bone model systems. In order to gain some insight into which signaling pathways are affected by ERRα, transcriptional profiling experiments were performed in mouse calvarial explant tissue. Neonatal mouse calvarial explants were infected with adenovirus over-expressing ERRα or a GFP control. Growth was assayed, and mRNA was harvested for transcriptional profiling. We observed approximately a 75% enhancement in total bone area upon over-expression of ERRα compared with GFP-infected control (Fig. 1A and B), demonstrating successful infection with ERRα. Upon analysis of transcriptional profiling data, we observed modest induction of some bone regulatory genes by ERRα, including Bmp1 (1.7-fold, \( P<0.008 \) by Welch test) and Sox9 (twofold, \( P<0.008 \) (Pappano et al. 2003, Pan et al. 2008; complete data set available in Supplementary Table 1, see section on supplementary data given at the end of this article).

The data set was then subjected to two different types of functional analyses to search for groups of functionally related genes with altered transcription when ERRα is over-expressed. In the first analysis, SigPathway software was used to identify functional categories changing within the entire data set (Tian et al. 2005). The second analysis platform, Ingenuity Pathway Analysis (IPA), searches for functional pathways that are over-represented in a set of genes considered to have altered transcription with adenoviral ERRα treatment. For this analysis, genes were considered as significantly changed if they exhibited a minimum fold change of 1:5 and passed a Welch test significance cutoff of \( P<0.01 \) vs adeno-GFP.

As shown in Fig. 1C, Wnt/β-catenin signaling was found to be enriched by SigPathway analysis (false discovery rates, FDR=0.000435). This pathway was among the most over-represented functional pathways (\( P=2.53^{-4} \)) by IPA of the set of genes significantly changed in ERRα vs GFP. Additionally, genes involved in cartilage development were found to be upregulated (FDR=0; Supplementary Figure 1, see section on supplementary data given at the end of this article), supporting previous suggestions for a role of ERRα in this process (Bonnelye et al. 2007).

**Over-expression of ERRα+PGC-1α activates a similar profile of Wnt pathway genes as Wnt3a**

Following the observation of global modulation of Wnt pathway genes in an intramembranous bone system, we sought to gain a more detailed understanding of which Wnt genes are altered by ERRα over-expression and/or activation. To this aim, we examined the transcription of a panel of ~80 Wnt pathway and target genes using the TaqMan Low Density Array (Applied BioSystems) real-time PCR platform (complete data set available in Supplementary Table 2, see section on supplementary data given at the end of this article). C3H10T1/2 cells, a model system for osteoblast differentiation, were infected with adenovirus expressing GFP, ERRα, and mRNA was harvested for transcriptional profiling. For this analysis, genes were considered as having altered transcription with adenoviral ERRα and mRNA was harvested for transcriptional profiling. We observed approximately a 75% enhancement in total bone area upon over-expression of ERRα compared with GFP-infected control (Fig. 1A and B), demonstrating successful infection with ERRα. Upon analysis of transcriptional profiling data, we observed modest induction of some bone regulatory genes by ERRα, including Bmp1 (1.7-fold, \( P<0.008 \) by Welch test) and Sox9 (twofold, \( P<0.008 \) (Pappano et al. 2003, Pan et al. 2008; complete data set available in Supplementary Table 1, see section on supplementary data given at the end of this article).

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PGC-1α, ERRz+PGC-1α, or WNT3A, an activator of canonical Wnt signaling. Table 1 lists the genes considered to have significantly altered transcription (P<0.05 by t-test) in C3H10T1/2 cells treated with either ERRz alone or ERRz+PGC-1α. Two internal ERRz-positive controls, GAPDH and VEGFA (Aranaly et al. 2008, Stein et al. 2008), were induced approximately three- and twofold, respectively, in ERRz+PGC-1α treatment, indicating successful over-expression of ERRz and PGC-1α; two genes known to be direct targets of WNT3A, Axin2 and Wnt11 (Dao et al. 2007, Zhou et al. 2007), were induced more than 20-fold in WNT3A treatment, serving as controls for expression of WNT3A (Supplementary Figure 2, see section on supplementary data given at the end of this article).

The WNT11 gene, a known target of ERRz and β-catenin regulation in cancer cells (Dwyer et al. 2010), was strikingly upregulated by ERRz+PGC-1α over-expression in C3H10T1/2 cells. ERRz alone did not upregulate WNT11 (Table 1). Id2, a known target of ERRz in mouse kidney (Tremblay et al. 2010), was also upregulated by both ERRz+PGC-1α and ERRz alone.

Table 1 Wnt/β-catenin pathway genes significantly regulated by ERRz or by ERRz+PGC-1α in C3H10T1/2 cells. Both conditions (ERRz alone and ERRz+PGC-1α) showed significant overlap with WNT3A over-expression, while PGC-1α alone did not (see text).

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*a Fold change was calculated from TaqMan Low-Density Array data from RNA collected 24 h after infection.

*b P values are obtained using Student’s t-test with unequal variance.

*c Genes also regulated by Wnt3a.
In order to examine whether the upregulation of these genes by ERRα + PGC-1α could involve Wnt-signaling pathway(s), we next compared these data with the set of genes significantly changing when cells are treated with WNT3A (P = 0.0006 by Fisher's exact test). Genes induced and repressed by ERRα expression alone also gave significant overlap with the profile observed in WNT3A over-expression (P = 0.0002 overall, P = 0.046 for genes up in both, and P = 0.0001 for genes down in both). No significant overlap was observed with PGC-1α alone. These results support our hypothesis that expression of ERRα and the co-activator PGC-1α causes similar gene changes within the Wnt pathway as activation by Wnt3a.

**ERRα co-activated by PGC-1α specifically activates canonical Wnt signaling**

Results from calvaria tissue and the C3H10T1/2 cell line suggested that ERRα could regulate the canonical Wnt/β-catenin pathway. In order to examine this
ERRα can both enhance and repress Wnt signaling in C3H10T1/2 cells, depending on state of activation

We next investigated the effect of modulating ERRα during activation of canonical Wnt signaling. We co-expressed ERRα, ERRα+PGC-1α, or PGC-1α in C3H10T1/2 cells with a known β-catenin-dependent signaling molecule, Wnt3a. In the 16×TCF-luc reporter assay, ERRα+PGC-1α potentiated luciferase signal activation induced by expression of Wnt3a (Fig. 2D). In control experiments, expression of ERRα with Wnt3a, but without PGC-1α, had no effect in this cell type (data not shown), further suggesting that ERRα activation is required for these transcriptional events. To test whether ERRα was required for β-catenin-dependent activation, we knocked down endogenous ERRα levels with shERRα infection (Fig. 2E). Knockdown of ERRα enhanced luciferase signaling by Wnt3a, suggesting that endogenous ERRα (without its co-activator PGC-1α) represses Wnt-driven gene transcription.

In summary, we have observed that ERRα can both repress and, when co-activated, promote transcription from the 16×TCF reporter, as depicted in the summary cartoon in Fig. 2F. In conjunction with the global modulations of Wnt gene transcription observed in transcriptional profiling and real-time analysis, these data suggest that ERRα is an important player in the transcriptional regulation of canonical Wnt/β-catenin pathway targets in osteoblastic and pre-osteoblastic cell lines.

Activation of Wnt signaling by ERRα + PGC-1α is cell intrinsic

Wnt signaling is modulated by a number of secreted proteins, including the WNTs themselves, the Frizzled proteins, and DKK1 (reviewed in Gordon & Nusse (2006)). In order to determine whether the activation of Wnt signaling by ERRα + PGC-1α was due to transcriptional modulation of a secreted factor, further studies with the 16×TCF reporter were performed. First, media collected from U2OS cells infected with GFP, Wnt3a, or ERRα+PGC-1α were added to cells expressing the 16×TCF reporter (Fig. 3A). Heparin was used in this experiment to release Wnt3a into the CM; no significant increase in luciferase signal was observed.

![Image 336x273 to 407x324](https://www.endocrinology-journals.org/)

Figure 3 Activation of Wnt signaling by ERRα and PGC-1α is cell intrinsic. (A) Average counts per second of luciferase from U2OS cells treated with 50% conditioned media from cells infected with GFP, ERRα+PGC-1α, or Wnt3a is graphed as the average and S.D. of four replicates. Wnt3a significantly induced expression by Welch t-test. (B) Average counts per second of luciferase from C3H10T1/2 cells infected with GFP, ERRα+PGC-1α, or Wnt3a and treated with 200 mg/ml recombinant mouse DKK1 is graphed as the average and S.D. of three replicates. Wnt3a signal is significantly reduced with DKK1 treatment compared with no DKK1 by Welch t-test. (C) U2OS cells infected with 16×TCF were cocultured with increasing numbers of cells expressing Wnt3a or ERRα+PGC-1α and luciferase activity was measured. Bars represent the average and S.D. of four replicates.
observed in the WNT3A control or ERRα+PGC-1α without heparin treatment (Supplementary Figure 4, see section on supplementary data given at the end of this article). While transfer of WNT3A-infected control conditioned media caused a significant induction of luciferase levels, we found that conditioned media transfer from cells infected with ERRα+PGC-1α was not sufficient to confer induction. Secondly, we tested whether the addition of recombinant DKK1, which blocks the interaction of Wnts with their cell surface receptors, blocked activation by ERRα+PGC-1α. Treatment with 200 ng/ml recombinant DKK1 fully inhibited activation by WNT3A but did not affect activation by ERRα+PGC-1α in C3H10T1/2 cells (Fig. 3B).

Finally, a coculture experiment was performed, in which a fixed number of U2OS cells infected with 16×TGF were combined with increasing numbers of cells infected with either Wnt3a or ERRα+PGC-1α. Again, the Wnt3a-positive control was able to activate Wnt signaling, whereas expressing ERRα+PGC-1α failed to induce TCF-luc transcription in coculture (Fig. 3C). These results from three orthogonal assays indicate that ERRα and PGC-1α can induce transcription of the Wnt reporter only when expressed in the same cell as the reporter construct and suggest that the observed induction of Wnt signaling by ERRα+PGC-1α is not due to expression changes of a secreted factor.

Activation of Wnt signaling by ERRα+PGC-1α is dependent on ERRα DNA binding

One possible mechanism of regulation of the WNT pathway is by affecting nuclear translocation of β-catenin. To determine whether active ERRα or knockdown of ERRα caused activation of the WNT pathway in this manner, high content analysis of the nuclear localization of β-catenin was performed 8, 16, and 24 h post-infection of C3H10T1/2 cells with adenovirus expressing ERRα, PGC-1α, ERRα+PGC-1α, shControl, or shERRα. Only WNT3a expression (positive control) caused nuclear translocation of β-catenin (Fig. 4A), with translocation slightly increased, but not significantly, at 8 h, peaking at 16 h and decreasing toward baseline by 24 h. None of the treatments for which activation of the WNT pathway by ERRα had been seen earlier resulted in an increase

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**Figure 4** Activation of Wnt signaling by ERRα and PGC-1α is dependent on ERRα DNA binding.

(A) The fold change in the ratio between nuclear and cytoplasmic β-catenin is graphed 8, 16, and 24 h post-infection of C3H10T1/2 cells with the indicated adenoviruses. Results represent the average and s.d. amongst eight replicates. *P<0.05 by t-test compared with GFP-infected cells. (B) Sites of mutation in ERRα and ERRγ DNA-binding domain (DBD) are noted in this cartoon (NTD, N-terminal domain; LBD, ligand-binding domain). Protein lysates from 293 cells transfected with GFP, ERRα, or each mutant were probed with anti-FLAG on this western blot. Anti-actin was used as a loading control. (C) Counts per second of luciferase were normalized to renilla after transfection of C3H10T1/2 cells with the indicated plasmids. Bars represent the average and s.d. of four replicates.
in β-catenin nuclear translocation. This strongly suggests that ERRz regulation of WNT target genes is independent of β-catenin translocation.

DNA-binding-independent activities through kinase interactions have been demonstrated for the estrogen receptor (ER) in osteoblast differentiation (Kousteni et al. 2007). To test whether ERRz DNA binding was required for activation of the TCF reporter, we generated point mutants in the DNA-binding domain of ERRz. The mutations, C99G in the first zinc finger and R150A in the A-box, were chosen based on corresponding mutations previously shown to ablate DNA binding in mouse ERRγ (Huppunen et al. 2004; Fig. 4B, top). The mutated proteins were expressed at the same size and similar levels to wild-type ERRz, as shown by western blot (Fig. 4B, bottom). Each mutation effectively blocked ERRz’s ability to transactivate two reporter constructs known to be ERRz responsive, the SHP promoter and 2×ERE (Supplementary Figure 5, see section on supplementary data given at the end of this article; Zhang & Teng 2000, Sanval et al. 2002).

Figure 4C shows the luciferase activity from C3H10T1/2 cells transfected with PGC-1α and either ERRz or each of these mutants. While the wild-type ERRz control gave strong induction, we found that neither DNA-binding domain mutant was capable of activating transcription from the 16×TCF reporter (Fig. 4C). Further, the ERRz mutants appear to act as dominant negative to endogenous ERRz in the cell. The mild activation with PGC-1α alone, which is shown in Fig. 2B to be dependent on the presence of endogenous ERRz in the cells, was ablated by both DNA-binding domain (DBD) mutants. Similar results were observed in U2OS and 293 cells (data not shown). Taken altogether, results using a Wnt-luciferase reporter suggest that active forms of ERRz are capable of inducing transcription of Wnt target genes through a cell-intrinsic mechanism requiring ERRz DNA binding, whereas endogenous ERRz can repress Wnt signaling (Fig. 2F).

ERRz modulation of osteoblast differentiation reflects changes in Wnt signaling

Next, we were interested in determining whether ERRz expression and activation affects Wnt pathway regulation of osteoblastogenesis. In order to test this, we examined effects of ERRz and PGC-1α activity in two different cell types that can undergo osteoblast differentiation, C3H10T1/2 and hMSCs. Treating C3H10T1/2 cells with WNT3A, thereby activating Wnt/β-catenin signaling, has been shown to induce

![Figure 5](https://example.com/figure5.png)

**Figure 5** Modulation of ERRz activity enhances osteogenic differentiation in C3H10T1/2 cells. (A) Alkaline phosphatase activity in C3H10T1/2 cells infected with VP16 or VP-ERRz after 13 days in growth media (GM) was normalized to total protein and graphed as the average and s.d. of six replicates. (B) Alkaline phosphatase activity in C3H10T1/2 cells after 10 days in culture in either GM or osteogenic differentiation media was graphed as in A. A Welch t-test was performed for ERRΔz vs shCtl in each condition to determine significance. (C) Osteocalcin transcription in C3H10T1/2 cells after 10 days in the indicated media was measured by TaqMan real-time PCR and reported as the average fold change of three biological replicates in shERRΔz vs shCtl. (D) Alkaline phosphatase activity in C3H10T1/2 cells is graphed as the average and s.d. of six samples. Cells were incubated for 13 days in GM with over-expression of the indicated protein from adenoviral infection.
alkaline phosphatase activity (Winkler et al. 2005), whereas WNT3A suppresses osteogenesis in hMSCs (Boland et al. 2004). We tested whether expression and activation of ERRα would elicit the same effects as canonical Wnt signaling in these cell types.

We first examined alkaline phosphatase activity in C3H10T1/2 cells in GM or osteogenic differentiation media (DM). Over-expression of the activated VP-ERRα induced alkaline phosphatase activity compared with the VP16 control after 10–13 days in GM (Fig. 5A), similar to the effect of WNT3A. Based on our results with the 16×TCF reporter in this cell type (Fig. 2E), if this effect was due to Wnt signaling, we would expect knockdown of ERRα to behave similarly to transcriptionally active ERRα. Indeed, knockdown of ERRα using shERRα virus induced differentiation in both GM and DM (Fig. 5B). Alkaline phosphatase levels of shERRα-infected cells in GM were elevated almost to those of the controls in DM after 10 days. Osteocalcin, another marker of osteoblast differentiation, was also induced three- to fourfold by shERRα in both conditions, as indicated by real-time PCR analysis of RNA samples at the same time point (Fig. 5C).

As the ERRα DNA-binding domain mutants were shown to act as dominant-negative proteins (Fig. 4C), we next tested whether the R150A mutant affected osteoblast differentiation. If endogenous ERRα represses Wnt pathway gene transcription via DNA binding, then we would expect the DNA-binding domain mutants to behave similarly to ERRα knockdown in the C3H10T1/2 alkaline phosphatase assay. Indeed, we observed a modest, but significant, enhancement of alkaline phosphatase with the DNA-binding mutant ERRα-R150A compared with GFP, and a more significant enhancement relative to WT ERRα (Fig. 5D shows results from GM, similar results were seen in DM, data not shown).

Overall, we observed the same pattern of osteogenic effects in C3H10T1/2 cells as was observed in the Wnt reporter assay: both knockdown and activation of ERRα caused enhanced osteogenesis in this system, via a DNA-binding-dependent mechanism. Importantly, both knockdown and activation of ERRα behave similarly to induction of Wnt/β-catenin signaling by Wnt3a in this cell type. As seen with the Wnt reporter assay, this set of data indicates that ERRα in its non-active state serves to repress canonical Wnt signaling, but when in the presence of its co-activator PGC-1α it is then able to potentiate canonical Wnt signaling.

We next tested the effects of ERRα over-expression and knockdown on mineralization of hMSCs. In this system, activation of the Wnt pathway has been shown to suppress osteogenesis (Liu et al. 2009), in contrast to the effects observed in C3H10T1/2 cells. Thus, if ERRα affects osteogenesis by modulating the Wnt pathway, we would expect opposite results to those seen in C3H10T1/2 cells. Indeed, in this system, over-expression of ERRα alone caused almost a threefold enhancement of mineralization, indicating Wnt pathway repression (Fig. 6A). Addition of the co-activator PGC-1α reduced the ability of ERRα to promote mineralization, indicating Wnt pathway repression.

**Figure 6** ERRα knockdown inhibits and over-expression promotes mineralization in hMSCs. (A) Human MSCs were infected with GFP, ERRα, and/or PGC-1α adenoviruses and incubated in MSCGM supplemented with 0.05 mM AA and 10 mM β-GP. After 24 days, cells were subjected to alizarin red-S staining for matrix mineralization. The graph provides quantification of the alizarin red-S staining from three independent samples. (B) Human MSCs were infected with adenoviruses containing shRNA specific for ERRα or scramble control shRNA and incubated in MSCGM supplemented with 0.05 mM AA, 10 mM β-GP, and 100 nM dexamethasone. After 18 days, cells were subjected to alizarin red-S staining for matrix mineralization. The graph provides quantification of the alizarin red-S staining from three independent samples.
activation. We also observed a 75% decrease in mineralization with knockdown of ERRα in hMSCs treated with dexamethasone to induce mineralization (Fig. 6B), similar to Wnt pathway activation in this cell type. The results in Figs 5 and 6 together suggest that ERRα’s effects on osteogenesis are likely due to changes in Wnt signaling. In summary, these results from osteogenic differentiation assays correlate well with our data from reporter assays (Fig. 2): the presence or over-expression of native (non-activated) ERRα represses, while over-expression of activated forms of ERRα, or loss of endogenous repressive ERRα, activates Wnt signaling (see model in Fig. 2F).

Discussion

In this study, we have examined mechanisms of action of the nuclear hormone receptor ERRα in osteogenesis. First, transcriptional profiling suggested the Wnt pathway as an important regulatory target of ERRα in calvarial explants. Subsequent studies then illustrated that ERRα is capable of both potentiating and repressing transcription of a TCF-luciferase reporter construct, depending on the presence or absence of PGC-1α co-activator. The activation observed in the presence of the co-activator PGC-1α is cell intrinsic and DNA binding dependent but does not result in (or require) nuclear translocation of β-catenin. Finally, modulation of ERRα activity affected osteogenesis in ways consistent with a role for Wnt signaling in these effects. These studies have significantly enhanced our mechanistic understanding of the role of ERRα in bone and as a transcription factor, both by linking ERRα to the Wnt pathway, and further by illustrating that ERRα can both repress and activate transcription of Wnt target genes, depending on its state of activation.

ERRα as a regulator of Wnt signaling

Cross talk between Wnt pathways and nuclear hormone receptors has been well documented (Mulholland et al. 2005). More recently, ERRα has been shown to physically interact with β-catenin and LEF1 in cancer cells and enhance β-catenin-dependent transcriptional activity in cancer cell lines (Dwyer et al. 2010). In the current study, only active forms of ERRα were able to potentiate transcriptional activity induced by WNT3A over-expression, while ERRα itself was shown to repress transcription. This difference could perhaps be the result of different levels of various co-activator and co-repressor proteins in the cell types examined in each study.

Another nuclear receptor, NURR1, has been shown to both repress and activate transcription of Wnt targets via interactions with the TCF/LEF complex and β-catenin (Kitagawa et al. 2007). In that case, binding of β-catenin to NURR1 disrupts co-repressor complexes, allowing co-activators to bind and activate. Our results demonstrating cell-intrinsic, DNA-binding-dependent activation and repression, in concert with the physical interaction between ERRα and β-catenin observed in Dwyer et al. (2010), suggest that a similar mechanism could be at play here. The forced activation of ERRα by over-expression of PGC-1α or fusion to the VP16 activation domain could function similarly to β-catenin binding, explaining why the activation of TCF/LEF reporter occurs in the absence of β-catenin nuclear translocation (Fig. 4A).

Overall, further studies will be required to determine the mechanism of Wnt activation observed in osteoblasts.

The analysis of transcriptional effects of ERRα and PGC-1α on Wnt pathway genes by TaqMan low-density array included in this work (Table 1) suggests potential novel targets of ERRα and Wnt co-regulation. Wnt11, the most strongly upregulated gene, has previously been shown by others to be regulated in this manner (Dwyer et al. 2010). A few genes that were found to be upregulated in both WNT3A and ERRα+PGC-1α over-expression are also predicted to have conserved (between human and mouse) ERRα response elements in their promoters: VEGFA, MESDC2, and Wnt5a. Of particular interest is VEGFA, which encodes a protein with a role in angiogenesis in bone. VEGFA has independently been shown to be a direct target of ERRα and Wnt/TCF regulation (Zhang et al. 2001, Stein et al. 2008), and it remains to be seen whether there is an interplay between the two pathways in regulating expression of this gene. MESDC2 encodes a chaperone protein for LRP6, a receptor in the canonical Wnt-signaling pathway. Studies have shown that its co-expression is required for LRP6 function (Li et al. 2006). Wnt5a has been shown to both inhibit and activate canonical β-catenin/TCF signaling depending on the receptor context (Mikels & Nusse 2006), as well as to activate the non-canonical Wnt pathway, a role that has been shown to be important for promoting osteoblastogenesis from mesenchymal progenitors (Takada et al. 2007).

Role of ERRα in bone differentiation

The precise role of ERRα in bone differentiation and mineralization remains unclear. Some previous studies on mammalian osteoblast and chondrocyte cell lines, and primary RC cell cultures, have pointed to ERRα as a positive factor in osteoblast and chondrocyte differentiation (Bonnelye et al. 2001, 2007). However, two separate mouse studies have shown increased mineral density with deletion of ERRα, suggesting that the in vivo function of ERRα in bone may be a suppressor of osteoblast differentiation (Delhon et al.
This model correlates with our data in C3H10T1/2 cells (Fig. 5). We here demonstrate that ERRz can behave very differently in bone systems depending on the presence or absence of PGC-1α or other activating factors, which may in part account for the apparent discrepancies in previous studies. Further work is required to specifically investigate the effects of this interaction in rodent model systems.

The key contribution of this work to understanding ERRz’s role in bone biology is that at least part of its effects may be due to regulation of Wnt signaling. ERRz not only affects a Wnt reporter construct but also affects differentiation of two different cell types in ways that track with the effects of Wnt activation. Given the broad importance of both these factors in development and adult physiology, it will be important to examine whether this cross talk also occurs in other biological processes where ERRz has known roles, for example, during adipogenesis. In addition, the contribution of various co-activators and co-repressors to the mechanism is an interesting topic for further examination.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-11-0140.

Declaration of interest
K L A, Y L, S F, C C, B M B, R A B, and E L B were previous Wyeth (now Pfizer, Inc.) employees and S P B, M C, Y Z, C H, W Z, J Z, and R V M are current Pfizer employees.

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