The Orphan Nuclear Receptor Estrogen Receptor-related Receptor γ Negatively Regulates BMP2-induced Osteoblast Differentiation and Bone Formation*

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Estrogen receptor-related receptor γ (ERRγ/ERR3/NR3B3) is a member of the orphan nuclear receptor with important functions in development and homeostasis. Recently it has been reported that ERRs are involved in osteoblast differentiation and bone formation. In the present study we examined the role of ERRγ in osteoblast differentiation. Here, we showed that ERRγ is expressed in osteoblast progenitors and primary osteoblasts, and its expression is increased temporarily by BMP2. Overexpression of ERRγ reduced BMP2-induced alkaline phosphatase activity and osteocalcin production as well as calcified nodule formation, whereas inhibition of ERRγ expression significantly enhanced BMP2-induced osteogenic differentiation and mineralization, suggesting that endogenous ERRγ plays an important role in osteoblast differentiation. In addition, ERRγ significantly repressed Runx2 transactivity on osteocalcin and bone sialoprotein promoters. We also observed that ERRγ physically interacts with Runx2 in vitro and in vivo and competes with p300 to repress Runx2 transactivity. Notably, intramuscular injection of ERRγ strongly inhibited BMP2-induced ectopic bone formation in a dose-dependent manner. Taken together, these results suggest that ERRγ is a novel negative regulator of osteoblast differentiation and bone formation via its regulation of Runx2 transactivity.

Bone formation is a series of well orchestrated lineage-specific differentiation events (1). Osteoblasts, which play key roles in bone formation, are derived from pluripotent mesenchymal stem cells that have the capacity to differentiate into myocytes, adipocytes, and chondrocytes (2). Osteoblasts possess the necessary components to form bone matrix, which allows subsequent mineralization. Several hormones, growth factors, cytokines, and nuclear receptor proteins regulate these sequential events to trigger a complex network of signaling pathways.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β family and were originally identified by their capacity to induce ectopic bone formation (3, 4). Among the BMP family members, the action of BMP2 has been studied extensively in embryonic skeletal development, postnatal bone remodeling, and bone repair (5, 6). BMP2 promotes the commitment of pluripotent mesenchymal cells to the osteoblast lineage by regulating the signals that stimulate the specific transcriptional programs required for bone formation (5, 7).

A master regulator of osteoblasts, Runx2, is indispensable for a skeletal development and maturation. Targeted disruption of Runx2 results in a complete lack of functional osteoblasts (8, 9). Runx2 directly regulates osteoblast-specific genes such as osteocalcin (OC), bone sialoprotein (BSP), osteopontin, and type I collagen through binding to specific DNA enhancer elements of its target gene promoters (4). In addition, Runx2 interacts with a variety of transcription factors (10) and recruits both co-activators (11–13) and co-repressors (14, 15) to form a complex on its target promoter. Therefore, it is important to identify the possible partners of Runx2 to understand the mechanism of Runx2-dependent osteoblast differentiation.

Estrogen receptor-related receptors (ERRs) are closely related to estrogen receptor (ER) without binding to the classical ER ligand but share high homology in their DNA binding domain (16). To date, three subtypes of ERR have been identified as ERRα, β, and γ, based on their sequence similarity to ERα, and have been shown to regulate a broad spectrum of genes in their target cells. Recently, it has been reported that ERRα is involved functionally in bone differentiation. It is strongly expressed throughout the osteoblast differentiation process and plays a physiological role in differentiation and bone formation (17). It also regulates osteopontin expression through a non-canonical ERRα response element (18). ERRγ is the most recently described member of the ERR subfamily. It

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5 The abbreviations used are: BMP, bone morphogenetic protein; OC, osteocalcin; BSP, bone sialoprotein; ERR, estrogen receptor-related receptors; Ad, adenovirus; AR-S, alizarin red stain; GST, glutathione S-transferase; PN, particle number; µCT, microCT; sh-, short hairpin; RT, reverse transcription; ALP, alkaline phosphatase; HA, hemagglutinin.
ERRγ Regulates BMP2-induced Bone Formation

**A**

Primary osteoblasts | MC3T3-E1 | C2C12
---|---|---
- | + | -
- | + | -

BMP2 | ERRγ | β-actin

**B**

C2C12 + BMP2

(days)

ALP | OC | ERRγ | β-actin

0 | 2 | 3 | 4 | 5

**C**

Primary osteoblasts + BMP2

<table>
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<th>(days)</th>
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| 0 | 1 | 2 | 3 | 4 | 5

ERRγ | β-actin

**D**

C2C12 + BMP2

(days)

ERRγ | β-actin

0 | 2 | 3 | 4 | 5

**E**

ERRγ-Luc / C2C12

Fold Activity

BMP2 | 0 | 5 | 50 | 500 (ng/ml)

**F**

ERRγ-Luc / MC3T3-E1

Fold Activity

BMP2 | 0 | 5 | 50 | 500 (ng/ml)

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**FIGURE 1. Expression profiles of the ERRγ in osteoblast progenitor cells.**

A, expression of ERRγ mRNA in osteoblast progenitor cells. Primary osteoblasts, MC3T3-E1, and C2C12 cells were cultured in the absence or presence of BMP2 (200 ng/ml) for 24 h. RT-PCR was carried out with the indicated primers. B, expression of ERRγ during osteoblast differentiation by BMP2. C2C12 cells were cultured in osteogenic medium containing ascorbic acid (50 μg/ml) and β-glycerophosphate (5 mM) in the presence of BMP2 (200 ng/ml) for 5 days. At the designated time points, cells were harvested for total RNA isolation, and RT-PCR was performed with the indicated primers. C and D, Western blot analysis of ERRγ expression. Primary osteoblasts and C2C12 cells were cultured in osteogenic medium with BMP2 for 5 days. At the indicated time points, the protein extracts were used for Western blot analysis with the indicated antibody. E and F, transactivation of ERRγ promoter by BMP2. C2C12 and MC3T3-E1 cells were transfected with 200 ng of ERRγ-Luc reporter plasmid and 100 ng of pCMV-β-galactosidase as an internal control with the indicated amounts of BMP2, respectively. Data are expressed as the mean ± S.D. of three independent experiments.

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differs from the other family members in that it is a constitutively active nuclear receptor with high basal transcriptional activity (19).

In this study we examined the role of ERRγ in osteoblast differentiation in vitro and ectopic bone formation in vivo, and our results show that orphan nuclear receptor ERRγ plays an inhibitory role in BMP2-induced osteoblast differentiation and bone formation.

**EXPERIMENTAL PROCEDURES**

**Recombinant Proteins**—Recombinant human BMP2 (rhBMP2) was obtained from R&D Systems (Minneapolis, MN). The stock solution (1 mg/ml) was prepared in phosphate-buffered saline containing 0.1% bovine serum.

**Plasmids and Adenoviruses**—The ERRγ promoter was PCR-amplified from mouse genomic DNA (Novagen) and inserted into the pGL3 basic vector (Promega) using the MluI and XhoI restriction enzyme site (ERRγ-Luc). pcDNA3/HA-ERRγ and GST-ERRγ are previously described (20). 6×OSE-Luc reporter construct, pcDNA3/Runx2, GST-Runx2, and pcDNA3/p300 from 10-day-old neonatal mice and digested with 0.1% collagenase (Roche Applied Science) at 37 °C for 30 min. The collagenase digest was then discarded and replaced with fresh collagenase solution. After 30 min the cells were collected by a sedimentation step and centrifuged twice at 400 × g for 10 min. Finally, cells in the pellet fraction were used for primary culture.

**RNA Preparation and Semi-quantitative RT-PCR**—Total RNA was extracted from the cells using the TRIzol reagent (Invitrogen) and RNase-free DNase (Qiagen) according to the manufacturer's instructions. The complementary DNA was synthesized from the total RNA using a random primer and reverse transcriptase (Invitrogen). Each reaction consisted of initial denaturation at 94 °C for 1 min followed by three-step cycling: denaturation at 94 °C for 30 s, annealing at a temperature optimized for each primer pair for 30 s, and extension at 72 °C for 1 min. After the requisite number of cycles (27–30 cycles), the reactions underwent a final extension at 72 °C for 5 min. The following primer sequences, designed based on published cDNA sequences, were used for PCR: ERRα, forward 5'-CAGGAAAGTGAAATGCCCAGG-3' and reverse 5'-CTTGTG-