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Nuclear Receptor Retinoid-Related Orphan Receptor α1 Modulates the Metabolic Activity of Human Osteoblasts

Mohamed Benderdour,[1*] Hassan Fahmi,[2] François Beaudet,[3] Julio C. Fernandes,[1] and Qin Shi[1]

[1] *Orthopaedics Research Laboratory, Research Centre, Hôpital du Sacré-Coeur de Montréal, University of Montreal, 5400 Gouin Blvd. West, Montreal, Quebec, Canada H4J 1C5*

[2] *Osteoarthritis Research Unit, Research Centre of the University of Montreal Hospital Center, Notre-Dame Hospital, Montreal, Quebec, Canada*

[3] *Department of Rheumatology, Hôpital du Sacré-Cœur de Montréal, University of Montreal, 5400 Gouin Blvd. West, Montreal, Quebec, Canada H4J 1C5*

ABSTRACT

Nuclear receptor retinoid-related orphan receptor alpha (RORα1) is a member of ROR-family receptors. It is broadly expressed in various tissues and organs during embryonic development. However, so far, little is known about its function in bone. Here, we have elucidated the expression and function of RORα1 in human MG-63 osteoblast-like cells. Reverse transcriptase-polymerase chain reaction and immunocytochemical analysis revealed that human MG-63 osteoblasts expressed and produced RORα1. Other cell lines, such as THP-1 monocytes expressed also RORα1. RORα1 over-expression increased alkaline phosphatase, osteocalcin, cell mineralization, and collagen type I mRNA and protein expression, while RORα1 RNA silencing inhibited these responses. In addition, RORα1 over-expression suppressed the tumor necrosis factor-alpha (TNFα)-induced production of cyclooxygenase-2, prostaglandin E_2, and metalloproteinase-9. Examination of the signaling pathways disclosed that RORα1 was able to block TNFα-evoked nuclear factor-kappaB activation. In conclusion, this study demonstrates that RORα1 is involved in human osteoblast metabolism by stimulating osteoblast marker expression and inhibiting inflammatory responses. The results may encourage further exploration of RORα1 as a potential target for the treatment of bone disorders related to inflammation. J. Cell. Biochem. 112: 2160–2169, 2011.

KEY WORDS: RORα1; OSTEOBLAST METABOLISM MARKERS; INFLAMMATORY RESPONSES

Retinoid-related orphan receptors (RORs) constitute a subfamily of nuclear receptors that include three members (isotypes): RORα, RORβ, and RORγ. RORα, β, and γ expression varies from tissue to tissue, indicating that ROR subtypes play different biological roles. RORα is expressed in various organs, including the brain, skeletal muscle, kidneys, testis, and hair follicles [Giguere, 1999; Lau et al., 1999; Jetten and Ueda, 2002]. RORβ is brain-specific and highly expressed in the pineal gland, suggesting its involvement in the control of circadian rhythm. RORγ expressed most highly in the thymus, has been shown to be important in thymopoiesis [Giguere, 1999; Jetten and Ueda, 2002]. RORα binds as monomer to the ROR response element (RORE) sequence in the promoter of target genes composed of a 6-bp A/T rich region immediately preceding a half core AGGTCA motif [Giguere et al., 1994; McBroom et al., 1995]. RORE sites have been identified in many gene promoters [Schrader et al., 1996]. RORα-deficient mice exhibit abnormalities in bone formation and bone tissue maintenance, denoting that it functions as a positive regulator of osteogenesis with participation in bone metabolism [Meyer et al., 2000]. The RORα gene generates four splicing isoforms: RORα1, α2, α3, and α4 [Schrader et al., 1996; Jetten and Ueda, 2002]. These receptors have different tissue distributions: while RORα1 and RORα4 are expressed ubiquitously, RORα2 and RORα3 are expressed in a more restricted manner. RORα2 was recently reported in cells of the immune system [Pozo et al., 2004]. RORα3 has so far been detected only in the human testis [Jetten and Ueda, 2002]. However, little is known about the role of RORα1 in human osteoblasts.

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*Correspondence to: Mohamed Benderdour, Orthopaedics Research Laboratory, Research Centre, Hôpital du Sacré-Cœur de Montréal, University of Montreal, 5400 Gouin Blvd. W., Montreal, Quebec, Canada H4J 1C5.
E-mail: mohamed.benderdour@umontreal.ca

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ROR null mice display skeletal and immune abnormalities associated with abnormal mineralization and increased mRNA levels of interleukin-1beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNFα) [Kopmels et al., 1992; Lyashenko et al., 2010]. Under the influence of an adenovirus encoding for RORα1, RORα1 negatively regulates the inflammatory response by interfering with the nuclear factor-kappaB (NF-κB) signaling pathway in primary smooth muscle cells [Delerive et al., 2001]. This action of RORα1 on NF-κB is likely due to the induction of inhibitor kappa B-alpha (IκBα), a major inhibitory protein of the NF-κB signaling pathway, whose expression is transcriptionally up-regulated by RORα1 via a RORE in the IκBα promoter. A role for RORα in bone metabolism has suggested by experiments showing that $RORα^{sg/sg}$ mice are osteopenic [Meyer et al., 2000]. Total mineral content and bone density are significantly reduced in bones of RORα-deficient mice compared to those of wild-type mice. These findings indicate an imbalance between bone formation and bone resorption, implying a positive regulatory function for RORα in bone development. This is supported by studies demonstrating that RORα mRNA is significantly enhanced during the differentiation of mesenchymal stem cells into osteoblasts, and RORα is able to activate the promoter of bone sialoprotein gene [Meyer et al., 2000]. Although these observations are in agreement with the hypothesis that RORα regulates osteoblast function and activity, further studies are needed to determine its precise role in bone metabolism.

Subchondral bone is specialized connective tissue formed by a mineralized matrix containing specific collagen type I (COL 1), proteoglycans, and several growth factors and cytokines, as well as bone-specific cell types (osteoblasts, osteocytes, and osteoclasts) [Karsenty, 1999; Iqbal and Zaidi, 2005]. Osteoblasts and osteoclasts, either alone or in combination, contribute to the bone remodeling process, and disturbance in the activities of these cells is responsible for altered bone metabolism. Under the action of pro-inflammatory cytokines, degradation of bone extracellular matrix (e.g., native collagen) is initiated by the combined influence of several proteases, such as matrix metalloproteinases (MMPs). MMP-9, in both human osteoblasts and osteoclasts, is likely crucial in bone pathophysiological processes [Cunnane et al., 2001; Kaneko et al., 2001; Lu and Rabie, 2006]. Other pro-inflammatory factors, such as prostaglandins (PGs) and leukotrienes, cause an imbalance in bone metabolism favoring bone resorption. PGE_2 is produced by bone cells largely through the action of inducible cyclooxygenase-2 (COX-2), stimulating either bone resorption or formation [Raisz et al., 1993]. Many inflammatory factor genes and MMPs involved in bone damage are regulated by inducible transcription factors, such as NF-κB, activator protein-1, and cyclic AMP response element-binding protein. The NF-κB signaling pathway appears to be critical in inflammation and bone destruction, particularly for osteoclast differentiation [Ahn and Aggarwal, 2005; Jimi and Ghosh, 2005].

The aim of this study was to investigate RORα1 functional in human osteoblast metabolism. The isoform expression pattern of RORα in human MG-63 osteoblast-like cells was examined. We also evaluated the effects of RORα1 over- and down-expression on osteoblast metabolism markers, such as alkaline phosphatase (ALP), osteocalcin (OC), and COL I, in MG-63 cells. The influence of RORα1 on TNFα-induced MMP-9 and PGE_2 (COX-2) expression as well as the NF-κB signaling pathway was also examined in these cells.

MATERIALS AND METHODS

CELL CULTURE

Human MG-63 osteoblast-like cells, obtained from the American Type Culture Collection (ATCC, Rockville, MD), were grown in HAMF12/Dulbecco's modified Eagle's medium (HAMF12/DMEM) (Sigma–Aldrich Canada Ltd, Oakville, ON, Canada) containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 1% penicillin–streptomycin (Invitrogen Life Technologies, Burlington, ON, Canada) mixture and 50 μg/ml ascorbic acid (Sigma–Aldrich Canada Ltd). The cells were seeded at high density in culture flasks at 37°C in a humidified atmosphere of 5% CO_2/95% air until they were confluent and ready for the experiments. They were utilized within 13 passages. Human acute monocytic leukemia THP-1 cells were obtained from ATCC (Manassas, VA) and cultured according to the supplier's recommendations. They were maintained in RPMI 1640 cell culture medium containing 10% FBS, L-glutamine, and 5×10^{-5} M β-mercaptoethanol. Cells were suspended in 12-well tissue culture plates and exposed to either medium alone.

TRANSFECTION EXPERIMENTS

RORα1 small interfering RNA (siRNA, sc-38864) and a random sequence siRNA serving as negative control (sc-37007) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). pCMX-RORα1 expression plasmid and pCMX empty vector were kindly provided by Dr. V. Giguere (McGill University, Montreal, QC, Canada). MG-63 cells were seeded in 12-well plates at a density of 1×10^5 cells per well. The next day, the cells were transfected with RORα siRNA or randomly sequenced siRNA at a concentration of 100 nM with siRNA Oligofectamine™ transfection reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. RORα1 plasmid transfection was performed with Lipofectamine^{2000} transfection reagent (Invitrogen Life Technologies). A typical reaction mixture consisted of 0.2 μg pCMX-RORα1 expression vector and 2 μl Lipofectamine^{2000} transfection reagent as per the manufacturer's protocol. pCMX empty vector was the negative control.

IMMUNOCYTOCHEMICAL STAINING

MG-63 cells (5×10^3/well) were transferred to eight-well culture chamber slides (Lab-Tek, Nalge Nunc International, Naperville, IL) and incubated for 24 h, as described previously [Shi et al., 2007]. Then, the cells were washed twice with phosphate-buffered saline (PBS) and fixed for 15 min at room temperature with PBS containing 10% formaldehyde (Tissufix, Laboratoire Gilles Chaput, Montreal, QC, Canada). Cellular RORα expression was analyzed by immunocytochemical staining with specific antibody against RORα (1:100, Novus Biologicals, Inc., Littleton, CO). Proteins were visualized with secondary fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibodies (1:1,000, Santa Cruz Biotechnology, Inc.) by Zeiss LSM 510 Meta Confocor24 confocal microscope (Carl Zeiss Microimaging, Inc., Jena, Germany). The nucleus was stained by 4'-6-diamidino-2-phenylindole (DAPI) (blue).

CELLULAR ALP ACTIVITY, OC, PGE_2, AND MMP-9 LEVELS

Forty-eight hours after transfection (RORα1 siRNA, scrambled siRNA control, empty vector, or RORα1 expression plasmid), the cell supernatants were collected and frozen at −80°C before assay. The cells were washed twice with PBS (pH 7.4) and lysed in ALP buffer (100 mM glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 1% Triton X-100, pH 10.5) for 60 min with agitation at 4°C. ALP activity in cellular lysates was quantified as the release of *p*-nitrophenol hydrolyzed from *p*-nitrophenyl phosphate (final concentration 12.5 mM) at 37°C for 30 min [Shi et al., 2006]. Proteins were quantified by the bicinchoninic acid method [Smith et al., 1985]. Nascent OC levels in culture media were analyzed by specific enzyme immunoassay (Biomedical Technologies, Inc., Stoughton, MA). The detection limit of this assay was 0.4 ng/ml.

In another set of experiments and 24 h after transfection of RORα1 siRNA or expression plasmid, MG-63 cell culture medium was replaced by fresh medium containing 0.5% FBS with or without 10 ng/ml TNFα. PGE_2 and MMP-9 were assessed, respectively, with enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI) and MMP-9 ELISA kit from R&D Systems (Minneapolis, MN). This assay is based on competition between PGE_2 and a PGE_2-acetylcholinesterase conjugate (PGE_2 tracer) for a limited amount of PGE_2 monoclonal antibody. Its sensitivity was 9 pg/ml.

PROTEIN DETECTION BY WESTERN BLOTTING

Twenty micrograms of total proteins from osteoblast lysates treated under the indicated conditions were subjected to discontinuous 4–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Protein transfer, immunodetection, and semiquantitative measurements were performed as described previously [Shi et al., 2006]. Polyclonal rabbit anti-human COX-2 and COL I (1:1,000, Oncogene Research Products, San Diego, CA), anti-human RORα (1:1,000, Sigma–Aldrich Canada Ltd), and anti-human β-actin (Santa Cruz Biotechnology, Inc.) were used. Nuclear and cytosolic fractions of osteoblasts were prepared for NF-κB/p65 and phosphorylated IκBα determination [Shi et al., 2006]. Ten μg nuclear and cytosolic proteins were subjected to Western blotting with specific rabbit antibodies, anti-NF-κB/p65, and anti-phospho IκBα (Santa Cruz Biotechnology, Inc.).

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS

Total RNA was extracted from osteoblasts with TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's specifications. RNA was quantified with the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR).

One microgram of total RNA was taken for semi-quantitative RT-PCR of RORα isoforms in a Thermocycler (Waterman, Biometra GmbH, Göttingen, Germany), as indicated in our previous report [Shi et al., 2007]. RT-PCR assays were conducted with SuperScript 1-step RT-PCR kit from Invitrogen Life Technologies. The sequences of the forward primer were: RORα1, 5′-AAA CAT GGA GTC AGC TCC G-3′; RORα2, 5′-CTC CAA ATA CTC CAT CAG TGT ATC C-3′; RORα3, 5′-CAA CTT GAG CAC ATA AAC TGG-3′; RORα4, 5′-GCA CCG CGC TTA AAT GAT GT-3′. Four primers were combined with a common reverse primer with the following sequence: 5′-CAT ACA AGC TGT CTC TCT GC-3′. The sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control were: 5′-CCA CCC ATG GCA AAT CCA TGG CA-3′ (forward) and 5′-TCT AGA CGG CAG GTC AAG GTC CAA CC-3′ (reverse) (BioCorp, Inc., Montreal, QC, Canada). Negative controls for RT-PCR without primers were included in each experiment. RT-PCR products were separated on 2.2% agarose gel and scanned in a digital imaging system (G-image 2000, Canberra Packard Canada, Mississauga, ON, Canada).

For real-time RT-PCR analysis, 1 μg total RNA was reverse transcripted with reverse transcription kit (Qiagen Inc., Mississauga, ON, Canada), as per the manufacturer's guidelines. One-fifth of the reverse transcriptase reaction was analyzed by real-time quantitative PCR [Shi et al., 2006]. The following primers were deployed: ALP, forward 5′-ATG GTG GAC TAT GCT CAC AAC-3′, reverse 5′-GAC GTA GTT CTG CTC GTG GA-3′; OC, forward 5′-GCA GCG AGG TAG TGA AGA-3′, reverse 5′-TCC TGA AAG CCG ATG TGG-3′; COL I, forward 5′-GAG AGC ATG ACC GAT GG-3′, reverse 5′-GTG ACG CTG TAG GTG AA-3′; COX-2, forward 5'-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3', reverse 5'-AGT TCA TCT CTG CCT GAG TAT CTT-3'; MMP-9, forward 5'-GCC AAT CCT ACT CCG C-3', reverse 5'-GTC GCT GTC AAA GTT CG-3'; and GAPDH, forward 5′-CAG AAC ATC ATC CCT GCC TCT-3′, reverse 5′-GCT TGA CAA AGT GGT CGT TGA G-3′. Preliminary experiments showed that the amplification efficiency of RORα1, ALP, OC, COL I, COX-2, MMP-9, and GAPDH was similar. Relative ALP, OC, COL I, COX-2, or MMP-9 mRNA expression in cultured osteoblasts was analyzed as fold changes relative to control conditions (unstimulated cells) according to the $\Delta\Delta C_T$ method, as detailed in the manufacturer's guidelines (Stratagene, La Jolla, CA). A ΔC_T value was first calculated by subtracting the C_T value for the GAPDH housekeeping gene from each sample. A $\Delta\Delta C_T$ value was then obtained by subtracting the ΔC_T value of the control from the ΔC_T value of each treatment. Fold changes compared with the control (unstimulated cells) were then quantified by raising 2 to the $\Delta\Delta C_T$ power. Each PCR was performed in triplicate on two separate occasions in at least three independent experiments.

NF-κB PROMOTER

NF-κB luciferase plasmids containing five NF-κB consensus sequences were obtained from Stratagene. NF-κB luciferase plasmids were transiently transfected with or without RORα1 expression plasmid into MG-63 osteoblasts (approximately 50% confluence) in 12-well cluster plates according to Lipofectamine2000^{TM} reagent methods (Invitrogen Life Technologies) following the manufacturer's protocol. Briefly, transfections were conducted for 6 h with DNA–Lipofectamine complexes containing 2 μl of Lipofectamine reagent and 2 μg DNA plasmid. After washing, the medium was replaced by fresh medium containing 1% FBS, and the experiments were performed in this medium supplemented with the factors under study. Luciferase activity was quantified in cellular extracts with luciferase assay kit (Promega Corporation) in a microplate luminometer (Applied Biosystems, Foster City, CA) and normalized to protein level.

EVALUATION OF MINERALIZATION

MG-63 osteoblasts were seeded in eight-well culture chamber slides (Lab-Tek, Nalge Nunc International) at a density of 2×10^4 per chamber and incubated in BGJb medium containing 10% FBS, 50 μg/ml ascorbic acid, and 50 μg/ml β-glycerophosphate. The next day, the cells were transfected with scrambled siRNA or RORα1 siRNA, as described above. Then after, the medium was changed medium was changed every 2 days until day 28. On day 28, mineralization of cell cultures was evaluated by alizarin red staining [Gregory et al., 2004]. Quantification of alizarin red staining was also performed, following the extraction procedure described by Gregory et al. [2004].

STATISTICAL ANALYSIS

The data are expressed as means ± standard error of the mean (SEM). Assays were performed in triplicate in at least three independent experiments. Statistical significance was assessed by unpaired Student's *t*-test, and $P < 0.05$ was considered significant.

RESULTS

RORα1 EXPRESSION IN HUMAN MG-63 OSTEOBLAST-LIKE CELLS

With specific primers for RORα isoforms in 1-step semi-quantitative RT-PCR assay, we determined that RORα1, RORα2, and RORα4, but not RORα3, were expressed in MG-63 osteoblast-like cells (n = 3) (Fig. 1A). The amplicons size for RORα1, RORα2, RORα4, and GAPDH were 340, 405, 310, and 500 bp, respectively. RT-PCR performed without primers in negative control experiments yielded no PCR products (Fig. 1A). Immunocytochemistry demonstrated that RORα protein was localized in the nucleus of MG-63 cells (Fig. 1B, green). RT-PCR data showed that RORα1 was also expressed in THP-1 monocytes (Fig. 1C). We then evaluated the transfection efficiency of RORα1 siRNA and expression vector in MG-63 cell culture. Figure 2 shows that siRNA decreased RORα1 mRNA and protein expression by 62 ($P < 0.05$) and 90%, respectively, compared to the scrambled siRNA control (Fig. 2A,B). Western blot analysis with polyclonal anti-RORα antibody revealed that transient transfection with RORα1 expression vector increased RORα expression in MG-63 osteoblasts by 380% (Fig. 2B). The empty vector-treated control presented no effect.

OSTEOBLAST METABOLISM MARKERS

In another set of experiments, we assessed the role of RORα1 in the modulation of osteoblast metabolism markers. Compared to the scrambled siRNA control, RORα1 siRNA treatment significantly decreased ALP mRNA and activity levels by 69% (Fig. 3A, $P < 0.05$) and 40% (Fig. 3B, $P < 0.001$), OC mRNA and protein levels by 66% (Fig. 3C, $P < 0.05$), and 22% (Fig. 3D, $P < 0.001$), and COL I mRNA and protein levels by 60% (Fig. 3E, $P < 0.05$) and 90% (Fig. 3F), respectively. In contrast, RORα1 over-expression increased ALP ($P < 0.05$), OC ($P < 0.05$), and COL I ($P < 0.05$) mRNA expression by about 900%, 135%, and 140% compared to empty-vector controls, respectively (Fig. 3A,C,E). RORα1 over-expression augmented ALP activity ($P < 0.05$), OC ($P < 0.05$), and COL I protein levels by about 15%, 25%, and 1.5-fold, respectively, compared to the empty-vector

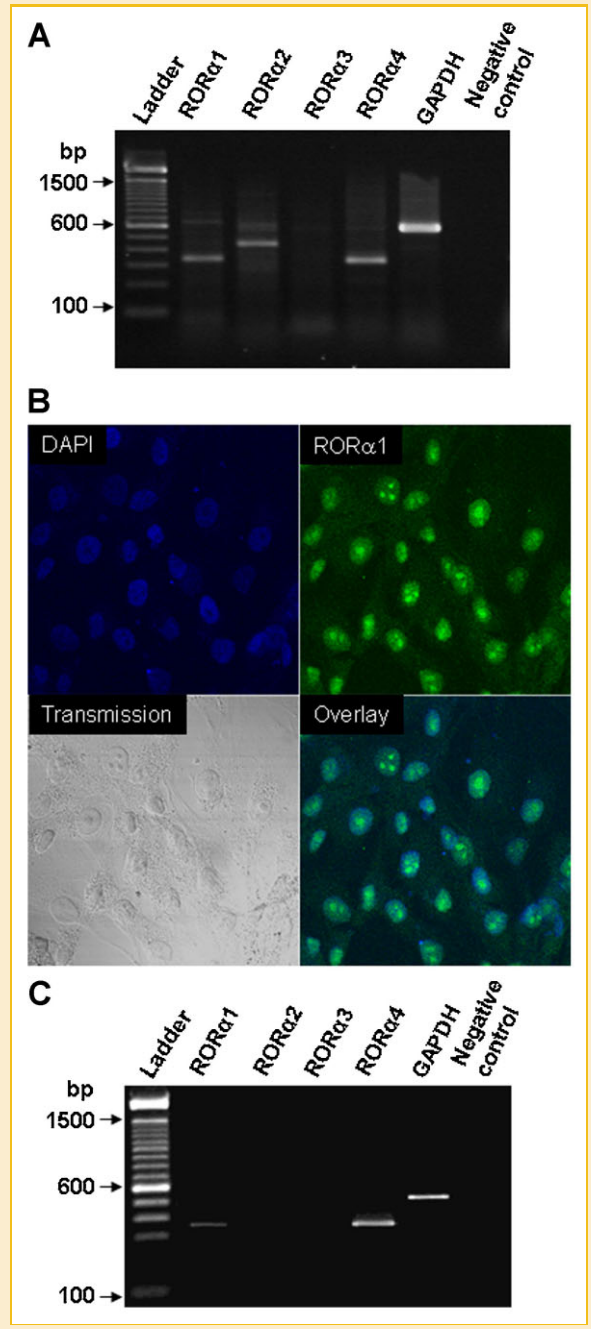

Fig. 1. RORα expression in human MG-63 osteoblast-like cells. A,C: Total RNA was extracted from MG-63 osteoblasts and THP-1 monocytes. One-step semi-quantitative RT-PCR analysis of RORα isoforms and GAPDH was performed, as described in the Materials and Methods Section. RT-PCR without primers served as negative control. B: Immunocytochemistry was undertaken as explained in the Materials and Methods Section with rabbit polyclonal anti-RORα antibody (1:100, Novus Biologicals, Inc.). Top left: The nucleus was visualized by DAPI (blue). Top right: Proteins (green) were visualized with secondary FITC-conjugated anti-rabbit antibodies. Bottom left: Control image without any staining. Bottom right: Co-localization of DAPI (blue) and RORα protein staining (green). RORα protein (top and bottom right) was localized in the nucleus.

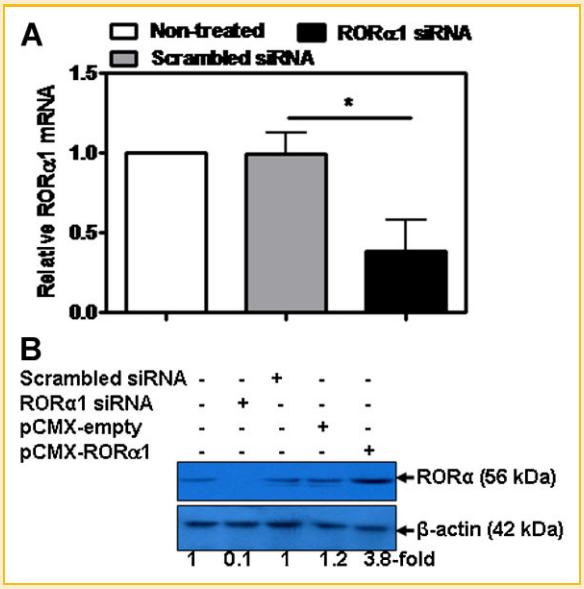

Fig. 2. Efficiency of RORα1 down- and over-expression. MG-63 cells were transfected with scrambled siRNA control, RORα1 siRNA, empty-vector or RORα1 expression plasmid, as described in the Materials and Methods Second. A: Total RNA was isolated and RORα1 mRNA expression analyzed by quantitative real-time RT-PCR. RORα1 mRNA levels were normalized to GAPDH mRNA expression. The data are means ± SEM of n = 3 and expressed as relative to scrambled siRNA control. Student's unpaired *t*-test: $^{*}P < 0.05$. B: Total cell lysates (approximately 20 μg) were prepared and subjected to Western blotting with rabbit polyclonal anti-RORα antibody (1:100, Sigma–Aldrich Canada Ltd).

control (Fig. 3B,D,F). Taken together; these results indicate that RORα1 was involved in the regulation of osteoblast metabolism markers in MG-63 cells.

IN VITRO MINERALIZATION POTENTIAL

To determine the involvement of RORα in osteoblasts mineralization in bone tissue, we incubated confluent MG-63 osteoblasts for 28 days in a culture medium that promotes mineralization, with or without siRNA against RORα1 or expression vector of RORα1, and determined their mineralization potential by alizarin red staining. As indicated in Figure 4A,B, transfected cells with RORα1 siRNA showed reduced alizarin staining compared to scrambled siRNA. In contrast, the overexpression of RORα1 in osteoblasts stimulated cell mineralization when compared to controls.

PGE_2 RELEASE AND COX-2 EXPRESSION

To establish whether RORα1 modulates inflammatory responses, we evaluated the effects of RORα1 down- and over-expression on COX-2 mRNA and protein expression and related PGE_2 release in MG-63 osteoblasts. Basal PGE_2 production was increased significantly after RORα1 siRNA treatment by 312% ($P < 0.001$) (57.52 ± 6.79 ng/mg prot., n = 4) compared to scrambled siRNA control untreated cells (18.42 ± 1.13 ng/mg prot., n = 4) (Fig. 5A). Heightened PGE_2 production was directly related to elevate basal COX-2 mRNA ($P < 0.05$) and protein level by about 1.52- and 2.2-fold compared to siRNA control cells, respectively (Fig. 5B,C). However, RORα1 over-expression had no effect on basal PGE_2, COX-2 mRNA, and protein levels (Fig. 5A,B). In the presence of TNFα, the addition of RORα siRNA increased PGE_2 ($P < 0.01$), COX-2 mRNA ($P < 0.01$), and protein levels by 300%, 250%, and 145% compared to siRNA cells (Fig. 5A,B). RORα1 over-expression significantly lowered TNFα-induced PGE_2 levels by 20% ($P < 0.05$) in comparison to MG-63 osteoblasts transfected by empty-vector followed by TNFα treatment (Fig. 5A). This reduction was accompanied by decreased COX-2 mRNA ($P < 0.01$) and protein level by 69% and about 1.3-fold, respectively (Fig. 5B,C). These results suggested that RORα1 siRNA interacted synergistically with TNFα-induced inflammatory responses, but RORα1 over-expression had a protective effect against inflammation.

MMP-9 EXPRESSION

MMP-9, expressed in both human osteoblasts and osteoclasts, is likely to be a crucial factor in bone pathophysiological processes [Bordji et al., 2000; Cunnane et al., 2001]. To study the regulation of MMP-9 expression by RORα1, osteoblasts were transfected by RORα1 siRNA or expression vector and then stimulated for 24 h with TNFα (10 ng/ml). Compared to control cells, TNFα significantly induced MMP-9 mRNA and protein levels in MG-63 by 450% and 250% (Fig. 6A,B, $P < 0.05$). Basal MMP-9 mRNA and protein production was increased significantly after RORα1 treatment by 155% and 166% ($P < 0.05$) compared to scrambled siRNA control untreated cells. This increment was completely inhibited by RORα1 over-expression by 75% when compared to empty-vector control cells ($P < 0.05$).

EFFECT OF RORα1 OVER-EXPRESSION ON TNFα-INDUCED NF-κB ACTIVATION

The transcription factor NF-κB is believed to mediate the induction of several pro-inflammatory target genes, such as COX-2 and MMP-9 [Yang and Karsenty, 2002; Ahn and Aggarwal, 2005; Jimi and Ghosh, 2005]. To identify the mechanism by which RORα1 inhibits TNFα-evoked COX-2 and MMP-9 expression, we examined whether RORα1 suppresses NF-κB activation. First, we investigated the influence of RORα1 on the transcriptional activity of the pNF-κB-Luc gene construct, which contains five copies of NF-κB response elements. We found that RORα1 siRNA treatment led to basal and TNFα-induced transcriptional activation of luciferase activity by 121% and 121%, respectively, compared to scrambled siRNA (Fig. 7A). RORα1 overexpression plasmid resulted in a decrease of basal and TNFα-induced transcriptional activation of luciferase activity in MG-63 cells by 76% and 83%, respectively (Fig. 7A). Western blotting showed that RORα1 down- and over-expression had no significant effect on basal NF-κB/p65 level in the nucleus of osteoblasts (Fig. 7B). Combined with TNFα, RORα1 over-expression strongly inhibited TNFα-induced NF-κB/p65 protein translocation in the nucleus. RORα1 silencing by siRNA treatment increased basal IκBα phosphorylation and interacted synergistically with TNFα-induced IκBα phosphorylation and NF-κB/p65 protein translocation in the nucleus. In contrast, RORα1 over-expression decreased TNFα-induced phosphorylated IκBα.

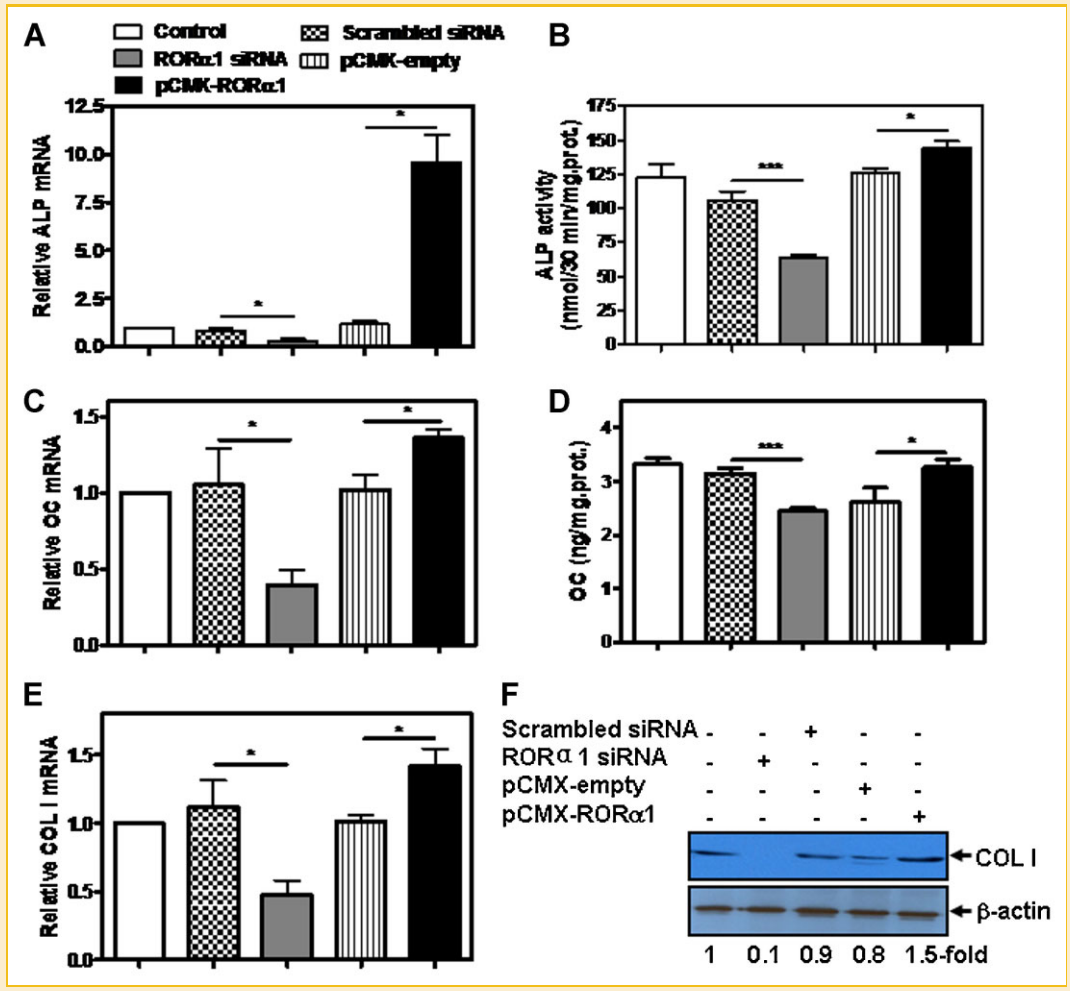

Fig. 3. Effect of RORα1 on modulation of the osteoblast metabolism markers ALP, OC, and COL I. MG-63 cells were transfected with scrambled siRNA control, RORα1 siRNA, empty-vector, or RORα1 expression plasmid, as described in the Materials and Methods Section. ALP mRNA (A) and activity (B), OC mRNA (C) and protein (D), COL I mRNA (E) and protein (F) were analyzed by quantitative real-time RT-PCR, ELISA, or Western blotting, respectively. ALP, OC, and COL I mRNA levels were normalized to GAPDH mRNA expression. The data are means ± SEM of n = 3 and expressed as relative to scrambled siRNA or empty vector-treated control. Student's unpaired *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

DISCUSSION

In the present experiments, we characterized RORα1 and investigated its role in human osteoblast metabolism. The presence of RORα in rat chondrocytes from articular cartilage was demonstrated by Bordji and coworkers [Bordji et al., 2000]. Heightened RORα mRNA expression was observed in human mesenchymal stem cells undergoing osteogenic differentiation [Bordji et al., 2000; Meyer et al., 2000]. However, our study was the first to document RORα1 expression in human osteoblasts, the principal bone-forming cell type present in bone. RORα1 expression was low in osteoblasts isolated from rheumatoid arthritis (RA) patients undergoing knee joint replacement, compared to normal human osteoblasts or MG-63 osteoblast-like cells (data not included). The variation in RORα1 expression could be attributed to the physiological or pathological conditions of osteoblasts. Up to now, there are four known human RORα isoforms. The expression and precise role of RORα1 in RA disease and in joint tissues still remain to be elucidated. Considering recent articles reporting the ability of RORα to control inflammation [Delerive et al., 2001] and bone metabolism [Meyer et al., 2000], the finding of novel mechanisms regulating its expression in osteoblasts could lead to pharmacological targeting of RORα1 in RA.

Bone formation is a process involving the synthesis and deposition of extracellular matrix by osteoblasts with its subsequent mineralization. The differentiation and proliferation of osteoblasts can be regulated by numerous growth factors, hormones, and cytokines. Osteoblast function is also under the control of multiple transcription factors at various stages [Yang and Karsenty, 2002]. Core binding factor a1 and osterix have recently been considered as important osteoblast-specific transcription factors [Ducy et al., 1997; Nakashima et al., 2002]. Other transcriptional factors, such as Dlx5, Msx 2, Fos, and Twist, are not bone cell-specific but are predominantly expressed in the skeleton during development [Morsczeck, 2006]. The coordinated action of these factors affects the expression of osteoblast metabolism marker genes. To widen our knowledge and understanding of the role of RORα1 in osteoblast

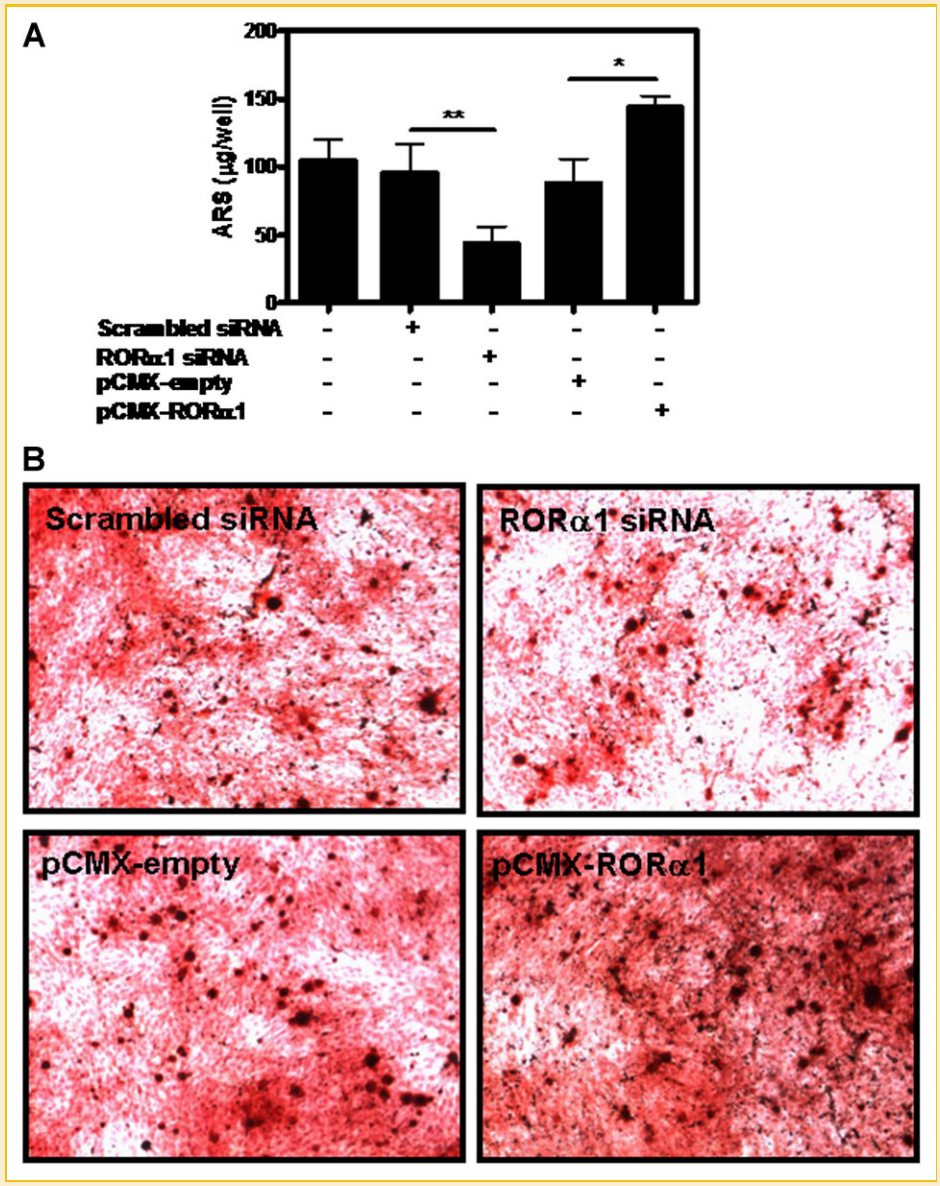

Fig. 4. Evaluation of in vitro mineralization of MG-63 osteoblasts. Confluent MG-63 osteoblasts were incubated in BGJb medium containing 10% fetal bovine serum, 50 μg/ml ascorbic acid, and 50 μg/ml β-glycerophosphate for 28 days, in the presence of scrambled siRNA, RORα1 siRNA, p-CMX-empty vector, or pCMX-RORα1 expression vector. Representative (A) and quantitative (B) mineralization of cell cultures was evaluated by alizarin red staining according to the method described by Gregory et al. [2004]. Values are the mean and SEM results from n = 4. Student's unpaired *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$.

metabolism, we first investigated the impact of its expression on the osteoblast metabolism markers ALP, OC, and COL I in human MG-63 osteoblast-like cells. Our results revealed that reduced RORα1 expression affected osteoblast mineralization as well as ALP, OC, and COL I expression at the mRNA level, as measured by quantitative real-time RT-PCR. In contrast, RORα1 over-expression increased osteoblast mineralization as well as ALP, OC, and COL I mRNA expression. ALP activity, OC, and COL I protein expression changed in a similar manner. ALP and COL I are osteoblast markers of early-stage cellular differentiation, and OC is a marker of late-stage cellular differentiation. By affecting these osteoblast markers, we suggest that RORα1 might be involved in both early- and late-stage osteoblast differentiations. In a recent study, Lyashenko et al. [2010] reported that loss of the ROR1 results in a variety of phenotypic defects within the skeletal, including growth retardation and abnormal mineralization.

RORα can directly regulate gene expression by binding to RORE in target gene promoters in a monomeric fashion and appears to act as a constitutive transcriptional activator in the absence of exogenously added ligand [Enmark and Gustafsson, 1996; Schrader et al., 1996]. In particular, this RORE specifically binds RORα1 and RORα4, but not RORα2 and RORα4 [Steinhilber et al., 1995; Meyer et al., 2000]. RORE sites have been identified in many gene promoters, namely, human retinoic acid receptor β, 5-lipoxygenase,

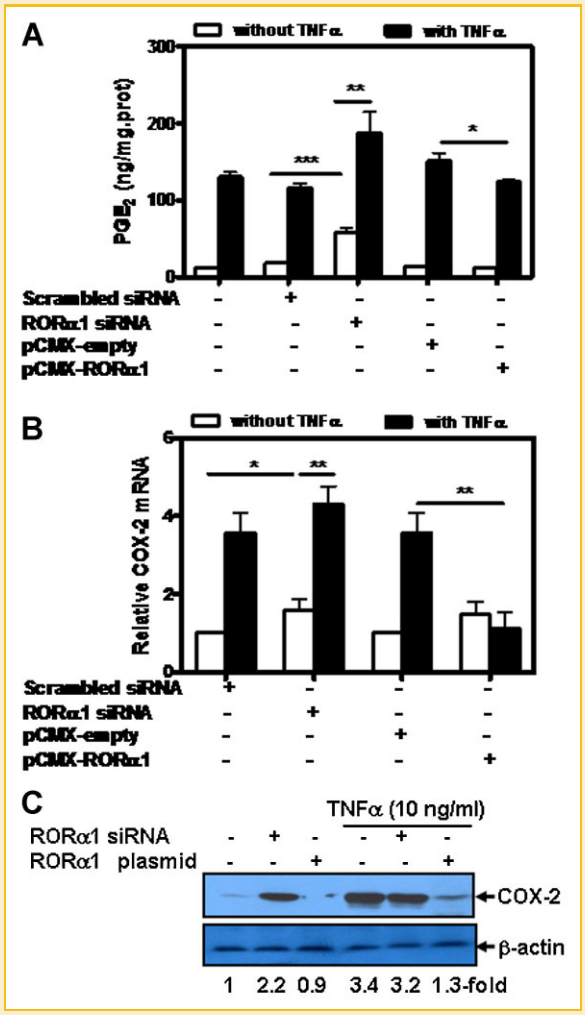

Fig. 5. Effect of RORα1 on PGE_2 release and COX-2 expression. MG-63 cells were transfected with scrambled siRNA control, RORα1 siRNA, empty-vector, or RORα1 expression plasmid, as described in the Materials and Methods Section. Six hours after transfection, the cells were incubated in the presence or absence of TNFα (10 ng/ml) for 48 or 8 h for PGE_2 and COX-2 mRNA and protein measurement. A: PGE_2 release was evaluated in culture medium with PGE_2 enzyme immunoassay kit. B: COX-2 mRNA levels were analyzed by quantitative real-time RT-PCR and normalized to GAPDH mRNA expression. C: COX-2 protein levels were quantified by Western blotting. The data are means ± SEM of n = 3. Student's unpaired *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

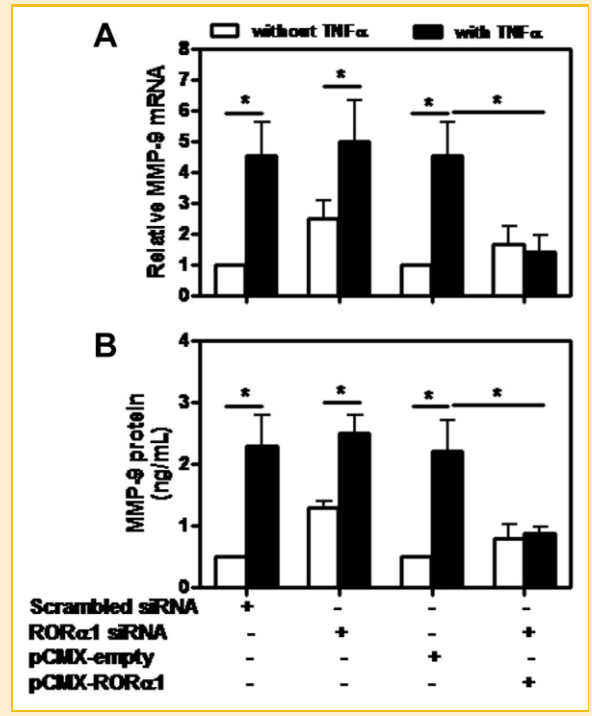

Fig. 6. Effect of RORα1 on MMP-9 expression. MG-63 cells were transfected with scrambled siRNA control, RORα1 siRNA, empty-vector, or RORα1 expression plasmid, as described in the Materials and Methods Section. Six hours after transfection, the cells were incubated in the presence or absence of TNFα (10 ng/ml) for 8 or 24 h for MMP-9 mRNA (A) and protein (B) levels which were analyzed by quantitative real-time RT-PCR and ELISA, respectively. The data are means ± SEM of n = 4. Student's unpaired *t*-test: $^{*}P < 0.05$.

bone sialo protein, mouse Purkinje cell protein-2, and chicken γF-crystalline. Moreover, apolipoprotein, Rev-erbα, and peroxisome proliferator-activated receptor-gamma are especially induced by RORα1 [Sundvold and Lien, 2001]. Recently, Meyer et al. [2000] reported that RORα regulates the OC gene in osteoblasts, by direct binding to the OC promoter region in ROS 17/2.8 cells. We conducted a search in the Ensemble database (www.ensembl.org) to obtain sequence information on the 5-kb 5'-flanking region of ALP and COL I gene. Our analysis disclosed the presence of several potential RORα-binding sites in the promoter region of both ALP and COL I. Thus, it is conceivable that RORα1 might regulate the expression of ALP, OC, and COL I genes at the transcriptional level. However, considering the distal locations of RORE in osteoblast metabolism marker genes whose expression is affected by RORα1, the effect of RORα1 might be secondary. RORα1 could control transcription of another master osteoblast differentiation gene, which needs to be identified and, in turn, could regulate the expression of osteoblast marker genes.

The mechanisms causing bone damage in RA are complex and involve the association of many pro-inflammatory mediators and degradative enzymes [Kaneko et al., 2001; Ram et al., 2006]. Thus, we tested the hypothesis that MMP-9 and COX-2 expressions are under RORα1 control. MMP-9 and COX-2 are important in the local regulation of bone formation and bone resorption [Kaneko et al., 2001; Lu and Rabie, 2006]. We demonstrated that RORα1 siRNA treatment increased basal and TNFα-induced COX-2 at the mRNA level in MG-63 cells. PGE_2 was also augmented by RORα1 gene silencing. In contrast, RORα1 over-expression inhibited basal and TNFα-induced PGE_2 release and COX-2 mRNA expression. We observed that primary osteoblasts derived from RA patients expressed elevated MMP-9 mRNA levels (data not shown), indicating a role of inflammation in the induction of this degradative enzyme. The reduced expression of RORα1 by siRNA treatment increased basal and TNFα-induced MMP-9 mRNA and basal MMP-9 protein, but not TNFα-induced MMP-9 protein level. RORα1 over-expression repressed both basal and TNFα-induced MMP-9 at the protein and mRNA levels. Moreover, no NF-κB/p65

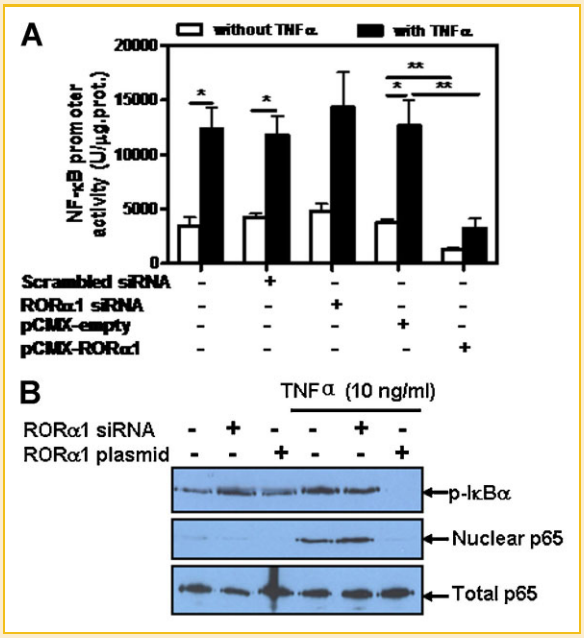

Fig. 7. Effect of RORα1 over-expression on TNFα-induced NF-κB activation. MG-63 cells were transfected with scrambled siRNA control, RORα1 siRNA, empty-vector, or RORα1 expression plasmid, as described in the Materials and Methods Section. Six hours after transfection, the cells were incubated in the presence or absence of TNFα (10 ng/ml) for 1 h. A: NF-κB-Luc gene constructs were co-transfected in MG-63 cells. Luciferase levels were then measured in cellular extracts with specific commercial kits and normalized to protein levels. B: Total cell lysates or nuclear extracts (approximately 50 μg) were prepared and analyzed by Western blotting with anti-phospho-IκBα or anti-NF-κB p65 antibody. The data are means ± SEM of n = 3. Student's unpaired *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

translocation from cytosol to the nucleus and phosphorylated IκBα were observed in osteoblasts transfected with RORα1. This finding was confirmed by NF-κB binding activity. RORα has been shown to be a negative regulator of inflammatory responses [Delerive et al., 2001].

In many cell types, the NF-κB pathway is one of the transcription factors involved in IL-1β- and TNFα-induced COX-2 and MMP-9 [Crofford et al., 1997; Yan et al., 2004]. RORα1 has been demonstrated to suppress the TNFα-induced expression of pro-inflammatory genes in part by inhibiting NF-κB signaling pathways in vascular smooth muscle cells [Delerive et al., 2001; Migita et al., 2004]. In addition, in human umbilical vein endothelial cells, the inhibition of NF-κB signaling pathways by RORα1 and RORα4 l leads to the suppression of TNFα-induced adhesion molecule expression [Migita et al., 2004]. Unexpectedly, sequence information on the 5-kb 5'-flanking region of COX-2 and MMP-9 revealed the absence of RORα-binding sites in these sequences. RORα1 repression at the COX-2 and MMP-9 promoter may occur through a RORE-independent mechanism. Another possibility is that RORα1 does not bind directly to the COX-2 or MMP-9 promoter; it may mediate repression by interacting with other proteins bound to the promoter.

In summary, we demonstrated that RORα1 was involved in the regulation of osteoblast mineralization and of osteoblast marker genes ALP, OC, and COL I in vitro. Further studies are required to address the regulation of these biomarkers and their impact on bone formation in vivo. Thus, a RORα1 conditional knockout animal model should help to clarify this issue. We also observed that RORα1 repression of TNFα-induced COX-2 and MMP-9 expression and PGE_2 release required inhibition of the NF-κB signaling pathway. All our findings suggest that RORα1 may play an important protective role in bone metabolism in RA. The exact molecular mechanism by which it regulates the expression and activity of these pro-inflammatory factors needs further investigation.

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