

Synergistic Antitumor Effect of the Activated PPAR γ and Retinoid Receptors on Human Osteosarcoma

Bai-Cheng He^{1,2,4}, Liang Chen^{1,4}, Guo-Wei Zuo^{1,4}, Wenli Zhang^{4,5}, Yang Bi^{1,4}, Jiayi Huang^{1,4}, Yi Wang^{1,4}, Wei Jiang⁴, Qing Luo^{1,4}, Qiong Shi^{1,4}, Bing-Qiang Zhang^{1,4}, Bo Liu^{1,4}, Xia Lei^{4,3}, Jinyong Luo^{1,4}, Xiaoji Luo^{1,4}, Eric R. Wagner⁴, Stephanie H. Kim⁴, Connie J. He⁴, Yawen Hu⁴, Jikun Shen⁴, Qixin Zhou², Farbod Rastegar⁴, Zhong-Liang Deng^{1,4}, Hue H. Luu⁴, Tong-Chuan He^{1,4}, and Rex C. Haydon⁴

Abstract

Purpose: Osteosarcoma is the most common primary malignancy of bone. The long-term survival of osteosarcoma patients hinges on our ability to prevent and/or treat recurrent and metastatic lesions. Here, we investigated the activation of peroxisome proliferator-activated receptor γ (PPAR γ) and retinoid receptors as a means of differentiation therapy for human osteosarcoma.

Experimental Design: We examined the endogenous expression of PPAR γ and retinoid receptors in a panel of osteosarcoma cells. Ligands or adenovirus-mediated overexpression of these receptors were tested to inhibit proliferation and induce apoptosis of osteosarcoma cells. Osteosarcoma cells overexpressing the receptors were introduced into an orthotopic tumor model. The effect of these ligands on osteoblastic differentiation was further investigated.

Results: Endogenous expression of PPAR γ and isotypes of retinoic acid receptor (RAR) and retinoid X receptor (RXR) is detected in most osteosarcoma cells. Troglitazone, 9-*cis* retinoic acid (RA), and all-*trans* RA, as well as overexpression of PPAR γ , RAR α , and RXR α , inhibit osteosarcoma cell proliferation and induce apoptosis. A synergistic inhibitory effect on osteosarcoma cell proliferation is observed between troglitazone and retinoids, as well as with the overexpression pairs of PPAR γ /RAR α , or PPAR γ /RXR α . Overexpression of PPAR γ , RAR α , RXR α , or in combinations inhibits osteosarcoma tumor growth and cell proliferation *in vivo*. Retinoids (and to a lesser extent, troglitazone) are shown to promote osteogenic differentiation of osteosarcoma cells and mesenchymal stem cells.

Conclusions: Activation of PPAR γ , RAR α , and RXR α may act synergistically on inhibiting osteosarcoma cell proliferation and tumor growth, which is at least partially mediated by promoting osteoblastic differentiation of osteosarcoma cells. *Clin Cancer Res*; 16(8); 2235–45. ©2010 AACR.

Osteosarcoma is the most common nonhematologic malignant tumor of bone in children and adults, with its peak incidence in the teens (1–4). Osteosarcoma is characterized by a high propensity for lung metastasis, with

10% to 20% having detectable metastases at diagnosis (5). Without systemic treatment, few patients with osteosarcoma achieve long-term disease-free status, even with optimal local treatment (6). Certain genetic or acquired conditions increase the risk for osteosarcoma (1–4). Patients with hereditary retinoblastoma have a high risk for osteosarcoma (7). We have shown that Wnt/ β -catenin and S100A6 are frequently upregulated in human osteosarcoma tumors (8–12). Cytogenetic studies on osteosarcoma have documented a variety of genetic alterations resulting in inactivation of tumor suppressor genes and overexpression of oncogenes (1–4). However, it is unclear how much these genetic changes contribute to osteosarcoma development (3, 4).

Increasing evidence suggests that disruptions of osteogenic differentiation may lead to osteosarcoma development (3, 4, 13). Mesenchymal stem cells are adherent bone marrow stromal cells that can differentiate into osteogenic, chondrogenic, adipogenic, and myogenic lineages (14–16). Osteogenic differentiation is a multistep process that requires a balanced regulation of proliferation and

Authors' Affiliations: ¹Key Laboratory of Diagnostic Medicine designated by the Chinese Ministry of Education and the Affiliated Hospitals; ²Department of Pharmacology, School of Pharmacy, Chongqing Medical University; ³Department of Dermatology, Daping Hospital of the Third Military Medical University, Chongqing, China; ⁴Molecular Oncology Laboratory, Department of Surgery, The University of Chicago Medical Center, Chicago, Illinois; and ⁵Department of Orthopaedic Surgery, Huaxi Hospital Affiliated with Sichuan University School of Medicine, Chengdu, Sichuan, China

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

Corresponding Authors: Rex C. Haydon, Molecular Oncology Laboratory, The University of Chicago Medical Center, 5841 South Maryland Avenue, MC 3079, Chicago, IL 60637. Phone: 773-702-5263; Fax: 773-834-4598; E-mail: rhaydon@surgery.bsd.uchicago.edu and Tong-Chuan He, Molecular Oncology Laboratory, The University of Chicago Medical Center, 5841 South Maryland Avenue, MC 3079, Chicago, IL 60637. Phone: 773-702-5263; Fax: 773-834-4598; E-mail: tche@surgery.bsd.uchicago.edu.

doi: 10.1158/1078-0432.CCR-09-2499

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Translational Relevance

Osteosarcoma is the most common nonhematologic malignant tumor of bone in children and adults, with its peak incidence in the teens. The long-term survival of osteosarcoma patients depends on our ability to prevent and/or treat recurrent and metastatic lesions. Clinical management of osteosarcoma has met numerous challenges in chemoresistance, recurrence, and pulmonary metastasis. In this study, we show that the activation of PPAR γ , retinoic acid receptor α , and retinoid X receptor α acts synergistically on inhibiting osteosarcoma proliferation *in vitro* and tumor growth *in vivo* by promoting terminal differentiation. Thus, agonists for these nuclear receptors may represent an attractive alternative of differentiation therapy and/or adjuvant therapy to conventional chemotherapy in treating osteosarcoma. Further investigations should focus on testing these nuclear receptor agonists for their chemopreventive effect on metastatic and/or recurrent osteosarcoma in animal models.

differentiation of osteoprogenitors (14–17). It is conceivable that osteoprogenitor cells harboring defects at early stages in osteogenic pathway may lead to the development of less differentiated and more aggressive osteosarcoma tumors. Likewise, osteosarcoma tumors may be lower grade if they are caused by defects later in osteogenic pathway.

Clinical management of osteosarcoma faces numerous challenges, including adverse effects associated with chemotherapies, chemoresistance, recurrence, and pulmonary metastasis (2–4). To overcome or circumvent some of these challenges, differentiation therapy may represent an attractive alternative and/or adjuvant therapy to conventional chemotherapy (18–21). Agonists of several members of the nuclear receptors have been shown to inhibit proliferation and promote differentiation in cancer cells (20, 22–26). Among them, PPAR γ and retinoid receptors [retinoic acid receptors (RAR) α , β , or γ and retinoid X receptors (RXR) α , β , or γ] attract the most attention. One important biological function of PPAR γ , RAR, and RXR is to promote the terminal differentiation of many precursor cells, including mesenchymal progenitor cells (20, 23–26).

Here, we investigate the anti-osteosarcoma potential of activated PPAR γ and retinoid receptors in human osteosarcoma cells. We have found that endogenous expression of PPAR γ and isotypes of RAR and RXR is readily detected in most osteosarcoma cells and that troglitazone, 9-*cis* RA, and all-*trans* RA can inhibit osteosarcoma cell proliferation and induce apoptosis *in vitro*. Overexpression of PPAR γ , RAR α , and RXR α effectively inhibits osteosarcoma cell proliferation. A synergistic inhibitory effect on osteosarcoma cell proliferation is observed between troglitazone and 9-*cis* RA or troglitazone and all-*trans* RA, as well as overex-

pression of the pairs PPAR γ /RAR α and PPAR γ /RXR α . Overexpression of PPAR γ , RAR α , or RXR α , alone or in combination, effectively inhibits osteosarcoma tumor growth *in vivo*, possibly by inhibiting osteosarcoma cell proliferative activity. Further analysis indicates that retinoids (and to a lesser extent, troglitazone) promotes osteogenic differentiation of osteosarcoma cells and mesenchymal stem cells, suggesting that the activation of their receptors may contribute to their ability to inhibit osteosarcoma proliferation and *in vivo* tumor growth.

Materials and Methods

Cell culture and chemicals. Human osteosarcoma lines MG63, TE85, U2OS, SaOS2, and 143B, as well as HEK293, C3H10T1/2, and C2C12 cells, were from American Type Culture Collection. Primary human osteosarcoma cells were isolated from resected osteosarcoma specimens according to the approved Institutional Review Board protocol (13). Cell lines were maintained in the conditions as described (13, 18, 27, 28). Troglitazone (Rezulin) was provided by Warner-Lambert. All-*trans* and 9-*cis* RA were purchased from BIOMOL. Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich or Fisher Scientific.

Recombinant adenoviruses expressing red fluorescent protein, PPAR γ 2, RXR α , and RAR α . Adenoviruses expressing PPAR γ 2, RXR α , and RAR α were generated using AdEasy technology as described (27, 29–32). The resulting adenoviruses also coexpress red fluorescent protein (RFP) and were designated as AdR-PPAR γ 2, AdR-RAR α , and AdR-RXR α . An analogous adenovirus expressing only monomeric RFP (AdRFP) was used as a control (27, 29, 32).

RNA isolation, quantitative real-time PCR, and semi-quantitative reverse transcriptase-PCR (RT-PCR) analyses. Total RNA was isolated using TRIZOL Reagents (Invitrogen). Semiquantitative RT-PCR and quantitative real-time PCR were carried out as described (13, 30, 33–37). PCR primers (Supplementary Table S1) were designed by using the Primer3 program to amplify the genes of interest (~120–150 bp). All samples were normalized by the expression level of glyceraldehyde-3-phosphate dehydrogenase.

Hoechst 33258 staining. Cells were plated in 12-well plates at a subconfluent condition and treated with troglitazone or RAs. The cells were collected and stained with Hoechst 33258 (Molecular Probes) to visualize apoptotic cells as described (18).

Crystal violet staining. Cell lines were plated in 12-well tissue culture plates at a subconfluent condition (~10⁵ cells per well) and were treated with troglitazone or RAs. The ligand-treated cells were maintained for 4 d and stained with crystal violet to visualize cell viability (18).

Cell proliferation assay. Cells were plated at subconfluent conditions in 24-well tissue culture plates and treated with troglitazone, RAs, or dimethyl sulfoxide (DMSO). The cells were collected by trypsinization at the indicated time

after treatment. Viable cells were counted in the presence of trypan blue (18). Each assay condition was done in triplicate.

Alkaline phosphatase assay. Alkaline phosphatase activity was assessed by colorimetric assay as described (27, 30, 31, 33, 34, 38, 39).

Establishment of osteosarcoma lines stably expressing firefly luciferase. Stable MG63-Luc and 143B-Luc were generated by using a retroviral vector expressing firefly luciferase as described (13, 40).

Luciferase reporter assay. Cells were seeded in 12-well plates and transfected with 0.5 μ g per well of luciferase reporter or a control reporter using Lipofectamine (Invitrogen). At the end of transfection, cells were infected with Ad-PPAR γ 2 or AdRFP or treated with ligands. At 48 h, cells were lysed, and cell lysates were subjected to luciferase assays using Luciferase Assay kit as described (30, 34, 35). Each assay condition was done in triplicate.

Intratibial tumor injection. MG63-Luc and 143B-Luc cells were infected with adenoviruses. Cells were harvested and resuspended in PBS to a final density of 2×10^7 cells/mL. Cells (1×10^6) in 50 μ L of PBS were injected into the proximal tibiae of athymic mice as described (41). Animals were sacrificed after 5 wk, and tumor samples were retrieved for histologic examination.

Xenogen bioluminescence imaging. Animals were anesthetized with isoflurane attached to a nose-cone mask within Xenogen IVIS 200 imaging system. Animals were injected (i.p.) with D-Luciferin sodium salt (Gold Bio-Technology) at 100 mg/kg in 0.1 mL sterile PBS. The pseudomages were obtained by superimposing the emitted light over the grayscale photographs of the animal. Quantitative analysis was done with Xenogen's Living Image V2.50.1 software as described (13).

Histologic evaluation and immunohistochemical staining. Retrieved tissues were fixed in 10% formalin (decalcified if necessary) and embedded in paraffin. Serial sections of the embedded specimens were stained with hematoxylin and eosin. Paraffin-embedded sections were deparaffinized and then rehydrated in a graduated fashion. The deparaffinized samples were subjected to antigen retrieval and fixation. Slides were blocked and probed with an anti-proliferating cell nuclear antigen (PCNA) antibody (Santa Cruz Biotechnology). PCNA was visualized by 3,3'-diaminobenzidine staining. Mouse IgG was run as a negative control (8, 12).

Statistical analysis. Microsoft Excel was used to calculate SDs and statistically significant differences between samples using the two-tailed Student's *t* test.

Results

Endogenous expression of PPAR γ and retinoid receptors in human osteosarcoma lines and primary tumor cells derived from osteosarcoma patients. The presence of PPAR γ and retinoid receptors in human osteosarcoma cells is a prerequisite for any potential effects mediated by their ligands. We first determined the endogenous expression level of PPAR γ and the α , β , and γ isotypes of RARs and RXRs

in a panel of four commonly used human osteosarcoma lines (Fig. A) and 10 primary human osteosarcoma lines (Fig. 1B) through semiquantitative RT-PCR analysis. As shown in Fig. 1, PPAR γ expression was readily detected in almost all tested lines but with different levels of expression between cell lines. Among the RXR isotypes, RXR α was widely expressed, and RXR β was highly expressed in most of the 14 lines, whereas RXR γ was not expressed or weakly expressed in most of the 14 cell lines. For RARs, it was noted that all three RAR isotypes were highly expressed in almost all primary osteosarcoma lines, whereas their expression in the four commonly used osteosarcoma lines was readily detected. These results show that the 14 tested osteosarcoma lines express PPAR γ and at least one isotype of RXRs and RARs. This indicates that human osteosarcoma cells should be responsive to the ligand stimulation of these nuclear receptors.

Troglitazone and retinoids inhibit osteosarcoma cell proliferation in a synergistic fashion. We next assessed the effect of PPAR γ , RXR, and RAR activation on osteosarcoma cell proliferation. As shown in Fig. 2A, cell proliferation of 143B cells was inhibited by troglitazone, 9-*cis* RA, and all-*trans* RA in dose-dependent and time-dependent manners. Similar results were obtained using MG63 cell line (Fig. 2B). The ligand-mediated inhibition of osteosarcoma cell growth and viability was also confirmed by crystal violet staining assays (Supplementary Fig. S1A and B).

We next tested suboptimal concentrations of troglitazone (40 μ mol/L) with various concentrations (20 and 40 μ mol/L) of 9-*cis* or all-*trans* RA. As shown in Fig. 2C, combinations of suboptimal doses of troglitazone and 9-*cis* RA effectively inhibited 143B and MG63 cell proliferation. Likewise, suboptimal doses of troglitazone and all-*trans* RA also suppressed osteosarcoma proliferation (Fig. 2D). In both cases, the synergy was more pronounced at day 3 after combined treatments. Thus, these results collectively show that troglitazone and retinoids inhibit osteosarcoma cell proliferation in a synergistic fashion.

Troglitazone and retinoids induce apoptosis of osteosarcoma cells. We further tested whether these nuclear receptor ligands could induce apoptosis of osteosarcoma cells. Subconfluent 143B and MG63 cells were treated with troglitazone, 9-*cis* RA, all-*trans* RA (each at 40 μ mol/L), or DMSO control. At 3 days after treatment, all cells were collected and subjected to Hoechst 33258 staining. It was shown that the three ligands were able to induce apoptosis in 143B and MG63 cells (Supplementary Fig. S1C). Combinations of troglitazone and 9-*cis* RA or troglitazone/all-*trans* RA induced more pronounced apoptosis of osteosarcoma cells (data not shown). Thus, it is conceivable that these ligands may inhibit osteosarcoma proliferation through inducing apoptosis.

Inhibition of osteosarcoma cell proliferation by adenovirus-mediated overexpression of PPAR γ 2, RXR α , and RAR α . Although nuclear receptor ligands are important resources for studying ligand-mediated activities, they have relative specificities with possible off-target effects. Moreover, these ligands are not convenient for *in vivo* studies. To

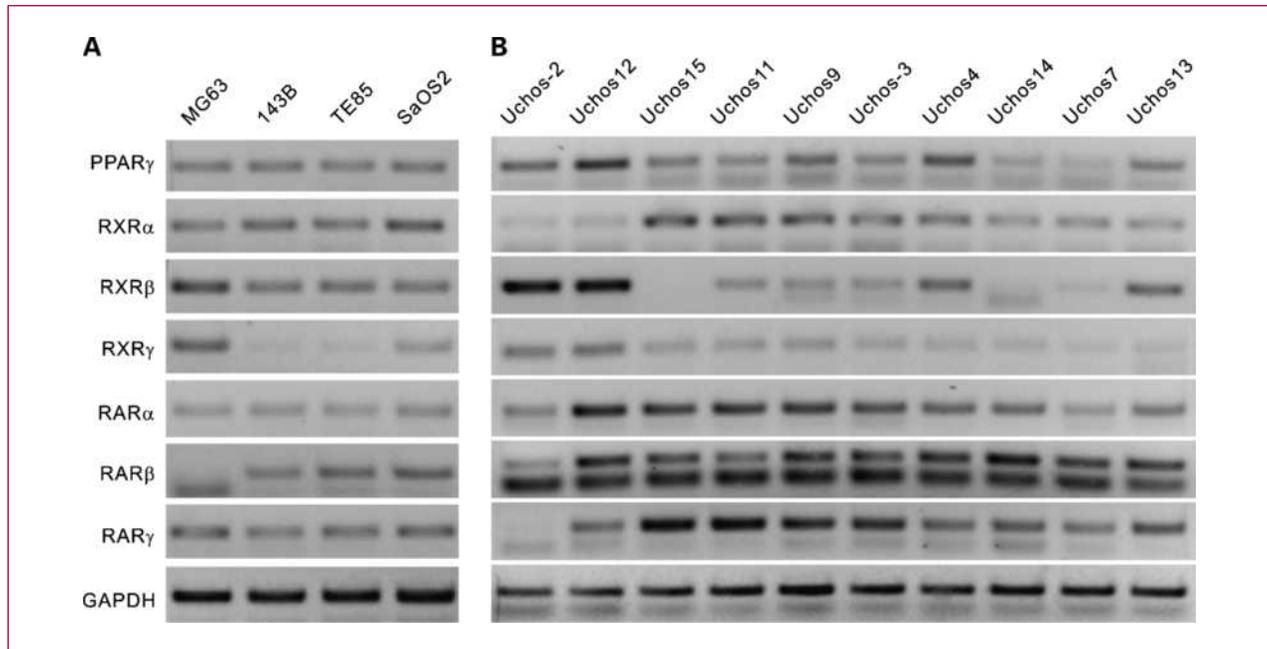


Fig. 1. Endogenous expression of nuclear receptors in human osteosarcoma lines and primary cells derived from osteosarcoma patients.

A, semiquantitative analysis of basal expression of the nuclear receptors in four commonly used osteosarcoma lines. Subconfluent cells were cultured in 1% fetal bovine serum medium for 36 h and collected for total RNA isolation as described in Methods. The total RNA was subjected to reverse transcription and subsequently semiquantitative RT-PCR analysis. Gene-specific primers (listed in Supplementary Table S1) were used to determine the expression levels of PPAR γ and the α , β , and γ isotopes of RARs and RXRs. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B, semiquantitative analysis of basal expression of the nuclear receptors in 10 primary osteosarcoma-derived lines. Primary osteosarcoma cells were cultured in 1% FCS for 36 h. Total RNA was isolated, and semiquantitative PCR was carried as described in (A). All RT samples were normalized with the expression level of glyceraldehyde-3-phosphate dehydrogenase. Each condition was done in triplicate (see Methods).

determine whether inhibition of osteosarcoma cell proliferations was receptor dependent, we overexpressed the PPAR γ 2, RXR α , and RAR α using recombinant adenoviral vectors and tested whether they could function in a ligand-independent manner. Using the AdEasy system (32), we generated adenoviral vectors expressing PPAR γ 2 (AdR-PPAR γ 2), RXR α (AdR-RXR α), and RAR α (AdR-RAR α), all of which were shown to effectively transduce 143B cells (Supplementary Fig. S2A). Using quantitative real-time PCR analysis, we showed that adenovirus-mediated exogenous expression of PPAR γ 2, RAR α , and RXR α were 35- to 47-fold higher than the endogenous levels (e.g., AdRFP control) in osteosarcoma 143B cells (Supplementary Fig. S2B). We further showed that overexpression of PPAR γ 2 effectively activated its reporter pTK-PPREx3-Luc (Supplementary Fig. S2C). Likewise, adenovirus-mediated expression of RXR α or RAR α was shown to induce the expression of several known RAR and RXR target genes, including EGFR, CRABP2, RIP140, and ErbB2 (refs. 42, 43; Supplementary Fig. S2D). Therefore, these results indicate that adenovirus-mediated overexpression of PPAR γ 2, RXR α , and RAR α can function in a ligand-independent fashion.

We next tested the functional activity of these adenoviral vectors in osteosarcoma cells. 143B and MG63 cells were infected with the three nuclear receptor viruses or AdRFP control. We found that overexpression of PPAR γ 2, RXR α , and RAR α inhibited osteosarcoma cell proliferation

in a dose-dependent fashion (Fig. 3A). We further showed that the proliferation inhibitory activities of adenovirus-mediated nuclear receptor expression were potentiated by their ligands (Fig. 3B). In addition, when 143B and MG63 cells were coinfecting with a low titer of AdR-PPAR γ 2 and various titers of AdR-RXR α , AdR-RAR α , or AdRFP, we found that coexpression of either PPAR γ 2/RXR α or PPAR γ 2/RAR α significantly inhibited osteosarcoma cell proliferation and viability (Fig. 3C). Taken together, these results suggest that adenovirus-mediated overexpression of PPAR γ 2, RXR α , and RAR α may recapitulate the functions of their respective ligands, although the overexpressed receptors can be functionally potentiated by their ligands.

Inhibition of *in vivo* osteosarcoma tumor growth by exogenous expression of PPAR γ 2, RXR α , and RAR α , individually or in combination. We sought to test if overexpression of PPAR γ 2, RXR α , and RAR α , individually or in combination, would inhibit osteosarcoma tumor growth *in vivo*. We used our previously established orthotopic xenograft model of osteosarcoma tumors (40, 41). Briefly, luciferase-tagged 143B-Luc and MG63-Luc cells were infected with AdR-PPAR γ 2, AdR-RXR α , AdR-RAR α , AdRFP, or combinations of AdR-PPAR γ 2 + AdR-RXR α or AdR-PPAR γ 2 + AdR-RAR α , and injected into athymic mice intratibially (40, 41). Animals were subjected to Xenogen imaging weekly (Fig. 4A). When the Xenogen data were analyzed, we found that osteosarcoma tumor sizes (in photons per

second per square centimeter per steradian) derived from 143B cells infected with AdR-PPAR γ 2, AdR-RXR α , AdR-RAR α , AdR-PPAR γ 2 + AdR-RXR α , or AdR-PPAR γ 2 + AdR-RAR α were significantly smaller than with AdRFP control starting week 2 (at least, $P < 0.05$; Fig. 4B). Among the three nuclear receptors, AdR-RAR α alone was associated with the lowest inhibition of tumor growth at the early stages (e.g.,

weeks 2 and 3), whereas the AdR-RXR α was the most potent inhibitor at all time points (Fig. 4B). Likewise, a similar inhibitory trend of osteosarcoma tumor growth was found in the mice injected with adenovirus-infected MG63 cells (Fig. 4C). Consistent with our previous findings, MG63 cells formed orthotopic tumors at a much slower pace than that of 143B cells (40, 41). AdR-PPAR γ 2, AdR-RXR α ,

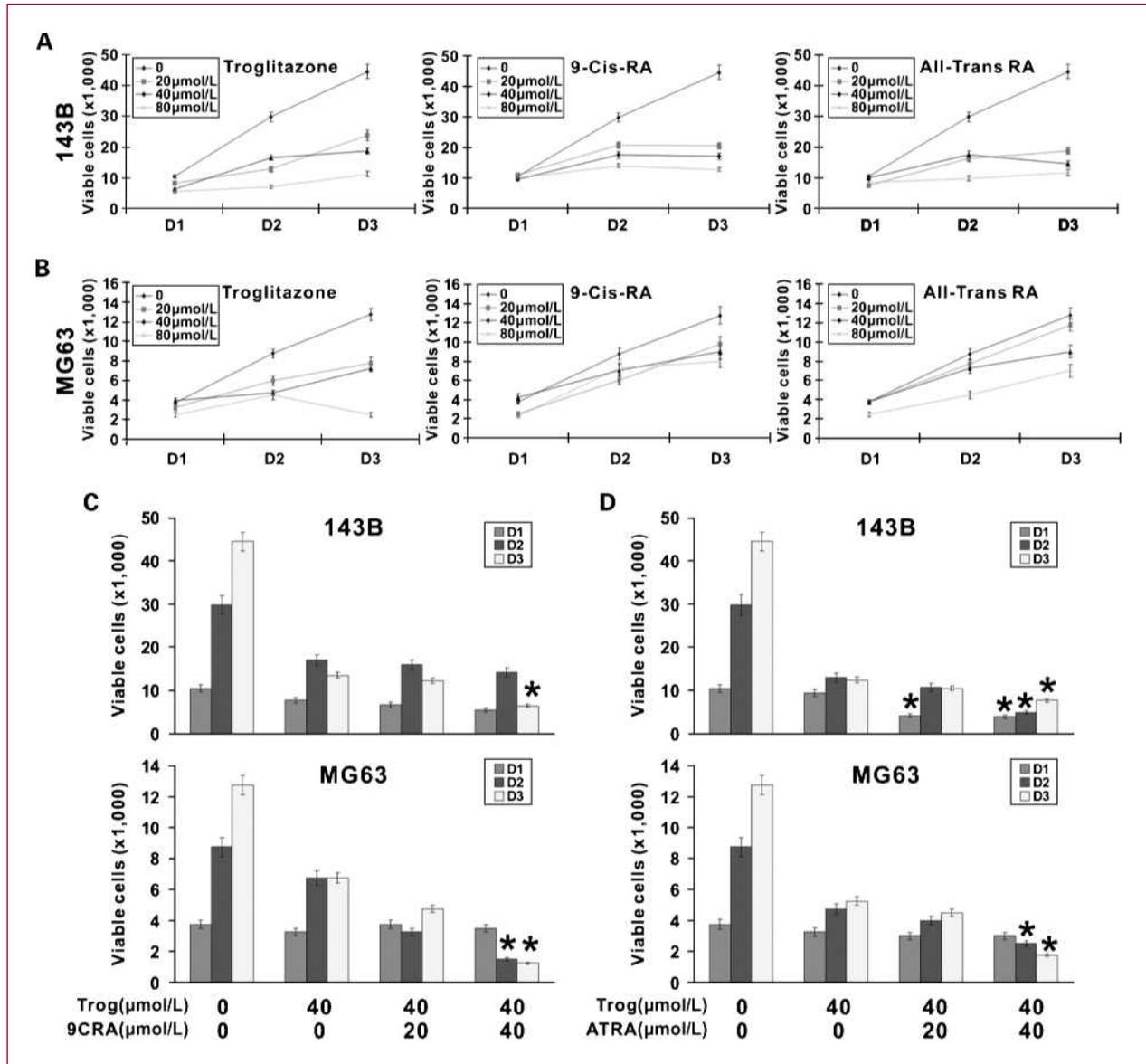


Fig. 2. PPAR γ agonist and retinoids inhibit osteosarcoma cell proliferation in a synergistic fashion. A and B, inhibition of osteosarcoma cell proliferation by PPAR γ agonist troglitazone, 9-*cis* RA, and all-*trans* RA. Osteosarcoma cell lines 143B (A) and MG63 (B) cells were seeded at low density and were treated with various concentrations (0–80 $\mu\text{mol/L}$) troglitazone, 9-*cis* RA, and all-*trans* RA. Viable cells were counted at the indicated time points. Each assay condition was done in triplicate. C, troglitazone and 9-*cis* RA act synergistically in inhibiting cell proliferation of human osteosarcoma cells. 143B and MG63 cells were treated with 0 or 40 $\mu\text{mol/L}$ troglitazone and various concentrations (up to 40 $\mu\text{mol/L}$) of 9-*cis* RA. Viable cells were counted at the indicated time points. Each assay condition was done in triplicate. Trog, troglitazone; 9CRA, 9-*cis* RA. *, $P < 0.05$. D, troglitazone and all-*trans* RA act synergistically in inhibiting cell proliferation of human osteosarcoma cells. 143B and MG63 cells were treated with 0 or 40 $\mu\text{mol/L}$ troglitazone and various concentrations (up to 40 $\mu\text{mol/L}$) of all-*trans* RA. Viable cells were counted at the indicated time points. Each assay condition was done in triplicate. ATRA, all-*trans* RA. *, $P < 0.05$.

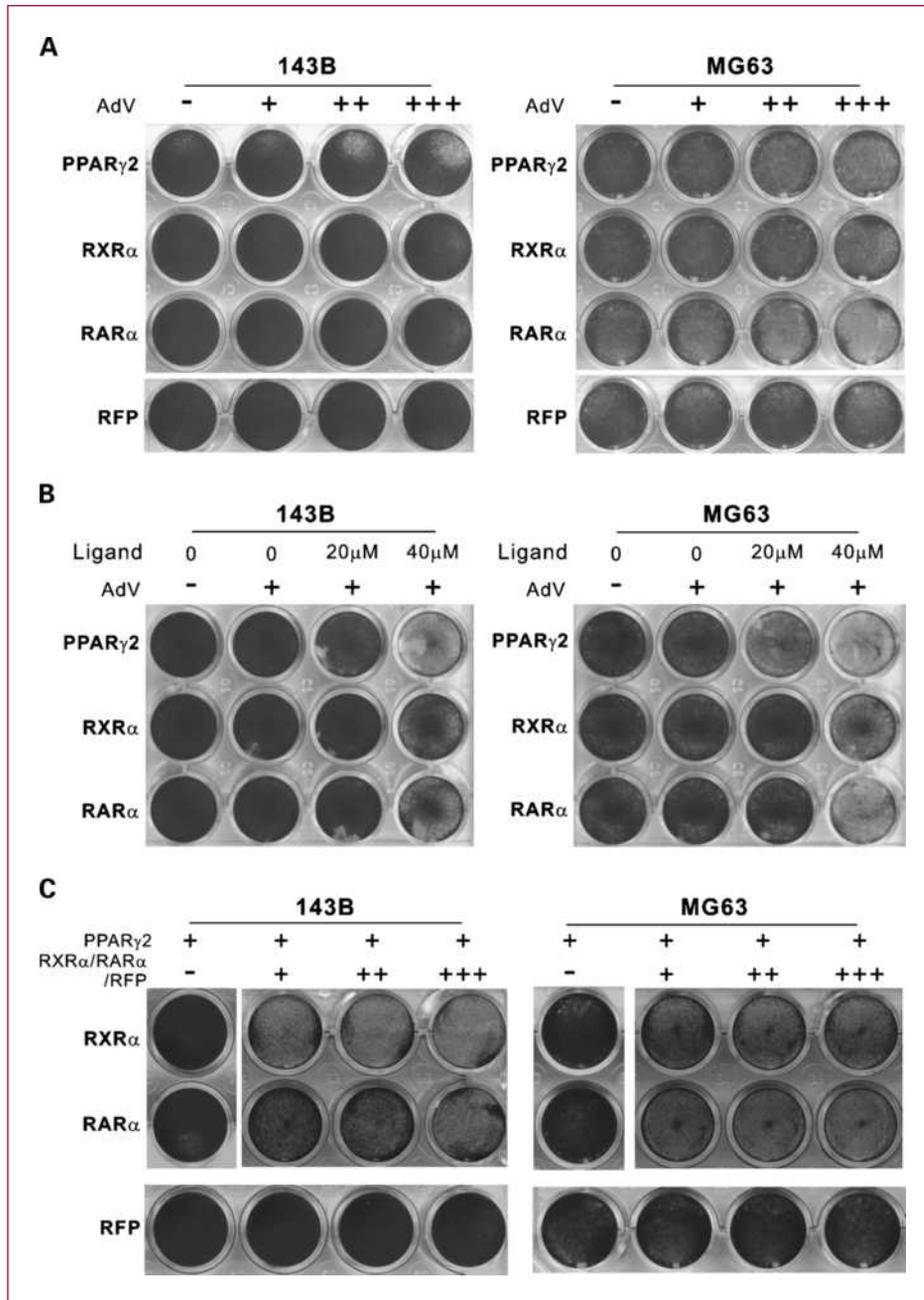


Fig. 3. Inhibition of osteosarcoma cell proliferation by adenovirus(AdV)-mediated overexpression of PPAR γ 2, RXR α , and RAR α .

A, adenovirus-mediated expression of PPAR γ 2, RXR α , and RAR α inhibits cell proliferation of human osteosarcoma cells in a dose-dependent fashion. 143B and MG63 cells were infected with the three nuclear receptor viruses or AdRFP control. At 4 d postinfection, cells were subjected to crystal violet staining.

B, proliferation inhibitory activities of adenovirus-mediated nuclear receptor expression can be potentiated by their ligands. 143B and MG63 cells were infected with a low dose of the adenoviruses and treated with various concentrations of respective ligands for the nuclear receptors. At 4 d, cells were subjected to crystal violet staining.

C, synergistic inhibitory activities in human osteosarcoma cells between PPAR γ 2 and RXR α or RAR α . Cells were infected with a low dose of AdR-PPAR γ 2 and various doses of AdR-RXR α , AdR-RAR α , or AdRFP. At 4 d, cells were subjected to crystal violet staining. Each assay condition was done in duplicate and carried out in at least two independent batches of experiments.

AdR-PPAR γ 2 + AdR-RXR α , or AdR-PPAR γ 2 + AdR-RAR α were shown to inhibit the growth of osteosarcoma tumors derived from MG63 cells at all time points (at least, $P < 0.05$), whereas AdR-RAR α only inhibited tumor growth at week 4 (Fig. 4C). The results from both osteosarcoma lines indicate that RAR α itself may be a weak inhibitor of osteosarcoma tumor growth *in vivo*.

It is noteworthy that coexpression of PPAR γ 2 and RXR α or PPAR γ 2 and RAR α (at half dose for each adenovirus) achieves a significant inhibition of osteosarcoma tumor growth *in vivo*, consistent with the results from *in vitro*

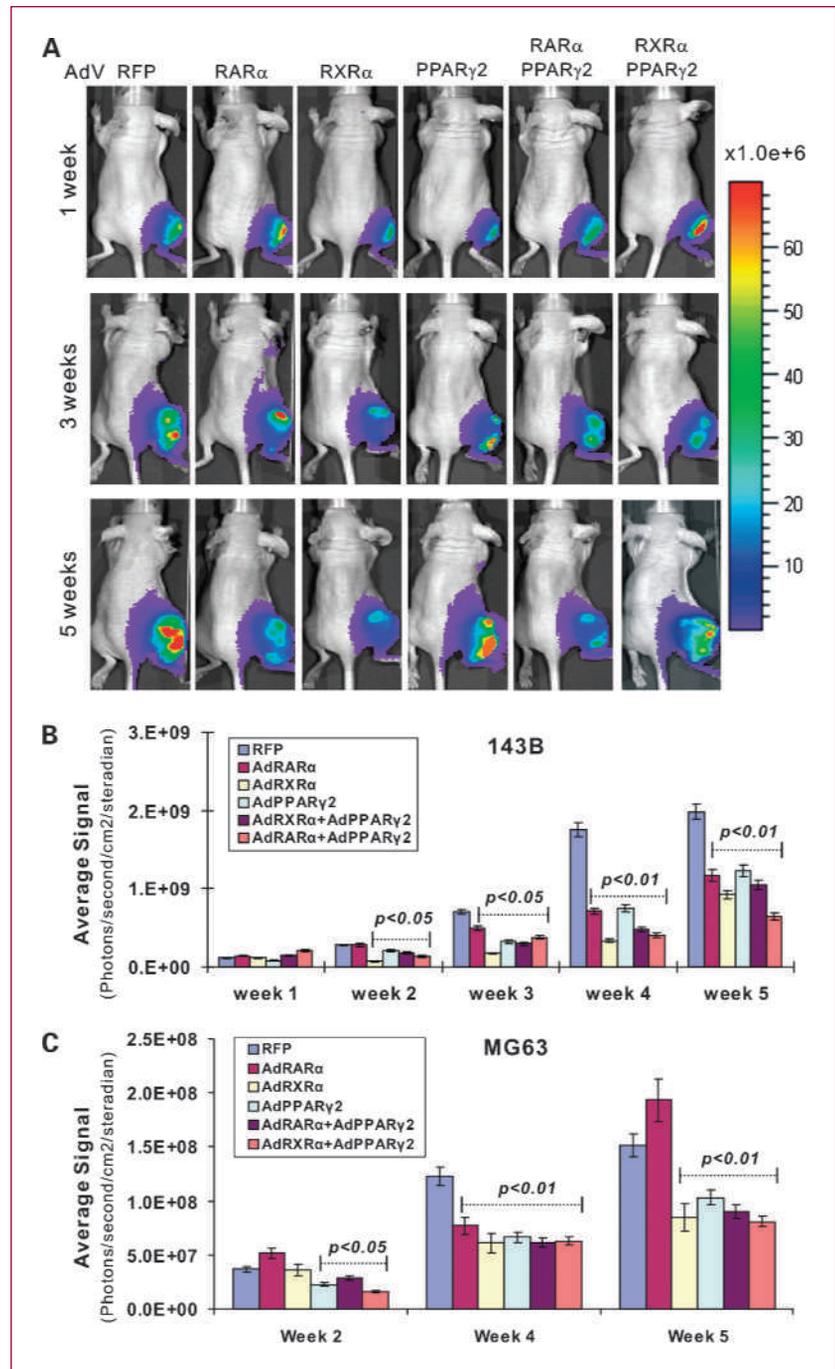
studies (Figs. 2C and 3C). Nonetheless, the adenovirus-mediated coexpression of two nuclear receptors may be further optimized. It is noted that overexpression of these nuclear receptors, alone or in combination, only slowed down the osteosarcoma tumor growth but did not halt osteosarcoma tumor growth. These findings indicate that overexpression of the three nuclear receptors, alone or in combination, may not be sufficient to halt osteosarcoma tumor growth, suggesting that the ligands for these nuclear receptors may not be used as sole therapeutic agents but rather as adjuvant chemotherapeutic agents. This limited

inhibitory effect may also be caused by the decreased adenovirus-mediated expression of the nuclear receptors over the course of the *in vivo* experiments. Nonetheless, our *in vivo* results have shown that the activation of PPAR γ , RAR α , and/or RXR α inhibits osteosarcoma tumor growth in an orthotopic tumor model.

Exogenous expression of PPAR γ , RAR α , and/or RXR α inhibits proliferation of osteosarcoma *in vivo*. The retrieved samples derived from 143B were subjected to hematoxylin

and eosin staining. We found that the tumor cells were modestly to highly proliferative in the RFP control group (Fig. 5A). However, in the PPAR γ , RAR α , and/or RXR α expressing groups, there was significant necrosis in the tumor tissues (Fig. 5A). Immunohistochemical staining with the cell proliferation marker PCNA revealed that most of the tumor cells had intense staining in the RFP control group, whereas much weaker staining and lower percentage of positive staining were observed in the PPAR γ ,

Fig. 4. Inhibition of *in vivo* osteosarcoma tumor growth by exogenous expression of nuclear receptors. A, exogenous expression of PPAR γ , RXR α , RAR α , or in combination, inhibits osteosarcoma tumor growth. The animal experiments were carried out as described in Methods. Briefly, luciferase-tagged 143B and MG63 cells were infected with AdR-PPAR γ 2, AdR-RXR α , AdR-RAR α , AdRFP, or a combination (a half dose of each adenovirus was used) of AdR-PPAR γ 2 + AdR-RXR α , or AdR-PPAR γ 2 + AdR-RAR α . At 16 h after infection, cells were collected and injected into athymic mice intratibially (10^6 cells/injection; five animals per assay condition). Animals were subjected to Xenogen imaging weekly. Representative Xenogen images of the athymic mice intratibially injected with 143B cells are shown. B, sizes of tumors derived from 143B cells (in photons per second per square centimeter per steradian) were calculated by using Xenogen's Living Image software as described in Methods. C, sizes of tumors derived from MG63 cells were calculated by using Xenogen's Living Image software. Statistical significance was calculated by comparing the treated group versus the RFP control group.



RAR α , and RXR α groups (Fig. 5B). These results indicate that exogenous expression of these nuclear receptors can inhibit osteosarcoma cell proliferation *in vivo*.

Retinoids (and to a lesser extent, troglitazone) promote osteogenic differentiation of osteosarcoma cells and mesenchymal stem cells. One of the important functions for PPAR γ , RAR, and RXR is to promote the terminal differentiation of many precursor cells, including mesenchymal stem cells (20, 23–26). Here, we sought to determine if these nuclear receptor ligands would promote terminal differentiation of osteosarcoma cells. RA (9-*cis* all-*trans*) induced alkaline phosphatase activity in 143B and MG63 osteosarcoma cells in a dose-dependent manner, although troglitazone was not effective in inducing alkaline phosphatase activity, suggesting possible defects may lay downstream of alkaline phosphatase marker (Fig. 6A). Alkaline phosphatase is widely used as an early osteogenic marker (27, 31). We next tested the effect of these ligands on the Runx2-binding sites (OSE2)–luciferase reporter, p6OSE2-Luc. Runx2 is a critical regulator of osteoblastic differentiation (44). Our results indicated that 9-*cis* and all-*trans* RA activated p6OSE2-Luc reporter in 143B and MG63 cells in a dose-dependent manner, whereas troglitazone was unable to activate p6OSE2-Luc reporter (Fig. 6B).

We further examined the effect of retinoids and troglitazone on osteogenic differentiation of normal mesenchymal stem cells. Using mesenchymal progenitor cell line C3H10T1/2 and preosteoblast C2C12 cells, we found that 9-*cis* and all-*trans* RA drastically induced alkaline phosphatase activity in C3H10T1/2 cells and C2C12 cells in a dose-dependent fashion, whereas troglitazone exhibited a modest ability to induce alkaline phosphatase activity in C2C12 cells (Fig. 6C). Taken together, we have shown that retinoids (and to a lesser extent, troglitazone) promote osteogenic differentiation of osteosarcoma cells and mesenchymal stem cells, suggesting that the activation of their receptors may contribute to their ability to inhibit osteosarcoma proliferation and *in vivo* tumor growth.

Discussion

Osteosarcoma is a heterogeneous group of malignancies characterized by varying degrees of mesenchymal differentiation. The unifying histologic features of osteosarcoma are the presence of malignant osteoid produced by neoplastic cells (45). Successful clinical management of osteosarcoma faces two major challenges. First, the toxic and adverse effects associated with chemotherapy can

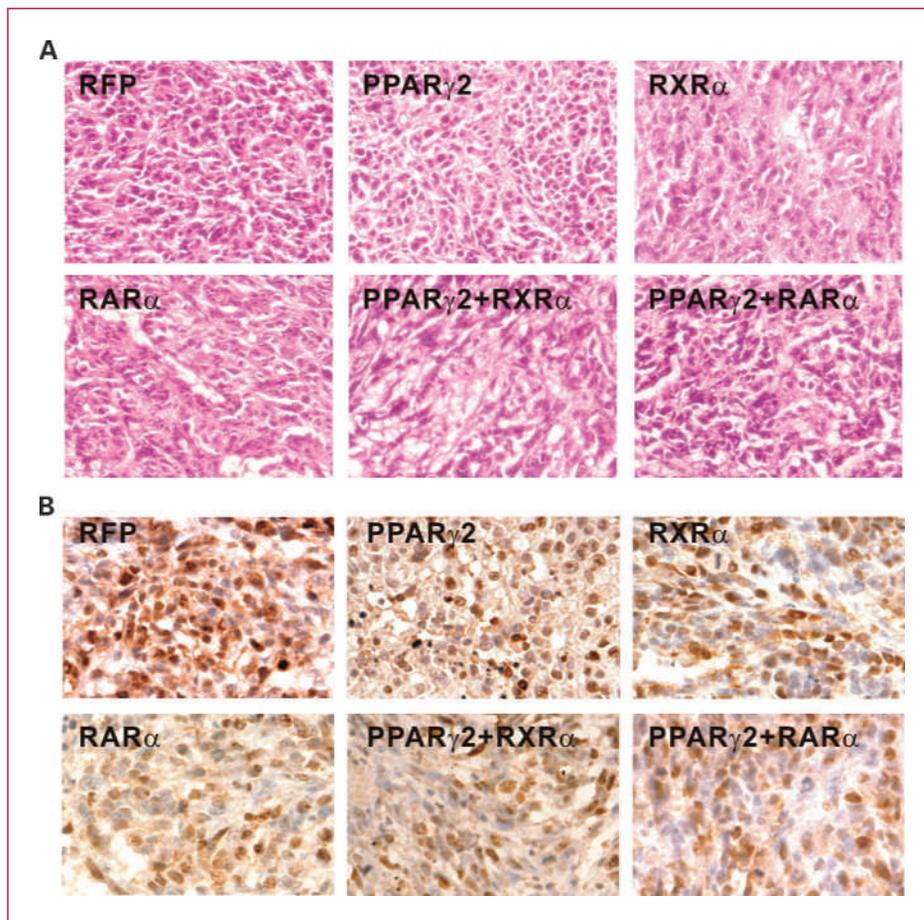


Fig. 5. Exogenous expression of nuclear receptors inhibits proliferation of osteosarcoma cells *in vivo*. A, hematoxylin and eosin staining. Samples retrieved from 143B formed tumors were fixed and paraffin embedded. Sections were used for hematoxylin and eosin staining. Original magnification, $\times 200$. B, reduced expression of PCNA marker in nuclear receptor–expression osteosarcoma tumors. Sections of the embedded tumor samples were subjected to immunohistochemical staining using an anti-PCNA. Original magnification, $\times 200$.

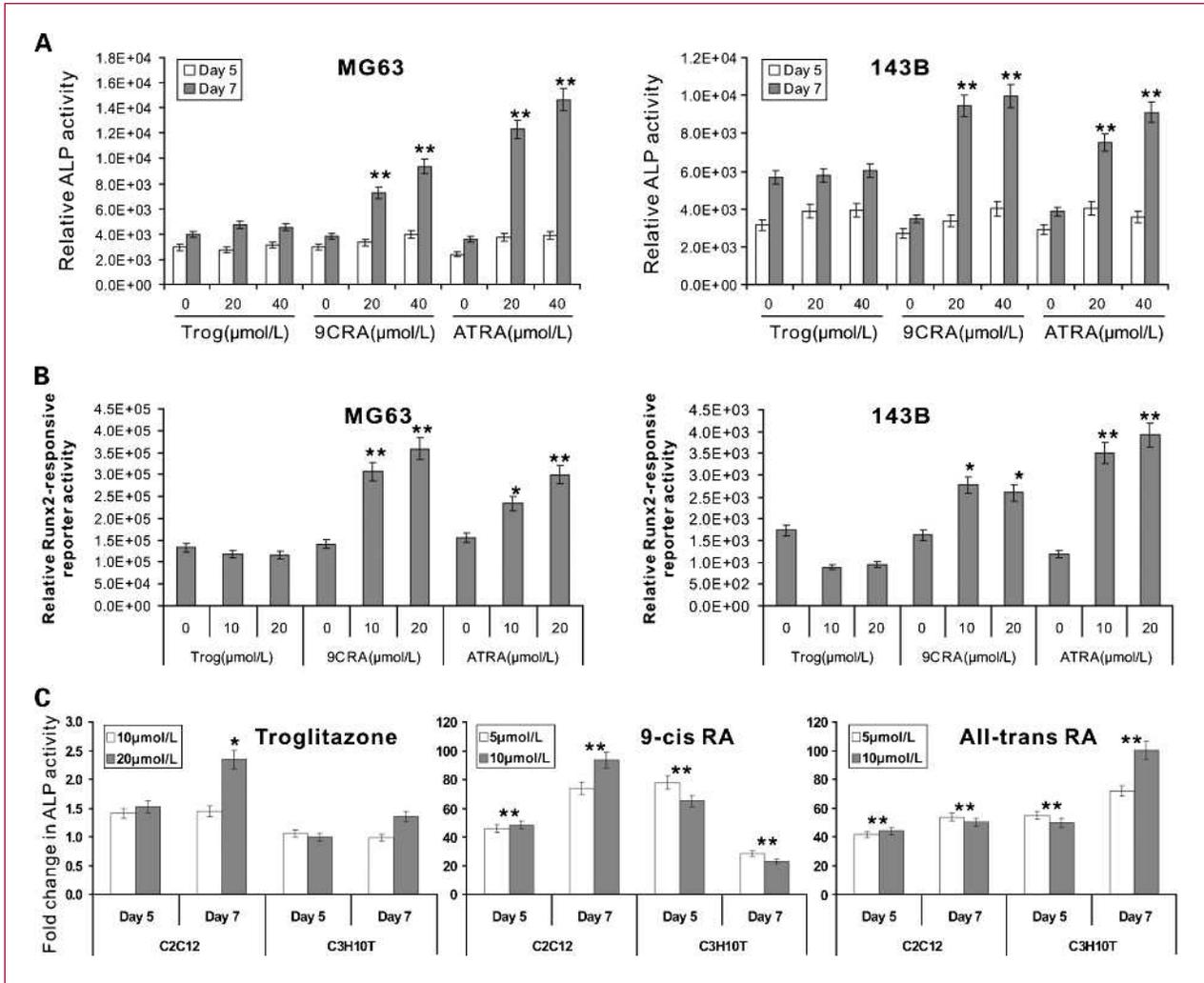


Fig. 6. Nuclear receptor agonists promote osteogenic differentiation. A, nuclear receptor ligands induce alkaline phosphatase activity in osteosarcoma cells. 143B and MG63 cells were treated with the indicated concentrations of troglitazone, 9-cis RA, or all-trans RA. Alkaline phosphatase activity was measured at days 5 and 7. ALP, alkaline phosphatase. B, effect of nuclear ligands on Runx2-responsive reporter. 143B and MG cells were transfected with Runx2-binding sites (OSE2)-luciferase reporter, p6OSE2-Luc, for 16 h; replated in 24 wells; and treated with the indicated concentrations of troglitazone, 9-cis RA, or all-trans RA. Luciferase activity was determined at 48 h posttreatment as described in Fig. 4C. C, retinoids (to a lesser extent, troglitazone) promote osteogenic differentiation of mesenchymal progenitor cells. Mesenchymal stem cell C3H10T1/2 and preosteoblast C2C12 cells were treated with the indicated concentrations of troglitazone, 9-cis RA, or all-trans RA. Alkaline phosphatase activity was measured at days 5 and 7. Each assay condition was done in triplicate. *, $P < 0.05$; **, $P < 0.001$.

significantly reduce the quality of a patient's life, although preoperative and postoperative chemotherapies have improved the 5-year survival rate. Second, osteosarcoma possesses a high rate of recurrence and metastasis, which causes most osteosarcoma-related mortality. Thus, there is an urgent need to find less toxic and more efficacious treatment alternatives.

Induction of terminal differentiation may represent a promising alternative to conventional chemotherapy for certain malignancies. For example, the all-trans RAR has been targeted for intervention in acute promyelocytic leukemia, and differentiation therapy with all-trans RA has become the standard treatment. PPAR γ agonists exert inhibitory activity in human breast cancer, colon cancer, and

liposarcoma cells (23). Nonetheless, most current chemotherapies and radiation therapies target the rapidly proliferative tumor cells, with little consideration of promoting tumor cell differentiation. It is conceivable that a combined therapeutic approach targeting proliferation and differentiation phases of tumor cells would be more efficacious and less prone to inducing chemoresistance.

Some genetic and/or molecular changes in osteoprogenitors may disrupt osteogenic differentiation pathway, leading to the osteosarcoma development. This differentiation defect model is supported by following facts. (a) Osteosarcoma cells exhibit the characteristics of undifferentiated osteoblasts (3, 4, 46). (b) We have shown that most osteosarcoma cells fail to undergo terminal

osteogenic differentiation (13). Osteogenic bone morphogenetic proteins fail to induce bone formation from human osteosarcoma cells and instead promote osteosarcoma tumor growth (13). (c) Retinoblastoma functions as a direct transcriptional coactivator promoting osteoblast differentiation and loss of pRb blocks late osteoblast differentiation (47). (d) Runx2 and p27^{KIP1}-mediated osteoblast terminal differentiation is disrupted in osteosarcoma (46). (e) In Ewing's sarcoma (EWS), the second most common malignant pediatric bone tumor caused by EWS/FLI-1 or EWS/E-twenty six (ETS), EWS/ETS proteins block differentiation along osteogenic and adipogenic lineages (48). (f) EWS-FLI1-silenced Ewing cells differentiate along adipogenic or osteogenic lineage when stimulated with appropriate differentiation cues (49). (e) Expression of EWS/FLI-1 oncogene in murine primary mesenchymal stem cells results in EWS/FLI-1-dependent, Ewing's sarcoma-like tumors (50).

In summary, we have investigated the anti-osteosarcoma potential of activated PPAR γ and retinoid receptors in human osteosarcoma cells and found that troglitazone, 9-*cis* RA, and all-*trans* RA inhibit osteosarcoma cell proliferation and induce apoptosis *in vitro*, which can be recapitulated by overexpression of PPAR γ , RAR α , and RXR α in osteosarcoma cells. We have shown that overexpression of PPAR γ , RAR α , or RXR α , alone or in combination, can inhibit osteosarcoma tumor growth *in vivo*. Further studies revealed that retinoids (and to a lesser extent, troglitazone) promote osteogenic differentiation of osteosarcoma cells and mesenchymal stem cells, suggesting that the activation

of their receptors may contribute to their ability to inhibit osteosarcoma proliferation and *in vivo* tumor growth. It should be pointed out that PPAR γ may function in a ligand-independent fashion and that retinoid receptors are known to function as homodimers or heterodimers in a PPAR-independent fashion. Future investigations should focus on testing these agonists for their chemopreventive effect on metastatic and/or recurrent osteosarcoma in animal models.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. Michael Simon and Terrance Peabody of The University of Chicago for their assistance on collecting human osteosarcoma samples, and Ronald Evans of Salk Institute and Hiroyuki Kawashima of Niigata University, Japan, for their kind provision of the pTK-PPREx3-Luc and p6OSE2-Luc plasmids, respectively.

Grant Support

American Cancer Society (T-C. He), Brinson Foundation (T-C. He), and NIH (CA106569, AT004418, AR50142, and AR054381; T-C. He, R.C. Haydon, and H.H. Luu).

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Received 09/14/2009; revised 01/21/2010; accepted 02/10/2010; published OnlineFirst 04/06/2010.

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