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SECTION III
REGULAR AND SPECIAL FEATURES

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Osteosarcoma and Osteoblastic Differentiation

A New Perspective on Oncogenesis

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In addition to changes in cellular pathways, loss of differentiation is a notable feature of osteosarcoma. We hypothesized that blocks to normal differentiation may be a common feature of osteosarcoma, and may be one of many critical events that occur during oncogenesis in osteosarcoma. Furthermore, therapies that restore normal programs of differentiation may be attractive new treatment strategies for chemotherapy and/or chemoprevention. We exposed an osteosarcoma cell line to two highly osteogenic bone morphogenetic proteins and noted increased tumor volume and no evidence of osteoinduction in vivo. We then used expression profile analysis to identify downstream targets of the osteogenic bone morphogenetic proteins, revealing up-regulation of the inhibitor of differentiation genes 1, 2, and 3, and the nuclear receptor, peroxisome proliferator activated receptor γ. We then evaluated the use of nuclear receptor agonists, including peroxisome proliferator activated receptor γ, to circumvent the apparent block to bone morphogenetic protein-induced differentiation in osteosarcoma cell lines. The peroxisome proliferator activated receptor γ/retinoid X receptor agonists induced terminal differentiation in all four osteosarcoma cell lines and were synergistic when combined. In osteosarcoma cells, there are inherent blocks to normal bone morphogenetic protein-induced differentiation; however, they do not prevent nuclear receptor agonists from inducing terminal differentiation.

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Oncogenesis is the culmination of a multiple-step process in which cellular pathways critical for normal cell function are disrupted through genetic alterations. Dysregulation of pathways controlling cell proliferation, genetic stability, cell senescence/apoptosis, and motility can occur through various genetic mechanisms.[9] Studies have shown that osteosarcoma possesses various cytogenetic and genetic abnormalities.[36,42] However, there is no common mechanism for the development of osteosarcoma. The most commonly dysregulated genes in osteosarcoma are p53 and retinoblastoma, although other alterations have been identified in MDM2, beta-catenin, and pathways directly related to cell proliferation and genome stability.[13,15,28,29,36,42] Less well understood is the role of pathways regulating normal osteoblastic differentiation in osteosarcoma cells.

Bone formation is a well-orchestrated process in which pluripotent mesenchymal stem cells pass through successive stages of differentiation with a decreasing propensity for proliferation. Each step is carefully regulated by various endogenous and environmental factors. The precise mechanisms underlying normal osteoblastic differentiation

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are unclear. Bone morphogenetic proteins (BMPs) play a central role in regulating osteoblast differentiation and bone formation during development, bone turnover, and healing.[17,32] Bone morphogenetic proteins originally were identified by Marshall Urist in 1965.[41] They are members of the TGF-β superfamily and encompass at least 15 multifunctional morphogens (BMP-1–15) in humans, many of which currently are used for clinical applications in orthopaedics.[10,44] With respect to sarcomas, some investigators have suggested that the BMPs may promote cell proliferation in osteosarcoma, in contrast to what occurs in nonneoplastic osteoprogenitor cells.[14,20,47]

We hypothesized that alterations in normal pathways of osteoblastic differentiation exist in osteosarcoma. An improved understanding of these blocks may reveal key mechanisms of oncogenesis in osteosarcoma, and lead to new treatments that focus on differentiation therapy. To study these questions, we evaluated the impact of the most osteogenic BMPs on osteosarcoma cell lines in vivo. We used expression profile analysis to define potential mechanisms that promote dedifferentiation and cell proliferation in osteosarcoma, and to identify strategies to circumvent the underlying blocks to normal BMP-induced differentiation.

MATERIALS AND METHODS

To address the above hypotheses, we first performed a comprehensive evaluation of all BMPs to determine which of the BMPs are the most osteogenic in osteoprogenitor cells, using in vitro and in vivo assays. We then transduced the osteosarcoma cell line, 143B, with an adenoviral vector expressing BMP-2 and BMP-9, and evaluated growth differences between the mock virus control and the experimental groups.

To explain the differences between osteosarcoma and osteoprogenitor cells after exposure to osteogenic BMPs, we compared differences in the expression profile of C2C12 cells exposed to osteogenic BMPs versus nonosteogenic BMPs. The immediate downstream targets of BMP activation included at least one nuclear receptor that has been used in other neoplasms to induce differentiation. We studied the use of PPARγ agonists to promote differentiation using in vitro assays with four osteosarcoma cell lines.

The C2C12 cell line and human osteosarcoma cell lines 143B, MG-63, and TE-85 were obtained from the American Type of Culture Collection (ATCC, Manassas, VA). Human osteosarcoma line MNNG/HOS was provided by Dr. Sabine Krueger of the Otto-von Guericke (University of Magdeburg, Magdeburg, Germany). All cells were maintained in complete Minimum Essential Medium Eagle (EMEM) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine (Mediatech, Herndon, VA), nonessential amino acids diluted to ×1 from the stock solution (Mediatech), 1 mmol/L sodium pyruvate (Mediatech), 100 units penicillin, and 100 μg streptomycin at 37°C in 5% CO_2. Troglitazone (Rezulin®) was provided by Parke-Davis/Warner-Lambert (Ann Arbor, MI). Ciglitazone and 9 cis-retinoic acid were purchased from BIOMOL (Plymouth Meeting, PA). Unless indicated otherwise, all other chemicals were purchased from Sigma-Aldrich (St Louis, MO).

Our study was conducted following IACUC guidelines for the use of animals. We used 5 to 6-month-old male athymic nude mice (Frederick Cancer Research Center, Frederick, MD). Each experimental group had four animals. For the injection with adenovirus-transduced C2C12 cells, subconfluent C2C12 cells were infected with AdBMPs or AdGFP at preoptimized titers (multiplicity of infection [MOI], 50–100 virus-to-cell ratio) overnight. Cells were collected and resuspended in phosphate buffered saline (PBS) at an approximate density of 1×10^8 cells/mL. Fifty microliters of cell suspension (5×10^6 cells) were used for intramuscular injection to the right quadriceps. Injected animals resumed activities immediately without any food or drink restraints. In the experiment with C2C12 cells, the animals were sacrificed at 3 and at 5 weeks after injections. Plain radiographs were obtained followed by histologic evaluation (hematoxylin & eosin, Masson's trichrome) of the injected sites. In the experiment with transduced osteosarcoma cell lines, a cell suspension containing 1×10^6 cells was injected directly into the proximal tibia. The animals were sacrificed at 4 weeks and analyzed histologically.

Subconfluent C2C12 cells were seeded in 25 cm^2-cell culture flasks for 12 hours in complete Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 0.5% fetal calf serum (FCS), and infected with an optimal and compatible titer of AdBMP2, AdBMP3, AdBMP6, AdBMP9, AdBMP12, or AdGFP. Thirty hours after infection, total RNA was isolated using RNAgent Total RNA Isolation kit (Promega, Madison, WI) according to the manufacturer's instructions.

The microarray analysis was performed at The University of Chicago Functional Genomics Facility, Chicago, IL. Fully characterized total RNA samples were used for target preparation and then subjected to hybridizations to Affymetrix mouse genechip, U74Av2 (Affymetrix, Santa Clara, CA). The target preparation protocol followed the Affymetrix GeneChip Expression Analysis Manual. The quality of hybridizations initially was evaluated at our microarray facility by examining the MAS 5.0 Report file for housekeeping gene hybridization, Spike control hybridization, percentage of genes present, 5′ to 3′ ratio, signal to background ratio, and scale factor ratio, and then by using DNA-Chip (dCHIP) analysis to eliminate regional image contamination and/or sample contamination. Significance Analysis of Microarrays (SAM) analysis was performed as described previously.[40] We performed a model-based expression analysis using dCHIP with the CEL files obtained from MAS 5.0.[24] The model-based approach allowed probe level analysis and facilitated automatic probe selection in the analysis stage to reduce errors caused by outliers, cross-hybridizing probes, and image contamination. We selected 200 of the most differentially expressed genes using the dCHIP filter function to perform a cluster analysis.

Cell lines were plated in 12-well tissue culture plates at a subconfluent condition (~10^5 cells/well). They were treated with troglitazone, ciglitazone, or 9-cis retinoic acid at the final concentrations of 0 μm (control), 20 μm, 50 μm, and 100 μm. The

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ligand-treated cells were maintained at 37°C in 5% CO_2 for 5 days, and then stained with Crystal Violet to observe cell viability.

Cell lines were plated in 12-well tissue culture plates at a subconfluent condition (~10^5 cells/well), and treated with troglitazone at the final concentrations of 0 μm (control) and 100 μm. After being maintained at 37°C in 5% CO_2 for 72 hours, the treated cells were collected and stained with Hoechst 33258 (Molecular Probes, Eugene, OR) to observe the apoptotic cells under fluorescence microscopy.

Exponentially growing TE-85 cells were plated in a 12-well tissue culture plate at a subconfluent condition (~10^5 cells/well), and were treated with troglitazone at the final concentrations of 0 μm (control), 20 μm, and 50 μm. Seventy-two hours after treatment, cells were fixed with 0.05% (v/v) glutaraldehyde (Sigma-Aldrich) at room temperature for 10 minutes. After being washed with PBS, cells were stained with a mixture of 0.1 mg/mL naphthol AS-MX phosphate and 0.6 mg/mL Fast Blue BB salt (Sigma-Aldrich). Histochemical staining was performed using bright field microscopy.

RESULTS

To identify the BMPs that exhibit the most potent osteoinductive activity, we evaluated the effects of individual BMPs on osteoblast lineage-specific differentiation in mesenchymal progenitor cells C3H10T1/2 and C2C12 by measuring the osteogenic markers such as alkaline phosphatase (ALP) and osteocalcin. Only BMP-2, BMP-6, and BMP-9 induced a significant increase ALP activity in C3H10T1/2 cells[7] (Fig 1A). Bone morphogenic protein-6 induced ALP activity as early as 5 days after infection (greater than a 13-fold increase), and the ALP activity continued to increase at 9 days after infection (greater than a 292-fold increase). In C2C12 cells, BMP-2, BMP-4, BMP-6, BMP-7, and BMP-9 all induced ALP activity[7] (Fig 1B). There was a longer latency in ALP induction in C3H10T1/2 cells than in C2C12 cells. The time difference in BMP-induced ALP activity in C3H10T1/2 and C2C12 cells may reflect their different pluripotency. The histochemical assays of ALP activity were consistent with those obtained from colorimetric assays.

We then determined the relative in vivo osteogenic activity of 14 types of BMPs.[19] We used a nude mouse model in which C2C12 cells were first transduced with AdBMPs and subsequently implanted into the quadriceps of athymic nude mice by intramuscular injection (~ 5 ×

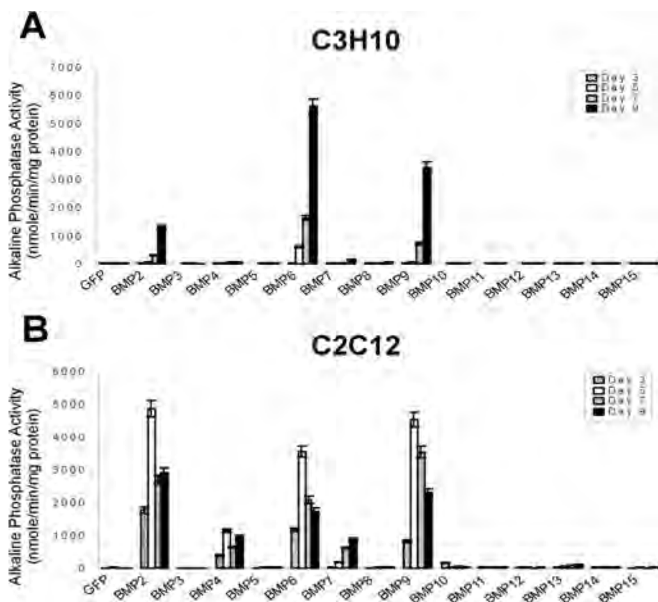

Fig 1A–B. A comprehensive comparison of the osteogenic activity of all BMPs was performed on osteoprogenitor cell lines: (A) C3H10T1/2 and (B) C2C12 are shown. Cells were infected with AdBMPs and a control AdGFP were lysed at indicated times for a colorimetric assay of ALP activity by using p-nitrophenyl phosphate as a substrate. Alkaline phosphatase activity was used to assess osteoblastic differentiation. Representative results from at least two independent experiments are shown. Bone morphogenetic protein-2, BMP-6 and BMP-9 seemed to be the most osteogenic BMPs in both cell lines tested.

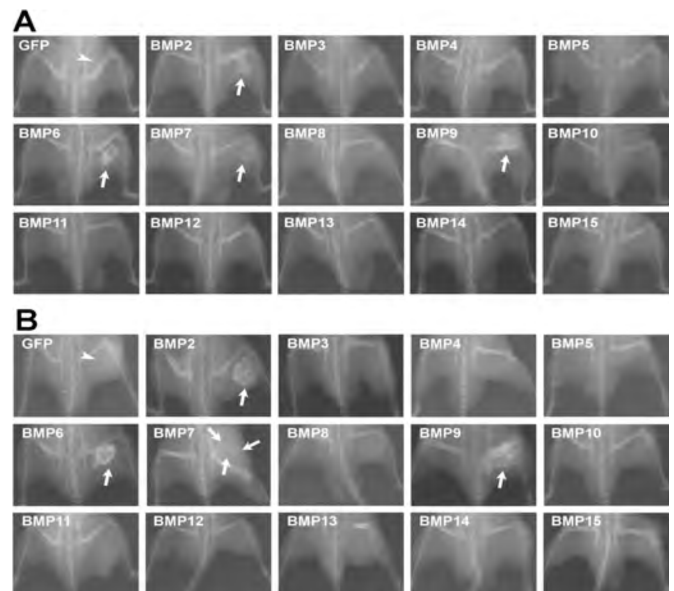

Fig 2A–B. We compared the in vivo osteoinductive activity of all BMPs in a heterotopic bone formation model using athymic mice. The C2C12 cells were infected with AdBMPs or the control AdGFP for 15 hours. Approximately 5 × 10^6 of the infected cells were injected into the right quadriceps of athymic nude mice (an arrow shows where the injection occurred in the GFP group as an example). (A) Three weeks and (B) 5 weeks after injections, the mice were sacrificed and radiographs were taken, with arrows indicating areas of bone formation. Each experimental group had four mice. The in vivo data suggest the BMP-2, BMP-6, and BMP-9 are the most potent osteoinductive BMPs.

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10^6 cells per injection). Bone formation was readily detected on plain radiographs from the animals injected with AdBMP-2, AdBMP-6, AdBMP-7, and AdBMP-9 transduced C2C12 cells at 3 weeks (Fig 2A) and 5 weeks (Fig 2B).[19] The histologic evaluations of the retrieved specimens correlated well with the findings on plain radiographs. At 3 weeks, BMP-2, BMP-6, BMP-7, and BMP-9 showed varying degrees of ossification. Bone morphogenic protein-2 and BMP-7 were the least developed with small foci of woven bone (Fig 3A). Bone morphogenic protein-2, BMP-6, BMP-7, and BMP-9-induced osteogenesis was further confirmed by Masson's trichrome staining (Fig 3B).[19]

To study the impact of osteogenic BMPs on osteosarcoma, we transduced the 143B osteosarcoma cell line with adenoviral vectors expressing either BMP-2, BM-P9 or GFP (mock virus control), and injected subcutaneously in a nude mouse model. Four weeks after implantation, the tumor volume was larger ($p < 0.001$ at 27 days) (Fig 4A). Histologic evaluation showed no evidence of bone formation (Fig 4B). The histologic appearance of the cells was identical to that of the control infected with the mock virus (AdGFP) and did not stain for ALP suggesting that 143B cells were refractory to the osteogenic effects of BMP-2, BMP-6, and BMP-9.

To examine the molecular basis of BMP-mediated osteogenesis, we performed an expression profile analysis of genes whose expression was affected by osteogenic BMPs.[34] Briefly, exponentially growing C2C12 cells were infected with three osteogenic BMP viruses (AdBMP-2, AdBMP-6, and AdBMP-9), along with AdGFP (mock infection), AdBMP-3 (a negative BMP), and AdBMP-12 (a nonosteogenic BMP). Thirty hours after infection, total RNA was isolated and subjected to microarray hybridization analysis using the Affymetrix's mouse genechip, U74Av2. Hierarchical clustering was used to observe overall similarities in expression patterns. The three osteogenic BMPs (BMP-2, BMP-6, and BMP-9) induced a similar overall expression pattern that was distinct from BMP-3, BMP-12, and the GFP control (Fig 5). This suggested that BMP-3 and BMP-12 behaved differently from the three osteogenic BMPs. The similarities in gene expression pattern among the three osteogenic BMPs may underscore a fundamental mechanism behind bone formation. Using SAM analysis, there were 203 genes whose expression changed by at least twofold; 105 were up-regulated and 98 were down-regulated by osteogenic BMPs. The microarray data provided important information regarding early signaling events of osteogenesis.[34] Several transcription regulators were induced among the top 30 up-regulated genes. These included Inhibitor of DNA binding (Id) 1, Id2, Id3, peroxisome proliferator activated receptor (PPAR) γ, GIF (TIEG1), junB, and homeobox genes (Dlx-3 and Prx2). The top 30 up-regulated genes from SAM analysis also included several growth factors/cytokines (eg, hepatocyte growth factor, brain-derived neurotrophic factor, and connective tissue growth factor) and signaling molecules (eg, Grb10, receptor-type protein tyrosine phosphatase M, and interferon activated gene 202). Other notable down-regulated targets were

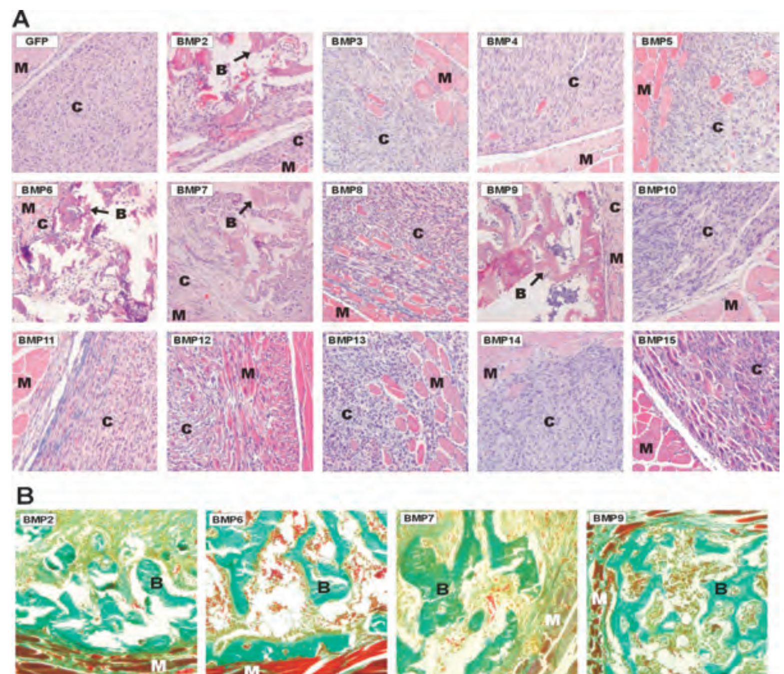

Fig 3A–B. Histologic evaluations of AdBMP-induced orthotopic ossification are shown. (A) The specimens show AdBMP-transduced C2C12 injection sites at 5 weeks (Stain, hematoxylin and eosin; original magnification, ×200) and (B) at 3 weeks (Stain, Masson's trichrome; original magnification, ×200). Muscle fibers and cytoplasm were stained red and collagen of osteoid matrix was stained blue (B = osteoid matrix; C = injected C2C12 cells; M = muscle cells). The injection sites from the BMP-2, BMP-6, and BMP-9 experimental groups showed more robust and mature bone formation.

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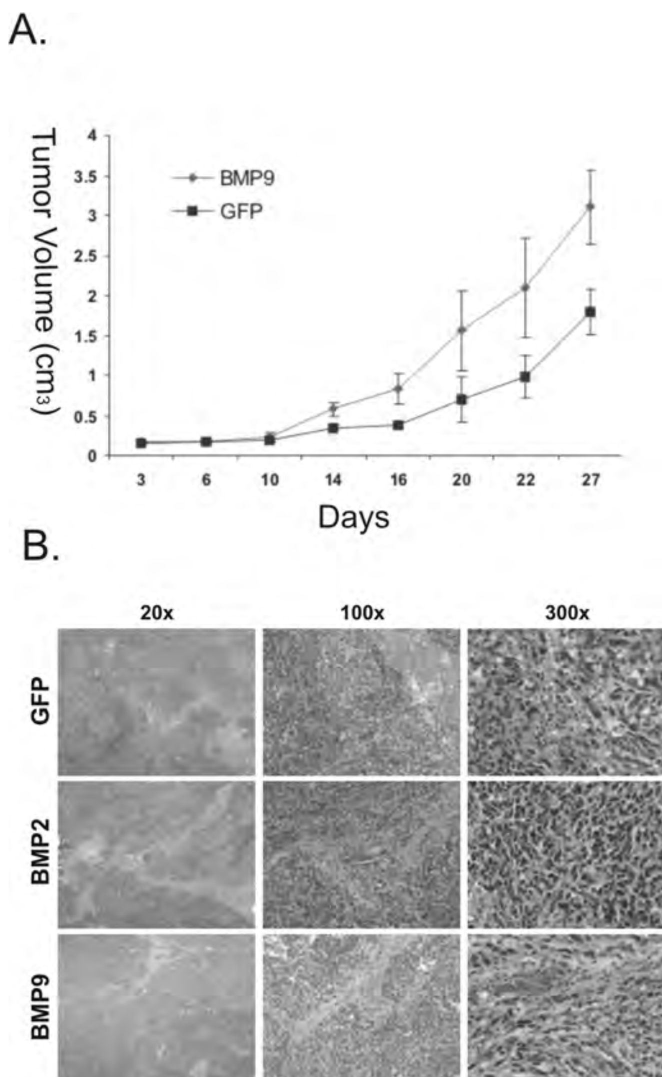

Fig 4A–B. (A) A graph shows the results of tumor volume in vivo using 143B cells transduced with an adenoviral vector expressing BMP-9. (B) The 143B cells transduced with BMP-2 or BMP-9, or the mock virus control AdGFP, did not reveal any ossification or morphologic changes consistent with increasing differentiation.

transmembrane protein Wfs1, integrin α7, and several muscular/cytoskeletal structure proteins (eg, myosin binding protein H, M-protein, microtubule associated protein tau, α-actinin 3, myosin alkali light chain, and a-actinin-2 associated LIM protein). These up-regulated and down-regulated target genes may belie a general trend in the early stage of BMP-mediated osteogenic signaling, indicating an increase in potential toward osteoblast lineage and a decrease in myogenic potential.

After the microarray data showed up-regulation of PPARγ after BMP stimulation, we next measured the ex-

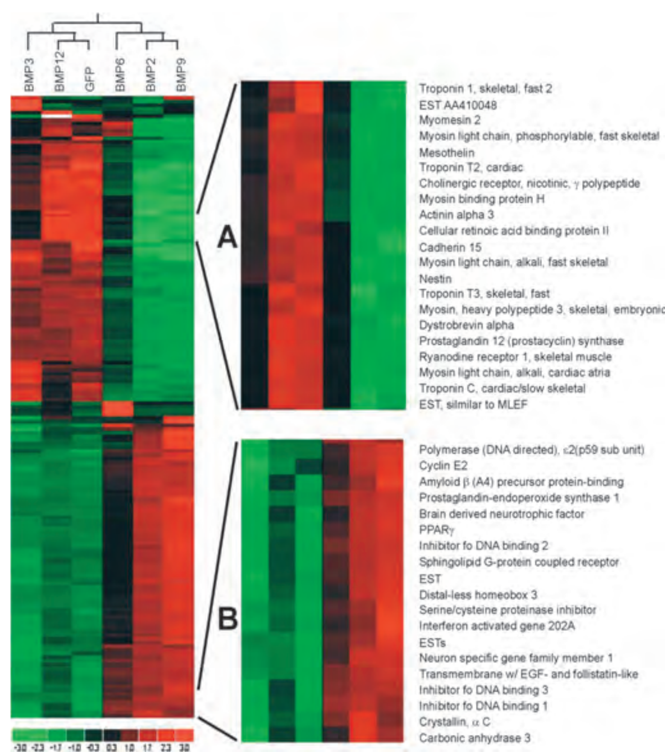

Fig 5A–B. Approximately 200 genes were selected using dCHIP data filtration default settings and used for hierarchical clustering analysis by dCHIP. Two representative subhierarchical clusters of (A) down-regulated and (B) up-regulated genes are shown. The expression level matrix is shown in a log ratio representing normalized values from –3 (green, below the mean) to 3 (red, above the mean). The mean (0 value) is black.

pression of PPARγ in four osteosarcoma cell lines and compared them with the positive control cell line HT-29. All cell lines expressed PPARγ, although variability in expression was detected using reverse transcription- polymerase chain reaction (RT-PCR) and Western blots.[16] To assess the effect of PPARγ activation on cell proliferation, the four osteosarcoma lines were treated with two PPARγ ligands (troglitazone and ciglitazone) or a RXR ligand (9-cis retinoic acid) at various concentrations. The cell lines exhibited various degrees of responsiveness to the three ligands (Fig 6A). As assessed by the Crystal Violet viability staining assays 5 days after the ligand treatment, complete inhibition of cell growth was observed in all four lines treated with 100 μm of troglitazone, whereas cell death also was observed in the MNNG/HOS cells treated with 50 μm of troglitazone. Inhibition of cell proliferation also was apparent in 143B, MG-63, and TE-85 cells treated with 50 μm of troglitazone. Cell death was observed in 143B and MNNG/HOS cells treated with 100 μm of 9-cis retinoic acid, whereas MG-63 and TE-85 cells exhibited a marginal responsiveness to the same dose. Cell

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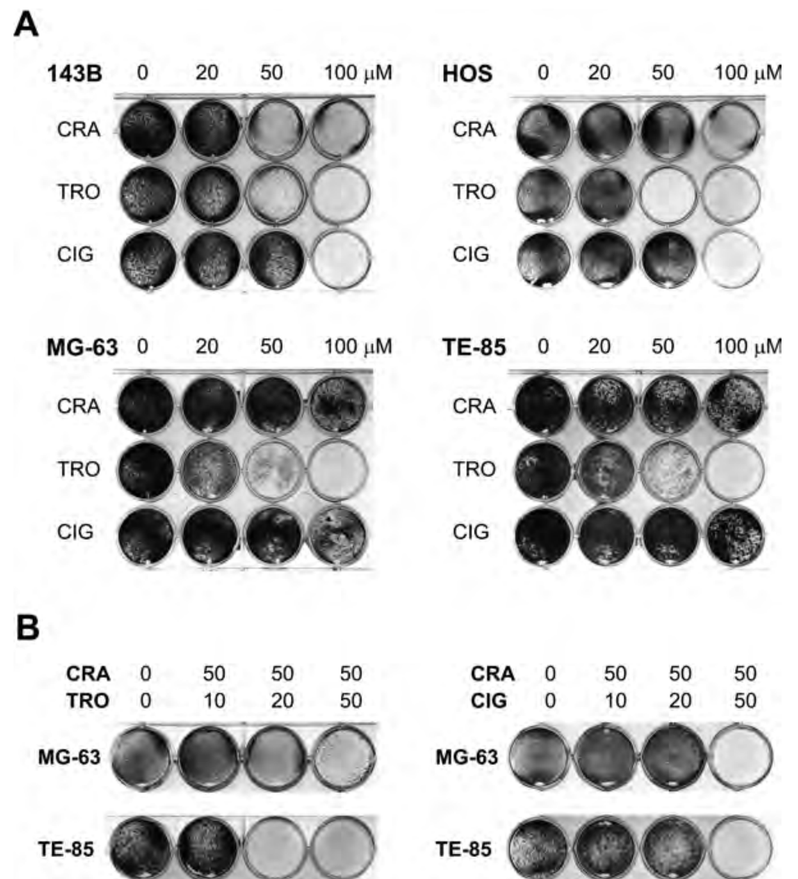

Fig 6A–B. Osteosarcoma cells affected by PPARγ and RXR ligands are shown. (A) Inhibition of the viability of osteosarcoma cells is shown after being treated with the three ligands individually. Exponentially growing cells were treated with 9-cis retinoic acid, troglitazone, or ciglitazone at the indicated concentrations. Five days after treatment, the cells were fixed and stained with crystal violet (original magnification) to observe the viable cells. Darker staining represents more viable cells. Each assay condition was performed in at least three independent experiments. (B) The synergistic effect between PPARγ and RXR agonists is shown. Subconfluent MG-63 and TE-85 cells were treated with a combination of 9-cis retinoic acid/troglitazone or 9-cis retinoic acid/ciglitazone at the indicated concentrations. Cell viability was seen by crystal violet staining (original magnification) 5 days after treatment. Each assay condition was performed in at least three independent experiments (CRA = 9-cis retinoic acid; TRO = troglitazone; CIG = ciglitazone).

death also was observed in 143B and MNNG/HOS cells treated with 100 μm of ciglitazone, and only a marginal effect on MG-63 and TE-85 cells was obtained when they were treated with the same dose of ciglitazone. A higher dose (200 μm) of ciglitazone was able to completely inhibit cell proliferation of MG-63 and TE-85 cells. The use of 9-cis retinoic acid with troglitazone or ciglitazone reduced the concentrations required to achieve cell death, suggesting synergy between the agonists (Fig 6B).[16]

To confirm that these ligands could induce apoptosis in the treated cells, the four osteosarcoma lines were plated at subconfluency and treated with 0 μm and 100 μm of troglitazone. Seventy-two hours after treatment, cells were collected and stained with Hoechst 33258. Apoptosis was observed in all four lines treated with 100 μm of troglitazone; indicating that troglitazone is an effective apoptosis-inducing agent for osteosarcoma cells (Fig 7A). A DNA ladder assay also was used to verify apoptosis (Fig 7B). Based on our preliminary experiments, TE-85 cells were chosen to test whether troglitazone could induce a differentiation phenotype. Subconfluent TE-85 cells were treated with 0 μm, 20 μm, and 50 μm of troglitazone. Seventy-two hours after the treatment, cells were fixed and stained with a mixture of naphthol AS-MX phosphate/Fast Blue BB salt. Induction of ALP activity was evident in the cells treated with 20 μm of troglitazone, and increased in those treated with 50 μm of troglitazone (Fig 7C). This suggests that PPARγ ligands can induce a lineage-specific differentiation phenotype in human osteosarcoma cells.[16]

DISCUSSION

The genetic changes that occur during the development of osteosarcoma are poorly understood. Dysregulation of tumor suppressor genes and oncogenes such as retinoblastoma, p53, and various genes critical to normal cell cycle control, genetic stability, and apoptosis/senescence have been identified in osteosarcoma.[15,28,29,36,42] However, causative mutations in these genes occur in no more than 30% to 35% of patients.[13,36,42] This suggests that there is not one common pathway underlying the development of osteosarcoma. Multiple interacting pathways are implicated in its pathogenesis, many of which become dysregulated as epiphenomena of other, as yet undefined, genetic alterations. Because osteosarcoma often shows evidence of dedifferentiation,[42] we hypothesized that defects in dif-

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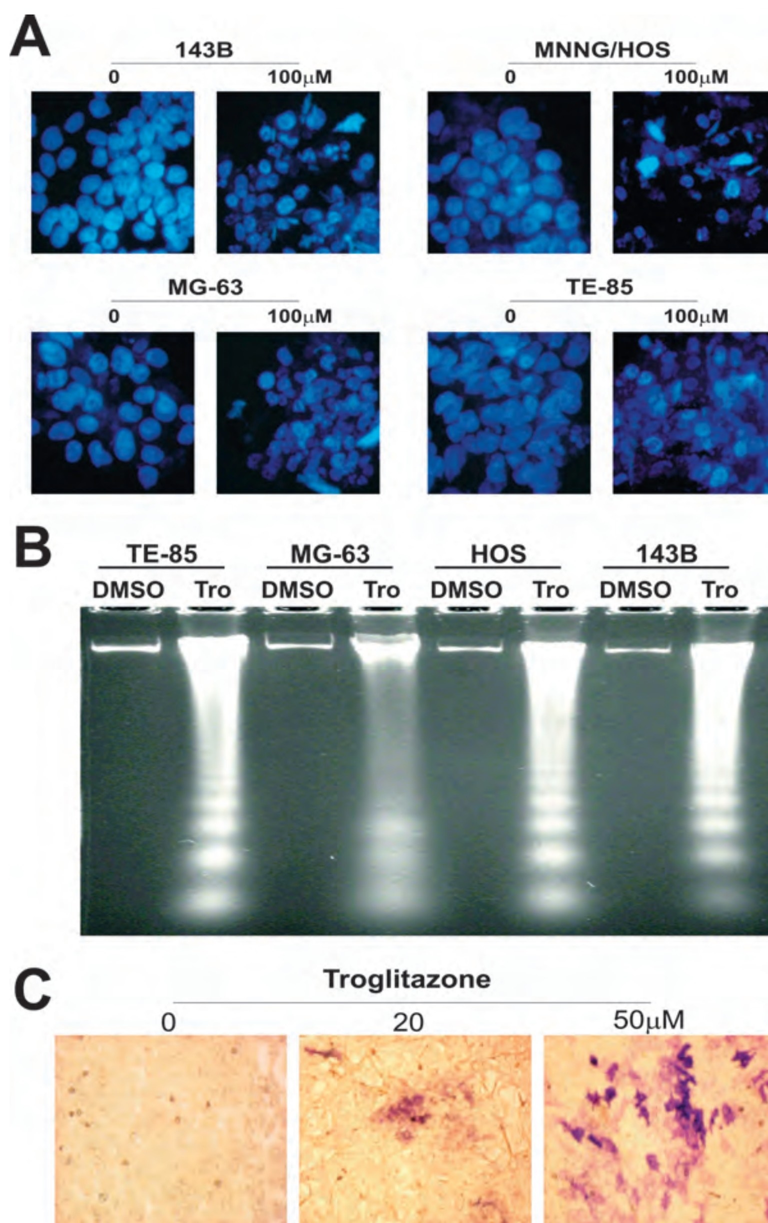

Fig 7A–C. Troglitazone-induced apoptosis and differentiation are shown in osteosarcoma cells. (A) The images show induction of apoptosis in osteosarcoma cells by PPARγ ligand troglitazone. The subconfluent osteosarcoma cells were treated with troglitazone at the indicated concentrations. Seventy-two hours after treatment, cells were harvested and stained with Hoechst 33258 (Molecular Probes, Eugene, OR) (×20 magnification) to observe the presence of apoptotic nuclei under the fluorescence microscopy. (B) Troglitazone-induced DNA fragmentation is shown. Subconfluent cells were treated with troglitazone (100 μmol/L) or DMSO for approximately 48 hours. The cell-containing medium was collected along with the attached cells by trypsinization. The cell pellets were lysed in DNA extraction and digested with proteinase K. Genomic DNA was isolated by phenol chloroform extraction and ethanol precipitation. The samples were resolved on a 1.4% agarose gel and observed by ethidium bromide staining. (C) The images show differentiation of the osteosarcoma line TE-85 by PPARγ ligand troglitazone. Subconfluent TE-85 cells were treated with troglitazone at the indicated concentrations. Three days after treatment, the cells were fixed with 0.05% (v/v) glutaraldehyde (Sigma-Aldrich). Induction of ALP activity was measured using histochemical staining (×20 magnification) with a mixture of 0.1 mg/mL naphthol AS-MX phosphate and 0.6 mg/mL Fast Blue BB salt (Sigma-Aldrich). Histochemical staining was recorded by using bright field microscopy.

ferentiation may play an important role in the oncogenesis of osteosarcoma. Furthermore, an improved understanding of the relationship between osteoblastic differentiation and osteosarcoma may lead to novel treatments for this challenging neoplasm.

The current study has certain limitations that must be considered. First, the impact of osteogenic BMPs on osteosarcoma was evaluated in a commercial cell line that may differ from primary cell lines. Variability in the expression of the BMPs and their receptors has been encountered in primary cell lines, suggesting that the impact of BMP expression may be uneven.[14] Tumors that express BMPs and their receptors have been associated with a poorer clinical outcome and an increased metastatic potential compared with tumors that do not express these proteins.[45] Therefore, even though the 143B cell line may not be representative of osteosarcoma in general, there is significant evidence that BMP activation supports an aggressive pattern of growth and metastasis in clinical studies. Another limitation of this study is the lack of correlative studies of the expression patterns of the Id-genes in osteosarcoma. The expression profile data in C2C12 suggest that this is an important downstream target of osteogenic BMPs, but this has not been documented in osteosarcoma cell lines. Given that the emphasis of this study was to identify therapeutic targets, we focused our fol-

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lowup experiments on PPARγ. These included only in vitro experiments. In vivo experiments using PPARγ agonists are needed to show efficacy with respect to primary tumor growth and/or reduction in the rate of metastasis.

In this study, we showed that BMP-2, BMP-6, and BMP-9 (and to a lesser extent, BMP-4 and BMP-7), were the most osteogenic BMPs in the in vitro and in vivo assays. When osteosarcoma cell lines were exposed to this panel of osteogenic BMPs, the tumor failed to undergo differentiation as was observed in osteoprogenitor cells and remained in the early proliferative phase (Fig 8). This implies that inherent blocks to the effects of osteogenic BMPs exist in certain osteosarcoma cell lines. Our expression profile analysis of the downstream targets of osteogenic BMPs revealed significant up-regulation of Id1, Id2, and Id3. The burgeoning family of helix-loop-helix (HLH) transcriptional regulatory proteins has been recognized to play an essential role in regulating cell growth and differentiation.[26,31,35,43] Most HLH transcription factors exemplified by MyoD possess a highly basic domain adjacent to the HLH region. It frequently is referred to as the bHLH domain, in which the basic region binds to DNA and the HLH domain is responsible for dimerization.[26,43] The Id proteins lack such a basic region, and function as dominant-negative regulators of bHLH proteins through formation of Id-bHLH heterodimers.[31] Our findings suggest that blocks to osteoblastic differentiation may be important features of osteosarcoma, essentially arresting tumor cells during the proliferative phase of osteoblastic differentiation. The Id genes play a key role in promoting cell proliferation during this phase of osteoblastic differentiation,[35] and also may play an important role in supporting cell proliferation and actively inhibiting differentiation in osteosarcoma.

Another important finding in expression profile analysis was the up-regulation of PPARγ. Peroxisome proliferators activated receptors originally were identified as nuclear receptors that mediate the biologic effects of a group of synthetic compounds called peroxisome proliferators.[23] Three subtypes of PPARs have been identified in mammalian cells: PPARα, PPARγ, and PPARδ (PPARβ or NUC1).[1,18,23,37] Peroxisome proliferator activated receptor α is a critical mediator of the hepatocarcinogenic effects of certain peroxisome proliferators in rodents.[5,46] Peroxisome proliferator activated receptor γ is expressed mainly in adipose tissue, and to a lesser extent in colon and other tissues.[23] Peroxisome proliferator activated receptor δ is ubiquitously expressed with the highest level found in the gut, kidney, brain, and heart, and has been implicated in colorectal tumorigenesis.[23] Similar to other nuclear receptors, PPARs are ligand-activated transcription factors in which transcriptional activation of target genes depends

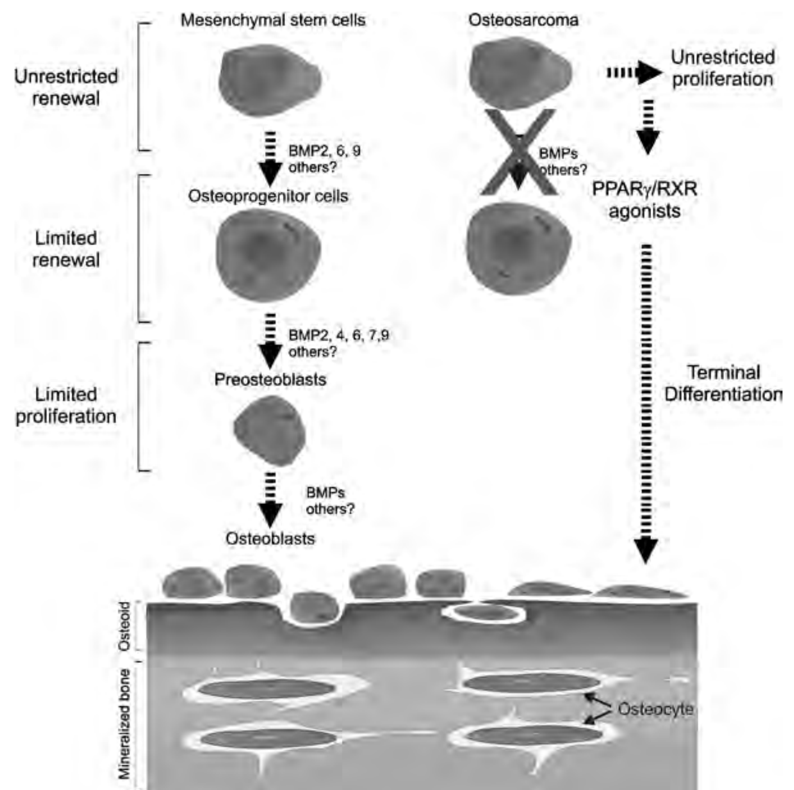

Fig 8. A conceptual diagram of differences between osteoprogenitor cells and osteosarcoma is shown, particularly with respect to their response to BMP-induced osteogenic differentiation. Blocks to BMP-induced differentiation may suspend cells in an early proliferative phase, supported in part through up-regulation of factors such as the Id-family of genes. Other downstream genes such as PPARγ may be attractive targets for therapy, and can be used to circumvent these blocks to differentiation, and induce terminal differentiation.

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on the binding of ligand to the receptor.[25] Peroxisome proliferators activated receptors are fully functional only when they heterodimerize with the retinoid X receptor, RXR.

One study showed that activation of PPARγ receptors in nonadipocytes leads to growth arrest and a differentiation phenotype.[33] In particular, exposure of several types of tumors to PPARγ ligands has led to terminal differentiation and apoptosis.[2,6,8,11,12,21,22,27,30,38,39] Adipocytes and osteoblasts are derived from the same bone marrow stromal progenitor cells;[3,4] therefore, we hypothesized that activation of PPARγ/RXR activity by their agonists such as troglitazone, ciglitazone, and/or 9-cis retinoic acid would induce terminal differentiation and apoptosis in osteosarcoma cells. To test the therapeutic potential of these agonists for human osteosarcoma, we confirmed that the expression of PPARγ was readily detected in human osteosarcoma cell lines. When the osteosarcoma lines were treated with various concentrations of PPARγ agonists, troglitazone, and ciglitazone, RXR ligand, and 9-cis retinoic acid, all three ligands exhibited the ability to inhibit cell proliferation and to induce apoptosis, with troglitazone as the most potent of these agents. A synergistic effect on the induction of cell death was observed when troglitazone and 9-cis retinoic acid or ciglitazone and 9-cis retinoic acid were administered. Troglitazone effectively induced the activity of ALP, a hallmark of lineage-specific osteoblastic differentiation.[16] Our findings suggest that ligands for PPARγ and/or RXR may be used as safer, more efficacious treatment alternatives for primary osteosarcoma, and may promote differentiation in osteosarcoma through alternate pathways distinct from osteogenic BMPs.

In tumors such as osteosarcoma, where long-term survival is largely determined by prevention and/or treatment of metastatic disease, nuclear receptor agonists are a particularly attractive alternative to current chemotherapeutic modalities. Our findings suggest that PPARγ/RXR agonists may induce differentiation in osteosarcoma despite critical blocks to normal BMP-regulated pathways (Fig 8), an approach that could be used as a promising chemotherapeutic and/or chemopreventive agent for this challenging clinical problem.

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References

1. Amri EZ, Bonino F, Ailhaud G, Abumrad NA, Grimaldi PA. Cloning of a protein that mediates transcriptional effects of fatty acids in preadipocytes: homology to peroxisome proliferator-activated receptors. *J Biol Chem*. 1995;270:2367–2371.
2. Asou H, Verbeek W, Williamson E, Elstner E, Kubota T, Kamada N, Koeffler HP. Growth inhibition of myeloid leukemia cells by troglitazone, a ligand for peroxisome proliferator activated receptor gamma, and retinoids. *Int J Oncol*. 1999;15:1027–1031.
3. Aubin JE. Bone stem cells. *J Cell Biochem Suppl*. 1998;30-31: 73–82.
4. Caplan AI, Bruder SP. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. *Trends Mol Med*. 2001;7: 259–264.
5. Cattley RC, DeLuca J, Elcombe C, Fenner-Crisp P, Lake BG, Marsman DS, Pastoor TA, Popp JA, Robinson DE, Schwetz B, Tugwood J, Wahli W. Do peroxisome proliferating compounds pose a hepatocarcinogenic hazard to humans? *Regul Toxicol Pharmacol*. 1998; 27:47–60.
6. Chang TH, Szabo E. Induction of differentiation and apoptosis by ligands of peroxisome proliferator-activated receptor gamma in non-small cell lung cancer. *Cancer Res*. 2000;60:1129–1138.
7. Cheng H, Jiang W, Phillips FM, Haydon RC, Peng Y, Zhou L, Luu HH, An N, Breyer B, Vanichakarn P, Szatkowski JP, Park JY, He TC. Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). *J Bone Joint Surg Am*. 2003;85: 1544–1552.
8. Clay CE, Namen AM, Atsumi G, Willingham MC, High KP, Kute TE, Trimboli AJ, Fonteh AN, Dawson PA, Chilton FH. Influence of J series prostaglandins on apoptosis and tumorigenesis of breast cancer cells. *Carcinogenesis*. 1999;20:1905–1911.
9. Coleman WB, Tsongalis GJ. Molecular mechanisms of human carcinogenesis. *EXS*. 2006;96:321–349.
10. De Biase P, Capanna R. Clinical applications of BMPs. *Injury*. 2005;36(Suppl 3):S43–S46.
11. Demetri GD, Fletcher CD, Mueller E, Sarraf P, Naujoks R, Campbell N, Spiegelman BM, Singer S. Induction of solid tumor differentiation by the peroxisome proliferator-activated receptor-gamma ligand troglitazone in patients with liposarcoma. *Proc Natl Acad Sci USA*. 1999;96:3951–3956.
12. Elstner E, Muller C, Koshizuka K, Williamson EA, Park D, Asou H, Shintaku P, Said JW, Heber D, Koeffler HP. Ligands for peroxisome proliferator-activated receptorgamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. *Proc Natl Acad Sci USA*. 1998;95: 8806–8811.
13. Gokgoz N, Wunder JS, Mousses S, Eskandarian S, Bell RS, Andrulis IL. Comparison of p53 mutations in patients with localized osteosarcoma and metastatic osteosarcoma. *Cancer*. 2001;92: 2181–2189.
14. Guo W, Gorlick R, Ladanyi M, Meyers PA, Huvos AG, Bertino JR, Healey JH. Expression of bone morphogenetic proteins and receptors in sarcomas. *Clin Orthop Relat Res*. 1999;365:175–183.
15. Haydon RC, Deyrup A, Ishikawa A, Heck R, Jiang W, Zhou L, Feng T, King D, Cheng H, Breyer B, Peabody T, Simon MA, Montag AG, He TC. Cytoplasmic and/or nuclear accumulation of the beta-catenin protein is a frequent event in human osteosarcoma. *Int J Cancer*. 2002;102:338–342.
16. Haydon RC, Zhou L, Feng T, Breyer B, Cheng H, Jiang W, Ishikawa A, Peabody T, Montag A, Simon MA, He TC. Nuclear receptor agonists as potential differentiation therapy agents for human osteosarcoma. *Clin Cancer Res*. 2002;8:1288–1294.
17. Hogan BL. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev*. 1996;10:1580–1594.
18. Jow L, Mukherjee R. The human peroxisome proliferator-activated receptor (PPAR) subtype NUC1 represses the activation of hPPAR alpha and thyroid hormone receptors. *J Biol Chem*. 1995;270: 3836–3840.
19. Kang Q, Sun MH, Cheng H, Peng Y, Montag AG, Deyrup AT, Jiang W, Luu HH, Luo J, Szatkowski JP, Vanichakarn P, Park JY, Li Y, Haydon RC, He TC. Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery. *Gene Ther*. 2004;11:1312–1320.
20. Khurana JS, Ogino S, Shen T, Parekh H, Scherbel U, Delong W,

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Feldman MD, Zhang PJ, Wolfe HJ, Alman BA. Bone morphogenetic proteins are expressed by both bone-forming and non-bone-forming lesions. *Arch Pathol Lab Med*. 2004;128:1267–1269.

21. Kitamura S, Miyazaki Y, Shinomura Y, Kondo S, Kanayama S, Matsuzawa Y. Peroxisome proliferator-activated receptor gamma induces growth arrest and differentiation markers of human colon cancer cells. *Jpn J Cancer Res*. 1999;90:75–80.
22. Kubota T, Koshizuka K, Williamson EA, Asou H, Said JW, Holden S, Miyoshi I, Koeffler HP. Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo. *Cancer Res*. 1998;58:3344–3352.
23. Lemberger T, Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: a nuclear receptor signaling pathway in lipid physiology. *Annu Rev Cell Dev Biol*. 1996;12:335–363.
24. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci USA*. 2001;98:31–36.
25. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. The nuclear receptor superfamily: the second decade. *Cell*. 1995; 83:835–839.
26. Massari ME, Murre C. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Mol Cell Biol*. 2000;20:429–440.
27. Mehta RG, Williamson E, Patel MK, Koeffler HP. A ligand of peroxisome proliferator-activated receptor gamma, retinoids, and prevention of preneoplastic mammary lesions. *J Natl Cancer Inst*. 2000;92:418–423.
28. Mendoza S, Konishi T, Dernell WS, Withrow SJ, Miller CW. Status of the p53, Rb and MDM2 genes in canine osteosarcoma. *Anticancer Res*. 1998;18:4449–4453.
29. Miller CW, Aslo A, Won A, Tan M, Lampkin B, Koeffler HP. Alterations of the p53, Rb and MDM2 genes in osteosarcoma. *J Cancer Res Clin Oncol*. 1996;122:559–565.
30. Mueller E, Sarraf P, Tontonoz P, Evans RM, Martin KJ, Zhang M, Fletcher C, Singer S, Spiegelman BM. Terminal differentiation of human breast cancer through PPAR gamma. *Mol Cell*. 1998;1: 465–470.
31. Norton JD. ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. *J Cell Sci*. 2000;113:3897–3905.
32. Olsen BR, Reginato AM, Wang W. Bone development. *Annu Rev Cell Dev Biol*. 2000;16:191–220.
33. Park BH, Breyer B, He TC. Peroxisome proliferator-activated receptors: roles in tumorigenesis and chemoprevention in human cancer. *Curr Opin Oncol*. 2001;13:78–83.
34. Peng Y, Kang Q, Cheng H, Li X, Sun MH, Jiang W, Luu HH, Park JY, Haydon RC, He TC. Transcriptional characterization of bone morphogenetic proteins (BMPs)-mediated osteogenic signaling. *J Cell Biochem*. 2003;90:1149–1165.
35. Peng Y, Kang Q, Luo Q, Jiang W, Si W, Liu BA, Luu HH, Park JK, Li X, Luo J, Montag AG, Haydon RC, He TC. Inhibitor of DNA binding/differentiation helix-loop-helix proteins mediate bone morphogenetic protein-induced osteoblast differentiation of mesenchymal stem cells. *J Biol Chem*. 2004;279:32941–32949.
36. Sandberg AA, Bridge JA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: osteosarcoma and related tumors. *Cancer Genet Cytogenet*. 2003;145:1–30.
37. Schmidt A, Endo N, Rutledge SJ, Vogel R, Shinar D, Rodan GA. Identification of a new member of the steroid hormone receptor superfamily that is activated by a peroxisome proliferator and fatty acids. *Mol Endocrinol*. 1992;6:1634–1641.
38. Takahashi N, Okumura T, Motomura W, Fujimoto Y, Kawabata I, Kohgo Y. Activation of PPARgamma inhibits cell growth and induces apoptosis in human gastric cancer cells. *FEBS Lett*. 1999; 455:135–139.
39. Tontonoz P, Singer S, Forman BM, Sarraf P, Fletcher JA, Fletcher CD, Brun RP, Mueller E, Altiok S, Oppenheim H, Evans RM, Spiegelman BM. Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor gamma and the retinoid X receptor. *Proc Natl Acad Sci USA*. 1997;94:237–241.
40. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA*. 2001;98:5116–5121.
41. Urist MR. Bone: formation by autoinduction. *Science*. 1965;150: 893–899.
42. Wang LL. Biology of osteogenic sarcoma. *Cancer J*. 2005;11: 294–305.
43. Weintraub H, Davis R, Tapscott S, Thayer M, Krause M, Benezra R, Blackwell TK, Turner D, Rupp R, Hollenberg S, Zhuang Y, Lassar A. The myoD gene family: nodal point during specification of the muscle cell lineage. *Science*. 1991;251:761–766.
44. Yoon ST, Boden SD. Osteoinductive molecules in orthopaedics: basic science and preclinical studies. *Clin Orthop Relat Res*. 2002; 395:33–43.
45. Yoshikawa H, Takaoka K, Masuhara K, Ono K, Sakamoto Y. Prognostic significance of bone morphogenetic activity in osteosarcoma tissue. *Cancer*. 1988;61:569–573.
46. Youssef JA, Badr MZ. Aging and enhanced hepatocarcinogenicity by peroxisome proliferator-activated receptor alpha agonists. *Ageing Res Rev*. 2005;4:103–118.
47. Yu Y, Harris RI, Yang JL, Anderson HC, Walsh WR. Differential expression of osteogenic factors associated with osteoinductivity of human osteosarcoma cell lines. *J Biomed Mater Res A*. 2004;70: 122–128.

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