Osterix, a Transcription Factor for Osteoblast Differentiation, Mediates Antitumor Activity in Murine Osteosarcoma

Ying Cao, Zhichao Zhou, Benoit de Crombrugghe, Kazuhisa Nakashima, Hui Guan, Xiaoping Duan, Shu-Fang Jia, and Eugenie S. Kleinerman

Abstract

Osterix is a novel zinc finger–containing transcription factor that is essential for osteoblast differentiation and bone formation. We hypothesized that osterix might have a role in osteosarcoma tumor growth and metastasis. Northern blot analysis showed that the mRNA level of osterix was decreased in two mouse osteosarcoma cell lines compared with its level in normal mouse osteoblasts. Osterix expression was also decreased in three human osteosarcoma cell lines. Transfection of the osx gene into the mouse osteosarcoma cells inhibited tumor cell growth in vitro and in vivo and significantly reduced tumor incidence, tumor volume, and lung metastasis following intratibial injection. Osterix expression was also associated with decreased osteolysis. Using an in vitro migration assay, osterix suppressed the migration of tumor cells to lung extracts. These results suggest that osterix expression may play a role in osteosarcoma tumor growth and metastasis.

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Introduction

Although osteosarcoma is a rare malignant disease, it is the most common primary malignant bone tumor in both children and adults (1). The disease initially presents in the distal femur, proximal tibia, or proximal humerus (2). The pathogenesis of osteosarcoma has been linked to alterations in several genes (3), including mutations in Rb (4) and p53 (5). However, mutation in these genes are not consistent, suggesting that other genes may contribute to the development of this tumor. Osterix, a recently identified zinc finger–containing transcription factor, is expressed in the osteoblasts of all endochondral and membranous bones. In osx-null mutant mice, neither endochondral nor intramembranous bone formation occurs and osteoblast differentiation is arrested (6). Because osx is an essential gene for osteoblast differentiation, we postulated that osterix down-regulation might be involved in the pathogenesis of osteosarcoma. We used a mouse model of osteosarcoma to investigate the role of osterix in the pathogenesis of this tumor. Osterix was down-regulated in two mouse osteosarcoma cell lines, and its expression was inversely correlated with metastatic potential. Replacement of osterix by transfection inhibited tumor cell growth in vitro and in vivo and reduced the metastatic potential of the tumor cells. Our results showed that osterix may be important in the etiology of osteosarcoma.

Materials and Methods

Cell Lines and Mouse Model. The clonally related variants K7 and K7M2 (obtained from Dr. C. Khanna, Pediatric Oncology Branch, National Cancer Institute, Bethesda, MD) were derived from a spontaneously occurring murine osteosarcoma cell (7). K7 and K7M2 differ in pulmonary metastatic potential, with K7M2 having more metastatic potential. These mouse osteosarcoma cells and the MC3T3 normal murine osteoblasts were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum. Human osteosarcoma cell lines HOS, Krib, and MG were also maintained in DMEM supplemented with 10% fetal bovine serum. Normal human osteoblasts were purchased from Clonetics, Inc. (San Diego, CA), and maintained in the special medium provided by Clonetics. Male, 4-week-old, specific pathogen-free BALB/c mice were purchased from Charles River Breeding Laboratories (Kingston, MA). The mice were maintained in an animal facility approved by the American Association of Laboratory Animal Care in accordance with the current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and the NIH. Mice were housed five per cage and kept in a laminar flow cabinet under specific pathogen-free conditions for 2 weeks before being used for any experiments.

Cell Transfection. K7M2 cells were transfected with either an osterix expression vector (pTriEx-1.1 hygro-osx) or the control vector pTriEx-1.1 hygro (Novagen, EMD Biosciences, Inc., San Diego, CA) using FuGene 6 (Roche Applied Sciences, Indianapolis, IN) and selected in 100 μg/mL hygromycin (Roche Applied Sciences). The resulting K7M2 clones (K7M2-osx-1 and K7M2-osx-2, respectively) were then maintained in complete medium containing 100 μg/mL hygromycin for 3 months and then screened by Northern blot analysis to assess osterix expression.

Northern Blot Analysis. Total RNA was isolated from cells using a TRIZOL RNA isolation kit (Life Technologies, Inc., Grand Island, NY). Twenty micrograms of total RNA was separated by electrophoresis under denaturing conditions and then transferred to a Hybond N+ membrane. Mouse and human osx cDNAs (6) were labeled with 32P using the Rediprime DNA labeling system (Amersham Biosciences, Piscataway, NJ). After prehybridization with 10 mL Rapidhyb buffer (Amersham Biosciences), the resulting K7M2 clones (K7M2-osx-1 and K7M2-osx-2, respectively) were then maintained in complete medium containing 100 μg/mL hygromycin for 3 months and then screened by Northern blot analysis to assess osterix expression.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay. Cells (5 × 104) were plated in 96-well culture plates in 0.2 mL culture medium. At 24, 48, and 72 hours after plating, 60 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (2.5 mg/mL, Sigma Chemical Co., St. Louis, MO) was added to each well. Cells were cultured for an additional 2 hours, the supernatant was removed, and 100 μL dimethyl sulfoxide was added to each well. After shaking for 30 seconds, absorbance was measured at 570 nm.

In vivo Analyses. K7M2, K7M2-neo, K7M2-osx-1, and K7M2-osx-2 cells in separate single-cell suspensions (1 × 106) in HBSS (4°C) were injected into the left tibia of the mice. Briefly, mice were anesthetized with Nembutal anesthetic mix, and a 30G1/2 needle was inserted into the proximal end of the left tibia followed by injection of 10 μL cell suspension. Animals were sacrificed 35 days after tumor inoculation. Tumor incidence was determined...
Osterix Inhibits Osteosarcoma Tumor Growth and Metastasis

The tumor volume was calculated using the following formula: volume = \text{length} \times \text{width}^2/2. The grading scheme for quantification of bone lysis has been described previously (8). The lungs were resected and fixed in 10% formalin for 2 days, dissected into five lobes, paraffin embedded, divided into 5 μm sections, and stained with H&E to evaluate metastatic foci.

In vitro Migration Assay. The migration assay was done in 24-well chambers with an 8 μm pore polycarbonate filter (Corning, Inc., Corning, NY). Cells from each cell line were seeded into the top chamber. The bottom chamber was filled with 10% fetal bovine serum containing DMEM or lung extract. The lung extract was prepared from lungs of normal mice homogenized in complete DMEM. The chambers were incubated at 37°C for 4 hours. The cells were then fixed and visualized using a Hema-Diff staining kit (Statlab Medical Products, Inc., Lewisville, TX). The number of cells that migrated to the bottom of the filter were counted (five high-power fields per membrane). Experiments were set up in triplicate, and the average ± SD was determined.

Histologic Assessment of Bone. Bone specimens were fixed in 10% formalin for 24 hours, then decalcified for 14 days using 10% EDTA (pH 7.4). The specimens were then paraffin embedded, divided into 5 μm sections, and stained with H&E to for histologic assessment.

Reverse Transcription-PCR. Total RNA was extracted from different cell lines using a Reverse Transcription System (Promega Corp., Madison, WI). Reverse transcription products were amplified by PCR using specific primers for murine receptor activator of NF-κB ligand (RANKL; sense 5′-GGTCGGGCAATTCTGAATT-3′; antisense 5′-GGAATTACAAAGTGCACCAG-3′). The initial denaturation was done at 94°C for 5 minutes. Then, the products were subjected to denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 1 minute for 28 cycles, and a final elongation at 72°C for 10 minutes. The PCR products were subjected to electrophoresis on 1% agarose gel with ethidium bromide and visualized under UV light. The glyceraldehyde-3-phosphate dehydrogenase primers and competimers (Ambion, Austin, TX) were used as the internal controls.

Statistics. After performing a one-way ANOVA to compare the means, comparisons among groups were made using the two-tailed, unpaired Student’s t test. A difference was considered significant at \( P < 0.05 \).

Results

Expression of Osterix Was Down-regulated in K7 and K7M2 Murine Osteosarcoma Cells and in HOS, Krib, and MG Human Osteosarcoma Cells. On Northern blot analysis, osterix expression was shown to be significantly decreased in K7 cells and nearly absent in K7M2 cells compared with its expression in normal murine osteoblast MC3T3 cells. Osterix expression was inversely correlated with the metastatic potential of the cells. Similarly, human osterix expression was almost absent in HOS, Krib, and MG cells compared with its expression in normal human osteoblast cells (Fig. 1A). Because osteosarcoma is a malignant tumor that originates within bone (9), this result suggested that decreased osterix expression might be involved in osteosarcoma pathogenesis and metastasis.

Restoration of Osterix Inhibited K7M2 Cell Growth In vitro and In vivo. Having determined that osterix expression was down-regulated in K7M2, we then investigated whether increasing the expression of osterix affected cell growth in vitro (Fig. 1B). The growth rates of K7M2-osx-1 and K7M2-osx-2 were slower than that of K7M2 and the K7M2-neo control but similar to the growth rate of the normal MC3T3 cells (data not shown). The percentage of apoptotic cells was assessed using flow cytometry. No significant apoptosis was shown in any of the cell populations (data not shown), indicating that apoptosis does not play a role in osterix-mediated growth inhibition.

We next investigated whether osterix expression affected tumor cell growth in vivo. K7M2-osx-1, K7M2-osx-2, K7M2-neo, and K7M2 cells were injected intratibially. After 35 days, the tumor incidence was determined by radiography. The mice injected with K7M2-osx-1 or K7M2-osx-2 cells had a lower incidence of tumor development than the mice injected with the vector control and parental cells (Fig. 2A). The tumors that developed in mice injected with K7M2-osx-1 and K7M2-osx-2 were substantially smaller (Fig. 2B).

Osterix Expression Suppressed Tumor-Induced Osteolysis. Osteosarcoma lesions are characterized by bone destruction (9). Osterix is specifically expressed in osteoblast cells (6); therefore, expression of osterix may inhibit the bone destruction caused by osteosarcoma. Leg tumors were examined by radiography 35 days following the intratibial injection of tumor cells. As shown in Fig. 3A, osteolytic lesions developed in mice injected with K7M2 cells. Of the 10 mice inoculated with K7M2 cells, 6 exhibited osteolytic lesions detectable by radiographs (Table 1). The histologic analysis also revealed that tumor cells had infiltrated the bone cortex in the mice with K7M2 tumors (Fig. 3B).
inoculated with K7M2-neo control cells had a similar phenotype (Fig. 3A-C; Table 1). By contrast, when mice were inoculated with K7M2-osx-1 and K7M2-osx-2 cells, only 10% and 18%, respectively, developed osteolysis (Fig. 3A; Table 1). Bone cortex infiltration was not observed in the osterix-expressing tumors (Fig. 3B). Furthermore, lung metastases developed in only 30% to 35% of mice injected with K7M2-osx-1 and K7M2-osx-2 cells, respectively, developed lung metastases (Table 1). By analysis, 60% and 68% of mice injected with K7M2 or K7M2-neo cells, respectively, developed lung metastases (Table 1). Based on the histologic examination for lung metastasis. Mice were sacrificed 35 days after the intratibial injection of tumor cells. The lungs were resected and histologically examined to see if metastatic potential in lung was expressed. Because our data showed that the expression of osterix was decreased in K7 and near absent in K7M2 cells (Fig. 1A), a possible link with metastasis as well as tumor formation was hypothesized. We therefore assessed the metastatic potential of the osterix-transfected clones. Mice were sacrificed 35 days after the intratibial injection of tumor cells. The lungs were resected and examined for lung metastasis. Based on the histologic analysis, 60% and 68% of mice injected with K7M2 or K7M2-neo cells, respectively, developed lung metastases (Table 1). By contrast, lung metastases developed in only 30% to 35% of mice inoculated with K7M2-osx-1 or K7M2-osx-2 cells (Table 1). The incidence of metastasis was similar to that in mice injected with poorly metastatic K7 cells (i.e., 33%). Therefore, transfection of osterix also decreased the metastatic potential of K7M2 cells.

**Osterix Expression Suppressed Lung Metastasis In vivo.** K7M2 cells were derived from the K7 cell line (10) by repeated cycling of cells from pulmonary metastases into the orthotopic site (7). K7M2 cells are more aggressive locally and have a greater potential to metastasize to lung than K7 cells (11). Because our data showed that the expression of osterix was decreased in K7 and near absent in K7M2 cells (Fig. 1A), a possible link with metastasis as well as tumor formation was hypothesized. We therefore assessed the metastatic potential of the osterix-transfected clones. Mice were sacrificed 35 days after the intratibial injection of tumor cells. The lungs were resected and examined for lung metastasis. Based on the histologic analysis, 60% and 68% of mice injected with K7M2 or K7M2-neo cells, respectively, developed lung metastases (Table 1). By contrast, lung metastases developed in only 30% to 35% of mice inoculated with K7M2-osx-1 or K7M2-osx-2 cells (Table 1). The incidence of metastasis was similar to that in mice injected with poorly metastatic K7 cells (i.e., 33%). Therefore, transfection of osterix also decreased the metastatic potential of K7M2 cells.

**Osterix Suppressed the Migration of K7M2 to Lung Conditioned Medium.** The process of metastases involves the migration of cells from the primary tumor to distant organ sites. Because we showed the decreased metastatic potential of the osterix-transfected cells to the lung, we did an in vitro migration assay using lung homogenate as the chemotactic stimulant. The migration of K7M2-osx-1 and K7M2-osx-2 cells to medium containing a lung homogenate was significantly less (18 cells ± 1.5/5 high-power fields and 20 cells ± 0.71/5 high-power fields, respectively) than that of vector control (48 cells ± 3.62/5 high-power fields) and parental K7M2 cells (50 cells ± 5.74/5 high-power fields).

**Discussion**

Osterix is a newly discovered transcription factor that is essential for osteoblast differentiation and bone formation (6). However, its role in bone tumor development has not been explored. In the present study, osterix expression was decreased in murine osteosarcoma cells compared with normal mouse osteoblasts. Human osterix expression was also nearly absent in human osteosarcoma cells compared with normal human osteoblasts. Furthermore, osterix expression was inversely correlated with the metastatic potential of the cells. Although K7 osteosarcoma cells, which form primary bone tumors but rarely metastasize to the lung, had decreased levels of osterix mRNA compared with the normal osteoblast cells, the highly metastatic K7M2 cells expressed little to no osterix. Restoration of osterix via gene transfection resulted in increased inhibition in vitro and in vivo, decreased tumor formation following intratibial injection, and decreased metastatic spread to the lungs. We also observed a reduced chemotactic response to lung extracts in the osterix-transfected cell lines.

![Figure 2](image-url)

**Figure 2.** Osterix expression suppressed tumorigenicity in vivo. K7M2, K7M2-neo, K7M2-osx-1, and K7M2-osx-2 cells were injected into the left tibia of BALB/c mice. Mice were sacrificed 35 days after tumor cell injection. Tumor size was measured and tumor volume calculated at the time of sacrifice. Mice injected with osterix-expressing K7M2 cells had a lower incidence of tumor formation and smaller tumors than those injected with K7M2 or K7M2-neo cells.

<table>
<thead>
<tr>
<th>Study group</th>
<th>Osteolytic lesion incidence (%)</th>
<th>Bone lysis (micro and macro, %)</th>
<th>Lung metastases incidence (%)</th>
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</thead>
<tbody>
<tr>
<td>K7M2</td>
<td>65</td>
<td>2.2</td>
<td>60</td>
</tr>
<tr>
<td>K7M2-neo</td>
<td>54</td>
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<td>68</td>
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<tr>
<td>K7M2-osx-1</td>
<td>10†</td>
<td>0.3‡</td>
<td>30i</td>
</tr>
<tr>
<td>K7M2-osx-2</td>
<td>18*</td>
<td>0.4*</td>
<td>35‡</td>
</tr>
</tbody>
</table>

* K7M2, K7M2-neo, K7M2-osx-1, and K7M2-osx-2 cells were injected into the left tibia of BALB/c mice. The mice (10 per group) were sacrificed 35 days after tumor cell injection. Results are one representative experiment of two.

† P < 0.05 compared with control groups.

‡ P < 0.01 compared with control groups.

§ P < 0.05 compared with control groups.

**P** < 0.01 compared with control groups.
In addition to inhibiting tumorigenicity, transfection of osterix into K7M2 cells altered the osteolytic morphology of the tumors. In comparison with K7M2 and K7M2-neo transfected cells, K7M2-osx cells did not result in lytic bone destruction. Osterix expression inhibited the infiltration of the tumor cells into the bone cortex, thus preserving the bone trabecula architecture (Fig. 3B and C).

Osteosarcoma, a primary malignant bone tumor, destroys the cancellous and cortical areas of bone as the tumor grows. This bone destruction is thought to be mediated by osteoclast activity (9). Decreased osterix expression may, therefore, play a role not only in tumor development but also in the tumor phenotype. Decreased osterix expression would result in decreased osteoblast differentiation and increased osteoclast activity leading to lytic destruction as the tumor cells invade the normal bone. Therefore, induction of osteoblastic differentiation by osterix and subsequent inhibition of osteosclerosis may be one mechanism by which osterix inhibits tumor growth. The mechanism of reduced osteolysis following osx gene transfection is unclear. RANKL has been shown to be critical to osteoclast formation. The expression of RANKL is high in undifferentiated osteoblasts compared with more differentiated osteoblasts. Higher expression of RANKL in osteosarcoma cells may stimulate more osteoclasts in the bone, resulting in increased osteolysis. We were, however, unable to show elevated RANKL expression in K7M2, K7M2-neo, K7M2-osx-1, and K7M2-osx-2. Total RNA was extracted from the indicated cells. RANKL expression was quantified by semiquantitative reverse transcription-PCR analysis. Glyceraldehyde-3-phosphate dehydrogenase was used as internal control.

Osteosarcoma is the most common primary bone tumor in both adults and children. Unfortunately, the 60% to 65% disease-free survival rate has remained stagnant for the past 15 to 20 years. The majority of cases of osteosarcoma are "sporadic," with there being no familial history and no consistent genetic alterations. Abnormalities in Rb and p53 have been described in some cases but more often patient tumors have normal Rb and p53 (3). Understanding the genes involved in tumor development, progression, and metastases are crucial to designing better and more specific therapeutic targets. Our data indicate that abnormalities in osterix may contribute to the...
tumorigenic phenotype of osteosarcoma. Restoration of osterix expression in osteosarcoma cells led to reduced tumor growth, decreased bone destruction, and fewer lung metastasis. Together, these data suggest that osterix might be a therapeutic target for osteosarcoma.

Acknowledgments

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