Overexpression of Smad7 blocks primary tumor growth and lung metastasis development in osteosarcoma

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Translational relevance

We have demonstrated that the TGF-β/Smad signaling pathway plays a crucial role in osteosarcoma metastatic progression. We have first shown that TGF-β levels are higher in the serum of osteosarcoma patients compared to healthy volunteers and that TGF-β/Smad3 signaling pathway is activated in clinical samples of patients. Second, using a murine model of osteosarcoma, we demonstrated that blocking the TGF-β/Smad signaling pathway via overexpression of inhibitory-Smad slows the growth of the primary bone tumor mainly by affecting the tumor microenvironment by controlling the "vicious cycle" established between tumor cells and bone cells. Third, blocking TGF-β signaling inhibits the development of lung metastasis at least by inhibition of cell migration and invasion. Here, we show for the first time that blocking TGF-β signaling represents a novel therapeutic approach for the treatment of lung metastasis in osteosarcoma patients, which has a poor prognosis.
Abstract

Purpose: Osteosarcoma is the main malignant primary bone tumor in children and adolescents for whom the prognosis remains poor, especially when metastasis are present at diagnosis. Because TGF-β has been shown to promote metastasis in many solid tumors, we investigated the effect of the natural TGF-β/Smad signaling inhibitor Smad7 and the TβRI inhibitor SD-208 on osteosarcoma behavior.

Experimental Design: By using a mouse model of osteosarcoma induced by paratibial injection of cells, we assessed the impact of Smad7 overexpression or SD-208 on tumor growth, tumor microenvironment, bone remodeling and metastasis development.

Results: First, we demonstrated that TGF-β levels are higher in the serum of osteosarcoma patients compared to healthy volunteers and that TGF-β/Smad3 signaling pathway is activated in clinical samples. Second, we showed that Smad7 slows the growth of the primary tumor and increases mice survival. We furthermore demonstrated that Smad7 expression does not affect in vitro osteosarcoma cell proliferation but affects the microarchitectural parameters of bone. In addition, Smad7-osteosarcoma bone tumors expressed lower levels of osteolytic factors such as RANKL, suggesting that Smad7 overexpression affects the "vicious cycle" established between tumor cells and bone cells by its ability to decrease osteoclast activity. Finally, we showed that Smad7 overexpression in osteosarcoma cells and the treatment of mice with SD208 inhibit the development of lung metastasis.

Conclusion: Taken together, these results demonstrate that the inhibition of TGF-β/Smad signaling pathway may be a promising therapeutic strategy against tumor progression of osteosarcoma specifically against the development of lung metastasis.
Introduction

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents with a second peak of incidence in adults over the age of 65 (1). Although several predisposing environmental (e.g. ionizing radiation) and genetic (e.g. TP53) factors have been identified (2), the exact etiology of this disease remains unknown (3). These rare tumors, believed to originate from mesenchymal cells forming the primitive bone, preferentially grow in the metaphysis of long bone (4). Approximately 20% of patients have lung metastasis at initial diagnosis and an additional 40% will develop metastasis during the later stages of disease. The presence of metastasis at diagnosis is the most important predictor of disease-free survival with a 5-year survival rate of only 20% for osteosarcoma patients with metastasis compared to 65% for patients with localized disease (5). The standard treatment of osteosarcoma consists of complete surgical resection associated with neoadjuvant and adjuvant chemotherapy composed of four agents: doxorubicin, cisplatin, methotrexate or ifosfamide (6). These combined treatment protocols have significantly improved survival of the non-metastatic patients over the past several decades (7,8). Unfortunately, such therapeutic strategies have a limited efficacy in the treatment of metastatic disease, and the metastatic relapse or recurrent conditions have remained unchanged over the last 3 decades (8). Treating metastatic osteosarcoma thus remains a challenge in bone cancer (9).

Transforming Growth Factor-β (TGF-β) family members are a class of cytokines that control a variety of biological processes, including proliferation, differentiation, extracellular matrix production and apoptosis. Three isoforms of TGF-β exist in mammals: TGF-β1, TGF-β2 and TGF-β3. Dimers of TGF-β initiate the canonical signaling cascade via the serine/threonine kinase receptor cell surface complexes (TβRI and TβRII) which phosphorylate the ligand-specific receptor-activated Smads (R-Smads: Smad2 or Smad3). Upon phosphorylation by type I receptors, R-Smads form a heteromeric complex with the Common-Smad, Smad4. The R-Smad/Smad4 complex then translocates to the nucleus to regulate gene transcription (10–13). A third group of Smad proteins, the inhibitory Smads (Smad7), inhibits the canonical Smad signaling (14,15) by different means; Smad7 i) binds TβRII and prevents R-Smad phosphorylation, ii) recruits E3-type ubiquitin ligases to the receptor complexes ultimately leading to their degradation, and iii) interacts with GADD34, the regulatory subunit of the protein phosphatase PP1 to inactive TβRI (10–16).
The role of TGF-β in cancer is complex. During the first stages of the development of primitive tumors from epithelial origin, the TGF-β/Smad cascade acts as a tumor suppressor mainly through inhibition of cell proliferation and/or promotion of cell apoptosis (11,12,17). Contrarily, during the later stages, the TGF-β cascade promotes tumor progression mainly by its ability to stimulate epithelial to mesenchymal transition, tumor invasion, metastatic dissemination and/or evasion of the immune system (12,13,17). With regard to bone cancers, most studies have focused on the role of TGF-β in the development of bone metastasis. The contributions of TGF-β to breast cancer and melanoma bone metastasis have been well described (18–21). TGF-β promotes bone metastasis by its ability to promote the metastatic process by stimulating MMP-2 production and thus promoting cellular invasion of melanoma (19,20). It has been reported that TGF-β contributes to the establishment of a vicious cycle between epithelial tumor cells and bone cells. Briefly, the tumor cells secrete osteoclast-activating factors that promote bone degradation thus stimulating the release of factors which in turn will promote bone metastasis development (22,23). In this context, it has been shown that overexpression of the TGF-β inhibitor Smad7 in melanoma cells reduces the development of melanoma bone metastasis, and that the use of chemical inhibitors targeting TβRI reduces the development and progression of both, melanoma and breast cancer bone metastasis (21). With regard to primary bone sarcomas, few studies have described the role of TGF-β on tumor development. It has been shown that the production of TGF-β1 is associated with high-grade osteosarcoma (24,25) and that TGF-β stimulates the growth of several osteosarcoma cell lines in culture (26) suggesting that TGF-β could favor osteosarcoma development.

In this study, we particularly demonstrate that the overexpression of the inhibitory Smad, Smad7, in osteosarcoma cells and treatment of mice with a specific inhibitor of TβRI (SD-208) block the development of osteosarcoma lung metastasis.
Materials and Methods

Measurement of circulating TGF-β levels in serum
The levels of circulating TGF-βs were measured in serum from healthy controls (n = 20) from the Etablissement Français du Sang (EFS) and osteosarcoma patients (n = 40) from the OS 2006 protocol (PAC SARCOME, Sarcome 09/0603, EudraCT n° 2006-00337727) with the Bio-Plex Pro™ Assay TGF-β Standard 3-Plex system (Bio-Rad, Richmond, CA, USA). Serum samples were obtained with written informed consent.

Cell cultures and reagents
HOS and SaOS2 osteosarcoma cells were purchased from ATCC (respectively CRL-1544 and HTB-85) and cultured in DMEM (Dulbecco’s Modified Eagle’s Medium, Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (Hyclone Perbio, Bezons, France). All cell lines were authenticated by short tandem repeat (STR) profiling. TGF-β1 and BMP-6, and G418 were respectively from R&D System, Inc (Minneapolis, MN) and Sigma (St Quentin-Fallavier, France). SD-208 has been synthetized by the laboratory CEISAM UMR6230, Nantes University (France).

Western blot analysis
Western blot analysis were performed as previously described (27). Membranes were immunoblotted with anti-phospho-Smad3 (Millipore, Temecula, CA, USA), anti-Smad3 (Millipore), anti-Smad7 (Santa Cruz Biotechnology, CA, USA), anti-phosphoErk (Cell Signaling Technology, Beverly, MA), anti-Erk (Cell Signaling) or anti-β-actin (Sigma) antibodies.

Proliferation Assay
Cell growth and viability were determined by using a 2,3-bis(2 methoxy-4 nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reagent assay kit (Roche Molecular Biomedicals, Mannheim, Germany).

Real-time polymerase chain reaction
Total RNA from cell lines was extracted using NucleoSpin®RNAII (Macherey Nagel, Duren, Germany).
Total RNA from tumors was extracted using the TRIzol reagent (Invitrogen Life Technologies) after mechanical grinding with Turrax (IKA, Staufen, Switzerland). qRT-PCR were performed as previously described (27). Primer sequences are provided in Table 1.

**Transient cell transfections, reporter assays and plasmid constructs**

Transient cell transfections were performed with jetPEITM (Polyplus-transfection, Illkirch, France). The phRLMLP-**Renilla** luciferase expression vector was cotransfected in all experiments to monitor transfection efficiencies. Luciferase activity was determined with the Dual-Luciferase reporter assay system (Promega, Charbonnieres, France). The (CAGA)r-Luc construct was used as a reporter construct specific for Smad3/4-driven signaling (28). The pcDNA-Smad7 vector is a kind gift from Dr Alain Mauviel (19).

**Osteosarcoma mouse model**

Four-week-old female Rj:NMRI-nude mice (Elevages Janvier, Le Genest Saint Isle, France) were maintained under pathogen-free conditions at the Experimental Therapy Unit (Faculty of Medicine, Nantes, France) in accordance with the institutional guidelines of the French Ethical Committee (CEEA Pays de la Loire n°06; project authorization n° CEEA-2010-23) and under the supervision of authorized investigators. The mice were anesthetized by inhalation of an isoflurane/air mixture (1.5%, 1 L/min) before receiving an intramuscular injection of 1.10⁶ HOS or SaOS2 osteosarcoma cells in close proximity to the tibia, leading to a rapidly growing tumor in soft tissue with secondary contiguous bone invasion. One day after HOS cells injection, some mice received different doses (20 or 60 mg/kg) of SD-208 or control vehicle by daily gavage. The tumor volume (V) was calculated from the measurement of two perpendicular diameters using a caliper, according to the following formula: \( V = 0.5 \times L \times (S)^2 \), as previously described (29). Mice were sacrificed when the tumor volume reached 2500 mm³ for ethical reasons. Under these conditions, pulmonary metastasis developed when tumor volumes were \( \geq 2000 \) mm³.

**Micro-CT analysis**

Analysis of bone microarchitecture was performed as previously described (27) at different tumor volumes (250, 1000 and 2500 mm³). All tibiae/fibulae were scanned using the same parameters (pixel
size 18 µm, 50kV, 0.5-mm Al filter and 0.8 degrees per rotation step). Three-dimensional reconstructions and analysis of bone parameters were performed using the CTvol and CTan software (Skyscan).

**Histologic Analysis**

After sacrifice, the tibiae were conserved and fixed in 10% buffered formaldehyde, decalcified (4% EDTA, 0.2% paraformaldehyde, pH 7.4), and embedded in paraffin. 3-µm sections of tumor-bearing tibiae were cut and stained for tartrate-resistant acid phosphatase (TRAP) to analyze osteoclast activity. Lungs were fixed in 10% buffered formaldehyde and embedded in paraffin. Lung sections (3µm-thick) were mounted on glass slides and stained with hematoxylin-eosin (HE).

**Immunohistochemistry of human and mice tumor samples**

3-µm sections of human tumor tissues (embedded in paraffin) were cut and stained for phosphoSmad3 using rabbit polyclonal anti-phosphoSmad3 antibody (Abcam). Patient tumor clinical samples collected at Nantes University Hospital (Nantes, France) were obtained following patient informed consent and after ethical approval by the Nantes University Hospital Ethics Committee. 3-µm sections of mice tumor tissues (embedded in paraffin) were cut and stained for osterix, RANKL, osteocalcin, caspase-3, Ki-67 and CD146 using respectively rabbit polyclonal anti-osterix (Abcam), anti-RANKL (Santa Cruz Biotechnology), anti-osteocalcin (Abcam), anti-caspase-3 (Cell Signaling), anti-Ki67 (Dako), anti CD146 (Abcam) antibodies. Immunodetection was performed using DAB Substrate-Chromogen (Dako) and counterstained with hematoxylin.

**Collagen degradation.** The degradation of collagen was evaluated by the measure of pyridinoline excretion in mice serum using the MicroVue Serum PYD EIA kit.

**Transwell™ motility and invasion.** Osteosarcoma cells (30 000 cells/well) were pre-treated with 5 ng/ml TGF-β in the presence or absence of SD-208 (10µM) for 24 h and seeded onto the upper surface of transwell inserts (Falcon, Franklin Lakes, NJ) coated with 0.1 mg/ml of growth factor-reduced Matrigel™ (Biocoat, BD Biosciences, SanJose, CA) for the invasion assay, or on uncoated transwells (migration assay) and incubated at 37°C for 48 h. At the end of the incubation period, cells
on the upper surface of the inserts were wiped off, and the cells on the underside of the membrane were fixed, stained with "cristal violet" and counted by bright-field microscopy in five random fields.

**Gelatin zymography.** Cells were cultured for 48 h without serum and their conditioned media were analyzed by gelatin zymography in 10% polyacrylamide gels containing 1 mg/ml gelatin (Sigma-Aldrich) as described previously (19).

**Statistical analysis**

All analyses were performed using GraphPad Prism 4.0 software (GraphPad Software, La Jolla, CA, USA). Results of *in vitro* experiments were analyzed with the unpaired t-test and are given as means ± SD. For *in vivo* experiments, results from groups overexpressing Smad7 were compared with control groups (parental and mock) and results from mice treated with SD-208 were compared with untreated mice using the unpaired t-test and are given as means ± SEM. Results of animal survival were analyzed using the log-rank test. Results with p<0.05 were considered significant.
Results

High levels of TGF-β1 and TGF-β2 are measured in serum of osteosarcoma patients

TGF-β1, TGF-β2 and TGF-β3 levels were measured in the serum of forty osteosarcoma patients and compared with twenty age-matched healthy volunteers. As shown in Figure 1A (upper panels), TGF-β1 (left panel) and TGF-β2 (right panel) concentrations measured in the serum were significantly higher in the serum of osteosarcoma patients compared to healthy volunteers (p<0.01 and p<0.0001 for TGF-β1 and TGF-β2 respectively). Note that the TGF-β3 serum levels were very low, under the limit of detection in our experimental conditions (not shown). Secondly, immunohistochemical experiments were performed to analyze the levels of phospho-Smad3 in six clinical samples of osteosarcoma patients. As shown in Figure 1A (lower panels), a high level of phospho-Smad3 was detected in the nucleus of osteosarcoma cells demonstrating the activation of the TGF-β1/Smad3 cascade. Interestingly, this level of phospho-Smad3 was higher in clinical samples from patients with pulmonary metastasis than without pulmonary metastasis at diagnosis (Fig1A right panel vs left panel).

Overexpression of Smad7 and a chemical inhibitor of TβRI (SD-208) block the TGF-β/Smad3 signaling pathway in osteosarcoma cells

To evaluate the effect of Smad7 overexpression on osteosarcoma growth and progression, two human osteosarcoma cell lines (HOS and SaOS2) were stably transfected with either empty pcDNA or pcDNA-Smad7 encoding Smad7. Firstly, endogenous Smad7 was not detectable in either parental or mock-transfected HOS and SaOS2 cells, whereas Smad7-transfected osteosarcoma cells expressed high levels of the protein (Figure 1B left panel and Supplementary Figure S1A). Secondly Smad7 expression inhibits the ability of TGF-β to induce the phosphorylation of Smad3 (Figure 1B middle panel), to transactivate the Smad3/4-specific reporter construct (CAGA)₉-luc (Fig. 1C, left panel), and to stimulate the expression of CTGF, PAI-1 and COL1A1 (Fig. 1D, upper panels). Similar results were obtained with the SaOS2 cell line (Supplementary Fig. S1). Note that the overexpression of Smad7 partially inhibits a BMP-specific target gene such as ID1 (Supplementary Fig. S1C) but not the ability of TGF-β to induce the activation of MAPKinases such as ERK₁/₂ (Supplementary Fig. S1D). To specifically target the TGF-β cascade, we secondly studied the effect of a TβRI inhibitor, the chemical
compound SD-208. As expected, SD-208 (10 μM) effectively blocks the ability of TGF-β to induce the phosphorylation of Smad3 (Figure 1B, right panel), to transactivate the Smad3/4-specific reporter construct (CAGA)$_9$-luc (Figure 1C, right panel) and to stimulate the expression of CTGF, PAI-1 and COL1A1 (Figure 1D, lower panels) in HOS cells and in SaOS2 cells (Supplementary Fig. S1). Note that in contrast to Smad7 overexpression, the SD-208 inhibitor is able to block the ability of TGF-β to activate the phosphorylation of ERK$_{1/2}$ (Supplementary Figure S1D, lower panel).

These results confirmed that both Smad7 overexpression and SD-208 block the TGF-β/Smad3 cascade in osteosarcoma cells.

**Overexpression of Smad7 in osteosarcoma cell lines dramatically inhibits in vivo tumor growth**

A preclinical experimental model of osteosarcoma induced by paratibial injection of osteosarcoma cells was developed. Smad7 overexpression in HOS or SaOS2 cells inhibited tumor growth in both models (Fig. 2A, upper panels). The mean tumor size at day 40 in mice injected with parental or mock-transfected HOS cells was $1531.8 \pm 73.4$ mm$^3$ and $1663.9 \pm 297.0$ mm$^3$ respectively, compared to only $341.5 \pm 43.4$ mm$^3$ in mice injected with HOS-S7 cells (means ± SEM, $p<0.005$; Fig. 2A, upper HOS panel). Similar results were obtained in the SaOS2 model (Fig 2A, upper SaOS2 panel). Consequently, Smad7 overexpression resulted in an increased animal survival in both models (Fig. 2A, lower panels). In this context, immunohistochemical staining for the proliferative marker Ki67 in tumor samples from mice showed that Smad7 overexpression decreased cell proliferation as compared with the mock-transfected group (Fig. 2B, upper panels) when the tumor sizes reached 250 mm$^3$. By contrast, caspase 3 immunostaining of the same samples showed no significant difference between mice injected with Smad7-transfected cells and animals receiving mock-transfected cells (Fig. 2B, lower panels). To better understand the mechanisms underlying the effect of Smad7 on osteosarcoma tumor growth, we next carried out in vitro experiments. Interestingly, treatment of osteosarcoma cells with TGF-β (5 ng/ml) did not affect osteosarcoma cell proliferation even after 6 days of TGF-β treatment, whether the cells expressed Smad7 or not (Fig. 2C). In contrast to the effect of Smad7 overexpression, the treatment of mice with SD-208 (20 or 60 mg/kg/day) does not affect the in vivo tumor growth of osteosarcoma (Fig. 2D).

These results demonstrate that overexpression of Smad7 reduced in vivo tumor growth and suggest that this effect is not due to a direct effect of Smad7 on osteosarcoma cell proliferation.
**Smad7 overexpression in HOS and SaOS2 osteosarcoma cells inhibits tumor associated bone resorption**

Since osteosarcoma-associated alteration of bone remodeling plays a central role in the development and progression of osteosarcoma bone tumors, we evaluated the ability of Smad7 and SD-208 to alter tumor-associated bone remodeling. The microarchitecture of bone in mice bearing osteosarcoma tumors was firstly examined when the tumor sizes reached 2500 mm\(^3\) using a high-resolution X-ray micro-CT system.

Visual inspection of the 3D reconstructions of the tibia suggests that Smad7 overexpression enhanced the tumor-associated bone formation in HOS osteosarcoma models (Fig. 3A). Indeed the total bone volume in mice injected with parental or mock-transfected HOS cells was 9.34 ± 0.08 mm\(^3\) and 8.77 ± 0.15 mm\(^3\) respectively, compared to 11.44 ± 0.28 mm\(^3\) in mice bearing HOS-S7 tumors (p<0.005; Fig. 3B HOS left panel). Similarly, Smad7 overexpression enhanced total bone volume in mice injected with SaOS2 cells (Fig.3B SaOS2 left panel).

We next determined whether this increase in total bone volume was due either to direct new bone formation (ectopic bone) and/or to an inhibition of bone resorption. As shown in Figure 3B (HOS right panel), the ectopic bone volume in mice injected with parental or mock-transfected HOS cells was significantly lower than in mice bearing HOS-S7 tumors (1.91 ± 0.11 mm\(^3\) and 1.43 ± 0.26 mm\(^3\) vs. 3.77 ± 0.18 mm\(^3\) respectively, p<0.005). Similarly, Smad7 enhanced ectopic bone volume in the SaOS2 model (p<0.005; Fig. 3B SaOS2 right panel). We then analyzed the ability of Smad7 to alter bone osteolysis by evaluating the trabecular number (Tb.N) and trabecular thickness (Tb.Th) when tumor sizes were around 250 mm\(^3\), since at higher volumes (1000 mm\(^3\) or 2500 mm\(^3\)) trabecular bone is completely destroyed by the tumor cells (data not shown). As shown in Figure 3C (upper panel), mice injected with mock-transfected HOS cells had a lower Tb.Th than mice bearing HOS-S7 tumors (0.15 ± 0.01 mm vs. 0.22 ± 0.01 mm, p<0.005). Moreover, the Tb.N was also lower in the mock group compared to the HOS-S7 tumor-bearing group (2.92 ± 0.03 /mm vs. 4.18 ± 0.02 /mm; p<0.005, Fig. 3C, lower panel). Note that Tb.Th and Tb.N in mice bearing HOS-S7 tumors are similar to those observed in healthy control mice. Interestingly, although the treatment of mice with SD-208 affects bone remodeling with a significant increase of tibia bone volume in the absence of tumor (Fig. 3D left...
panel) as previously described (30), the SD-208 does not affect significantly the tumor-associated bone remodeling (Fig. 3D right panel).

These results demonstrate that in contrast with SD-208 mice treatment, overexpression of Smad7 in osteosarcoma cells both decreased tumor-associated bone osteolysis and promoted tumor-associated bone formation.

**Overexpression of Smad7 in HOS and SaOS2 osteosarcoma cells reduces osteoclast activity**

The activity of osteoclasts and osteoblasts, two cell lineages implicated in bone remodeling, was then assessed during the early stages of tumor growth (tumor size ≤ 250 mm$^3$ or ≤ 1000 mm$^3$). Tartrate resistant acid phosphatase (TRAP) staining in sections of tumor-bearing tibia showed that HOS-S7 tumor cells reduced the number of TRAP+ multinucleated cells at the interface between tumor and cortical bone (Fig. 4A upper panels) and in the growth plate (not shown), relative to the control conditions at the same tumor size (1000 mm$^3$). Similar results were obtained when tumors had reached 250 mm$^3$ (data not shown). By contrast, osteocalcin (Fig. 4A middle panels) and osterix (data not shown) immunostaining of the same samples showed no significant difference between mice injected with Smad7-transfected cells and animals receiving mock-transfected cells.

To understand the effect of Smad7 on osteoclast activity, we analyzed RANKL gene expression in HOS osteosarcoma cell lines. qRT-PCR analysis indicated that the mRNA steady-state level of RANKL was increased in response to TGF-β and that Smad7 overexpression prevented such induction (Fig 4B, respectively upper and middle panels). Moreover, Smad7 overexpression decreased RANKL production by HOS tumor cells (Figure 4A lower panels) evaluated by immunohistochemical experiments. In accordance with this observation, HOS-S7 bone tumors expressed significantly lower mRNA levels of RANKL (Fig. 4B, lower panel). Finally, the degradation of collagen evaluated by the measure of pyridinoline excretion in mice serum is decreased when Smad7 is overexpressed in osteosarcoma cells (Fig. 4C).

In addition to RANKL, the expression of other TGF-β target genes implicated in bone remodeling (18,20,21) such as interleukin-11 (IL-11) and osteopontin (OPN) is decreased when Smad7 is overexpressed. As shown in Figure 4D, q-PCR analysis indicated that the *IL-11* and *OPN* expressions were increased in response to TGF-β and that Smad7 overexpression prevented such induction (Fig 4D, left panel). HOS-S7 bone tumors expressed significantly lower levels of IL-11 and osteopontin...
These results demonstrate that Smad7 overexpression decreases osteoclast activity and thus bone osteolysis at least in part via the modulation of osteolytic genes such as RANKL.

**Overexpression of Smad7 in HOS and SaOS2 osteosarcoma cells or treatment of mice with SD-208 inhibit dissemination of pulmonary metastasis**

To evaluate the effect of Smad7 on pulmonary metastasis development (Fig. 5A), the lungs of mice were removed when primary tumor volumes reached 2500 mm$^3$. As shown in Figure 5B, a high incidence of pulmonary metastasis was observed in mice inoculated with parental or mock-transfected HOS cells, respectively 9 of 10 (90%) and 4 of 4 (100%) mice. By contrast, only 1 of 7 (14%) mice bearing HOS-S7 cells developed lung metastasis (Fig. 5B, left panel). As shown in Figure 5B (middle panel), similar results were obtained in the SaOS2 model. Remarkably, the treatment of mice with SD-208 inhibits the development of lung metastasis. As shown in Figure 5B (right panel), 5 of 6 (83.3%) mice treated with vehicle (control group) developed lung metastasis. By contrast, only 1 of 6 (16.7%) mice treated with 20 mg/kg/day developed lung metastasis, and no mice treated with 60 mg/kg/day developed lung metastasis. Interestingly, immunohistochemical staining for the endothelial marker CD146 in mice tumor samples showed that Smad7 overexpression or SD-208 treatment dramatically decreased the angiogenic process as compared respectively with the parental and mock-transfected groups or with the untreated group (Fig. 5C). In addition, qPCR analysis from mice biopsies indicated that the expression by tumor cells of VEGF (implicated in the angiogenic process), and CXCR4 and ANGPTL4 (respectively identified as key players to prime breast cancer cells for metastasis towards the lungs and/or the bones) were both reduced when Smad7 was over-expressed or when mice were treated with SD-208 (Fig. 5D).

The influence of Smad7 overexpression in osteosarcoma cells or the treatment of tumor cells with SD-208 was then examined on several aspects of tumor cell behavior *in vitro*. As shown in Figure 6 and in Supplementary Figure S2, Smad7 overexpression in HOS and SaOS2 cells or treatment of HOS cells with SD-208 lead to a strongly reduced capacity of TGF-β to stimulate cell migration (Fig. 6A and supplementary Fig. S2A) and invasion (Fig. 6B and supplementary Fig. S2B). In addition, exogenous TGF-β induced secretion of the active form of matrix metalloproteinase MMP-2, was strongly diminished both in Smad7-transfected cells and in cells treated with SD-208 (Fig. 6C and
supplementary Fig. S2C). qRT-PCR analysis indicated a reduction of TGF-β-induced MMP-2 mRNA levels in Smad7 transfected- or SD-208-treated cells (Fig. 6D upper panels). MMP-2 mRNA steady-state levels were increased by approximately 5-fold in parental and mock-transfected HOS cells, but only by 2-fold in HOS-S7 cells after treatment of osteosarcoma cells with TGF-β (5 ng/ml) for 24h. Similar results were obtained when Smad7 was overexpressed in SaOS2 cells (Supplementary Fig. S2D) or when osteosarcoma cells were treated with SD-208 (Fig. 6D, right panel and Supplementary Fig. S2D right panel). In addition, both HOS-S7 bone tumors and HOS cells from mice treated with SD-208 expressed significantly lower levels of MMP-2 (Fig. 6D, lower panel). These results demonstrated that overexpression of Smad7 in osteosarcoma cells or treatment of mice with SD-208 blocks the formation of lung metastasis.
Discussion

Increased TGF-β1 mRNA and/or protein expression has been correlated with a wide range of cancers such as colorectal cancer, gastric carcinoma, or prostate cancer (31–34). This increase in serum TGF-β1 production and/or TGF-β1 staining in tumor cells has been associated with disease progression to metastasis in these carcinomas (31). Here, we demonstrated that high concentrations of TGF-β1 and TGF-β2 measured in serum of patients is associated with osteosarcoma disease. In addition, using clinical samples, we demonstrated that the Smad3 cascade is activated in osteosarcoma cells particularly in high-risk patients when lung metastasis are detected at diagnosis. Previous studies have reported that high levels of TGF-β1 mRNA in tumor cells are associated with high-grade osteosarcoma, which shows an aggressive behavior and frequently metastasizes to lung or other sites (24). These observations together with our results suggest that high levels of TGF-β1 in serum could be associated with a poor prognosis in osteosarcoma.

Following these observations, we firstly inhibited the Smad signaling cascade in osteosarcoma cells via the overexpression of the inhibitory Smad, Smad7, and we secondly used the chemical inhibitor of TβRI, SD-208, to specifically inhibit the signaling cascade downstream the receptor TβRI. By using various in vitro approaches, we demonstrated that Smad7 overexpression and SD-208 efficiently inhibit the TGF-β transcriptional response mediated by Smad3/4 in two human osteosarcoma cell lines, HOS and SaOS2.

Using a murine model of osteosarcoma induced by paratibial injection of osteosarcoma cells overexpressing Smad7, we then demonstrated that Smad7 overexpression slows primary tumor growth, a process associated with a reduction of the immunohistochemical staining for the proliferative marker Ki67. Since TGF-β is a cytokine widely implicated in the control of cell proliferation (17), the effect of TGF-β and overexpression of Smad7 was studied on osteosarcoma cell proliferation in vitro.

In contrast to previous observations that demonstrated an effect of TGF-β on cell proliferation (35,36), no effect of TGF-β and/or Smad7 overexpression was observed on the in vitro proliferation rate of osteosarcoma cells under our experimental conditions, suggesting that Smad7 does not directly affect the proliferation of osteosarcoma cells but rather affects the tumor microenvironment indirectly involved in the control of tumor cell proliferation.
In this context, since osteosarcoma-associated alterations of bone remodeling play a central role in the development and progression of osteosarcoma, we studied the effect of Smad7 on bone remodeling. We clearly demonstrated that Smad7 overexpression in osteosarcoma cells slows bone destruction associated with the tumor growth. In this context, we demonstrated that this process was mainly associated with a reduction of trabecular bone destruction during the early stages of tumor growth (when tumor volumes were below 250 mm$^3$) and with an increase of ectopic bone formation during the late stages of tumor growth (when tumor volumes were greater than 1000 mm$^3$). The presence of a "vicious cycle" established between tumor proliferation and paratumor osteolysis plays a crucial role in the development of primary bone tumors (37). Cancer cells produce soluble factors that activate directly or indirectly via osteoblasts, the osteoclast differentiation and maturation (38,39). In turn, during bone degradation, osteoclasts allow the release of growth factors stored in the mineralized bone matrix that are able to stimulate tumor growth. In this context, we demonstrated that the resulting increase in bone volume observed after Smad7 overexpression in osteosarcoma cells is due in large part to the inhibition of osteoclast activity. The decrease of TRAP activity at the growth plate level, which is not in direct contact with the tumor, suggests that Smad7 overexpression affects the ability of the tumor cells to produce a soluble factor able to regulate osteoclast activity. Here, we clearly demonstrated that Smad7 overexpression in osteosarcoma cells inhibits their ability to produce RANKL or IL-11, two cytokines which play a central role in bone osteolysis process (40). Together, these results suggest that Smad7 slows the tumor growth by acting at least at the tumor environment level, by inhibiting the tumor associated bone osteolysis.

Surprisingly, we did not observe a significant effect of SD-208 on tumor growth. Several hypotheses can be proposed to explain this difference between Smad7 and SD-208 effects on tumor growth. Firstly, we cannot rule out that Smad7 may also exert some of its action independently from its role as a TGF-β signaling inhibitor. Indeed, Smad7 is able to inhibit other signaling pathways such as the cascade of Bone Morphogenetic Protein family members (BMPs), highly involved in bone formation (41). This hypothesis seems unlikely since the BMPs are known to promote bone formation. Thus, we can hypothesize that BMP inhibitors should promote bone degradation by inhibiting the bone formation in contrast to the results observed by over-expression of Smad7. Secondly, Smad7 might have several distinct functions in cellular signaling (12). For example it has been shown that Smad7 overexpression is able to potentiate apoptosis in prostate carcinoma and in PC-3U cells (12).
addition, Smad7 is also able to activate some signaling pathways such as the JNK cascade (42). Although we have not observed an effect of Smad7 on the proliferation and apoptosis of osteosarcoma cells, or on the ability of TGF-β to stimulate the MAPK pathway, we cannot rule out this hypothesis. Thirdly, the inefficiency of SD-208 on tumor growth can be explained by its inability to reduce the tumor associated bone osteolysis. Indeed, although a systemic treatment of mice with SD-208 promotes bone formation in absence of tumor as previously described (30), this systemic treatment does not seem to reduce the tumor associated bone osteolysis in contrast with the overexpression of Smad7 effect in osteosarcoma cells. In addition, the HOS mice model used in these experiments is a high aggressive model with a fast bone degradation associated with tumor growth. The effectiveness of a local mice treatment with SD-208 directly into the tumor cells using a less aggressive model should be tested.

Finally, we showed that both Smad7 overexpression and SD-208 strongly affect the ability of the primary bone tumor to develop lung metastasis demonstrating the crucial role of TGF-β/Smad signaling pathway in the metastatic process of osteosarcoma. During the last decades, the role of TGF-β in the metastatic process of carcinomas has been widely described. A major step in this process is the ability of TGF-β to stimulate epithelial-to-mesenchymal transition and thus the ability of tumor cells to invade adjacent tissues (17). In the context of osteosarcoma cells which are from mesenchymal origin, we specifically demonstrated that Smad7 overexpression and SD-208 block the ability of TGF-β to stimulate osteosarcoma migration and invasion. In this context, we clearly demonstrated that Smad7 and SD-208 are able to block TGF-β-induced MMP-2 activity, mainly involved in the invasion process such as described in the context of melanoma bone metastasis (19,20). Another major step in the metastatic process is the ability of TGF-β to stimulate tumor associated angiogenesis and thus the dissemination of tumor cells into the bloodstream (17). In this context, we clearly demonstrated that both Smad7 and SD-208 reduce the angiogenic process as shown by immunohistochemical staining for the endothelial marker CD146. In addition, both Smad7 and SD-208 inhibit TGF-β-induced VEGF expression, mainly involved in the angiogenic process. Moreover, both Smad7 and SD-208 are able to inhibit the ability of TGF-β to stimulate the expression of ANGPTL4 and CXCR4 identified as key players to prime breast cancer cells for metastasis respectively towards the lungs (43) and towards the bones or the lungs (44).
In conclusion, this report provides evidence that blocking TGF-β signaling may represent a novel therapeutic approach to treat lung metastasis in osteosarcoma patients, which have a poor prognosis.

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References


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<th>Table 1. Primer sequence for quantitative RT-PCR.</th>
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Figure Legends

**Figure 1:** Evaluation of TGF-β levels in serum of osteosarcoma patients. Overexpression of Smad7 and SD-208 in HOS cells block the TGF-β/Smad3 cascade.

A) Upper panels: Comparison of TGF-β1 (left panel) and TGF-β2 (right panel) levels in serum from healthy age-matched controls (n = 20) or osteosarcoma patients (n = 40). (Median, ***p<0.005, **p<0.01). Lower panels: Clinical tumor samples of osteosarcoma patients with (n=3, right panel) or without (n=3, left panel) pulmonary metastasis at diagnosis were immunostained with phospho-Smad3 antibody. One representative photomicrograph per group is shown. Arrows indicate the localisation of P-Smad3 in the nucleus of osteosarcoma cells.

B) Left panel: Smad7 production was detected by Western Blot analysis in HOS cells (parental- (P), mock- (M) and Smad7-transfected cells (S7)). Middle panel: phospho-Smad3 and Smad3 were detected by Western-Blot analysis in parental (P), mock- (M) and Smad7-transfected (S7) HOS cells treated or not with TGF-β1 (5 ng/ml) for 15 min. Right panel: Parental HOS cells were treated with TGF-β (5 ng/ml, 15 min) in the presence or absence of SD-208 (10 μM). After incubation, Phospho-Smad3 and Smad3 levels were detected by Western Blot analysis of whole cell lysates.

C) Cells were transfected with the Smad3/4-specific construct (CAGA)_9-luc. 24h after transfection, TGF-β (5ng/ml) was added and incubation was continued for another 48h in the presence or absence of SD-208 (10 μM) as indicated. Bars indicate means ± S.D. of at least three independent experiments, each performed in duplicate (**p<0.005, *p<0.01).

D) Cells were treated with TGF-β1 (5 ng/ml) for 6h or 24h in the presence or absence of SD-208 (10 μM as indicated). After incubation, mRNA steady-state levels of the specific TGF-β target genes CTGF (6h), PAI-1 (24h) and COL1A1 (24h) were determined by quantitative RT-PCR. Bars indicate means ± S.D. of at least three independent experiments, each performed in duplicate (**p<0.005, *p<0.05).

**Figure 2:** Overexpression of Smad7 inhibits tumor growth in the HOS and SaOS2 osteosarcoma models and improves animal survival

A) Mice were injected either with 1.10^6 parental (P), mock- (M) and Smad7-transfected (S7) HOS cells (n = 10 for parental, n = 4 for mock and n = 7 for Smad7 cells, left panels), or with 1.10^6 parental (P), mock- (M) and Smad7-transfected (S7) SaOS2 cells (n = 22 for parental, n = 8 for mock and n = 22 for
Smad7 cells, right panels). The results are representative of 2 independent experiments. (Mean ± SEM;***p<0.005). Upper panels: The mean tumor volumes were calculated from day 1 to day 40 for HOS group (left panel) or from day 1 to day 30 for SaOS2 group (right panel). Lower panels: Overall survival rates for each groups. The survival rates were calculated from day 1 to day 90, when the last mouse was euthanized (in the Smad7 group).

B) Tumor samples (tumor sizes at 250 mm³) of the mock and Smad7 HOS group were fixed, embedded in paraffin, sectioned and stained with KI67 (upper panels) or caspase3 (lower panels). Representative photomicrographs per group for HOS osteosarcoma mice are shown.

C) Parental (P), mock- (M) and Smad7-transfected (S7) HOS cells were treated with TGF-β1 (5 ng/ml) for 6 days. After incubation, cell viability was evaluated by XTT test. Bars indicate means ± S.D. of two independent experiments, each performed in sixplicate.

D) Intramuscular paratibial injections of 1.10⁶ HOS tumor cells were performed in 3 groups of nude mice (vehicle, SD-208 20 mg/kg/day and SD-208 60 mg/kg/day, n=6 mice for each group). The mean tumor volumes were calculated from day 1 to day 28.

**Figure 3: Overexpression of Smad7 in tumor cells inhibits tumor associated bone resorption**

Mice were either injected with 1.10⁶ parental (P), mock- (M) and Smad7-transfected (S7) HOS cells (n = 10 for parental, n = 4 for mock and n = 7 for Smad7 cells), or with 1.10⁶ parental (P), mock- (M) and Smad7-transfected (S7) SaOS2 cells (n = 7 for parental, n = 7 for mock and n = 7 for Smad7). The results are representative of 2 independent experiments.

A) 3D reconstructions of one representative tibia/fibula of each group (parental, mock and Smad7 HOS cells) were performed when tumor sizes were approximately 2500 mm³, and compared to a healthy group bearing no tumors (control).

B) Left panels: Graphs represent the total bone volume of each individual animal in a given group injected with either HOS cells (upper panel) or with SaOS2 cells (lower panel) (***p<0.005). Right panels: Graph represents the mean ectopic bone volume of each individual animal in a given group injected with either HOS cells (upper panel) or with SaOS2 cells (lower panel) (***p<0.005).

C) Histograms represent the mean trabecular thickness (upper panel) and the number of bone trabecular (lower panel) in mock and Smad7 HOS groups compared to control group corresponding to mice bearing no tumors (***p<0.005, *p<0.05).
D) Intramuscular paratibial injections of $1.10^6$ HOS tumor cells were performed in 3 groups of nude mice (vehicle, SD-208 20 mg/kg/day and SD-208 60 mg/kg/day). The bone volumes of tibia were measured both at the leg having undergone the injection of tumor cells (right panel) and at the counterpart legs (left panel) when tumor volume reached 1000 mm$^3$. Graphs represent the bone volume of each individual animal in a given group (**p<0.01).

**Figure 4:** *Overexpression of Smad7 in osteosarcoma reduces osteoclast activity.*

A) Tumor samples (tumor sizes at 1000 mm$^3$) of the mock and Smad7 group were fixed, embedded in paraffin, sectioned and stained for TRAP (red stained cells, upper panels), osteocalcin (middle panels) and RANKL (lower panels). Representative photomicrographs per group for HOS osteosarcoma mice are shown.

B) *RANKL* mRNA steady-state levels were determined by quantitative RT-PCR in the presence or absence of TGF-β as indicated. Bars indicate mean ± S.D. of at least three independent experiments carried out in duplicate (upper panel). Parental (P), mock- (M) and Smad7-transfected (S7) HOS cells were treated with TGF-β1 (5 ng/ml) for 24h. After incubation, *RANKL* mRNA steady-state levels were determined by quantitative RT-PCR (middle panel). RNA was extracted from tumor biopsies of mice injected with mock- (black) or Smad7 (grey) transfected HOS cells. *RANKL* mRNA steady-state levels were determined by quantitative RT-PCR (lower panel). Bars indicate means ± S.D. of at least three independent experiments, performed in duplicate (*p<0.05, **p<0.01).

C) Concentrations of pyridinoline in mice serum of the mock and Smad7 groups were measured using the MicroVue Serum PYD EIA kit. Bars indicate means ± S.E.M of two independent experiments, performed in duplicate (*p<0.05).

D) Left panel: Treatment of mock- (black) and Smad7-transfected (grey) HOS cells with TGF-β1 (5 ng/ml) for 24h. After incubation, *IL-11* and *osteopontin (OPN)* mRNA steady-state levels were determined by quantitative RT-PCR. Bars indicate mean ± S.D. of at least three independent experiments carried out in duplicate (**p<0.005). Right panel: RNA was extracted from tumor biopsies of mice injected with mock- (black) and Smad7 (grey) transfected HOS cells. *IL-11* and *osteopontin (OPN)* mRNA steady-state levels were determined by quantitative RT-PCR. Bars indicate mean ± S.D. of two independent experiments carried out in duplicate (**p<0.005).
**Figure 5:** Overexpression of Smad7 in osteosarcoma and treatment of mice with SD-208 block lung metastasis development

Mice were either injected with parental (P), mock- (M) and Smad7-transfected (S7) osteosarcoma cells (HOS or SaOS2), or injected with parental-HOS cells and treated with vehicle or SD-208 as indicated. Mice were sacrificed when tumor sizes reached 2500 mm³ and lungs were removed.


**B)** Graphs indicate individual (dots) and mean (lines) numbers of lung metastasis measured in lungs from each group (**p<0.005; *p<0.01**), respectively for P, M and S7-HOS model (left panel), P, M and S7-SaOS2 model (middle panel) and for mice treated or not with SD-208 (right panel).

**C)** Tumor samples (tumor sizes at 2500 mm³) of the Parental (P), Mock (M), Smad7 (S7) group (upper panels) and from mice treated or not with SD-208 as indicated (lower panels) were fixed, embedded in paraffin, sectioned and stained with CD146. Representative photomicrographs per group for HOS osteosarcoma mice are shown.

**D)** RNA was extracted from tumor biopsies of mice injected with Parental (black), mock- (grey) and Smad7- (white) transfected HOS cells (left panel) or from tumor biopsies from mice treated with SD-208 at 20mg/kg/day (grey), SD-208 at 60mg/kg/day (white) or with vehicle (black) (right panel). VEGF, ANGPTL4 and CXCR4 mRNA steady-state levels were determined by quantitative RT-PCR. Bars indicate mean ± S.D. of two independent experiments carried out in duplicate.

**Figure 6:** Overexpression of Smad7 in osteosarcoma cells or treatment of cells with SD-208 inhibit the ability of TGF-β to induce osteosarcoma cell migration and invasion

**A and B)** Left panel: 30 000 parental, mock- or Smad7-transfected HOS cells pre-treated during 24 h with 5 ng/ml TGF-β were seeded onto the upper surface of uncoated (A) or transwell coated with 2µg Matrigel (B) inserts. 48h after incubation in the presence or absence of TGF-β (5 ng/ml), the cells on the underside of the membrane were fixed, stained with “cristal violet” and counted by bright-field microscopy in five random fields (magnification: X200). Bars indicate mean ± S.D. of at least three independent experiments carried out in duplicate (**p<0.005). Middle panel: 30 000 parental HOS cells pre-treated during 24 h with 5 ng/ml TGF-β in the presence or absence of SD-208 (as indicated) were seeded onto the upper surface of uncoated (A) or transwell coated with 2µg Matrigel (B) inserts.
48h after incubation in the presence or absence of TGF-β (5 ng/ml) and SD-208 (as indicated), the cells on the underside of the membrane were fixed, stained with "cristal violet" and counted by bright-field microscopy in five random fields (magnification: X200). Bars indicate mean ± S.D. of at least three independent experiments carried out in duplicate (**p<0.005). Right panel: Photographs of representative random fields (magnification: X200) of each group.

C) Upper panel: Zymography analysis of conditioned media from 48 h serum-free cultures of HOS-P, -M and -S7 cells treated with 5 ng/mL TGF-β or untreated. A Coomassie blue stained gel representative of three independent experiments is shown. Lower panel: Zymography analysis of conditioned media from 48 h serum-free cultures of HOS cells treated with 5 ng/mL TGF-β in the presence or absence of SD-208 (10 µM). A Coomassie blue stained gel representative of three independent experiments is shown.

D) Upper panels: HOS-P, -M and –S7 cells were incubated with TGF-β1 (5 ng/ml) for 24 h (left panel). HOS cells were incubated with TGF-β1 (5 ng/ml) in the presence or absence of SD-208 (10 µM) for 24 h (right). After incubation, MMP-2 mRNA steady-state levels were determined by quantitative RT-PCR. Bars indicate mean ± S.D. of at least three independent experiments carried out in duplicate (**p<0.05; ***p<0.005). Lower panel: RNA was extracted from tumor biopsies of mice injected with parental- (black), mock- (grey) and Smad7- (white) transfected HOS cells (left panel). RNA was extracted from tumor biopsies of mice injected with HOS cells and treated with SD-208 at 20mg/kg/day (grey), SD-208 at 60mg/kg/day (white) or with vehicle (black) (right panel). MMP-2 mRNA steady-state levels were determined by quantitative RT-PCR. Bars indicate mean ± S.D. of two independent experiments carried out in duplicate (**p<0.05).
Figure 1
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Figure 6
Overexpression of Smad7 blocks primary tumor growth and lung metastasis development in osteosarcoma

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