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► **To cite this version:**

Delphine Javelaud, Khalid S. Mohammad, Christopher R. Mckenna, Pierrick Fournier, Flavie Luciani, et al.. Stable overexpression of Smad7 in human melanoma cells impairs bone metastasis.. Cancer Research, American Association for Cancer Research, 2007, 67 (5), pp.2317-24. 10.1158/0008-5472.CAN-06-3950 . inserm-00147397

**HAL Id: inserm-00147397**

**<https://www.hal.inserm.fr/inserm-00147397>**

Submitted on 9 Sep 2008

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## **Stable Overexpression of Smad7 in Human Melanoma Cells Impairs Bone Metastasis**

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Running title: Autocrine TGF- $\beta$  and melanoma bone metastasis

Keywords: TGF- $\beta$ , Smad, Melanoma, Bone metastasis

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Abbreviations: CTGF: connective tissue growth factor; IL-11: interleukin-11; OPN: osteopontin; PTHrP: parathyroid hormone-related peptide; TGF- $\beta$ : transforming growth factor- $\beta$

## SUMMARY

Melanoma has a propensity to metastasize to bone, where it is exposed to high concentrations of TGF- $\beta$ . Because TGF- $\beta$  promotes bone metastases from other solid tumors such as breast cancer, we tested the role of TGF- $\beta$  in melanoma metastases to bone. 1205Lu melanoma cells, stably transfected to overexpress the natural TGF- $\beta$ /Smad signaling inhibitor Smad7, were studied in an experimental model of bone metastasis whereby tumor cells are inoculated into the left cardiac ventricle of nude mice. All mice bearing parental and mock-transfected 1205Lu cells developed osteolytic bone metastases 5 weeks post tumor inoculation. Mice bearing 1205Lu-Smad7 tumors had significantly less osteolysis on radiographs and longer survival compared to parental and mock-transfected 1205Lu mice. To determine if the reduced bone metastases observed in mice bearing 1205Lu-Smad7 clones was due to reduced expression of TGF- $\beta$  target genes known to enhance metastases to bone from breast cancer cells, we analyzed gene expression of osteolytic factors, PTHrP and IL-11, the chemotactic receptor CXCR4, and osteopontin in 1205Lu cells. Quantitative RT-PCR analysis indicated that PTHrP, IL-11, CXCR4, and osteopontin mRNA steady-state levels were robustly increased in response to TGF- $\beta$ , and that Smad7, and the T $\beta$ RI small molecule inhibitor, SB431542, prevented such induction. Also, 1205Lu-Smad7 metastases expressed significantly lower levels of IL-11, CTGF and PTHrP. These data suggest that TGF- $\beta$  promotes osteolytic bone metastases due to melanoma by stimulating the expression of prometastatic factors via the Smad pathway. Blockade of TGF- $\beta$  signaling may be an effective treatment for melanoma metastasis to bone.

## INTRODUCTION

Transforming growth factor (TGF)- $\beta$  is a prototypic multifunctional cytokine whose broad modulatory activity affects numerous biological functions. At the organism level, these include, but are not limited to, control of immune functions, embryogenesis, carcinogenesis and tissue responses to injury. At the cell level, TGF- $\beta$  controls proliferation, migration, as well as extracellular matrix (ECM) synthesis and degradation (1-5). The complexity of the role played by TGF- $\beta$  in cancer and metastasis is underscored by the duality of this growth factor, depending on the stage of the disease (6-8). Thus, even though the TGF- $\beta$  signaling cascade functions as a tumor suppressor pathway in early carcinogenesis, mainly through the ability of TGF- $\beta$  to inhibit the cell cycle, it paradoxically favors tumor progression during late-stage metastatic progression of tumors.

TGF- $\beta$  signal transduction occurs via ligand-activated heterotetrameric serine/threonine kinase receptors (T $\beta$ RI and T $\beta$ RII) on the cell surface, which phosphorylate the cytoplasmic proteins Smad2 and Smad3. These receptor-regulated Smads (R-Smads) are ligand-specific and, upon activation, associate with Smad4, a common partner to all receptor-regulated Smads activated by the various ligands of the TGF- $\beta$  family. R-Smad/Smad4 heterocomplexes then translocate into the cell nucleus to regulate target gene transcription (5, 9). The inhibitory Smad, Smad7, interferes with Smad signaling by various means: (a), it binds activated T $\beta$ RI to prevent phosphorylation of Smad2/3; (b), it recruits E3 ubiquitin-ligases such as Smurf1, Smurf2 and WWP1, to the activated TGF- $\beta$  receptor complexes, leading to their proteasomal degradation; (c), it interacts with GADD34, the regulatory subunit of the protein phosphatase PP1, thereby recruiting it to T $\beta$ RI to inactivate the latter (10-12).

The impact of TGF- $\beta$  on melanoma progression is just beginning to unravel. Malignant melanomas secrete high amounts of TGF- $\beta$ , whose autocrine and paracrine effects contribute directly and indirectly to tumor progression (13). Indeed, we previously identified that melanoma cell-derived TGF- $\beta$  results in high, ligand-dependent, constitutive Smad3-driven transcriptional activity (14). Also, we identified that overexpression of Smad7 inhibits melanoma cell MMP-2 and MMP-9 production, dramatically impairs their invasive capacity *in vitro*, reduces anchorage-independent growth, and delays subcutaneous tumor growth in nude mice (15). These findings underscore the notion that intact, or exacerbated, Smad signaling occurs throughout tumor progression in melanoma cells.

In experimental models of metastasis, it has been shown that TGF- $\beta$  is essential for breast cancer cells to form bone metastases (16, 17). Furthermore, the release of TGF- $\beta$  from the bone matrix upon activation of osteoclasts by soluble factors, such as parathyroid hormone-related protein (PTHrP) derived from cancer cells, further exacerbates the latter and enhances the growth of metastases, thereby establishing what has been viewed as a vicious cycle orchestrated by TGF- $\beta$  (18, 19).

In this report, we demonstrate that Smad7 overexpression delays the establishment and growth of melanoma bone metastases in a mouse model. Also, we determine that inhibition of the Smad cascade in melanoma cells represses the expression of a panel of TGF- $\beta$ -dependent genes that was previously identified as critical for the establishment of bone metastases by breast cancer cells (17).

## MATERIALS AND METHODS

### Cell cultures and reagents

Melanoma cell lines WM239-A, WM1341-A, WM983-A, WM793, WM983-B, WM852, and 1205Lu, kind gifts from Dr. M. Herlyn, Wistar Institute, Philadelphia, PA, have been previously described (14, 20, 21). They were grown in a composite medium (W489) consisting of three parts MCDB153 and one part L15 supplemented with 4% fetal calf serum (FCS) and antibiotics. Melanoma cell lines Dauv-1, 888-mel, 501-mel, SK-28, and FO-1 have been described previously (22). They were grown in RPMI medium supplemented with 10% FCS and antibiotics. Human lung fibroblasts (WI-26) were grown in DMEM containing 10% FCS and antibiotics. Generation of Smad7 expressing clones (S7.a and S7.c) and mock-transfected cells has been described previously (15). All cells were grown at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere. The Smad3/4-specific reporter plasmid (CAGA)<sub>9</sub>-luc (23) was a gift from S. Dennler (INSERM U697). The pRL-TK vector was from Promega (Madison, WI). TGF-β<sub>1</sub> was purchased from R&D Systems Inc. (Minneapolis, MN). The ALK5/TβRI inhibitor SB431542 was from Sigma-Aldrich (St-Louis, MO).

### Biochemical methods

Protein extraction and Western blotting were performed as previously described (24). Anti-Smad3 and anti-β-actin were from Zymed (San Francisco, CA) and Sigma-Aldrich, respectively. The rabbit anti-phospho-Smad2/3 antibody (25) was a generous gift from E. Leof (Mayo Clinic College of Medicine, Rochester, MN). Secondary anti-mouse and anti-rabbit HRP-conjugated antibodies were from Santa-Cruz Biotechnology Inc. (Santa-Cruz, CA).

### **Generation of melanoma cell conditioned media**

$3 \times 10^6$  cells of each cell type were plated in a 150-mm tissue culture dish and cultured in 16 ml W489 medium without FCS for 72 h. Supernatants were then collected and floating cells removed by centrifugation (420g, 5 min.). The corresponding cell layers were lysed and total protein concentration was determined to normalize each conditioned medium.

### **Cells transfections and luciferase assays**

Melanoma cells were seeded in 24-well plates and transfected at approximately 70–80% confluency with the polycationic compound Fugene™ (Roche Diagnostics, Indianapolis, IN, USA) in fresh medium containing 1% FCS. TGF- $\beta$  was added 4 h after transfection. WI-26 fibroblasts were seeded in 24-well plates and transfected at approximately 70–80% confluency with Jet-PEI™ (Polyplus-Transfection, Illkirch, France) in fresh W489 medium without FCS. Conditioned media were added 4h after transfection. Following a 16-h incubation, cells were rinsed twice with phosphate-buffered saline and lysed in passive lysis buffer (Promega). Luciferase activities were determined with a Dual-Glo™ luciferase assay kit according to the manufacturer's protocol (Promega).

### **Animal experiments**

The animal protocols for bone metastasis experiments were approved by the Institutional Animal Care and Use Committee at the University of Virginia in Charlottesville and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Athymic female nude mice 4 weeks of age were housed in laminar flow isolated hoods. Water supplemented with vitamin K and autoclaved mouse chow were provided *ad libitum*. Radiographs were taken under mouse anesthesia mixture (30% ketamine and 20% xylazine in 0.9% NaCl). Tumor inoculation into the left cardiac ventricle was performed on

anesthetized mice positioned ventral side up, as described previously (26). Briefly, the left cardiac ventricle was punctured percutaneously using a 26-gauge needle attached to a 1-ml syringe containing suspended tumor cells. Visualization of bright red blood entering the hub of the needle in a pulsatile fashion indicated a correct position in the left cardiac ventricle. Tumor cells ( $10^5$  in 0.1 ml of PBS) were inoculated slowly over 1 min. Mice were followed by radiography for the development of bone lesions throughout the experiments. Mice were x-rayed in a prone and lateral position using a Digital Faxitron MX-20 with digital camera (Faxitron x-ray, Wheeling, IL), as described previously (26). Radiographs were taken at 1X magnification and when a lesion was suspected, additional images with higher magnification (4X) were taken. Images were saved and lesion areas were measured and analyzed using MetaMorph software (Molecular Devices, Downingtown, PA).

**Bone Histology:** Forelimb and hind limb bones were removed from mice at the time of experimental termination. Tissues were fixed in 10% neutral buffered formalin for 48h, decalcified in 10% EDTA for 2 weeks, processed using an automated tissue processor (Excelsior, Thermoelectric), and embedded in paraffin. The sections were stained with hematoxylin and eosin with orange G and phloxine to visualize new bone.

### **RNA extraction and gene expression analysis**

*In vitro* experiments: Total RNA was isolated using an Rneasy™ kit (Qiagen GmbH, Hilden Germany). *In vivo* experiments: The femur and tibiae from mice were dissected and cleaned from adhering tissues. The cartilage ends were cut off and the tumor cells in the marrow cavity were flushed out using cold PBS in a syringe with a sterile needle. After centrifugation, cells were resuspended in RNeasy™ (Qiagen) and total RNA was isolated with TRIzol (Invitrogen, San Diego, CA), according to the manufacturer's instructions. Genomic DNA contaminations were eliminated by DNase I treatment. One microgram of RNA from each

sample was reverse transcribed using the ThermoScript kit (Invitrogen) following the manufacturer's instructions. The resulting cDNAs were then processed for Real-time PCR using SYBR Green technology. Reactions were carried out in a 7300 Real-time PCR System (Applied Biosystem) for 40 cycles (95°C for 15 sec/ 60 C for 1 min) after an initial 10-min incubation at 95°C. Primers were as follows: *in vitro* experiments, *TGFβ1* (sense, 5'-ctctccgacctgccacaga-3' ; anti-sense, 5'-aacctagatgggcgcatct-3'); *TGFβ2* (sense, 5'-ccfcccactttctacagacc-3' ; antisense, 5'-gcgctgggtgggagatgtaa-3'); *TGFβ3* (sense, 5'-ctggcctgtgaactttg-3' ; antisense, 5'-aaggtggtgcaagtggacaga-3'); *CXCR4* (sense, 5'-cagtgccgacctctctt-3' ; antisense, 5'-cagttgccacggcatca-3'); *IL-11* (sense, 5'-actgctgctgctgaagactc-3' ; anti-sense, 5'-ccaccctgctcctgaaata-3'); *PTHrP* (sense, 5'-tttacggcgacgattcttcc-3' ; anti-sense, 5'-ttctcccaggtgtcttgag-3'); *osteopontin* (sense, 5'-aggcagagcacagcatcgt-3' ; 5'-ttggctgagaaggctgcaa-3'); *in vivo* experiments, *IL-11* (sense, 5'-tgaagactcggctgtgacc-3' ; anti-sense, 5'-cctcacggaaggactgtctc-3') ; *PTHrP* (sense, 5'-actcgtctgcctggtaga-3' ; anti-sense, 5'-ggaggtgtcagacaggtggt-3'); *CTGF* (sense, 5'-gctaccacatttctacctagaaatca-3' ; anti-sense, 5'-gacagtcctgcaaacagattgtt-3'). Target gene expression was normalized against the endogenous control genes *cyclophylin A* (sense, 5'-caaatgctggaccaacaca-3' ; anti-sense, 5'-tgccatccaaccactcagtct-3') or *GAPDH* (sense, 5'-gctcctctgttcgacagtca-3' ; anti-sense, 5'-acctccccatggtgtctga-3'). Data were analyzed using Applied Biosystems Sequence Detections Software (version 1.2.1).

### Statistical analyses

Differences in osteolytic lesion areas between groups were determined by one-way ANOVA and two-way ANOVA. Kaplan-Meier survival curve data were analyzed by Logrank test. All results were expressed as mean ± SEM, and  $p < 0.05$  was considered significant (GraphPad Prism).

## RESULTS AND DISCUSSION

The role of TGF- $\beta$  in melanoma progression and metastasis is controversial. Using a specific Smad-dependent transcription assay as well as Smad/DNA interaction assays based upon our original characterization of *hCOL7A1* as a Smad3/4 gene target (27), we previously demonstrated that human melanoma cells secrete active TGF- $\beta$  and exhibit both high constitutive, ligand-induced, Smad signaling (14). Subsequently, others have shown that TGF- $\beta$  that is produced by melanoma cells contributes to peri-tumoral stroma remodeling, providing a survival advantage to melanoma cells (28). Contrasting with these results, it has also been proposed that the oncoproteins c-Ski and SnoN, known to interfere with Smad signaling (29), are expressed at high levels in melanoma cells, and may be responsible for the lack of growth inhibitory activity of TGF- $\beta$  in these cells (30, 31). Recently, we demonstrated that overexpression of Smad7 in melanoma cells inhibits endogenous constitutive Smad signaling, reduces MMP secretion, and delays tumorigenicity both *in vitro* and in a model of sub-cutaneous tumor formation in nude mice (15). These apparently contradictory results regarding the role of autocrine TGF- $\beta$  signaling in melanoma cells led us to re-evaluate the activation status of Smad signaling obtained in a number of available melanoma cell lines. Most transcriptional responses to TGF- $\beta$  in the adult are mediated by Smad3/4 while it is generally accepted that Smad2 is critical during embryonic life (9). We thus focused our attention on Smad3/4 status and activation. As shown in Fig. 1A, the various cell lines tested exhibited variable basal levels of P-Smad3, a marker of TGF- $\beta$  receptor activation (Fig. 1A). In all cases, exogenously added TGF- $\beta$  further induced a dramatic elevation of P-Smad3 levels, indicative of functional TGF- $\beta$  receptor complexes (Fig. 1B). Transient cell transfection experiments with the Smad3/4-specific reporter construct (CAGA)<sub>9</sub>-lux (23) indicated that most cell lines responded to exogenous TGF- $\beta$  with a robust transcriptional

response (Fig. 1C), indicative of functional TGF- $\beta$ /Smad signal transduction cascade all the way from the cell membrane to the nucleus. These results are in full agreement with our initial observations (14), and consistent with the accepted concept of an autocrine and oncogenic role for TGF- $\beta$  in late-stage carcinogenesis - reviewed in (32, 33).

TGF- $\beta$  expression is often increased in tumor cells and has been correlated with the advanced stage of melanoma progression (34). We thus wanted to determine whether an auto-regulatory loop controlled TGF- $\beta$  expression in melanoma cells. Firstly, using quantitative real-time-PCR, we determined that all melanoma cell lines available in the laboratory expressed the three TGF- $\beta$  isoforms (not shown). TGF- $\beta$ 1 expression was predominant, with relative expression levels 10- to 20-fold higher than those for TGF- $\beta$ 3 (not shown). TGF- $\beta$ 2 was expressed at very low levels (100- to 1000-fold less than TGF- $\beta$ 1). These results are consistent with previously published observations (35). Incubation of some of these cell lines, selected based on their robust Smad-specific transcriptional response to TGF- $\beta$  (see Fig. 1C) with exogenous TGF- $\beta$ 1 for 24h led to a consistently observed increase in the expression of TGF- $\beta$ 1, which was totally abrogated by the ALK5 inhibitor SB431542 (Fig. 2A). Expression of TGF- $\beta$ 2 mRNA was also induced by TGF- $\beta$ 1 treatment (Fig. 2B), however it only occurred in 50% of the cell lines tested (not shown). Again, incubation with the ALK5 inhibitor SB431542 led to a complete abrogation of TGF- $\beta$ 2 induction by exogenous TGF- $\beta$ 1. Of note, no modulation of TGF- $\beta$ 3 expression was observed after either TGF- $\beta$ 1 or SB431542 treatment in any of the cell lines tested (not shown), suggesting its independence from Smad signaling.

To determine whether melanoma cells secrete active TGF- $\beta$  that could induce stroma activation, melanoma cell culture supernatants were studied for their capacity to activate the Smad pathway in fibroblasts in culture. Incubation of WI-26 human lung fibroblasts with

conditioned media from four distinct melanoma cell lines exhibiting constitutive Smad3/4 activation and TGF- $\beta$ 1 expression induced both rapid and sustained Smad3 phosphorylation in WI-26 cells (Fig. 2C), and potent transactivation of the transfected (CAGA)<sub>9</sub>-lux construct (Fig. 2D). Incubation with W849 un-conditioned medium did not induce any of these TGF- $\beta$ -specific responses. Together, these results emphasize the fact that ligand-dependent constitutive activation of the Smad pathway in melanoma cells translates into production of active TGF- $\beta$  that is highly capable of inducing both autocrine and paracrine (stromal) responses.

It is expected from our data on the expression of the three TGF- $\beta$  isoforms (see above) that TGF- $\beta$ 1, and TGF- $\beta$ 3, represent the majority of constitutively produced isoforms capable of driving Smad signaling in melanoma cells, even in the presence of a T $\beta$ RI inhibitor. Furthermore, TGF- $\beta$ 1, and TGF- $\beta$ 2, is likely to be induced further in tissue environments rich in TGF- $\beta$ , such as in late-stage cancer progression.

We previously demonstrated that TGF- $\beta$  promotes bone metastases from solid tumors such as breast cancer (16, 17, 19). Thus, given our recent data linking autocrine TGF- $\beta$  signaling to melanoma aggressiveness (15), we decided to test the role of autocrine TGF- $\beta$  signaling in melanoma metastases to bone by overexpressing the inhibitory Smad7 in a cell line with high endogenous Smad activity. For this purpose, we used 1205Lu cells as, (a), they exhibit high autonomous, ligand-induced, constitutive TGF- $\beta$ /Smad signaling; (b), they exhibit a strong transcriptional response to exogenous TGF- $\beta$ ; (c) they are highly invasive in a Matrigel™ assay, and (d), they are highly tumorigenic *in vivo* (15). Furthermore, we previously demonstrated that Smad7 overexpression in 1205Lu melanoma cells reduces MMP-2 and MMP-9 production, as well as their capacity to invade Matrigel™ and to establish subcutaneous tumors in nude mice (15).

In a proof-of-concept experiment, parental 1205Lu cells rapidly established bone metastases in a model of bone metastasis whereby tumor cells are inoculated into the left cardiac ventricle of nude mice. Reasons for using the model include the facts that 1) no model exists whereby primary melanoma tumors metastasize to bone; 2) the left cardiac ventricle model was established with melanoma lines (36); 3) the relevance to bone metastases has been consistently demonstrated in the literature (37, 38). Specifically, this model addresses the process of metastasis from entry of tumor cells into the arterial circulation to the establishment of bone metastasis, and tumor-bone interactions.

Bone metastases appeared to be mostly osteolytic, but some osteoblastic lesions were also found (Supplementary data, Fig. 1). In addition, soft tissue metastases to various organs – adrenal glands, lungs, liver and skin - appeared in all test mice (n=6) 5-10 weeks after tumor inoculation (not shown).

Next, 1205Lu melanoma cells were stably transfected with pcDNA-Smad7. In the first experiment, Smad7-overexpressing clones were compared with parental 1205Lu. All mice (n=6) inoculated with parental 1205Lu cells developed osteolytic bone metastases 5 weeks post tumor inoculation (Fig. 3A, left panel), as well as metastases to adrenal glands and kidney (not shown). All mice bearing parental tumor cells had to be euthanized by week 5. In contrast, only 1 of 14 mice bearing Smad7-transfected 1205Lu cells (2 different clones, 7 mice for each group) had osteolytic bone lesions on radiographs 10 weeks post tumor inoculation (Fig. 3A, center and right panels), and only 5/14 had been euthanized after 15 weeks. The remaining animals were euthanized after 19 weeks because they all exhibited severe cachexia. Together, mice bearing Smad7-overexpressing 1205Lu tumors had significantly longer survival compared with mice bearing parental 1205Lu (Figure 3B). Quantitative computerized image analysis of osteolytic lesion area on radiographs indicated that at time of experiment termination (19 weeks), mice bearing Smad7-overexpressing

1205Lu had markedly less bone destruction than the mice inoculated with parental 1205Lu tumor cells after only 5 weeks (Figure 3C). This was remarkable in light of the fact that the mice bearing Smad7-overexpressing 1205Lu melanoma were euthanized 16-20 weeks following tumor inoculation compared with 5 weeks for mice bearing parental 1205Lu melanoma.

In a separate experiment, mice inoculated with Smad7-overexpressing 1205Lu melanoma cells (12 mice for each Smad7-overexpressing 1205Lu clone) were compared to mice inoculated with an empty vector-transfected control 1205Lu clone (11 mice) for the early establishment of bone metastases. For this purpose, the osteolytic lesion area was measured by x-ray, 4 and 6 weeks following intra-cardiac injection of the tumor cells. As shown in Figure 3D, while 10/11 mice injected with pcDNA-transfected tumor cells (EV) developed osteolytic metastases clearly detectable by x-ray as early as 4 weeks post-inoculation, none of the 24 mice inoculated with either of the Smad7-transfected clones had detectable bone lesions after 6 weeks.

Taken together, these experiments demonstrate (a), that 1205Lu-Smad7 tumors cause significantly less and delayed bone metastases than parental and mock-transfected cells, and (b), that mice bearing these Smad7 tumors live longer.

The contribution of TGF- $\beta$  to bone metastasis of breast cancer cells has been ascribed to what has been viewed as a vicious cycle whereby tumors homing favorably to bone secrete osteolytic factors such as PTHrP and IL-11. The latter activate osteoblasts and osteoclasts to cause the degradation of the bone matrix and subsequent release of soluble factors, including TGF- $\beta$  which, in turn, exacerbates tumor cells to produce more osteoclast-activating factors (19). A signature of TGF- $\beta$ -inducible genes was identified, comprising CTGF, CXCR4 and IL-11, which, when overexpressed in non-metastatic cells, induced them to metastasize to

bone (17, 39). We thus screened several melanoma cell lines for their expression of TGF- $\beta$ -dependent target genes known to enhance metastases to bone from breast cancer cells, namely the osteolytic factors PTHrP and IL-11, the chemotactic receptor CXCR4, as well as CTGF and osteopontin. Quantitative RT-PCR analysis indicated that PTHrP, IL-11, CXCR4, and osteopontin mRNA steady-state levels were robustly increased in all cell lines treated with TGF- $\beta$  *in vitro* (Table 2). The small molecule inhibitor ALK5/T $\beta$ RI, SB431542, efficiently prevented the induction of PTHrP, IL-11, CXCR4 and osteopontin by TGF- $\beta$  in all cell lines tested, and inhibited the basal expression levels of those genes, suggesting a role for autocrine TGF- $\beta$  signaling in controlling their basal level of expression.

Next, we examined whether Smad7 overexpression modulated the expression of bone metastasis-specific genes in 1205Lu cells *in vitro* and *in vivo*. As shown in Figure 4, stable Smad7 overexpression significantly reduced the extent of activation of IL-11, CXCR4, ONP, and to a lesser extent, PTHrP, gene expression by TGF- $\beta$  in two distinct clones. To determine whether such downregulation of bone metastasis-specific genes by Smad7 occurred *in vivo*, RNA was extracted from bone and subcutaneous metastases that developed in mice after intra-cardiac inoculation of either mock- or Smad7-transfected 1205Lu melanoma cells. Semi-quantitative RT-PCR was used to determine the respective steady-state mRNA levels for *IL-11*, *PTHrP*, *CTGF* and *GAPDH* using primers specific for the human ortholog of each gene. Gene expression of bone metastases genes *IL-11*, *PTHrP* and *CTGF* in RNA derived from melanoma tumors differed depending on the metastatic site and whether the tumors expressed Smad7. As shown in Figure 5, bone metastases from both Smad7 clones expressed little or no *IL-11*, *PTHrP* and *CTGF* while a bone metastasis from mock-transfected 1205Lu cells expressed these genes, all shown previously to drive the capacity of the latter to form bone metastases by MDA-MB-231 breast cancer (17). In contrast, *IL-11*, *PTHrP* and *CTGF*

were expressed in subcutaneous metastases regardless of whether the tumors overexpressed Smad7. One explanation for this differential expression may be due to different concentrations of TGF $\beta$  in the respective microenvironments. In bone, where TGF $\beta$  concentrations are high, Smad7 blocks the induction of bone metastases genes. In the subcutaneous site, *PTHrP* and *CTGF* may be induced by growth factors which do not signal through the TGF $\beta$ /Smad pathway. Thus, Smad7 does not block their induction. Together, these data suggest that attenuation of TGF- $\beta$  signaling by Smad7 results in tissue-specific regulation of genes involved in the process of bone metastasis by melanoma cells. It is thus likely that the pathophysiology of melanoma metastases to bone is similar to breast cancer, and that extinguished *IL-11*, *CTGF* and *PTHrP* expression likely contributes to the reduced capacity of Smad7-expressing clones to form bone metastases.

We previously demonstrated that expression of a dominant-negative T $\beta$ RII by MDA-MB-231 resulted in a reduced capacity for these aggressive breast cancer cells to form bone metastases, due to lesser bone destruction, less tumor with fewer associated osteoclasts, and prolonged animal survival compared with controls (26). PTHrP, produced by tumor cell under the control of TGF- $\beta$  signaling, was identified as critical for bone destruction. More recently, using both functional imaging of the Smad pathway in a mouse xenograft model and immunohistochemical analysis of human breast cancer bone metastases, Kang et al. provided evidence for active Smad signaling in both human and mouse bone metastatic lesions (40). Depletion of Smad4 using RNA interference demonstrated that the Smad pathway contributes to the formation of osteolytic bone metastases by MDA-MB-231 breast cancer cells, by controlling the expression of IL-11, CXCR4, and CTGF (41), thus reinforcing previous observations (40). Together, these studies suggest that a limited, yet highly significant gene signature is indicative of the bone metastasis potential of breast cancer cells. Many of these genes are regulated by TGF- $\beta$  via the Smad signaling pathway. Consistent with these

collective findings, our results demonstrate that targeting endogenous TGF- $\beta$ /Smad signaling in melanoma cells by mean of Smad7 overexpression is sufficient to dramatically delay bone metastasis formation in a mouse model of tumor cell inoculation in the left cardiac ventricle. Smad7 overexpression was shown to reduce the expression of the TGF- $\beta$ /Smad-dependent bone metastasis signature genes, PTHrP, IL-11, CTGF, osteopontin or CXCR4. We do not provide functional evidence for a causal role for each of these target genes in the melanoma metastatic process to bone. Yet, our results are consistent with the hypothesis that TGF- $\beta$  may promote melanoma bone metastases via mechanisms similar to those identified in breast cancer metastasis.

The TGF- $\beta$ /Smad pathway has also been implicated in other aspects of metastases. For example, Smad4 knock-down has reduced bone metastases due to MDA-MB-231 via interference with epithelial to mesenchymal transition (41). Moreover, it was recently shown that adenoviral delivery of Smad7 to JygMC(A) breast cancer cells significantly impairs their capacity to metastasize to lung and liver, possibly by altering their adhesive and migratory properties (42). TGF- $\beta$  blockade has been effective to reduce breast cancer metastases to bone and other sites in mouse models (41, 43-47). In concordance, our results support the notion that blockade, or attenuation, of intracellular TGF- $\beta$  signaling may be beneficial for the treatment of melanoma, another aggressive solid tumor able to generate bone metastases.

### *Acknowledgements*

The authors are thankful to Drs. Meenhard Herlyn (Wistar Institute, Philadelphia, PA; Sylviane Dennler (INSERM U697) and Jean-Michel Gauthier (Glaxo-Wellcome, Les Ulis, France), Edward Leof (Mayo Clinic, Rochester, MN), and Peter ten Dijke (Leyden, The Netherlands) for providing us with reagents essential for these studies. This work was supported by the Emile and Henriette Goutière donation as well as grants from INSERM, and Cancéropole Ile-de-France-INCa, France (to A.M.); CNRS, (to L.L.), the National Institutes of Health, National Cancer Institute (R01CA69158) and the U.S. Army Medical Research and Materiel Command, Department of Defense (award number 0410920) to T.A.G. D.J. is the recipient of post-doctoral fellowship awarded by Fondation pour la Recherche Médicale (France) and received a travel grant from the Fondation René Touraine (France).

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**Table 1: Modulation of osteolytic factor gene expression by TGF- $\beta$  in melanoma cells**

Sub-confluent melanoma cell cultures were pre-incubated for 6h in W489 medium without serum before stimulation with TGF- $\beta$ 1 (10 ng/ml), in the absence or presence of 5  $\mu$ M SB431542 (or DMSO) added 1h before addition of the growth factor. Total RNA was analyzed by quantitative RT-PCR with primers specific for *IL-11*, *PTHrP*, *CXCR4*, and osteopontin (*OPN*). Values are corrected for *cyclophilin A* mRNA levels in the same samples and expressed as fold induction relative to untreated (ut) cultures, after 8h (\*) or 24h or incubation, depending on the peak of gene expression induced by TGF- $\beta$ .

	cell line	dms0		SB431542	
		u	TGF-	u	TGF-
IL-11	1205Lu	0.55 $\pm$ 0.03	19.28 $\pm$ 2.06	0.32	0.46 $\pm$ 0.07
	fold induction		35		1.1
	WM983	0.02	1.36 $\pm$ 0.01	0.02 $\pm$ 0.005	0.02 $\pm$ 0.01
	fold induction		68		1
	WM983	0.05 $\pm$ 0.03	1.66 $\pm$ 0.15	0.52 $\pm$ 0.14	0.46 $\pm$ 0.13
	fold induction		31.9		0.9
PTHrP	1205Lu	0.41 $\pm$ 0.005	1.12 $\pm$ 0.2	0.41 $\pm$ 0.01	0.33 $\pm$ 0.25
	fold induction		2.69		0.8
	WM983	0.08	0.41 $\pm$ 0.01	0.03 $\pm$ 0.005	N/A
	fold induction		5.1		
	WM983	0.08 $\pm$ 0.005	0.31 $\pm$ 0.03	0.12 $\pm$ 0.02	0.13 $\pm$ 0.01
	fold induction		3.9		1.1
CXCR4	1205Lu	0.04	0.43	0.01	N/A
	fold induction				
	WM983	1.47 $\pm$ 0.03	35.4 $\pm$ 9.1	2.12 $\pm$ 0.01	2.01 $\pm$ 0.35
	fold induction		24.1		0.9
	WM983	1.38	14.37	0.78	1.49
	fold induction		10.4		1.9
OPN	1205Lu	0.65	4.65 $\pm$ 0.19	0.67 $\pm$ 0.03	1.04 $\pm$ 0.03
	fold induction	$\pm$ 0.08	7.1		1.5
	WM983	69.9 $\pm$ 2.4	155.3 $\pm$ 7.5	92.6 $\pm$ 2.9	88.8 $\pm$ 1.3
	fold induction		2.2		0.9
	WM85	0.64	11.09 $\pm$ 0.16	0.38 $\pm$ 0.01	0.25 $\pm$ 0.02
	fold induction		1.7		0.65

## Legends to Figures

### Figure 1

A, Total protein extracts (60  $\mu\text{g}$ ) from unstimulated cultured melanoma cell lines were analyzed by Western blotting for phospho-Smad3 content. An anti  $\beta$ -actin antibody was used for normalization.

B, Sub-confluent melanoma cell cultures were incubated for 6h in medium containing 1% serum. TGF- $\beta$ 1 (10 ng/ml) was added to the cultures 20 min before cell lysis for Western analysis (30  $\mu\text{g}/\text{lane}$ ) with specific antibodies directed against phospho-Smad3, Smad3, or  $\beta$ -actin.

C, Subconfluent melanoma cell cultures were transfected with 0.4  $\mu\text{g}$  of (CAGA)<sub>9</sub>-luc vector together with 0.2  $\mu\text{g}$  of pRL-TK Renilla luciferase expression vector. Four hours after transfection, cultures were left untreated or were stimulated with TGF- $\beta$  (10 ng/ml). Luciferase activities were measured in cell extracts 20h after transfection. Results are the means  $\pm$  s.e.m. of three independent experiments.

### Figure 2

Sub-confluent melanoma cell cultures were pre-incubated for 6h in medium containing 1% serum before stimulation with TGF- $\beta$ 1 (10 ng/ml), in the absence or presence of 5  $\mu\text{M}$  SB431542 (or DMSO) added 1h before addition of the growth factor. Total RNA was analyzed by quantitative RT-PCR with primers specific for *TGF- $\beta$ 1* (A) or *TGF- $\beta$ 2* (B). Values are corrected for *cyclophilin A* mRNA levels in the same samples.

C, Sub-confluent WI-26 human lung fibroblast cultures were incubated for 24 h in medium containing 0% serum, following which an equivalent volume of either W489 unconditioned or 1205Lu melanoma cell-conditioned medium was added. WI-26 cell layer

were then lysed for Western analysis with specific antibodies directed against phospho-Smad3 and Smad3.

D, Subconfluent WI-26 fibroblast cultures were transfected with 0.4  $\mu\text{g}$  of (CAGA)<sub>9</sub>-luc vector together with 0.2  $\mu\text{g}$  of pRL-TK Renilla luciferase expression vector in W489 medium without serum. Four hours after transfection, cultures were left untreated or were stimulated with TGF- $\beta$  (10 ng/ml), or an equivalent volume of various melanoma cell-conditioned media. Luciferase activities were measured in cell extracts 20h later. Results, expressed as relative reporter activation, are the means  $\pm$  s.e.m. of three independent experiments.

### Figure 3

A, Representative X-ray radiography of the hindlimbs of mice, 5 weeks (parental, n=6) or 14 weeks (clones S7.a and S7.b, 7 mice each) after tumor cell inoculation. Osteolytic lesions are highlighted with yellow lines. Note the dramatic difference in both size and number of lesions generated by parental 1205Lu cells vs. Smad7-overexpressing clones.

B, Survival of mice bearing S7.a ( $\nabla$ ) and S7.c ( $\circ$ ) tumors was significantly longer than that of mice bearing parental 1205Lu cells ( $\blacktriangle$ ).

C, Osteolytic lesion area ( $\text{mm}^2$ ) on radiographs was measured by computerized image analysis of forelimbs and hindlimbs at the time of death: 5 weeks post-inoculation for parental 1205Lu cells, 16 and 20 weeks after inoculation for S7.a and S7.c clones, respectively. Values represent mean  $\pm$  SEM (n=7).

D, Osteolytic lesion area ( $\text{mm}^2$ ) on radiographs was measured by computerized image analysis of forelimbs and hindlimbs at 4 and 6 weeks post-inoculation for both pcDNA transfected 1205Lu cells (n=11,  $\blacksquare$ ) and clones overexpressing Smad7 (12 mice for each clone,  $\blacktriangle$ ,  $\blacktriangledown$ ). Values represent mean  $\pm$  SEM. Note that lesion area was so small in both

Smad7-overexpressing 1205Lu-bearing mice that the data lines are indistinguishable from the x-axis.

#### Figure 4

Sub-confluent melanoma cell cultures (Mock-transfected and clones S7.a and S7.c) were pre-incubated for 6h in medium containing 1% serum before stimulation with TGF- $\beta$ 1. Twenty-four hours later, RNA was extracted and analyzed by quantitative RT-PCR with primers specific for *IL-11* (A), *PTHrP* (B), *CXCR4* (C), and *OPN* (D). Values are corrected for *cyclophilin A* mRNA levels in the same samples.

#### Figure 5

RNA was extracted from either bone or soft tissue (subcutaneous) metastatic lesions derived from mock (M) and Smad7 (S7) inoculated tumor cells, and analyzed by semi-quantitative RT-PCR with primers specific for human *IL-11* (*hIL-11*), human *PTHrP* (*hPTHrP*), human *CTGF* (*hCTGF*) or human *GAPDH* (*hGAPDH*). The left lane corresponds to PCR reactions run without cDNA templates. As a positive control, we used mRNA from cultured human breast adenocarcinoma cell line MDA-MB-231, known to express the genes of interest. Mouse bone marrow RNA was used as a negative control to ascertain the tissue specificity of the primers.