The Tumor Suppressor Smad4 Is Required for Transforming Growth Factor β–Induced Epithelial to Mesenchymal Transition and Bone Metastasis of Breast Cancer Cells

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Abstract

Transforming growth factor β (TGF-β) can act as suppressor and promoter of cancer progression. Intracellular Smad proteins (i.e., receptor regulated Smads and common mediator Smad4) play a pivotal role in mediating antimitogenic and proapoptotic effects of TGF-β, but their function in TGF-β-induced invasion and metastasis is unclear. Here, we have investigated the role of Smad4 in a cellular and mouse model for TGF-β-induced breast cancer progression. Consistent with its tumor suppressor function, specific silencing of Smad4 in NMuMG mammary gland epithelial cells using small hairpin RNA (shRNA)–expressing RNAi vectors strongly mitigated TGF-β-induced growth inhibition and apoptosis. Smad4 knockdown also potently inhibited TGF-β-induced epithelial to mesenchymal transition of NMuMG cells as measured by morphologic transformation from epithelial to fibroblast-like cells, formation of stress fibers, inhibition of E-cadherin expression, and gain of expression of various mesenchymal markers. Furthermore, we show that knockdown of Smad4 in MDA-MB-231 breast cancer cells strongly inhibited the frequency of bone metastasis in nude mice by 75% and significantly increased metastasis-free survival. Communication of MDA-MB-231 cells with the bone microenvironment, which is needed for optimal tumor cell growth and metastasis, may be affected in Smad4 knockdown cells as TGF-β-induced expression of interleukin 11 was attenuated on Smad4 knockdown. Taken together, our results show that Smad4 plays an important role in both tumor suppression and progression of breast cancer cells. (Cancer Res 2006; 66(4): 2202-9)

Introduction

The cytokine transforming growth factor β (TGF-β) plays a dual role in carcinogenesis, being a tumor suppressor in early stages and a tumor promoter in later stages of carcinogenesis (1–4). TGF-β elicits its cellular responses by binding to TGF-β type I and type II serine/threonine kinase receptors and phosphorylation of receptor-regulated (R-) Smad2 and Smad3. Activated R-Smads form heteromeric complexes with common mediator Smad4, which accumulates in the nucleus, where they control gene expression in a cell type–specific manner (reviewed in refs. 5, 6). Consistent with its role as a tumor suppressor are the potent antiproliferative and proapoptotic effects by TGF-β on many cell types. Moreover, results from clinical and preclinical data have shown that the TGF-β/Smad pathway plays a crucial role in suppressing primary tumor formation (7, 8). For example, Smad4 is frequently mutated in pancreatic and colorectal cancers and a subset of juvenile polyposis syndrome patients have inherited mutated alleles of Smad4 (9). In advanced disease, when cancer cells have become insensitive to TGF-β-induced growth inhibition and apoptosis, TGF-β can have direct pro-oncogenic effects on tumor cells by stimulating their invasion and metastasis. Ectopic expression of dominant negative TGF-β receptors in various tumor cell lines inhibits their invasion and metastasis (8). Moreover, colon cancers with inactivating mutations in TGF-β type II receptor show reduced level of metastasis and increased patient survival (9). The precise mechanism for this dichotomous function of TGF-β in cancer progression and, in particular, the downstream signaling pathways and role of Smads therein, by which TGF-β elicits pro-oncogenic responses, are poorly defined.

The dissemination of carcinoma cells from the primary tumor to distant organs is a stepwise, highly organized and nonrandom process (10). Epigenetic and genetic alterations in specific genes allow for the local dissemination of carcinoma cells to intravasate into blood and lymph vessels and survival in circulation, and to extravasate at secondary sites to form micrometastasis that develop into secondary carcinomas (11). The disruption of polarized epithelial morphology into cells with spindle-shaped morphology and the invasive properties during intravasation and extravasation are reminiscent of epithelial to mesenchymal transition (EMT) that occurs during embryogenesis (12). Hallmarks of carcinoma cells undergoing EMT, at least *in vitro*, are loss of polarity and cell-cell contacts, a remodeling of the cytoskeleton, and transdifferentiation into a migratory and scattering phenotype. Concurrent with the down-regulation of expression of epithelial markers, such as the E-cadherin, cells acquire expression of mesenchymal components such as fibronectin, α-smooth muscle actin, vimentin, and N-cadherin (11).

TGF-β is a potent stimulator of EMT and cooperates with Ras-Raf signaling in this response (13, 14). There has been debate whether or not Smads are required for the TGF-β-induced EMT response. It was previously shown in a frequently used model system of EMT (i.e., TGF-β-induced EMT of NMuMG cells) that the Smad pathway plays an important role. Overexpression of Smad2, Smad3, and/or Smad4 enhanced, whereas dominant negative Smads and wild-type inhibitory Smad7 blocked, TGF-β-induced EMT in NMuMG cells (15–17). Consistent with this notion, a mutated TGF-β type I receptor that cannot activate R-Smads was unable to induce an EMT response (16, 18). However, in contrast,

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

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two recent reports showed that overexpression of dominant negative Smads and Smad4 knockdown are efficient in blocking TGF-β-induced gene responses and growth inhibition; TGF-β-induced EMT was not affected (19, 20). In these latter studies, TGF-β-induced EMT has been attributed to Smad(4)-independent signaling pathways (18, 19, 21).

Clinical and preclinical data have shown that primary tumors disseminate to selective secondary organs, which cannot solely be explained by the anatomic distribution of the blood flow to the various organs (22). Whereas breast cancer has a predilection for the skeleton, uveal melanoma and colon cancer preferentially spread to liver (reviewed in refs. 23, 24), indicating that additional factors are present in the tumor microenvironment that contribute to this process (25). TGF-β may be one of the crucial factors driving bone metastasis of breast cancer cells. Recently, it has been recognized that the bone-specific dissemination of breast cancer cells is dictated by a distinct gene expression profile of these cells (26–28). Two of these genes that act cooperatively, osteolytic factor interleukin 11 (IL-11) and angiogenic factor connective tissue-derived growth factor, are direct target genes of TGF-β (29).

Smad4 is a central mediator of TGF-β intracellular signaling. However, the requirement of Smad4 and Smad-independent pathways in TGF-β-induced EMT and breast cancer metastasis remains unclear. Whereas loss of tumor suppressive functions of Smad4 has been proposed to be sufficient to maintain the tumor cell autonomous effects of TGF-β via Smad4-independent pathways, we and others have suggested that Smad4 signaling is needed for the tumor-promoting activity of TGF-β (15, 17). In this article, we have investigated the contribution of endogenous Smad4 in TGF-β-induced EMT *in vitro* and dissemination of breast cancer cells to bone *in vivo*. By investigating the phenotypic changes and alterations in TGF-β signaling responses on Smad4 knockdown using RNAi approaches in different breast cancer cells, we show a critical stimulatory function for Smad4 in TGF-β-driven breast cancer progression.

Materials and Methods

Cell lines. NMuMG cells were maintained as described (ref. 15; see Supplementary Fig. S1 for their characteristics). The human estrogen-independent breast carcinoma cell line MDA-MB-231 stably transfected with cytomegalovirus-luciferase was cultured as previously described (30). The human osteosarcoma cell line (U2OS) was cultured in DMEM containing 10% fetal bovine serum (FBS).

Cellular assays and immunodetection. Immunofluorescence, Western blotting, EMT, migration, and transcriptional reporter assays were done as previously described (15, 31). For details about these methods, see Supplementary Methods. Cellular proliferation was done using the MTS assay according to the instructions of the manufacturer (Promega, Leiden, the Netherlands).

Production of retroviral supernatants and construction of MDA-MB-231-Eco cell line. Retrovirus producing cells were transfected with small hairpin RNA (shRNA) constructs using the calcium precipitation technique as described (32). To improve the efficiency of retroviral infection of MDA-MB-231-luc cells, stable cell lines were created containing a LZRS-Eco receptor-internal ribosome entry site-green fluorescent protein (GFP) construct provided by Dr. N. Divecha (NKI, Amsterdam, the Netherlands). GFP-positive cells were sorted twice by GFP fluorescence-activated cell sorting to obtain a 99.9% pure GFP-positive population (MDA-MB-231-Eco).

Smad4 knockdown cell lines. For shRNA oligonucleotide design, see Supplementary Methods. MDA-MB-231-Eco cells or NMuMG cells were plated in six-well plates and incubated with 1 mL viral supernatants [pRetroSuper empty (pRS) or pRetroSuper-Smad4 (S4kd)] in the presence of polybrene (5 μg/mL) for 6 hours. Cells were subsequently propagated in selection medium containing puromycin.

RNA isolation, cDNA synthesis, reverse transcription-PCR, and ELISA. RNA was isolated using mini columns (Qiagen, Venlo, the Netherlands) according to the instructions of the manufacturer. One microgram of total RNA was transcribed into cDNA using Thermoscript cDNA synthesis kit (Promega). Primers used for reverse transcription-PCR (RT-PCR) and real-time PCR are depicted in Supplementary Methods. IL-11 protein content was assessed in supernatants of the indicated cell lines using an ELISA (R&D Systems, Abingdon, United Kingdom). IL-11 protein levels are depicted corrected for protein content.

Animals. Female BALB/c *nu/nu* mice were purchased from Charles River (L'Arbresle, France). Animals were housed in individually ventilated cages under sterile conditions containing five mice per cage. Sterilized food and water were provided ad libitum.

Metastasis assay of MDA cells. Metastasis of the various MDA-MB-231 cell lines to bone was studied using intracardial injection as previously described (30, 33). This method exclusively gives rise to bone metastasis (see Supplementary Methods for details).

X-ray and histology. The formation of osteolytic lesions was assessed by radiography (Kodak EDR-2 film, Eastman Kodak Co., Rochester, NY) using an X-ray system (Faxitron 43805, Hewlett Packard). Dissected bones were isolated, fixed in 4% formalin, and processed for paraffin embedding. Histology was done as described (ref. 33; see Supplementary Methods for details).

Results

TGF-β induces EMT of NMuMG cells. As previously reported, treatment of NMuMG cells (obtained from American Type Culture Collection, Manassas, VA) with TGF-β induced EMT (Fig. 1; refs. 15, 34). Plating NMuMG cells at low density revealed that this cell line consist of heterogeneous cell population (Supplementary Fig. S1; ref. 35). Two of twenty subclones (i.e., NM14 and clone NM18) were selected that maintained a stable epithelial morphology in the absence of TGF-β. Treatment of the two clones and parental NMuMG cells with TGF-β resulted in a strong morphologic epithelial to fibroblastic transdifferentiation response (Fig. 1*A*) and pronounced down-regulation of E-cadherin and up-regulation of N-cadherin and α-smooth muscle actin (Fig. 1*B*).

Specific silencing of Smad4 using shRNA-expressing RNAi vectors. Smad4 knockdown cell lines were created using retroviral infection. Three different shRNA constructs were designed (S4#1, S4#2, and S4#3; see Supplementary Methods). The knockdown efficiencies were subsequently tested in various cell lines using Western blotting (Fig. 2). Smad4 expression was attenuated in cells stably expressing constructs 2 and 3 and less efficiently with construct 1 (Fig. 2*A* and *B*; for quantification, see Supplementary Fig. S2). To further test the specificity of construct 3, endogenous Smad2 and Smad3 levels were assessed in NM18 control (Fig. 2*B, lane 1*) and Smad4 knockdown cells (*lane 2*). As expected, S4#3 specifically inhibited Smad4 levels without affecting Smad2 and Smad3 levels (Fig. 2*B, lane 2*). Similar results were obtained in MDA-231 cells (Fig. 5*A*) and NM14 (not shown).

Subsequently, the effect of stable Smad4 knockdown on TGF-β-induced $CAGA_{12}$-luc (36) activity was studied in stable U2OS Smad4 knockdown cells. This reporter construct requires Smad4 for activation. Consistent with the effects on Smad4 expression, knockdown of Smad4 attenuated TGF-β-induced $CAGA_{12}$-luc (Fig. 2*C*) with construct 3 being most efficient. Next, stable NM18 cells were transiently transfected with a Smad3/Smad4 dependent reporter $CAGA_{12}$-luc or a Smad2/Smad4 dependent activin-response element (ARE) reporter together with its transcription

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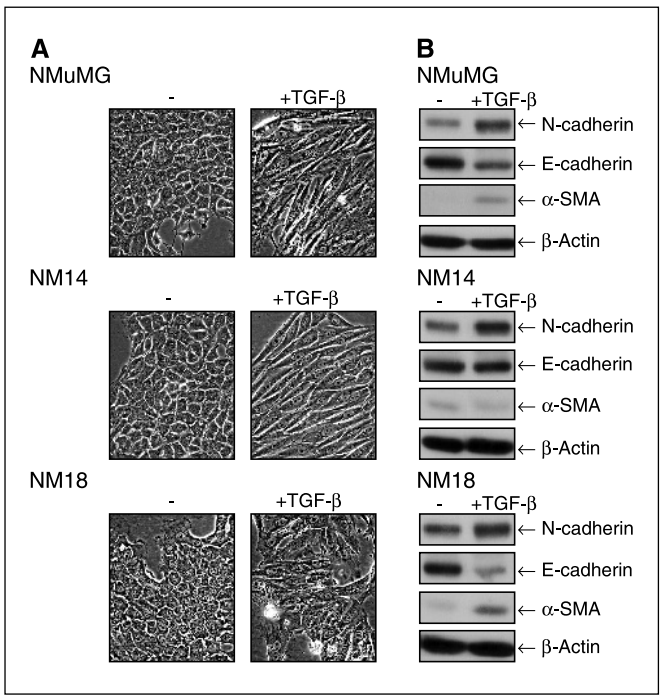

Figure 1. TGF-β induces EMT in NMuMG cells. Parental NMuMG cells (obtained from ATTC) and two subclones (NM18 and NM14) were cultured in the presence of absence of TGF-β (5 ng/mL) for 48 hours. *A,* in the presence of TGF-β, all cells transdifferentiated into a mesenchymal phenotype. *B,* analysis of EMT markers by Western blotting using E-cadherin antibody as epithelial marker and N-cadherin and α-smooth muscle actin (α-*SMA*) as mesenchymal markers. Treatment with TGF-β inhibits E-cadherin expression and up-regulates N-cadherin and α-smooth muscle actin expression. Among the various subclones isolated, NM18 showed strongest changes in EMT markers in the presence of TGF-β.

factor FAST-1 (37). NM18-S2#5 specifically inhibited endogenous Smad2 levels without affecting Smad3 or Smad4 levels (Fig. 2*B*). As expected, in NM18-S2#5 cells, TGF-β-induced $CAGA_{12}$ reporter activity was not affected whereas in NM18-S4#3 cells, this was strongly inhibited (Fig. 2*D, left*). Conversely, in NM18-S4#3, TGF-β-induced ARE-luc reporter activity was not affected while being partially inhibited in NM18-S2#5 cells (Fig. 2*D, right*). In conclusion, S4#3 is a highly specific siRNA construct to study the involvement of Smad4 in TGF-β-induced responses in both human and mouse cell lines.

Smad4 is required for TGF-β-induced EMT and TGF-β-induced growth inhibition and apoptosis. Two single-cell clones with strongest Smad4 knockdown were selected for both NM18 (clone 1 and clone 14; Supplementary Figs. S2*B*) and NM14 (clone 10 and clone 13; Supplementary Fig. S2*B*). TGF-β treatment of NMuMG clones NM18 and NM14 induced a down-regulation of E-cadherin and up-regulation of N-cadherin and α-smooth muscle actin (Fig. 3*A*, not shown), which was accompanied by formation of stress fibers (Supplementary Fig. S2*C*) and a morphologic change (Fig. 3*B*). In contrast, in both Smad4 knockdown clones of NM18 and NM14 clones, TGF-β largely failed to mediate all these changes (Fig. 3*A* and data not shown). These results indicate that Smad4 is crucial for TGF-β induced EMT.

To test if Smad4 is involved in TGF-β-induced growth arrest, a proliferation assay was done using a cell proliferation assay. Whereas empty vector control cells were growth inhibited in response to TGF-β, this was prevented in NM14 and NM18 knockdown cells (data not shown). Treatment of NM18-pRS cells (but not of NM14-pRS cells) for >72 hours with TGF-β induced an apoptotic response. NM-18 Smad4 knockdown cells were resistant to TGF-β-induced apoptosis and retained their epithelial morphology (Fig. 3*B*). To test if the responses induced by Smad4 knockdown were specific, NMuMG cells were infected with adenoviral Smad4. In Smad4 knockdown cells, TGF-β-induced Snail mRNA expression (a transcriptional repressor of E-cadherin) and plasminogen activator inhibitor (PAI)-1 mRNA expression (an extracellular matrix gene from which promoter the Smad binding elements to generate $CAGA_{12}$-luc reporter were derived) were attenuated. These responses were restored and even enhanced on Smad4 overexpression (Fig. 3*C*). Moreover, consistent with these results, on restoration of Smad4 expression in NM18 Smad4 knockdown cells, TGF-β induced EMT (24-48 hours after TGF-β treatment) and, subsequently, an apoptotic response was noted. In contrast, NM18 Smad4 knockdown cells infected with LAC-Z retained their epithelial morphology in the presence of TGF-β (Fig. 3*D*). Similarly, in NM14 cells infected with adenoviral Smad4, the TGF-β-induced EMT response was restored (Fig. 3*D*). In conclusion, these data indicate that Smad4 is required for TGF-β-induced EMT and TGF-β-induced growth arrest and apoptosis in NMuMG cells.

Model for bone-specific metastasis of breast cancer. To study whether the requirement of Smad4 has consequences on breast cancer metastasis to bone *in vivo*, we used the well-characterized metastasis model in which MDA-MB-231 breast cancer cells are intracardially injected into nude mice. MDA-MB-231 cells have been passaged multiple times *in vivo* to select for cells metastasizing to bone. As previously reported, the cells stably express luciferase, which enables detection of metastasis by bioluminescence (33). On successful injection, luciferase activity is detected throughout the body within 10 minutes, reminiscent of circulating tumor cells (not shown), which disappears within 24 hours (33). Discrete photon accumulation was detected in both femurs 14 days after injection as shown by whole body bioluminescent imaging (BLI; Fig. 4*A, left*), which substantially increased thereafter (Fig. 4*A, right*). Histologic and radiological analysis showed that bone metastases exclusively developed, on average, in 90% on the mice (n = 20). They were detected in the proximal tibia or the femur, spine, humerus, or scapulae with an average of six metastases per mouse within 35 to 42 days (data not shown). In line with our previous reports (30, 33, 38), these lesions showed osteolytic activity as illustrated by areas of low mineral content on radiography (Fig. 4*B, arrowhead*) and the presence of numerous osteoclasts located at the bone-tumor interface (Fig. 4*C, left* and *bottom*). Histologic examination of the affected and unaffected limb (compare Fig. 4*C, left* and *right*) confirmed that tumor cells had largely replaced the bone marrow. Analysis of expression of phosphorylated Smad2 using a phospho-Smad2–specific antibody (39) in an established MDA-MB-231 bone metastasis revealed prominent nuclear pSmad2 staining in tumor cells, indicating active TGF-β signaling surrounding the tumor cells (Fig. 4*D*).

Smad4 knockdown inhibits bone metastasis of MDA-MB-231 cells. Because retroviral infection of MDA-MB-231-luc by human retrovirus proved to be very inefficient, we increased this percentage by stably expressing the murine ecotropic receptor on MDA-MB-231-luc. Introduction of this receptor did not affect the metastatic potential of the cells (data not shown). To study the consequences of knockdown of Smad4 *in vivo*, a prolonged knockdown has to be guaranteed. For this, we created stable

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Smad4 knockdown cell lines using MDA-MB-231-luc expressing the ecotropic receptor (Fig. 5*A*). As can be appreciated from Figs. 2*A* and 5*A*, Smad4 shRNA construct 3 was most potent in inhibiting protein expression. For clarity, these Smad4 knockdown cells are referred to as MDA-S4kd and their respective empty vector controls as MDA-pRS.

An experimental setup was chosen in which one group of mice was intracardially injected with MDA-S4kd (without clonal selection) and the other group with MDA-pRS. Metastatic progression was assessed by BLI every 7 days. As expected, the first bioluminescent emission was detected in the mice injected with MDA-pRS 14 days after inoculation (data not shown). Four weeks after injection, photon accumulation, indicative of bone metastasis, was localized in the spine of all MDA-pRS injected mice and over the distal metaphyses of the femurs 5 weeks after injection (data not shown). Interestingly, in the mice injected with MDA-S4kd cells, the metastasis-free survival was prolonged (Fig. 5*B*). Moreover, the number of mice with detectable bone metastases was inhibited by 50% (not shown). Tumor cells were isolated from the tibia of a mouse injected with Smad4kd cells or MDA-pRS cells. Before injection, the level of Smad4 knockdown was >75% as shown by Western blot analysis (Fig. 5*C*, compare *lane 5* with *lanes 1-4*). Interestingly, in the MDA-S4kd tumor cells retrieved from the bone metastases (*lane 7*), Smad4 levels were comparable to the cells retrieved from mice injected with pRS cells (*lane 6*). The lack of knockdown in the cells was not due to unstable knockdown as, on passaging of the cells *in vitro*, no difference in knockdown was detected between early and late passages (not shown). However, analysis of single-cell clones from MDA-S4kd used for injection revealed that the extent of Smad4 knockdown among single-cell clones varied considerably (Fig. 5*D*). This suggests that there is a selective advantage for MDA-S4kd cells with inappropriate knockdown to metastasize as compared with MDA-S4kd cells with efficient knockdown. It should be noted that the reduction in the formation of metastatic lesions was not due to an intrinsic decrease in the growth rate of the cells (Fig. 5*E*) nor at the metastatic site because the increase in BLI intensity over time was similar between pRS and S4kd cells, both at the level of the spine as well as in the tibia (not shown).

In the following experiment the metastatic capacity of MDA-pRS, MDA-S4kd cl4 and MDA-S4kd cells were compared (n = 8, 9 and 8 per group respectively). The time point that the first metastasis was detected was significantly delayed from 16 days (± 3,4) in mice injected with MDA-pRS control cells to 38 days (± 4,9) days in mice injected with MDA-S4kd cl4 cells ($P < 0.01$; Fig. 6*A*). Consistent with the previous experiment, the frequency of metastasis was strongly reduced in the mice injected with the MDA-S4 knockdown pool and even further inhibited in the mice injected with MDA-S4kd cl4 (Fig. 6*B*). Although Smad4 knockdown inhibited the metastasis frequency (Fig. 6*A*), at the end of the experiment, in the BLI-positive mice the total number of metastases per mouse was not different from controls (Supplementary Table S1). Moreover, in all groups, osteolytic metastases were detected (Fig. 6*B*). In contrast to the increased lag time in knockdown cells, all cell lines exhibited a similar growth rate *in vivo* (Fig. 6*C*) and proliferation *in vitro* (Fig. 5*E*), indicating that the delay observed *in vivo* was not due to

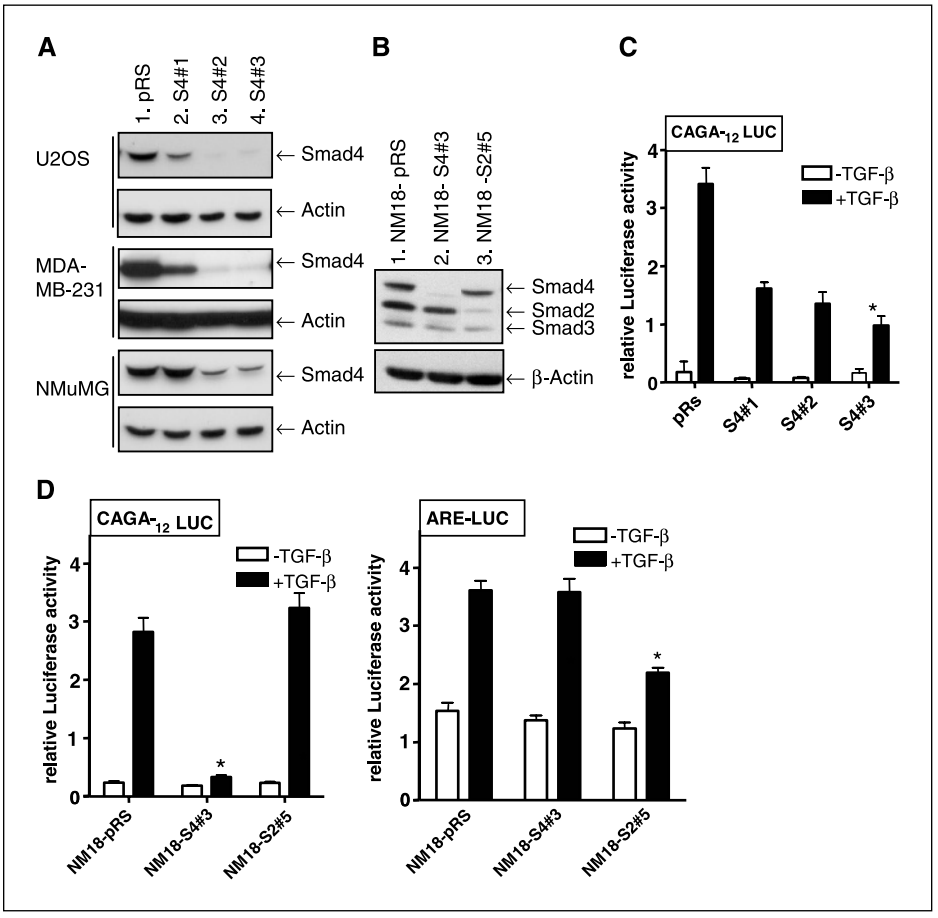

Figure 2. Specific silencing of Smad4 in NMuMG cells using shRNA expressing RNAi vectors strongly inhibits Smad4 expression and Smad4-dependent transcriptional activity. Indicated cell lines were infected with three different shRNA constructs targeting mouse and human Smad4 or empty vector (pRS) using retroviral infection. *A,* after selection on puromycin, protein was isolated and endogenous Smad4 expression was analyzed by Western blotting using anti-Smad4 antibody and β-actin as a loading control. *B,* stable Smad4 shRNA or Smad2 shRNA clones were generated by retroviral infection of U2OS cells and subsequent selection on puromycin. Cell extracts were analyzed for endogenous Smad2, Smad3, and Smad4 expression by Western blotting using anti-Smad2/3 and anti-Smad4 antibody and β-actin as a loading control. Smad4 shRNA construct 3 (S4#3) specifically inhibits Smad4 expression without affecting Smad2 or Smad3 expression (*lane 2*) whereas Smad2 shRNA construct 5 (S2#5) specifically inhibits Smad2 expression without affecting Smad4 or Smad3 (*lane 3*). *C,* four different U2OS lines stably expressing empty vector (pRS), SMAD4 shRNA construct 1 (S4#1), S4#2, or S4#3 were transiently transfected with $CAGA_{12}$-luc reporter construct in the presence or absence of TGF-β. *D,* TGF-β induced $CAGA_{12}$-luc and ARE-luc reporter activity was assessed in NM18 Smad2 and Smad4 stable knockdown cell lines by transient transfection.

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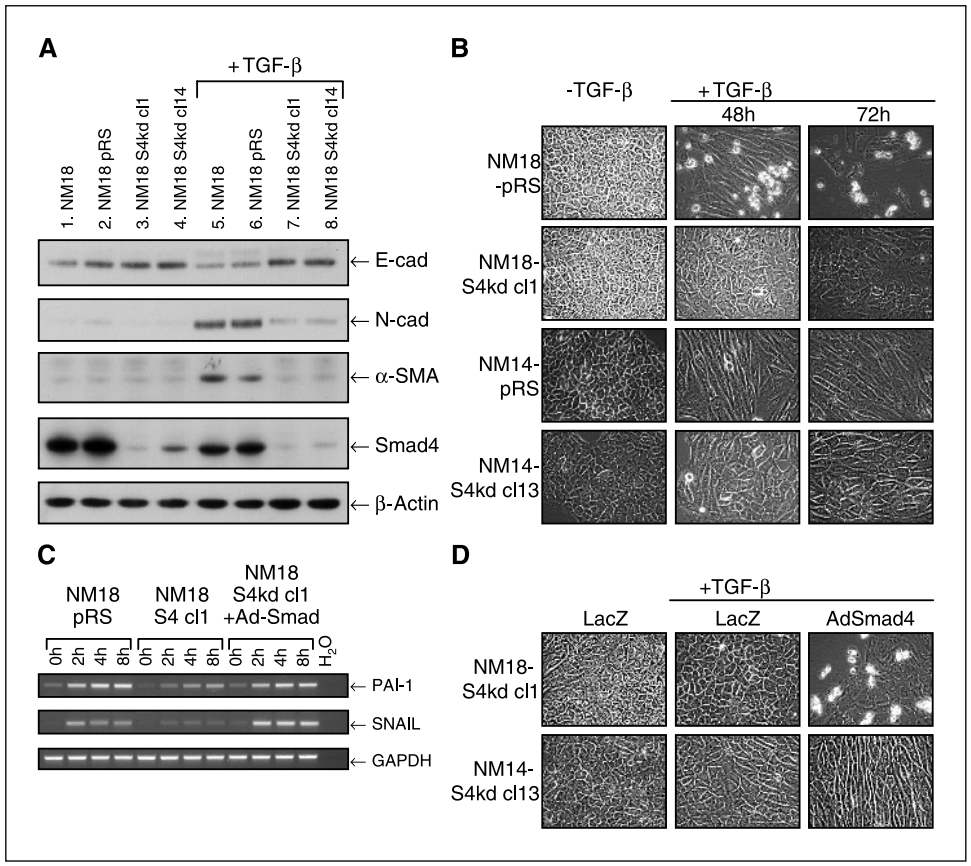

Figure 3. Smad4 is required for TGF-β induced EMT and apoptosis. Stable cell lines were generated using retroviral infection of NM-18. After selection on puromycin, single-cell clones were generated. Cells were plated in 3-cm culture dishes. After 24 hours, cells were treated with 5 ng/mL TGF-β for 48 and 72 hours of incubation as indicated. *A,* cell lysates of control cells (NM18, NM18-pRS) and Smad4 knockdown cells (NM18-S4kd cl1 and NM18-S4kd cl13) were analyzed by Western blotting after 72 hours using E-cadherin antibody (*E-cad*), N-cadherin (*N-cad*), and α-smooth muscle actin. β-Actin was used as loading control. TGF-β induced down-regulation of E-cadherin and up-regulation of N-cadherin and smooth muscle actin was abrogated in NM18-S4kd cl1 and NM18-S4kd cl14 cells. *B,* knockdown of Smad4 rescues NM-14 cells from TGF-β-induced EMT and NM-18 cells from apoptosis as shown by phase-contrast microscopy at 200-fold magnification. *C* and *D,* TGF-β responses are restored in knockdown cell lines after overexpression of Smad4. Smad4 was overexpressed in NM18-S4kd cl1 and NM14-S4kd cl13 using an adenoviral Smad4 construct (*Ad-Smad*). Cells were cultured in the presence of TGF-β for 2, 4, 8 (*C*), or 72 hours (*D*). Overexpression of Smad4 restored TGF-β-induced responses such as induction of PAI-1 and Snail mRNA expression (*C*) and sensitivity to TGF-β induced apoptosis and EMT in NM18-S4kd cl1 cells (*D*) as shown by RT-PCR and phase-contrast microscopy at 200-fold magnification.

intrinsic differences in proliferation. Moreover, analysis by Western blotting showed that Smad4 knockdown was maintained in the MDA-S4kd cl4 cells retrieved from the bone metastases (Fig. 6*D*). We next tested whether Smad4 knockdown has an effect on established bone macrometastases using intratibial injection. Only a marginal difference in tumor progression was observed among MDA-pRS, MDA-S4kd, and MDA-S4kd cl4 as measured by BLI (Supplementary Fig. S3).

Smad4 is required for efficient TGF-β-induced IL-11 expression. The SDF-CXCR4 axis is a chemokine-receptor couple that has been indicated to be involved in homing of breast cancer cells to bone. Smad4 knockdown did not affect the level of CXCR4 protein *in vitro* (Fig. 6*E*). Analysis of TGF-β-induced expression of IL-11 revealed that induction of IL-11 mRNA and protein was attenuated in MDA-S4 cl4 cells compared with MDA-pRS cells (Fig. 6*F* and *G*). These data suggest that Smad4 in breast cancer cells regulates the production of this osteolytic cytokine.

Discussion

ShRNA-expressing RNAi vectors specifically targeting mouse and human Smad4 have allowed us to study the requirement of Smad4 in two well-characterized experimental models in TGF-β-driven breast cancer progression: TGF-β-induced EMT of mouse NMuMG cells (34) and metastasis of human MDA-MB-231 cells to bone after intracardial injection in nude mice (40, 41). The models represent two different defined stages in breast cancer progression. EMT resembles the loss of polarity and cell-cell contacts and gain of invasive properties during intravasation whereas the metastasis of intracardially injected cancer cells recapitulates the steps after cells have gained access to circulation and ultimately form micro- and macro-metastases in selected organs. Here we show that both TGF-β-induced EMT of NMuMG cells and establishment of bone metastasis from circulating MDA-MB-231 cells require Smad4.

TGF-β induced a transformation from epithelial cuboidal cells into parallel aligned fibroblastic-like cells, down-regulation of E-cadherin (an epithelial maker), up-regulation of N-cadherin and α-smooth muscle actin (mesenchymal markers), and reorganization of cytoskeleton with the formation of stress fibers. Silencing of Smad4 in NMuMG cells strongly attenuated all these TGF-β-induced responses associated with EMT. As TGF-β also elicits an antimitogenic and apoptotic response in NMuMG cells (15), we also measured the need for Smad4 in this response. In line with its tumor suppressing activity, Smad4 was found to be required for this response.

The results that Smad4 is indispensable for TGF-β-induced EMT (and growth inhibition and apoptosis) confirm previous results from us (15, 16) and others (17, 18). Down-regulation of E-cadherin is mediated by up-regulation of its transcriptional repressor Snail (42). Similarly, in Smad4 knockdown cells, TGF-β-induced Snail expression is attenuated. Specificity of the Smad4 knockdown is shown by the rescue of the effect on Smad4 overexpression. In contrast to our findings using a conditional Smad4 shRNA-mediated knockdown approach, in HaCat and a pancreatic cancer cell line, Smad4 was reported to be dispensable for TGF-β-induced EMT (20). The discrepancy in both data might be explained by differences in cell types or efficiency in Smad4 knockdown. Our data certainly do not rule out an important role for Smad4-independent pathways in TGF-β-induced EMT.

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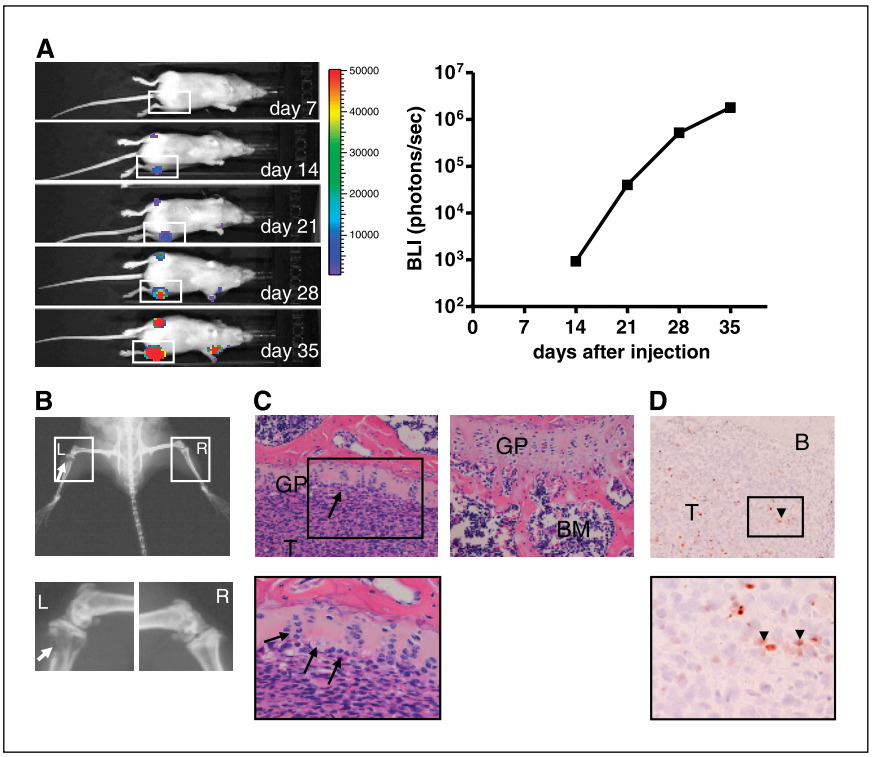

Figure 4. Intracardial injection of MDA-MB-231-luc cells in nude mice induces bone metastasis *in vivo*. MDA-MB-231-luc cells were intracardially injected in 5-week-old female nude mice. Every 7 days, whole body bioluminescence measurements were done. *A,* tumor burden as measured by optical imaging in the tibia (*left*); a ventral view of the whole body luminescent measurement with a pseudo-color overlay representing the photons emitted over time (*right*) on a scale of 500 to 500,000 photons/s. Bone metastases can be detected in both femurs and in the humerus. In all images, the maximum bar was set at 50,000 photons/s and the signal was normalized for a background of 5,000 photons/s. Radiographs (*B*) and the corresponding histologic sections (*C*) of affected (*left, middle*) and unaffected tibia (*right*). *B,* osteolytic areas in the left femur (*L*) are indicated by arrowheads. No sign of osteolysis is evident in the right femur (*R*). *C,* hind limbs were excised and embedded in paraffin and stained with H&E. Tumor cells have completely replaced the bone marrow in the affected tibia (*left, middle*) whereas the bone marrow of the unaffected tibia is intact. *Bottom,* a higher magnification of the *left. Arrows,* multinucleated osteoclasts at the interface between tumor and growth plate of the affected limb. *GP,* growth plate; *BM,* bone marrow. *D,* pSmad2 staining of an established bone metastasis. *Arrowheads,* nuclear pSmad2 staining in tumor cells. *Top,* ×100 magnification; *bottom,* ×2,000 magnification.

Apart from the requirement of Smad4 in TGF-β-induced invasion as measured by EMT, we showed that specific knockdown of Smad4 in human MDA-MB-231 breast cancer cells strongly mitigated the development of bone metastases in nude mice after intracardial injection. Injection of a heterogeneous pool of Smad4 knockdown cells resulted in a partial inhibition of metastasis as compared with MDA-MB-231-luc parental cells. Cells retrieved from bone metastases corresponded to the high Smad4 expressors in the heterogeneous

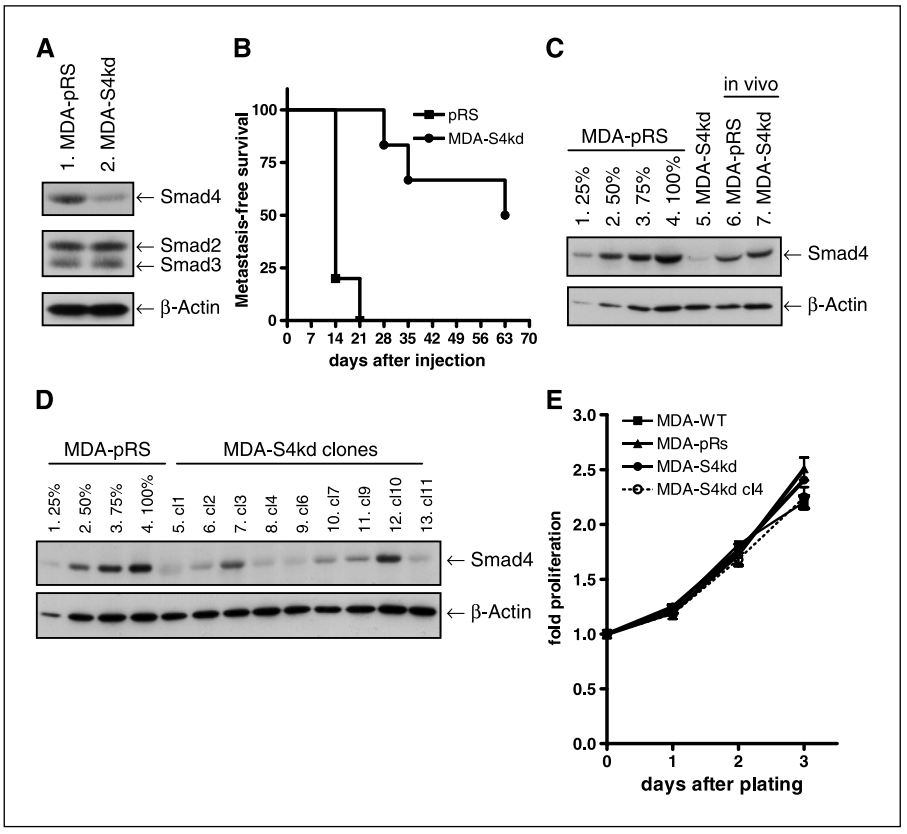

Figure 5. Knockdown of Smad4 inhibits bone metastasis *in vivo*. Stable MDA Smad4 knockdown cells were generated using retroviral infection of MDA-MB-231-Eco cells with empty vector or Smad4 shRNA construct 3 (MDA-S4kd) and subsequent selection with puromycin. *A,* endogenous Smad2, Smad3, and Smad4 levels were analyzed by Western blotting in control cells (MDA-pRS; *lane 1*) and knockdown cells (MDA-S4kd; *lane 2*), showing efficient and specific knockdown of Smad4 and not of Smad2 or Smad3. *B,* intracardial injection of Smad4 knockdown cells (MDA-S4kd) in 5-week-old female nude mice significantly prolonged the metastasis-free survival and inhibited the formation of bone metastases in the spine and in the tibia as compared with mice injected with control cells (MDA-pRS); $n = 5$ per group. *C,* analysis of Smad4 expression in the metastasized tumor cells. *Lanes 1* to *4,* serial dilutions of the lysates of the control cells before injection; *lane 5,* level of knockdown in the Smad4 knockdown cells before injection; *lane 6,* in the control cells obtained from the bone metastases; *lane 7,* in knockdown cells obtained from the bone metastases. β-Actin expression was used as a loading control. *D,* Smad4 expression in the various MDA-MB-231 single-cell clones. *Lanes 1* to *4,* serial dilution of control lysates; *lane 5,* 13 MDA-S4kd cl4 single-cell clones. *E,* cells were plated in 24-well plates and the proliferation rate was assessed using the MTS assay 24, 48, and 72 hours after plating.

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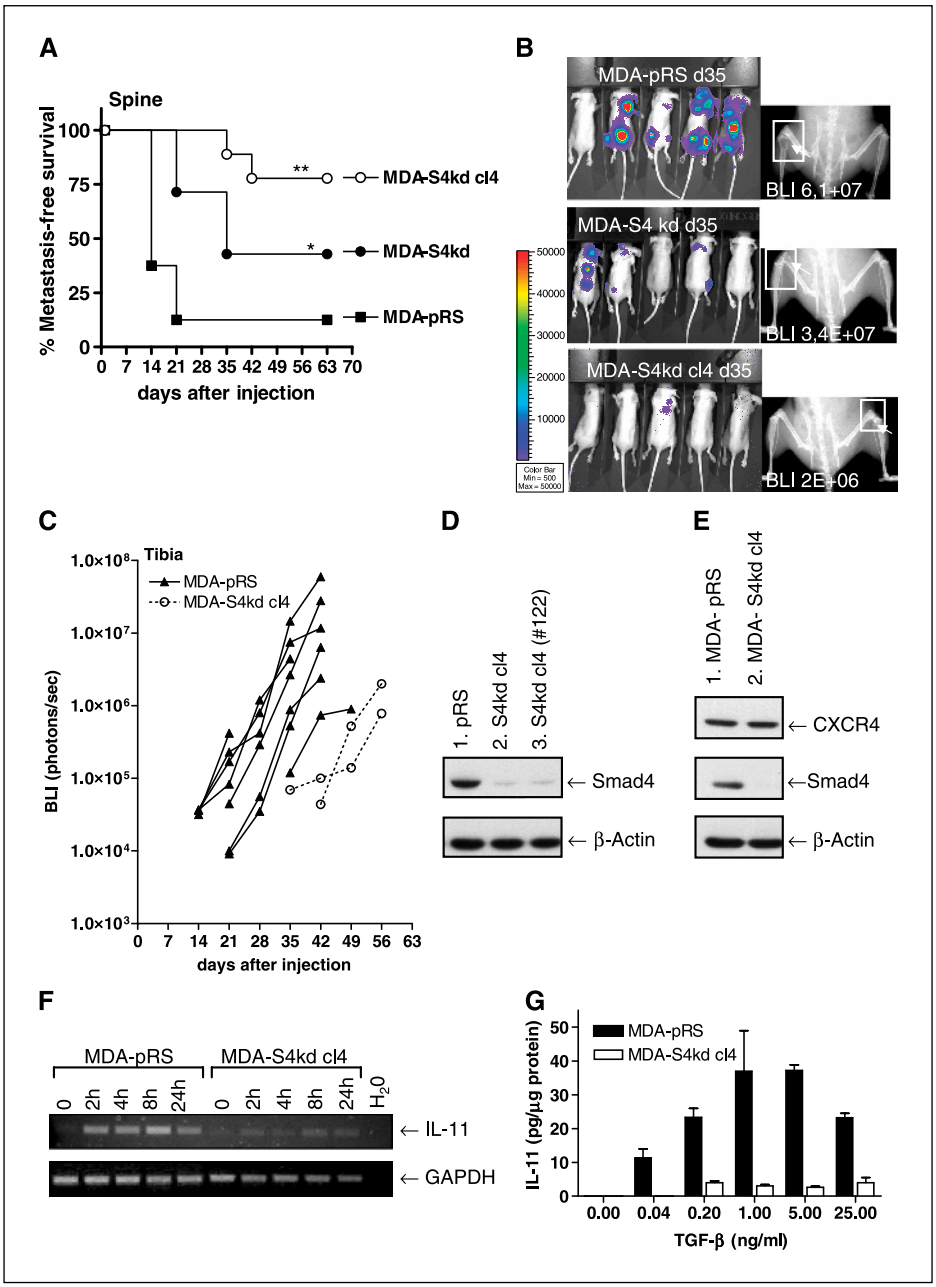

Figure 6. Knockdown of Smad4 inhibits bone metastasis *in vivo* and production of osteolytic cytokines. *A,* control cells (MDA-pRS), pooled Smad4 knockdown cells (MDA-S4kd), and clone 4 of MDA knockdown cells (MDA-S4kd cl4) were intracardially injected in 5-week-old female nude mice (n = 8, 8, and 9 per group, respectively). Metastasis-free survival for the spine is depicted. Statistical analysis using the log-rank test showed that survival is significantly prolonged in mice injected with Smad4 knockdown cells [*, $P < 0.05$ (MDA-S4kd); **, MDA-S4kd cl4 ($P < 0.002$)] as compared with mice injected with control cells. *B,* representative pictures of whole body bioluminescent imaging at day 35 (*left*) of mice injected with control cells (MDA-pRS; *top*) or knockdown cells (*middle* and *bottom*). Osteolytic lesions were shown in all three treatment groups at the end of the experiment (*right*). BLI measurement (number of photons per second) per mouse is indicated on the X-ray. *C,* development and progression of metastatic lesions in the tibia of mice injected with MDA-pRS or MDA-S4kd cl4 as measured by whole body bioluminescent imaging is depicted as number of photons/second. *D,* analysis of Smad4 expression in the metastasized tumor cells of mice injected with MDA-S4kd cl4. *Lane 1,* lysate of pRS control cells; *lane 2,* lysate of MDA-S4kd cl4 cells before injection; *lane 3,* lysate of MDA-S4kd cl4 retrieved from metastasis. β-Actin expression was used as a loading control. *E,* CXCR4 expression was analyzed by Western blotting in control cells (*lane 1*) or MDA-S4kd cl4 cells (*lane 2*). *F,* MDA-pRS or MDA-S4kd cl4 were plated in six-well plates and starved overnight in medium containing 0.3% serum. Cells were subsequently stimulated with TGF-β (5 ng/mL) for 2, 4, 8, and 24 hours. At the indicated time points, cells were lysed and RNA was isolated. Expression of IL-11 was analyzed by semiquantitative PCR. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as control for variation in total RNA content. *G,* MDA cells were plated in 24-well plates. After 24 hours, cells were starved in DMEM containing 0.3% FBS for 24 hours. Medium was aspirated and replaced by medium with or without TGF-β (5 ng/mL) as indicated. After 24 hours, supernatants were analyzed for IL-11 content by ELISA. Data are depicted corrected for protein content.

pool of Smad4 knockdown cells, indicating that the expression of Smad4 is beneficial for the development of bone metastasis. In line with this, using a clone in which Smad4 was stably knocked down, the frequency of metastasis was even further inhibited, emphasizing the requirement of tumor cell-autonomous Smad4 *in vivo*. This finding is consistent with the tumor-promoting role of TGF-β in late-stage tumor cells (8, 43, 44). Moreover, the presence of nuclear phosphorylated Smad2 (this work) and functional imaging of active TGF-β signaling in tumor cells in bone (29) show that the TGF-β signaling pathway is specifically activated in bone metastases.

To elucidate the underlying mechanism by which Smad4 is required for bone metastasis, we analyzed the expression of a number of key components previously implicated in this process. The increased lag time in metastasis formation *in vivo* suggests that Smad4 may affect homing of MDA-MB-231 cells to bone. One of the candidates is SDF-1, produced by osteoblasts lining the endosteal surface and in lung, and its receptor CXCR4, which is highly expressed on breast and prostate cancer cells (45, 46). We failed, however, to detect a difference in CXCR4 protein expression in Smad4 knockdown versus parental MDA-MB-231 cells *in vitro*. Importantly, we did observe that Smad4 was required for TGF-β-induced secretion of bone resorbing cytokine IL-11 by MDA-MB-231 cells. Thus, communication of MDA-MB-231 cells with the bone microenvironment, which is needed for optimal tumor cell growth and metastasis, may be affected in Smad4 knockdown cells. During the preparation of our article, Kang et al. (29) also reported that Smad4 is required for breast cancer bone metastasis. Their data and our are fully consistent with each other.

Our observation that Smad4 knockdown did not affect tumor growth of intraosseously transplanted MDA-MB-231 cells (reflecting

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to some extent a macro metastasis) suggests that Smad4 is most crucial during early stages of tumor invasion and micrometastasis formation. A similar escape was previously noticed in mice treated with bisphosphonates (38), indicating that only the formation of micro-metastases relies on active bone remodeling and probably active TGF-β. Once a critical mass is formed, tumor cells show uncontrolled growth independent of bone resorption. At later stages, tumor cells may thus use either alternative signaling pathways or SMAD-independent signaling for tumor growth. This dependency on active TGF-β in early lesions may explain the specific outgrowth of breast cancer cells in bone. It will be of great interest to examine whether the Smad4 dependency of breast cancer cells extends to their ability to metastasize to other organs using a similar approach.

In conclusion, we show that TGF-β-induced growth inhibition and apoptosis, TGF-β-induced EMT, and metastasis of breast cancer cells to bone are all critically dependent on Smad4. Thus, the dual role of TGF-β in carcinogenesis extends to its central intracellular mediator, Smad4. Our results are relevant for the therapeutic targeting of TGF-β for bone metastasis of breast cancer. We are currently exploring whether the repertoire of transcription factors that cooperate with Smad4 for its tumor suppressor and tumor promoting activities are distinct. If so, this may allow for the specific intervention of pro-oncogenic actions of Smad4 while leaving its antimitogenic activities on cancer and normal cells intact.

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The Tumor Suppressor Smad4 Is Required for Transforming Growth Factor β–Induced Epithelial to Mesenchymal Transition and Bone Metastasis of Breast Cancer Cells

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