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ORIGINAL ARTICLE

Transforming growth factor-β1 is the predominant isoform required for breast cancer cell outgrowth in bone

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Transforming growth factor (TGF)-β signaling is a potent modulator of the invasive and metastatic behavior of breast cancer cells. Indeed, breast tumor responsiveness to TGF-β is important for the development of osteolytic bone metastases. However, the specific TGF-β isoforms that promote breast cancer outgrowth in bone is unknown. We demonstrate that expression of a TGF-β ligand trap, which neutralizes TGF-β1 and TGF-β3, in MDA-MB-231 breast cancer cells diminished their outgrowth in bone and reduced the severity of osteolytic lesion formation when compared with controls. We further show that a reduction or loss of TGF-β1 expression within the bone microenvironment of TGF-β1 + /− and TGF-β1 −/− mice significantly reduced the incidence of breast tumor outgrowth compared with wild-type animals. Interestingly, those tumors capable of growing within the tibiae of TGF-β1-deficient mice had upregulated expression of all three TGF-β isoforms. Finally, breast cancer cells expressing the TGF-β ligand trap showed a pronounced reduction in their ability to form osteolytic lesions when injected into the tibiae of TGF-β1 + /− mice. Thus, our studies show that both host- and tumor-derived TGF-β expression plays a critical role during the establishment and outgrowth of breast cancer cells in bone.
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Introduction

The bone represents the most common site of metastasis in breast cancer patients (Mundy, 2002; Coleman, 2006; Hess *et al*., 2006). These skeletal metastases are typically characterized by excessive bone resorption (Kozlow and Guise, 2005). Breast tumor cells secrete factors that promote the differentiation and activity of multinucleated osteoclasts, which are the resident bone cells responsible for bone resorption (Yoneda and Hiraga, 2005; Rose and Siegel, 2006). In addition, the bone matrix is a reservoir of latent growth factors that can be released and activated during osteoclast-mediated bone resorption. These factors then function in a paracrine manner to promote breast cancer survival and growth within bone (Mastro *et al*., 2003; Kozlow and Guise, 2005).

All three transforming growth factor-β isoforms (TGF-β1, TGF-β2 and TGF-β3) are present in bone; however, TGF-β1 is the most abundant (Hering *et al*., 2001a, b). TGF-β isoforms bind to the TGF-β type II receptor (TβRII), which in turn recruits and activates the TGF-β type I receptor (TβRI) (Wrana *et al*., 1994; Groppe *et al*., 2008). TGF-β1 and TGF-β3 directly bind to the extracellular domain (ECD) of TβRII, whereas efficient binding of TGF-β2 to TβRII is facilitated by betaglycan (TβRIII) (Wang *et al*., 1991; Lopez-Casillas *et al*., 1993). The activated TGF-β receptor complex then initiates signaling through both Smad-dependent and -independent pathways (Moustakas and Heldin, 2005; Schmierer and Hill, 2007).

Mouse models of experimental breast cancer metastasis to bone show that TGF-β signaling in breast tumor cells is critical for this process. Expression of a dominant negative TβRII significantly reduces the ability of MDA-MB-231 breast cancer cells to form osteolytic bone metastases, which can be rescued by expression of constitutively active TβRI (Yin *et al*., 1999). *In vivo* selection approaches to isolate highly aggressive bone metastatic MDA-MB-231 breast cancer cells have identified TGF-β targets, such as parathyroid hormone-related protein (PTHrP), interleukin-11 (IL-11) and connective tissue growth factor, which play important roles in breast cancer metastasis to bone (Yoneda *et al*., 2001; Kang *et al*., 2003). Activation of both Smad-dependent and -independent pathways within breast cancer cells is important for the formation of osteolytic metastases (Kakonen *et al*., 2002). Stable knockdown of Smad4 expression in these cells further reinforces the importance of Smad-mediated TGF-β signaling for promoting breast cancer metastasis to bone

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(Kang *et al*., 2005; Deckers *et al*., 2006). Furthermore, kinase inhibitors targeting TβRI reduce the incidence of bone metastases following left cardiac ventricle injection of MDA-MB-435 and MDA-MB-231 breast cancer cells (Bandyopadhyay *et al*., 2006; Ehata *et al*., 2007). These animal-based studies support a role for TGF-β signaling within breast tumor cells that enable aggressive metastasis to bone. Finally, it was recently reported that greater than 50% of breast and prostate cancer patients with skeletal metastases show elevated serum levels of TGF-β1, which correlates with increased expression of markers indicative of enhanced TGF-β signaling (Baselga *et al*., 2008).

Although these studies show an important role for TGF-β signaling in breast cancer metastasis to bone, they do not identify the specific TGF-β isoforms that are most important for this process. Notably, they do not determine whether the primary source of TGF-β required for the development of osteolytic metastases originates from the bone matrix, resident bone cells or the tumor cells themselves. To address these issues, we have used a selective TGF-β isoform-neutralizing ligand trap and TGF-β1-deficient mice to examine the relative importance of tumor cell- or host-derived TGF-β in the establishment and outgrowth of osteolytic lesions in bone. Our results show that the removal of TGF-β1 and TGF-β3 isoforms significantly impairs the progression of breast cancer lesions in bone. Moreover, we conclusively show that the bone microenvironment provides a significant source of TGF-β1 and that its removal imposes a selective pressure on breast cancer cells to overexpress TGF-β isoforms, which promote the formation of osteolytic bone lesions.

Results

TGF-β-neutralizing ligand trap impairs signaling induced by TGF-β1 and TGF-β3

We first determined the expression pattern of each TGF-β isoform in parental MDA-MB-231 breast cancer cells that had been injected into the tibiae of athymic mice. MDA-MB-231 cells readily form osteolytic lesions (Figure 1a) that fill the marrow space (Figure 1b). We readily detected serine phosphorylated Smad2 throughout the tumor, suggesting that the TGF-β signaling pathway was active in MDA-MB-231 cancer cells (Figure 1c). Immunohistocytochemical analysis further showed that MDA-MB-231 breast cancer cells predominately expressed TGF-β1, with more moderate levels of TGF-β2 and very little TGF-β3 detected within osteolytic lesions (Figures 1d–f).

To assess the importance of the TGF-β isoforms for breast cancer cell outgrowth in bone, we first used a soluble ligand trap composed of the ECD of TβRII fused to the constant region of human IgG (Fc). This fusion protein has previously been used to study the impact of impaired TGF-β signaling on breast cancer progression and metastasis (Mourskaia *et al*., 2007). Pooled populations harboring an empty vector control (VC) or the ligand trap (Fc:TβRII(ECD)) were established in parental MDA-MB-231 cells possessing an

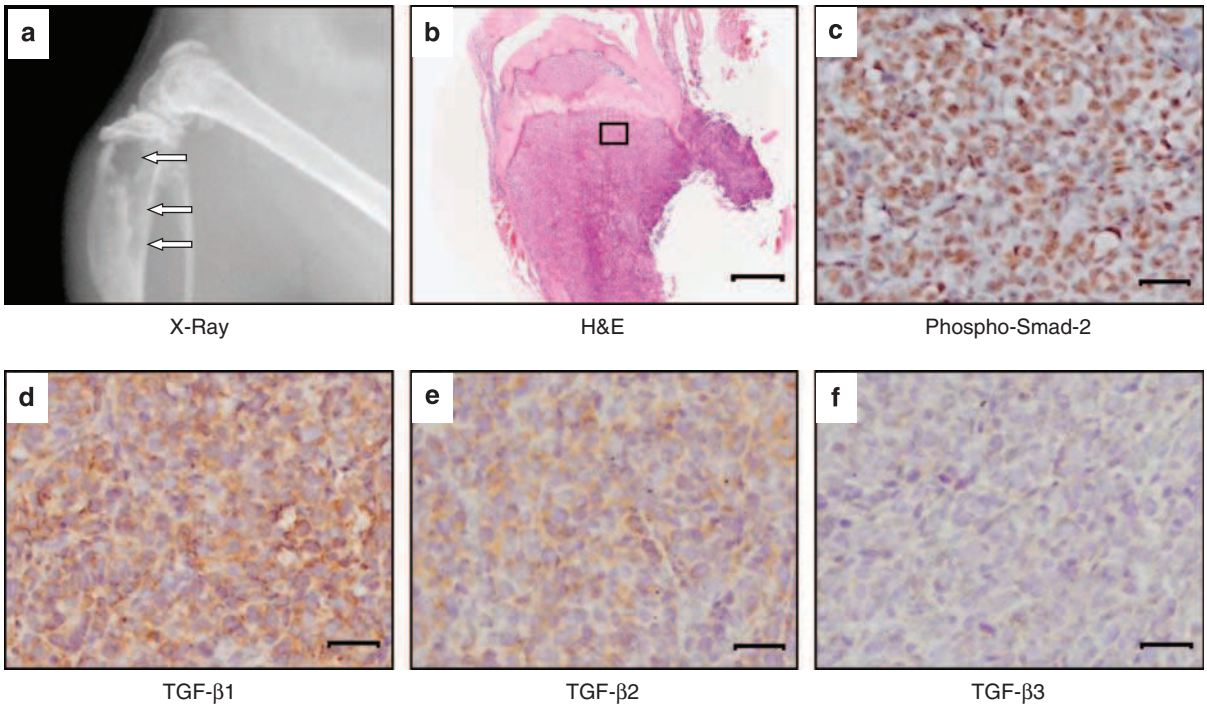

Figure 1 Transforming growth factor (TGF)-β1 is the predominant isoform expressed in MDA-MB-231-derived osteolytic lesions in the bone. (**a**) X-ray image illustrating an osteolytic lesion resulting from a tibial injection of MDA-MB-231 cells. Arrows indicate regions of extensive bone resorption. (**b**) Hematoxylin and eosin stain of an MDA-MB-231 tumor growing in the marrow space, the boxed area corresponds to the region displayed for immunohistocytochemical staining of phospho-Smad2 (**c**), TGF-β1 (**d**), TGF-β2 (**e**) and TGF-β3 (**f**). Scale bars represent 125 μm (panel b) and 25 μm (panels c–f), respectively.

imaging reporter system that includes firefly luciferase (Minn *et al*., 2005). To test the efficacy of the ligand trap, VC or Fc:TβRII(ECD)-expressing cells were allowed to condition media and subsequently challenged with increasing concentrations of TGF-β1, TGF-β2 or TGF-β3 (Supplementary Figure S1a–c). VC cells showed a concentration-dependent increase in phospho-Smad2 levels in response to stimulation by all three TGF-β isoforms (Supplementary Figure S1a–c). Relative to VC cells, MDA-MB-231 cells expressing the ligand trap were significantly diminished in their ability to activate Smad2-mediated signaling in response to both TGF-β1 and TGF-β3 (Supplementary Figure S1a–c). However, the ligand trap was incapable of neutralizing TGF-β2-induced signaling (Supplementary Figure S1b). Immunoblot analysis further confirmed that the fusion protein was expressed to similar levels by trap-producing MDA-MB-231 cells, but was absent in VC cells (Supplementary Figure S1a–c).

To confirm that the Fc:TβRII(ECD) fusion protein functioned as a soluble ligand trap, we asked whether conditioned media (CM) from VC and Fc:TβRII(ECD)-expressing MDA-MB-231 cells could block TGF-β isoform signaling in a TGF-β responsive cell line. To perform this experiment, we used NMuMG cells, an immortalized normal mouse mammary cell line, which exhibits numerous TGF-β-induced responses (Northey *et al*., 2008). NMuMG cells stimulated with 2 ng/ml of TGF-β1, TGF-β2 or TGF-β3, in the absence or presence of CM (1:10 dilution) from MDA-MB-231 VC cells, responded with robust Smad2 phosphorylation (Supplementary Figure S1d–f). However, CM (1:10 dilution) from Fc:TβRII(ECD)-expressing MDA-MB-231 cells effectively blocked Smad2 activation by TGF-β1 or TGF-β3, but remained ineffective against TGF-β2 (Supplementary Figure S1d–f). Immunoblot analyses of whole cell lysates and CM, using an antibody specific to the ECD of TβRII, confirmed expression of the ligand trap (Supplementary Figure S1g). These results show that the Fc:TβRII(ECD) fusion protein effectively neutralizes both TGF-β1 and TGF-β3 isoforms but fails to sequester TGF-β2.

Expression of the TGF-β ligand trap impairs breast tumor growth in the mammary fat pad

We first determined whether expression of the ligand trap altered the growth characteristics of MDA-MB-231 breast cancer cells, relative to VC cells. While VC and Fc:TβRII(ECD)-expressing MDA-MB-231 cells grew at the same rate *in vitro* (Supplementary Figure S2a), a clear and significant reduction in the growth of Fc:TβRII(ECD)-expressing tumors was observed in the mammary fat pad (Supplementary Figure S2b). Immunohistocytochemical analysis confirmed that the expression of the ligand trap was absent in VC-derived mammary tumors and maintained in Fc:TβRII(ECD)-expressing mammary tumors *in situ* (Supplementary Figure S3a). To investigate potential mechanisms for reduced mammary tumor outgrowth in cells expressing the ligand trap, we analysed the proliferative, angiogenic and apoptotic indices of these tumors by Ki67, CD31 and TUNEL staining, respectively. We did not observe statistically significant differences in any of these parameters between Fc:TβRII(ECD) and VC-derived end-stage mammary tumors (Supplementary Figure S3b–d). However, this does not preclude the possibility that these parameters are adversely affected by the ligand trap at earlier stages of tumor outgrowth.

Neutralization of TGF-β1 and TGF-β3 isoforms within the bone microenvironment impairs the formation of osteolytic lesions

We next determined the effects of ligand trap expression on the ability of breast cancer cells to grow in the bone, following direct tibial injection. The tibial injection model was specifically chosen to focus our investigations on the importance of TGF-β isoforms in mediating tumor cell interactions with the bone microenvironment, thereby avoiding possible TGF-β effects on tumor cell survival in circulation, endothelial adhesion and extravasation. Expression of the ligand trap significantly diminished the growth of osteolytic lesions within the bone, as measured by longitudinal bioluminescence imaging and X-ray imaging at end point (Figures 2a and b). While 100% of tibiae developed progressively growing lesions following injection of either VC or Fc:TβRII(ECD)-expressing MDA-MB-231 cells, the average normalized photon flux of lesions expressing the ligand trap (4.65×10^8 p/s/cm^2/sr) was approximately one-third the intensity compared with lesions derived from VC cells (1.45×10^9 p/s/cm^2/sr) at 28 days post-injection (Figure 2a). Moreover, analysis of blinded X-rays showed that the osteolytic lesion area was reduced threefold in mice injected with Fc:TβRII(ECD)-expressing MDA-MB-231 cells (7.7% of tibia occupied by osteolytic lesions) compared with animals receiving VC cells (21.4% of tibia occupied by osteolytic lesions) (Figure 2b).

Immunohistocytochemical staining with an antibody against the ECD of TβRII confirms that breast cancer lesions retain expression of the ligand trap *in vivo* (Supplementary Figure S4a). Examination of breast tumor cell proliferation, survival and recruitment of vasculature within osteolytic bone lesions failed to show statistically significant differences between VC and Fc:TβRII(ECD)-expressing MDA-MB-231 cells (Supplementary Figure S4b–d). Although the mechanisms remain unclear, these results indicate that neutralization of TGF-β1 and TGF-β3 isoforms significantly impairs the outgrowth of breast cancer cells in bone.

Transforming growth factor-β is known to induce the expression of factors important for osteoclast differentiation and function, such as *PTHrP* and *IL-11* (Yin *et al*., 1999; Kang *et al*., 2003, 2005; Deckers *et al*., 2006). Thus, we investigated whether expression of the neutralizing ligand trap could blunt TGF-β-induced expression of these two factors. Vector control (VC) or Fc:TβRII(ECD)-expressing MDA-MB-231 breast cancer cells were allowed to condition serum free media for 48 h and subsequently incubated in the absence or presence of TGF-β1 for a further 3 h. Quantitative

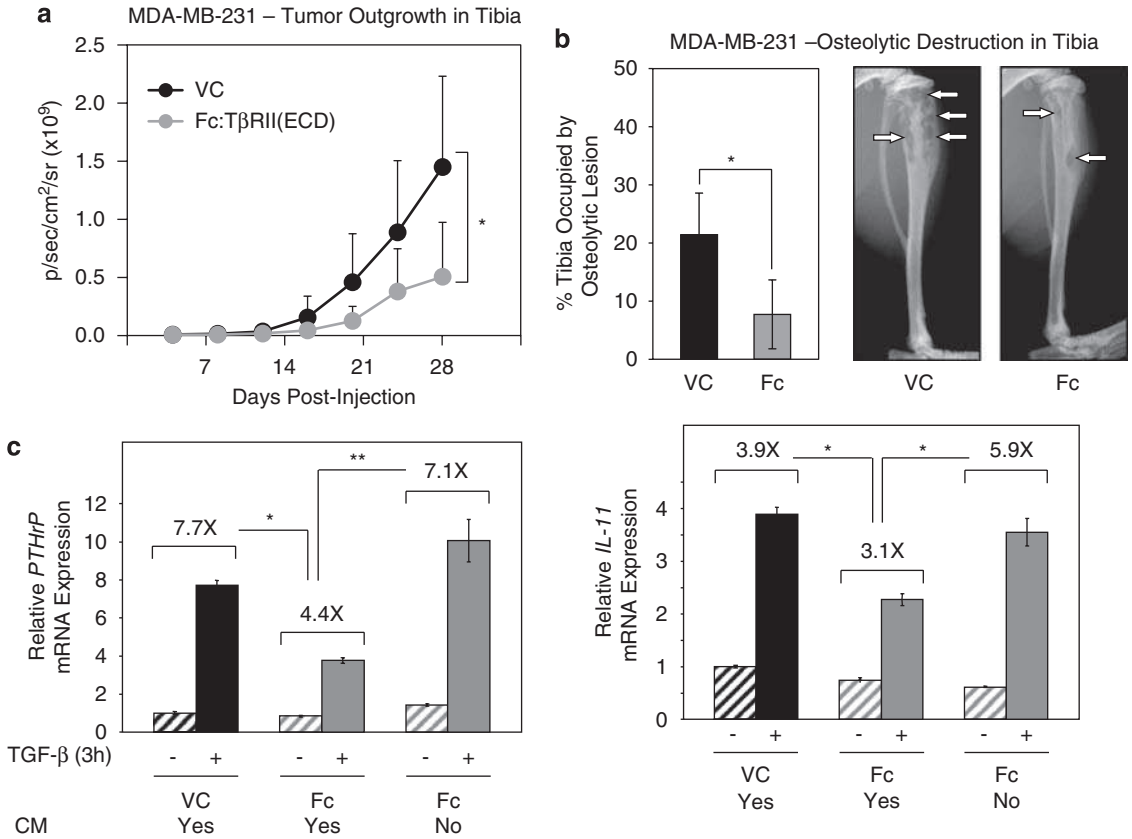

Figure 2 MDA-MB-231 breast cancer cells expressing Fc:TβRII(ECD) show reduced outgrowth and formation of osteolytic lesions in bone. (**a**) 1×10^5 vector control (VC) or Fc:TβRII(ECD)-expressing MDA-MB-231 cells were injected directly into the tibia of athymic female mice. Breast cancer cell outgrowth in bone was measured by bioluminescence imaging and quantified as the normalized photon flux (p/s/cm^2/sr) in each tibia over time. The averages (± s.d.) from 19 independent tibias are plotted (*$P<0.0001$) for each group. (**b**) Osteolytic destruction was measured in the tibiae of mice injected with VC or Fc:TβRII(ECD)-expressing MDA-MB-231 cells using blinded digital X-ray images. The data are presented as the percentage of the tibia occupied by osteolytic lesions (graph) (*$P<0.0001$). Representative X-ray images (day 28) are shown with arrows indicating regions of osteolytic bone resorption (right panels). (**c**) Expression of transforming growth factor (TGF)-β ligand trap diminishes TGF-β-induced expression of genes shown to be important for the formation of osteolytic bone metastasis. Quantitative reverse transcription (RT)–PCR was performed for *PTHrP* (left graph) and *IL-11* (right graph). VC and Fc:TβRII(ECD)-expressing MDA-MB-231 cells were allowed to condition media for 48 h before a 3 h incubation in the absence (−) or presence (+) of 5 ng/ml TGF-β1. Fc:TβRII(ECD)-expressing MDA-MB-231 cultures were changed to fresh media to remove any accumulated ligand trap and subjected to a 3 h incubation in the absence or presence of TGF-β1. The expression levels of each target gene were first normalized to β-actin and presented at a fold change relative to VC (−TGF-β1) (± s.d.). The data represents the average of two independent experiments, each performed in triplicate. TGF-β1-induced fold changes in *PTHrP* and *IL-11* expression were found to be statistically different in the absence of the ligand trap compared with conditions when the trap is present (*$P<0.0001$; **$P=0.0008$).

reverse transcription (RT)–PCR analysis showed that *PTHrP* expression was induced 7.7-fold in VC cells, but only 4.4-fold in ligand trap-producing cells following TGF-β1 stimulation (Figure 2c, left panel). Likewise, *IL-11* mRNA expression was induced 3.9-fold in MDA-MB-231 VC cells in response to TGF-β1, which dropped to a 3.1-fold induction in MDA-MB-231 cells expressing the Fc:TβRII(ECD) fusion (Figure 2c, right panel). TGF-β-induced expression of both *PTHrP* and *IL-11* was restored in Fc:TβRII(ECD)-expressing MDA-MB-231 cells, to the levels seen in MDA-MB-231 VC cells, when the ligand trap was removed before TGF-β1 stimulation (Figure 2c). Thus, the diminished capacity of MDA-MB-231 cells expressing a TGF-β-neutralizing ligand trap correlated with a statistically significant reduction in TGF-β-induced *PTHrP* and *IL-11* expression.

Loss or reduction of TGF-β1 levels in host-derived tissue prevents the efficient establishment of breast cancer cells in bone

To specifically interrogate the importance of bone-derived TGF-β1 on the ability of breast cancer cells to form osteolytic lesions, we used TGF-β1-deficient mice that were bred onto a RAG2-deficient background (Engle *et al*., 1999), which rescues the early lethality observed in TGF-β1-null mice due to widespread inflammatory disease (Shull *et al*., 1992; Kulkarni *et al*., 1993). To ensure that the RAG2−/− mice permitted xenografting of human breast cancer cells, we injected an MDA-MB-231 variant that aggressively metastasizes to bone (1833-TR cells) into mammary fat pads of RAG2−/− mice (Kang *et al*., 2003; Minn *et al*., 2005). Primary mammary tumors established and grew in 100% of TGF-β1+/+; RAG2−/−,

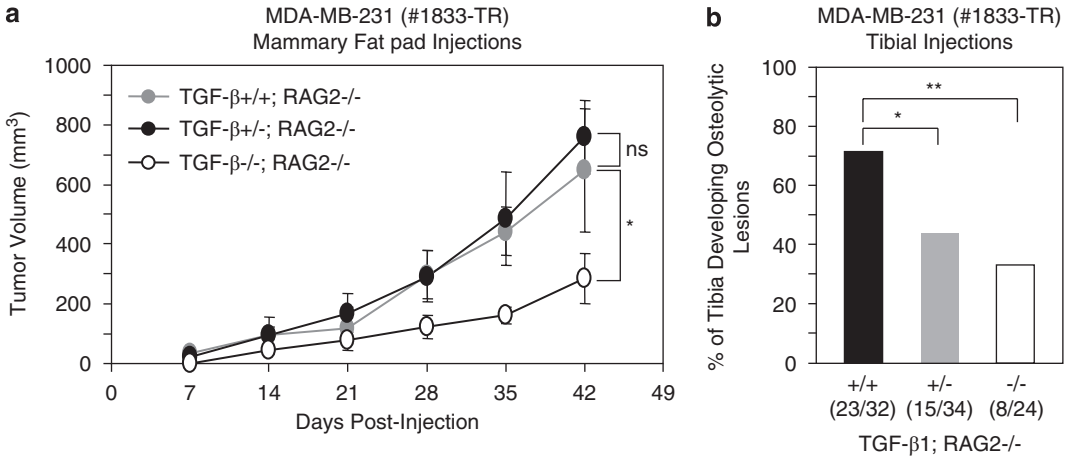

Figure 3 Reduced dosage of host-derived transforming growth factor (TGF)-β1 lowers the incidence of osteolytic breast cancer lesions in bone. (**a**) Mammary tumor outgrowth was measured by weekly caliper measurements following the injection of 1×10^6 1833-TR cells (MDA-MB-231) into the fourth abdominal mammary fat pad of TGF-β1 + / +, + / − and − / − female mice, all on the RAG2-deficient background. The average volumes (± s.d.) of 1833-TR-derived mammary tumors that grew out in TGF-β1 + / +; RAG2 − / − ($n = 8$), TGF-β1 + / −; RAG2 − / − ($n = 8$) and TGF-β1 − / −; RAG2 − / − ($n = 6$) are plotted (*$P < 0.0001$; NS, not significant). (**b**) 1×10^4 1833-TR cells were injected directly into the tibia of the TGF-β1 + / +, + / − and − / − female mice, all on a RAG2 − / − background. X-ray images of the indicated numbers of tibia were first blinded and scored for the presence of osteolytic bone lesions for each genotype (*$P = 0.027$, **$P = 0.006$).

TGF-β1 + / −; RAG2 − / − and TGF-β1 − / −; RAG2 − / − animals (Figure 3a). Tumor outgrowth was found to be similar in the TGF-β1 + / +; RAG2 − / − and TGF-β1 + / −; RAG2 − / − cohorts. However, there was a significant reduction in the rate of mammary tumor growth in TGF-β1 − / −; RAG2 − / − mice (Figure 3a).

To determine the effect of reduced host-derived TGF-β1 expression on the ability of breast cancer cells to grow in bone, we injected 1833-TR cells into the tibiae of TGF-β1 + / +; RAG2 − / −, TGF-β1 + / −; RAG2 − / − and TGF-β1 − / −; RAG2 − / − mice. X-rays were taken at 8 weeks post-injection and blinded before being scored for the presence of osteolytic lesions. Approximately 72% of the tibial injections in TGF-β1 + / +; RAG2 − / − gave rise to osteolytic lesions compared with only 44% in TGF-β1 + / −; RAG2 − / − mice and 33% in TGF-β1 − / −; RAG2 − / − animals (Figure 3b). The dramatic effect observed in TGF-β1 + / −; RAG2 − / − mice most likely reflects the fact that TGF-β1 protein levels are reduced up to 60–90% of the levels observed in wild-type mice (Tang *et al*., 1998). Interestingly, bioluminescence imaging showed that MDA-MB-231 breast cancer cells capable of establishing themselves in TGF-β1 + / +; RAG2 − / −, TGF-β1 + / −; RAG2 − / − and TGF-β1 − / −; RAG2 − / − mice grew out at similar rates (Figure 4a) and induced similar degrees of osteolytic bone destruction (Figure 4b). Importantly, our results argue that reduced TGF-β1 levels in the host microenvironment are sufficient to negatively impact the ability of breast cancer cells to establish osteolytic lesions. Nevertheless, a subset of animals do develop lesions in a TGF-β1-deficient or -null background, suggesting that these lesions have developed compensatory mechanisms in response to attenuated host-derived TGF-β1 signaling.

Breast cancer cells capable of establishing lesions in TGF-β1-deficient mice compensate by upregulating the expression of TGF-β isoforms within the tumor

Using an immunohistocytochemical approach, we examined the possibility that breast cancer cells emerging in a TGF-β1-deficient background could compensate for this loss by increasing tumor specific expression of the TGF-β isoforms. Osteolytic lesions formed in TGF-β1 + / +; RAG2 − / − mice showed readily detectable levels of TGF-β1, whereas TGF-β2 and TGF-β3 were very weakly expressed (Figure 5a, left panels). Interestingly, tumor cell-specific expression of both TGF-β1 and TGF-β2 was significantly increased in a TGF-β1 + / −; RAG2 − / − host microenvironment, whereas there was no detectable increase in TGF-β3 staining (Figure 5a, middle panels). A further upregulation of TGF-β1 and TGF-β2 expression was evident, with a more modest but significant increase in TGF-β3 staining, in TGF-β1 − / −; RAG2 − / − mice (Figure 5a, right panels). This pattern of TGF-β isoform expression correlated with similar levels of Smad2 phosphorylation in osteolytic lesions derived from all three genetic backgrounds (Figure 5b). These data show that a strong selective pressure is exerted upon tumor cells to retain TGF-β signaling during their outgrowth in the bone.

Simultaneous attenuation of TGF-β1 signaling within the breast tumor and the host microenvironment significantly impairs tumor outgrowth in bone

To determine whether tumor cell intrinsic TGF-β1 signaling could compensate for diminished TGF-β1 expression in the bone microenvironment, we injected MDA-MB-231 breast cancer cells expressing the ligand trap into the tibiae of TGF-β1 + / +; RAG2 − / − or TGF-β1 + / −; RAG2 − / − mice. We chose TGF-β1 + / −; RAG2 − / − mice given that the formation of

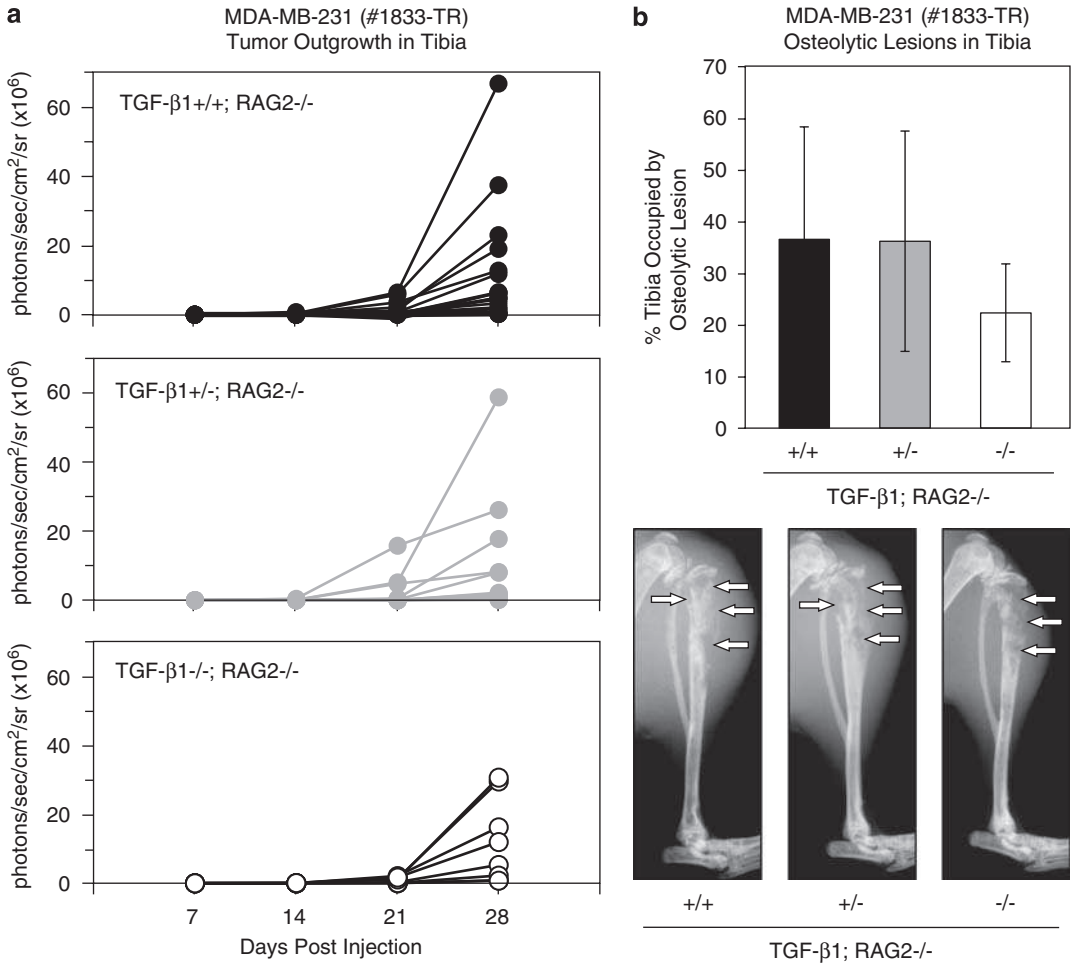

Figure 4 Established osteolytic lesions grow at similar rates in transforming growth factor (TGF)-β1+/+; RAG2−/−, TGF-β1+/−; RAG2−/− and TGF-β1−/−; RAG2−/− animals. (**a**) Breast cancer cell outgrowth in bone was followed by bioluminescence imaging and quantified as the normalized photon flux (p/s/cm²/sr) in each tibia over time in TGF-β1+/+; RAG2−/− (upper panel, $n=23$), TGF-β1+/−; RAG2−/− (middle panel, $n=15$) and TGF-β1−/−; RAG2−/− (lower panel, $n=8$) mice. (**b**) Osteolytic destruction was measured in the tibiae of TGF-β1+/+; RAG2−/−, TGF-β1+/−; RAG2−/− and TGF-β1−/−; RAG2−/− mice using blinded digital X-ray images. Data are expressed as the percentage of the tibia occupied by osteolytic lesions (graph). Corresponding representative X-ray images (weeks 7–8) are shown for each group with arrows indicating regions of osteolytic bone resorption (bottom panels). There was no statistical difference in breast tumor outgrowth in tibia of mice from the three genotypes or in the percentage of osteolytic lesion area.

osteolytic lesions was significantly reduced in TGF-β1+/− and TGF-β1−/− animals; however, mammary tumor outgrowth was only impaired in the latter (Figure 3). Injection of Fc:TβRII(ECD)-expressing MDA-MB-231 cells resulted in the development of osteolytic lesions in 34.6% (9/26) of TGF-β1+/+; RAG2−/− animals compared with 23.1% (6/26) in TGF-β1+/−; RAG2−/− mice. However, the growth of the resulting lesions was severely diminished when TGF-β1 signaling was attenuated both within the tumor cell and in resident bone cells (Figure 6a). Whereas most osteolytic lesions that developed in TGF-β+/+; RAG2−/− mice were large, tumors arising in TGF-β+/−; RAG2−/− animals showed significantly lower luminescence signals (Figure 6b). Indeed, the mean luminescence signal observed in tumors arising from TGF-β1+/+; RAG2−/− animals was 1.9×10^8 p/s/cm²/sr compared with 2.3×10^7 p/s/cm2/sr in TGF-β1+/−; RAG2−/− mice. Furthermore, the reduced ability of Fc:TβRII(ECD)-expressing MDA-MB-231 cells to grow in the tibiae of TGF-β+/−; RAG2−/− mice translated into a clear decrease in osteolytic lesion size when compared with TGF-β+/+; RAG2−/− animals (Figure 6c). The number of tibiae that developed small, medium and large lesions was determined based on the presence of bioluminescent signals. Those lesions with the lowest bioluminescent signals were not detectable by X-ray (compare Figures 6b and c) but were confirmed by histological analysis (Supplementary Figure S5).

Taken together, we show that neutralization of TGF-β1 and/or TGF-β3 signaling by the soluble ligand trap severely diminishes the formation of osteolytic bone lesions in a TGF-β1-deficient background. Given that tumor cell intrinsic TGF-β1, but not TGF-β3, is strongly overexpressed in osteolytic lesions that formed

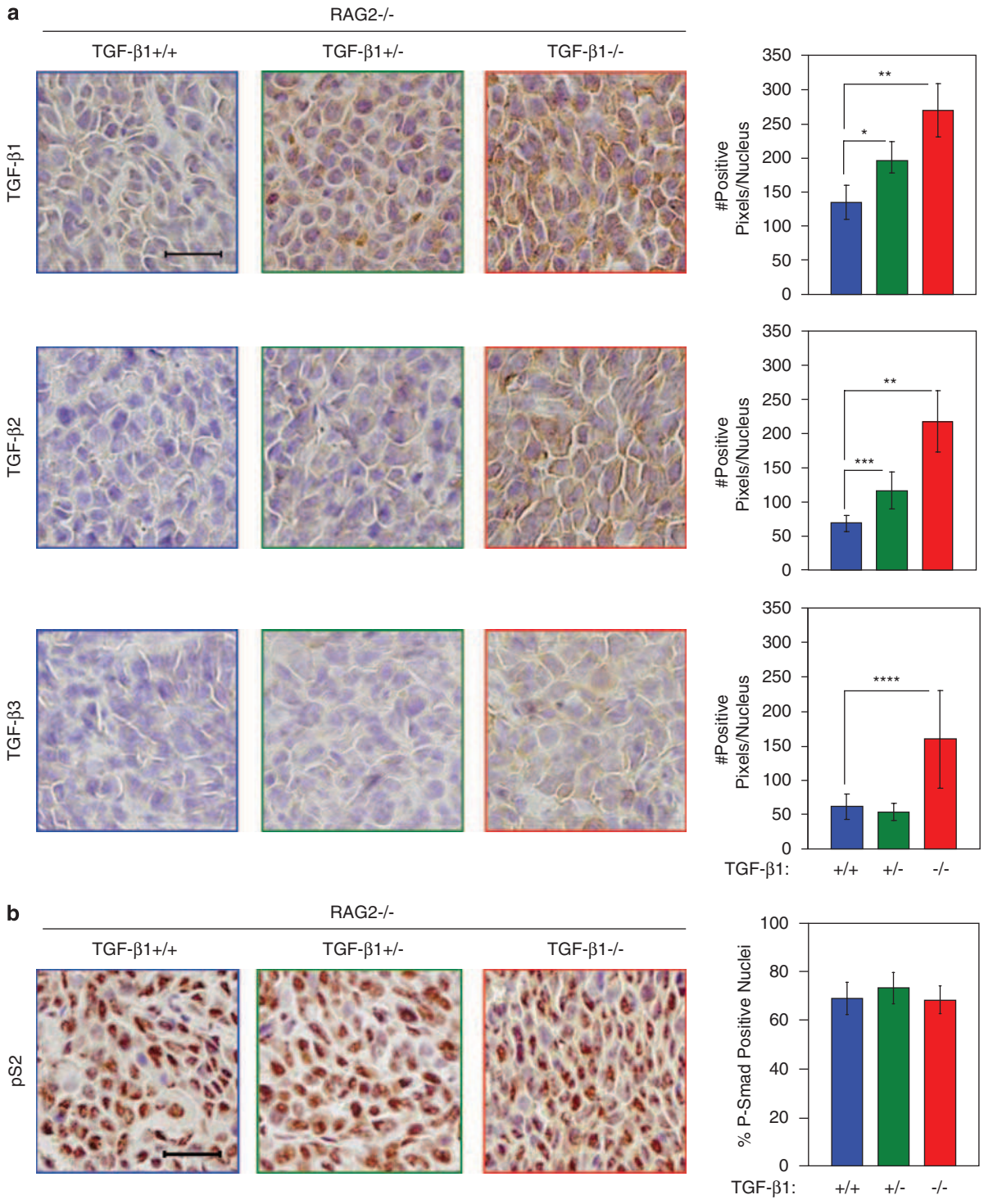

Figure 5 Upregulation of transforming growth factor (TGF)-β isoform expression in breast cancer cells compensates for loss of TGF-β1 in host tissue. (**a**) Immunohistocytochemical analyses of TGF-β1 expression (upper panels), TGF-β2 (middle panels) and TGF-β3 (lower panels) in breast cancer cells growing in TGF-β1 + / + ; RAG2−/− ($n=3$), TGF-β1 + /−; RAG2−/− ($n=3$) and TGF-β1−/−; RAG2−/− ($n=2$) animals following intra-tibial injection. Scale bar in the upper left panel represents 25 μm and applies to all panels. The number of positively stained pixels from 4 to 5 independent regions per osteolytic region were analysed for each TGF-β isoform (positive pixel count algorithm) and normalized to the number of nuclei in each region (*$P=0.034$; **$P<0.0001$; ***$P=0.025$; ****$P=0.0006$). (**b**) Immunohistocytochemical analysis of phospho-Smad2 (pS2) in TGF-β1 + / + ; RAG2−/−, TGF-β1 + /−; RAG2−/− and TGF-β1−/−; RAG2−/− mice following intra-tibial injection. Scale bar represents 25 μm and applies to all panels.

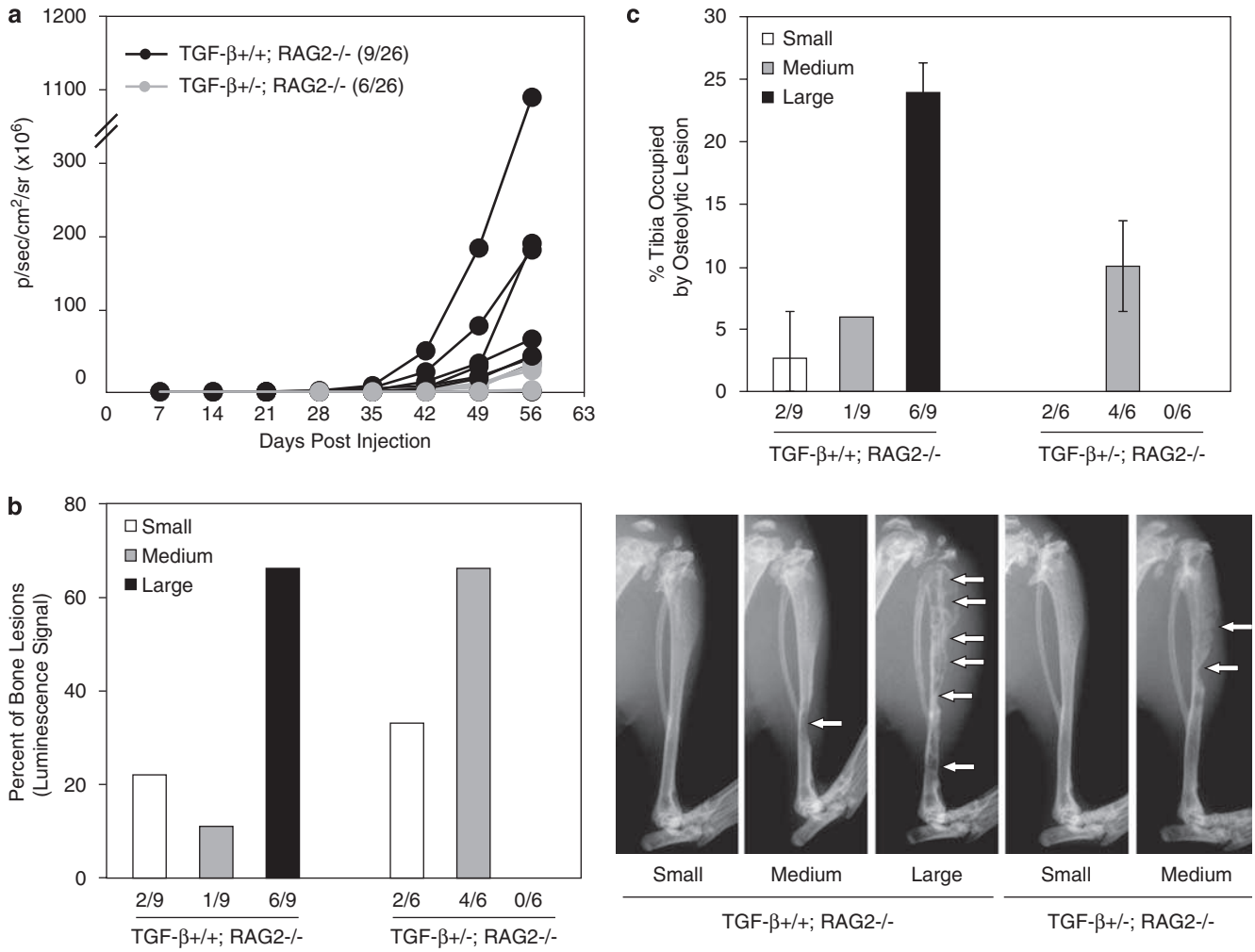

Figure 6 Loss of host-derived transforming growth factor (TGF)-β1 coupled with expression of the ligand trap significantly impairs osteolytic tumor outgrowth in bone. (**a**) 1×10^5 Fc:TβRII(ECD)-expressing MDA-MB-231 cells were injected directly into the tibia of the TGF-β1 + / + or + / − female mice, on a RAG2−/− background. Outgrowth in bone of Fc:TβRII(ECD)-expressing MDA-MB-231 cells was followed by bioluminescence imaging and quantified as the normalized photon flux (p/s/cm^2/sr) in each tibia over time in TGF-β1 + / +; RAG2−/− and TGF-β1 + /−; RAG2−/− mice. (**b**) The resulting lesions were grouped based on their luminescence signals into small ($<4 \times 10^6$ p/s/cm^2/sr), medium ($>4 \times 10^6$ and $<4 \times 10^7$ p/s/cm^2/sr) or large ($>4 \times 10^7$ p/s/cm^2/sr) lesions in TGF-β1 + / +; RAG2−/− and TGF-β1 + /−; RAG2−/− mice. The percentage bone lesions within each group are shown and are based on the presence of bioluminescent signals. (**c**) The size of osteolytic lesions (week 9) for each group is expressed as the percentage of each tibia occupied by osteolytic lesions. Note, the small lesions detectable by bioluminescence imaging could not be detected by X-ray imaging, thus osteolytic area could not be quantified. Corresponding representative images for each lesion size are presented in the bottom panels. The arrows indicate regions of osteolytic bone resorption.

in TGF-β1-deficient animals (Figure 5), these observations argue that establishment of a TGF-β1 autocrine loop, provided either from the breast cancer cells themselves or the host tissues, is crucial for breast cancer outgrowth in the bone microenvironment.

Discussion

Previous studies suggest that TGF-β1 is the predominant isoform present in bone (Hering *et al*., 2001a, b; Janssens *et al*., 2005). Here we show that MDA-MB-231 breast cancer cells express readily detectable levels of TGF-β1 and TGF-β2, but very low amounts of TGF-β3, within osteolytic lesions *in vivo*. The Fc:TβRII(ECD) ligand trap preferentially binds and neutralizes TGF-β1 and TGF-β3, but not TGF-β2 (Supplementary Figure S1) (Komesli *et al*., 1998), due to amino-acid substitutions in TGF-β2 that reduce its affinity for the TβRII (De Crescenzo *et al*., 2006). Expression of the ligand trap significantly inhibited the ability of breast cancer cells to grow effectively in the bone and suppressed TGF-β-induced expression of *PTHrP* and *IL-11*, two osteoclastic factors that are important for the formation of breast cancer bone metastases (Manolagas, 1995; Yin *et al*., 1999; Sotiriou *et al*., 2001; Kakonen *et al*., 2002; Kang *et al*., 2003, 2005; Deckers *et al*., 2006; Liao and McCauley, 2006). These results are consistent with the observation that a dominant negative TβRII or a TβRI inhibitor impaired the ability of breast cancer cells to metastasize to bone (Yin *et al*., 1999; Bandyopadhyay *et al*., 2006; Ehata *et al*., 2007). Thus, it is most likely

that an increase in the local concentration of the ligand trap would be achieved as Fc:TβRII(ECD)-producing tumors progressively grow in the bone. These increasing amounts of the secreted ligand trap would neutralize TGF-β1 and TGF-β3 that are (1) produced by resident bone cells, (2) released from the matrix during the induction of an osteolytic response or (3) secreted by the mammary tumor cells themselves. Ultimately, this would dampen the expression of pro-osteoclastic factors, resulting in diminished tumor growth of TGF-β ligand trap-expressing cells.

The expression of Fc:TβRII(ECD) is not sufficient to abrogate tumor growth in the bone, but results in a significant delay in outgrowth of osteolytic lesions. This may reflect the fact that expression of the various TGF-β isoforms, regardless of their source, is simply in excess of the neutralizing capacity of the ligand trap. The observation that expression of the ligand trap dramatically impairs tumor outgrowth in a TGF-β1-deficient background argues that TGF-β1 is the predominant isoform that drives the efficient establishment and growth of osteolytic lesions in bone.

To extend the results obtained with the ligand trap approach, we used TGF-β1-deficient mice that were bred onto a RAG2−/− background. We cannot rule out the possibility that delayed tumor outgrowth observed within the mammary gland and tibiae of TGF-β1−/−; RAG2−/− mice is a result of general failure to thrive observed in these animals relative to heterozygous and wild-type controls. Moreover, TGF-β1-null mice possess defects in bone quality, including reduced bone mineral content and bone elasticity in the tibiae compared with heterozygote and wild-type mice (Geiser *et al*., 1998). On the other hand, TGF-β1+/+; RAG2−/− and TGF-β1+/−; RAG2−/− mice are phenotypically indistinguishable with respect to overall health, bone quality and their ability to support mammary tumor growth in the primary site. Importantly, there remains a significant reduction in the ability of breast cancer cells to establish or grow in TGF-β1+/−; RAG2−/− tibiae compared with wild-type controls. This dramatic effect in TGF-β1+/−; RAG2 −/− mice may reflect the fact that TGF-β1 protein levels have been found to be reduced up to 60–90% of the levels observed in wild-type mice without a concomitant upregulation of TGF-β2 or TGF-β3 (Tang *et al*., 1998). Thus, our results show that the reduced levels of TGF-β1 can have a profound effect on the ability of breast cancer cells to establish or grow in bone.

Although the TGF-β ligand trap was able to diminish tumor outgrowth in the bone, all tibiae injected with Fc:TβRII(ECD)-expressing breast cancer cells developed osteolytic lesions. In contrast, TGF-β1 deficiency reduced the incidence of osteolytic lesion formation. This difference between the effects of a neutralizing ligand trap versus reduced TGF-β1 expression may reflect the fact that the bone microenvironment in TGF-β1+/− and −/− mice cannot support the early survival of breast cancer cells compared with wild-type mice. Such a result would not be expected when using a ligand trap, given that the bone microenvironment, and thus the putative 'niche' sites where breast cancer cells first establish, would be unaltered before the arrival of the ligand trap-expressing breast cancer cells. A careful examination of the number of breast cancer cells that seed the bone marrow and survive over the initial few days post-injection may shed light on these questions.

Once established, lesions in TGF-β1+/− and −/− mice grew out at the same rate as those in TGF-β1+/+ animals by virtue of their ability to upregulate tumor cell-specific TGF-β isoform expression and signaling. Interestingly, expression of the ligand trap in MDA-MB-231 breast cancer cells profoundly diminished their ability to grow in a TGF-β1-deficient background. In this respect, the TGF-β ligand trap functioned in a similar manner in breast cancer cells that established themselves as growing lesions within the bone, regardless of the experimental system employed. This argues that the predominant effect of the ligand trap is to neutralize TGF-β isoforms that are either released and activated from bone or secreted by cancer cells, which results in diminished growth of the breast cancer cells. However, tumor outgrowth of Fc:TβRII(ECD)-expressing MDA-MB-231 cells was not completely abrogated in the tibiae of TGF-β1+/− animals. This may indicate that the levels of the TGF-β isoforms upregulated in breast cancer cells were too high to be fully neutralized by the ligand trap. Alternatively, tumor-specific expression of TGF-β2, which cannot be neutralized by the ligand trap, may compensate in the formation of osteolytic lesions but is not sufficient to permit aggressive growth of breast cancer cells in bone. To address this possibility, a soluble ligand trap capable of neutralizing all three TGF-β isoforms will need to be tested in the context of reduced host-derived TGF-β1 levels. Thus, our data show that TGF-β signaling, predominantly initiated by TGF-β1, is not only required within the breast cancer cells themselves but also within the bone microenvironment to create favorable conditions that support breast tumor cell outgrowth.

Materials and methods

DNA constructs

The human TβRII ECD fused to the Fc portion of human IgG (Fc:TβRII(ECD)) (del Re *et al*., 2004) was PCR-amplified and subcloned into a lentiviral plasmid vector designated pCSII-CMV-mcs-ires-DsRed (proprietary vector).

Cell culture

Parental MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and transduced with a triple reporter system as described (Minn *et al*., 2005). The MDA-MB-231-derived bone metastatic cell population (1833-TR) has been described previously (Kang *et al*., 2003; Minn *et al*., 2005). The NMuMG normal murine mammary gland epithelial cell line was obtained from the American Type Culture Collection and cultured as previously described (Northey *et al*., 2008).

Lentivirus harboring the empty VC or Fc:TβRII(ECD) plasmids was produced and amplified in the packaging cell line 293SF-PacLV clone 29-6, as described previously (Broussau

et al., 2008). MDA-MB-231 cells were subjected to two rounds of infection with either VC or Fc:TβRII(ECD) carrying lentivirus (M.O.I. 10 transduction units/cell), as previously described (Broussau *et al*., 2008).

Bioluminescence and X-ray imaging
Tumor outgrowth within the bone was monitored using an IVIS 100 (Caliper Life Sciences, Mountain View, CA, USA) bioluminescence imaging system. Briefly, mice were anaesthetized by isoflurane inhalation, injected intraperitoneally with D-luciferin potassium salt (75 mg/kg; Xenogen/Caliper Life Sciences, Mountain View, CA, USA) and imaged 10 min following substrate injection. Images were analysed with Living Image Software. Bioluminescent signals within the tibiae were normalized to the background luminescence taken over the same region of interest from uninjected animals. Mice were killed between weeks 4 and 8 post-tibial injection and digital X-rays were obtained with a Faxitron Specimen Radiography System. All X-rays were blinded and evaluated by two readers (AAM and PMS). The size of osteolytic lesions was expressed as percentage area occupied by osteolytic lesions over the total tibial area measured from blinded X-rays. Both osteolytic area and total area of tibiae were quantified using ImageJ software. Animal experiments were conducted under a McGill University approved Animal Use Protocol in accordance with the guidelines established by the Canadian Council on Animal Care.

Immunohistocytochemistry
Bone samples were fixed overnight in 4% paraformaldehyde and further decalcified with 10% EDTA disodium salt. Immunohistocytochemistry was performed with the following antibodies: TGF-β1 (1:100 dilution; Santa Cruz Biotechnology; Santa Cruz, CA, USA), TGF-β2 (1:200 dilution; Santa Cruz Biotechnology), TGF-β3 (1:150 dilution; Santa Cruz Biotechnology) and Phospho-Smad2 (Ser465/467) (1:150 dilution; Cell Signaling; Danvers, MA, USA). Appropriate biotin-SP-conjugated anti-IgG secondary antibodies were purchased from Jackson Laboratories; Bar Harbor, ME, USA. Slides were first scanned using a Scanscope XT digital slide scanner (Aperio, Vista, CA, USA) and further analysed using Imagescope software (Aperio) using either positive pixel count or immunohistochemistry nuclear algorithms.

Quantitative RT–PCR
Total RNA was isolated using an RNeasy kit (Qiagen; Mississauga, ON, Canada) and 1 μg of RNA from each sample was reverse transcribed using the High capacity cDNA Reverse Transcription kit (Applied Biosystems; Foster City, CA, USA) in accordance with the manufacturer's instructions. The resulting cDNAs were used for real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems). Reactions were carried out in a 7500 Real-time PCR System (Applied Biosystems) for 40 cycles (95 °C for 15 s, 60 °C for 30 s and 72 °C for 45 s) after the initial 10-min incubation at 95 °C. See Supplementary Information for primer sequences. A C_t value for each reaction was calculated using Applied Biosystems Sequence Detections Software and the relative ratio of expression was determined using a previously described algorithm (Pfaffl, 2001).

Statistical analysis
Statistical significance values for primary tumor growth, bioluminescence, osteolytic lesion area, quantitative RT–PCR and TGF-β isoform staining data were obtained by performing a two-sample variance student's *t*-test. The Fisher's exact test was performed to analyse the probability of the occurrence of osteolytic lesions in TGF-β1 + / + ; RAG2−/−, TGF-β1 + /−; RAG2−/− and TGF-β1−/−; RAG2−/− mice.

Abbreviations

CM, conditioned media; ECD, extracellular domain; IL-11, interleukin-11; PTHrP, parathyroid hormone-related protein; TβRI, TGF-β type I receptor; TβRII, TGF-β type II receptor; TGF-β, transforming growth factor β; VC, vector control.

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