Targeted inactivation of β1 integrin induces β3 integrin switching, which drives breast cancer metastasis by TGF-β

Jenny G. Parvani[a], Amy J. Galliher-Beckley[b], Barbara J. Schiemann[c], and William P. Schiemann[c]
[a]Department of Pathology and [c]Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH 44106; [b]Department of Anatomy and Physiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506

ABSTRACT **Mammary tumorigenesis and epithelial–mesenchymal transition (EMT) programs cooperate in converting transforming growth factor-β (TGF-β) from a suppressor to a promoter of breast cancer metastasis. Although previous reports associated β1 and β3 integrins with TGF-β stimulation of EMT and metastasis, the functional interplay and plasticity exhibited by these adhesion molecules in shaping the oncogenic activities of TGF-β remain unknown. We demonstrate that inactivation of β1 integrin impairs TGF-β from stimulating the motility of normal and malignant mammary epithelial cells (MECs) and elicits robust compensatory expression of β3 integrin solely in malignant MECs, but not in their normal counterparts. Compensatory β3 integrin expression also 1) enhances the growth of malignant MECs in rigid and compliant three-dimensional organotypic cultures and 2) restores the induction of the EMT phenotypes by TGF-β. Of importance, compensatory expression of β3 integrin rescues the growth and pulmonary metastasis of β1 integrin–deficient 4T1 tumors in mice, a process that is prevented by genetic depletion or functional inactivation of β3 integrin. Collectively our findings demonstrate that inactivation of β1 integrin elicits metastatic progression via a β3 integrin–specific mechanism, indicating that dual β1 and β3 integrin targeting is necessary to alleviate metastatic disease in breast cancer patients.**

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INTRODUCTION

Transforming growth factor-β (TGF-β) is a pleiotropic cytokine that modulates all phases of mammary gland development, including branching morphogenesis, lactation, and involution (Taylor *et al.*, 2010; Moses and Barcellos-Hoff, 2011). In addition, TGF-β functions as a powerful tumor suppressor in normal mammary epithelial cells (MECs) but undergoes a dramatic functional transformation during mammary tumorigenesis that ultimately bestows TGF-β with tumor-promoting activities that drive malignant MEC invasion and metastasis (Taylor *et al.*, 2010; Tian *et al.*, 2011). These contrasting behaviors of TGF-β are known as the "TGF-β paradox" and represent the most confounding pathophysiological aspects of this cytokine in developing and progressing breast cancers. Indeed, elucidating the molecular mechanisms responsible for conferring oncogenic activities to TGF-β will undoubtedly provide new therapeutic opportunities to alleviate metastatic progression and disease recurrence of breast cancers (Taylor *et al.*, 2010; Tian *et al.*, 2011).

The acquisition of metastatic phenotypes by mammary tumors has been linked to the process of epithelial–mesenchymal transition (EMT) and its associated alterations in integrin expression (Taylor *et al.*, 2010; Keely, 2011; Nieto, 2011). EMT is a normal physiological process by which immotile, polarized, and cuboidal epithelial cells undergo transdifferentiation into fibroblastoid-like cells that possess heightened motility and spindle morphologies (Taylor *et al.*, 2010). Aberrant activation of EMT outside the context of development and wound resolution engenders the pathological features associated with metastasis (Kalluri, 2009; Micalizzi *et al.*, 2010). Tumorigenesis is also associated with dramatic changes in the

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Address correspondence to: William P. Schiemann (wps20@case.edu)

Abbreviations used: 3D, three-dimensional; EMT, epithelial–mesenchymal transition; FAK, focal adhesion kinase; MEC, mammary epithelial cell; TGF-β, transforming growth factor-β.

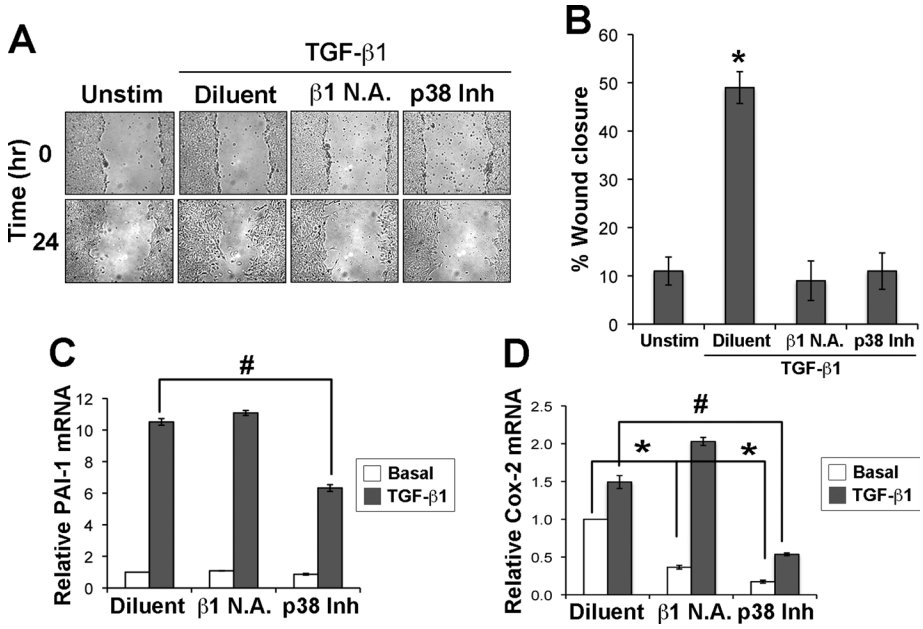

FIGURE 1: Functional disruption of β1 integrin attenuates TGF-β–mediated motility in normal NMuMG cells. (A) Confluent NMuMG cell monolayers were wounded and allowed to heal for 24 h in the absence (unstim) or presence of TGF-β1 (5 ng/ml), neutralizing β1 integrin antibodies (β1 N.A.; 5 μg/ml), or the p38 MAPK inhibitor SB203580 (p38 Inh; 10 μM) as indicated. Representative photomicrographs from a single experiment performed three times in triplicate. (B) Quantification of wounded NMuMG cultures at 24 h was conducted using ImageJ (v1.34S; National Institutes of Health, Bethesda, MD). Data are mean (±SE) percentage wound closure of three independent experiments completed in triplicate. (C, D) NMuMG cells were stimulated for 24 h with TGF-β1 (5 ng/ml) in the absence (diluent) or presence of either neutralizing β1 integrin antibodies (β1 N.A.; 5 μg/ml) or p38 MAPK inhibitor SB203580 (p38 Inh; 10 μM) as indicated. Afterward, total RNA was isolated to monitor changes in the expression of PAI-1 (C) or Cox-2 (D) by semiquantitative real-time PCR. Data are mean (±SE) of three independent experiments completed in triplicate. In B–D, $^{*,\#}p < 0.05$.

expression profiles of integrins, which sense and respond to mechanosensory stimuli provided by adjacent tumor microenvironments (Taylor *et al.*, 2010; Huttenlocher and Horwitz, 2011; Keely, 2011). Altered integrin expression profiles also facilitate the collaboration between integrins and growth factor and/or cytokine signaling systems (Sieg *et al.*, 2000), including those activated by TGF-β. Indeed, we demonstrated that TGF-β stimulation of MECs up-regulates their expression of β3 integrin, which forms a complex with TβR-II that is bridged by focal adhesion kinase (FAK) and functions in amplifying the activation of p38 mitogen-activated protein kinase (MAPK) necessary in driving EMT programs and breast cancer metastasis stimulated by TGF-β (Galliher and Schiemann, 2006, 2007; Galliher-Beckley and Schiemann, 2008). Conversely, others demonstrated that 1) inactivating β1 integrin uncouples TGF-β from the regulation of EMT and mammary tumorigenesis (Bhowmick et al., 2001; Huck et al., 2010; Lahlou and Muller, 2011) and 2) engagement of β1 integrin by collagen and fibronectin is essential in promoting pulmonary metastatic outgrowth (Barkan *et al.*, 2008, 2010; Shibue and Weinberg, 2009; Huck *et al.*, 2010). Thus β1 and β3 integrins both appear to play essential and potentially redundant roles in regulating the oncogenic activities of TGF-β.

Here we investigate the interplay between β1 and β3 integrins in promoting oncogenic TGF-β signaling and its stimulation of EMT and metastasis. In particular, the recent interest in using β1 integrin as a potential therapeutic target in breast cancers led us to determine how the inactivation of β1 integrin in metastatic breast cancers might affect their expression and activity of β3 integrin and, consequently, their tumorigenicity and metastasis in response to TGF-β. Collectively our findings demonstrate that the specific inactivation of β1 integrin failed to affect the growth and metastasis of breast cancers due to their compensatory up-regulation of β3 integrin, events that were alleviated by inactivation of β3 integrin in β1 integrin–deficient breast cancers. Thus our results highlight the inherent plasticity of integrin expression in metastatic breast cancers and indicate that dual targeting of β1 and β3 integrins may prove more efficacious in alleviating metastatic disease in breast cancer patients.

RESULTS

Functional disruption of β1 integrin attenuates TGF-β–mediated motility in normal NMuMG Cells

Previous studies demonstrated that administering neutralizing antibodies to β1 integrin prevents TGF-β from activating p38 MAPK and inducing EMT programs in NMuMG cells (Bhowmick *et al.*, 2001), a well-established model for studying EMT and its regulation by TGF-β (Miettinen *et al.*, 1994). Along these lines, we found a similar requirement for β3 integrin in mediating these same biological readouts in NMuMG cells stimulated by TGF-β (Galliher and Schiemann, 2006). These discrepant findings raised important questions as to whether the activities of β1 integrin lie upstream of β3 integrin in the TGF-β pathway, a notion that was speculated on previously (Galliher and Schiemann, 2006), or whether both β integrins lie in distinct branches of the TGF-β signaling system. As an initial attempt to address these questions, we inhibited the activities of β1 integrin and p38 MAPK to assess their function in coupling TGF-β to the motility of NMuMG cells. Under unstimulated conditions, wounded NMuMG cell monolayers exhibited minimal wound closure (~10%), whereas inclusion of TGF-β during the healing process significantly stimulated the closure of NMuMG cell wounds (Figure 1, A and B). Addition of either neutralizing β1 integrin antibodies or the p38 MAPK inhibitor SB203580 to TGF-β–treated NMuMG cultures abrogated their ability to initiate wound closure in response to TGF-β (Figure 1, A and B). Previous studies demonstrated that 1) elevated expression of the classical TGF-β gene target, plasminogen-activator inhibitor-1 (PAI-1), promotes integrin internalization and subsequent cell detachment (Czekay and Loskutoff, 2009), and 2) depleted expression of β1 integrin reduces breast cancer invasion and cyclooxygenase 2 (Cox-2) expression (Mitchell *et al.*, 2010). Thus we asked whether the diminished migration of NMuMG cells elicited by inactivating β1 integrin and p38 MAPK activity reflected alterations in PAI-1 expression stimulated by TGF-β. As shown in Figure 1C, PAI-1 expression induced by TGF-β was unaffected by the neutralization of β1 integrin activity but was significantly decreased in cells treated with the p38 MAPK inhibitor SB203580. Along these lines, the extent of cyclooxygenase 2 (Cox-2) expression induced by TGF-β was not significantly affected by β1 integrin inactivation, a cellular condition that did elicit diminished basal levels of Cox-2 expression

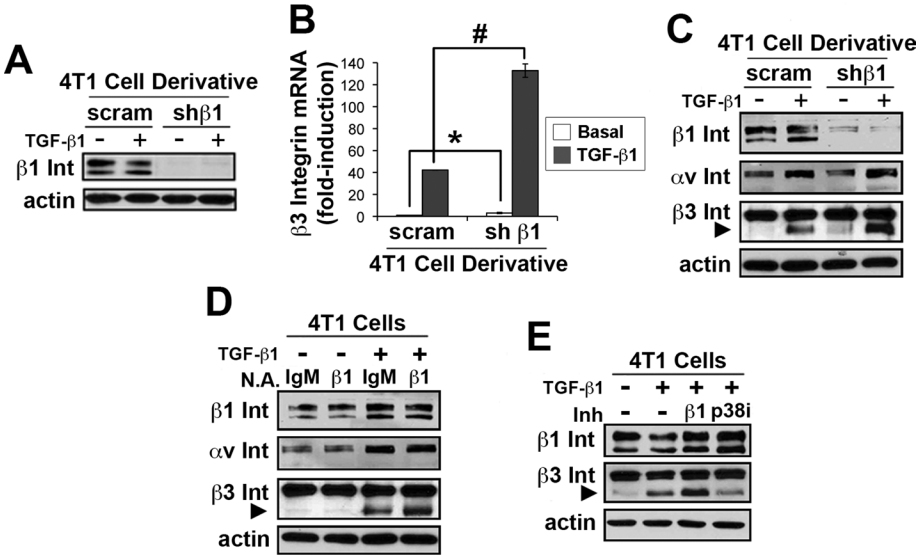

FIGURE 2: Inactivation of β1 integrin elicits compensatory expression of β3 integrin in metastatic 4T1 cells. Parental (scram) and β1 integrin–deficient (shβ1) 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for 24 h before monitoring alterations in integrin expression by immunoblotting or semiquantitative real-time PCR. (A) Monitoring the extent of β1 integrin deficiency. Data are representative of at least three independent analyses. (B–D) Inactivating β1 integrin expression (B, C) or activity by administration of neutralizing β1 integrin antibodies (β1; D) elicited compensatory up-regulation of β3 integrin transcripts (B) and protein (C, D). Shown in B is the mean (±SE) of three independent experiments completed in triplicate ($^{*,\#}p < 0.0005$), and results in C and D are representative of three independent experiments. (E) Parental 4T1 cells were stimulated for 24 h with TGF-β1 (5 ng/ml) in the absence or presence of neutralizing β1 integrin antibodies (β1; 5 μg/ml) or the p38 MAPK inhibitor SB203580 (p38i; 10 μM). Data are representative of three independent experiments. β1, neutralizing β1 integrin antibody; IgM, nonspecific control antibody; N.A., neutralizing antibody.

(Figure 1D). Finally, inhibiting the activity of p38 MAPK significantly decreased the coupling of TGF-β to Cox-2 expression in NMuMG cells (Figure 1D). Collectively these findings suggest that β1 integrin and p38 MAPK both play important roles in promoting TGF-β stimulation of cell motility and that the coupling of TGF-β to PAI-1 and Cox-2 expression occurs through a p38 MAPK-dependent process that is independent of β1 integrin activity.

We previously established the essential function of β3 integrin in mediating EMT and metastasis stimulated by TGF-β (Galliher and Schiemann, 2006, 2007; Galliher-Beckley and Schiemann, 2008). Mechanistically, TGF-β stimulation of EMT programs up-regulated β3 integrin expression, which then interacted physically with the TGF-β type II receptor (TβR-II) to drive breast cancer metastasis (Galliher and Schiemann, 2006, 2007; Galliher-Beckley and Schiemann, 2008). Of interest, we also found β1 integrin to be constitutively associated with TβR-II (Galliher and Schiemann, 2006), thereby implicating β1 integrin as a potential pathophysiological effector of TGF-β. Thus we sought to address the extent to which inactivation of β1 integrin affected the coupling of TGF-β to EMT programs, as well as the degree to which overexpression of β3 integrin could rescue these events. Of importance, stimulating β3 integrin–expressing NMuMG cells with TGF-β greatly exaggerated their EMT-associated morphologies irrespective of β1 integrin expression (Supplemental Figure S1). Similarly, we addressed how inactivation of β1 integrin and overexpression of β3 integrin affected the formation of focal adhesions, acinar structures, and mammospheres. In response to TGF-β, focal adhesions formed to various extents in all NMuMG derivatives, including those lacking β1 integrin (Supplemental Figure S2A). The magnitude of focal adhesion formation and vinculin staining, which was also readily apparent in the nucleus, was dramatically elevated in β1 integrin–deficient NMuMG cells that also stably expressed β3 integrin (Supplemental Figure S2A). Furthermore, any manipulation in the expression or activity of β1 or β3 integrins completely disrupted normal acinar hollowing, resulting in the appearance of uniformly filled acinar structures (Supplemental Figure S2B). Finally, β3 integrin drove mammosphere formation and β-catenin activation more effectively than β1 integrin in normal MECs (Supplemental Figure S3). Taken together, these findings suggest that β1 and β3 integrins fulfill similar functions in normal MECs stimulated by TGF-β and that robust compensatory expression of β3 integrin does not occur in normal MECs lacking β1 integrin activity. Thus β1 → β3 integrin switching is negligible in MECs that harbor normal genomes, a response that contrasts sharply with their malignant MEC counterparts.

Inactivation of β1 integrin elicits compensatory expression of β3 integrin in metastatic 4T1 cells

Although the aforementioned findings suggest that β1 and β3 integrins fulfill unique and overlapping functions in normal MECs, they fail to address the extent to which "integrin switching" occurs between these adhesion molecules during mammary tumorigenesis and metastatic progression. Indeed, TGF-β stimulation of EMT in MECs can induce integrin switching, such that pre-EMT cells preferentially signal through β4 integrins, whereas their post-EMT counterparts favor signaling by β1 integrins (Maschler *et al.*, 2005). However, the extent to which β1 and β3 integrins undergo integrin switching in metastatic breast cancers remains unknown. To address this question, we depleted β1 integrin expression in metastatic 4T1 breast cancer cells (Figure 2A) and subsequently determined the effect of this event on the expression of various integrin subunits. Rendering 4T1 cells deficient in β1 integrin expression had no effect on their basal or TGF-β–regulated expression of α3 or α6 integrins (unpublished data); however, this same cellular condition significantly enhanced the coupling of TGF-β to α5 integrin mRNA expression (scram, [1.77 ± 0.08]-fold; β1 integrin deficient, [3.18 ± 0.19]-fold; $n = 3$; $p = 0.002$). In addition, β1 integrin deficiency dramatically up-regulated β3 integrin mRNA and protein expression (Figure 2, B and C). To exclude potential off-target short hairpin RNA (shRNA) activities in eliciting up-regulated β3 integrin expression in 4T1 cells, we also conducted similar β1 integrin inactivation experiments by administering neutralizing β1 integrin antibodies to 4T1 cells, followed by analyses of β3 integrin expression. In doing so, we observed that neutralizing β1 integrin antibodies elicited robust compensatory expression of β3 integrin that depended on the protein kinase activity of p38 MAPK (Figure 2, D and E) and is consistent with previous reports (Pechkovsky *et al.*, 2008). Collectively these findings identify an integrin switching mechanism in metastatic breast cancer cells by which disruption of β1 integrin leads to compensatory expression of β3 integrin, an event mediated in part via p38 MAPK.

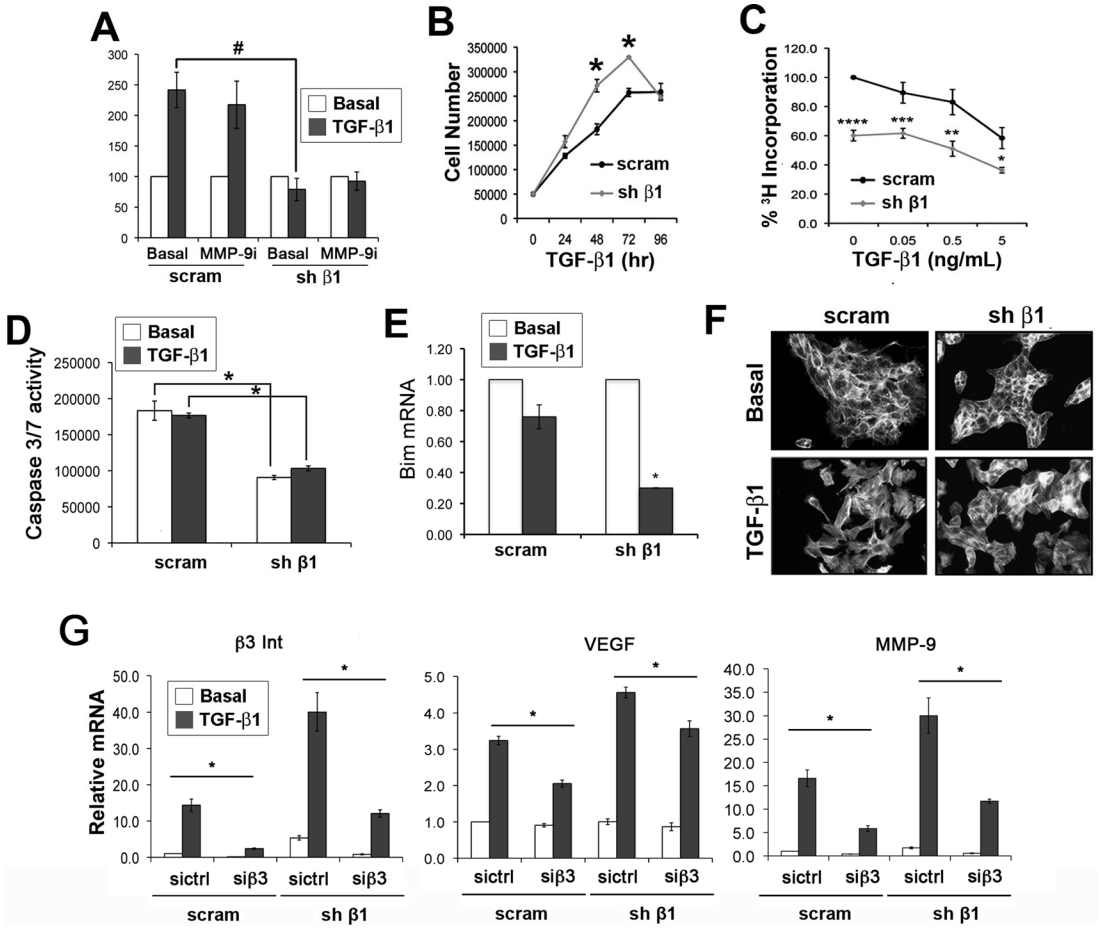

FIGURE 3: Heterogeneous invasive and EMT phenotypes elicited by β1 integrin deficiency in 4T1 cells. (A) Parental (scram) and β1 integrin–deficient 4T1 cells were allowed to invade reconstituted basement membranes in the absence or presence of either TGF-β1 (5 ng/ml) or the MMP-9 inhibitor (MMP-9i; 10 μM) as indicated. Data are mean (±SE) of three independent experiments completed in triplicate ($^{\#}p < 0.0008$). (B) Accumulation of TGF-β–stimulated (5 ng/ml) parental (scram) and β1 integrin–deficient 4T1 cells measured longitudinally by trypan blue exclusion. Data are mean (±SE) of four independent experiments. (C) Alterations in DNA synthesis of parental (scram) and β1 integrin–deficient 4T1 cells in response to increasing concentrations of TGF-β1 as determined by [^{3}H]thymidine incorporation assays. Data are mean (±SE) of three independent experiments completed in triplicate (****$p < 0.0001$, ***$p < 0.005$, **$p < 0.007$, and *$p < 0.02$). (D, E) Parental (scram) and β1 integrin–deficient 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for 48 h before monitoring caspase 3/7 activity by Caspase-Glo 3/7 assays (D) or Bim transcript expression by semiquantitative real-time PCR (E). Data are mean (±SE) of three (D) or two (E) independent experiments completed in triplicate. *$p < 0.005$. (F) Alterations in the actin cytoskeletons of parental (scram) and β1 integrin–deficient 4T1 cells determined by phalloidin immunofluorescence as indicated. Data are representative images (200×) of four independent experiments. (G) Parental (scram) and β1 integrin–deficient 4T1 cells were transfected with a control or β3 integrin–specific siRNA and subsequently stimulated with TGF-β1 (5 ng/ml) for 48 h before monitoring β3 integrin, VEGF, and MMP-9 transcript expression by semiquantitative real-time PCR. Data are mean (±SE) of three independent experiments completed in triplicate (*$p < 0.02$).

Heterogeneous invasive and EMT phenotypes elicited by β1 integrin deficiency in 4T1 cells

The foregoing findings identify β1 → β3 integrin switching in metastatic breast cancer cells. We next sought to characterize how compensatory expression of β3 integrin affects the behavior of β1 integrin–deficient 4T1 cells. Figure 3A shows that depleting β1 integrin expression inhibited the ability of 4T1 cells to invade in response to TGF-β, irrespective of MMP-9 activity. Similar results were obtained in 4T1 cells depleted of β1 integrin expression engendered by a second distinct shRNA construct (unpublished data), thereby demonstrating the necessity of β1 integrin during TGF-β–driven invasion of metastatic breast cancer cells. We also evaluated how the loss of β1 integrin expression affected the growth of 4T1 cells, and in doing so we observed that β1 integrin deficiency significantly enhanced the accumulation of 4T1 cells in response to TGF-β (Figure 3B). Of interest, 4T1 cells lacking β1 integrin expression synthesized significantly less DNA (Figure 3C), exhibited significantly less caspase 3/7 activity (Figure 3D), and expressed significantly less Bim transcripts (Figure 3E) than their parental counterparts. Thus compensatory expression of β3 integrin preferentially enhanced the accumulation of β1 integrin–deficient 4T1 cells in part by diminishing their sensitivity to apoptotic stimuli.

To determine the extent to which the attenuated invasion of β1 integrin–deficient 4T1 cells reflected defects in their ability to acquire EMT phenotypes, we first monitored alterations in the actin cytoskeletal systems of parental (scram) and β1 integrin–deficient 4T1 cells by subjecting them to phalloidin immunofluorescence. Indeed, parental 4T1 cells exhibited scant stress fiber formation under

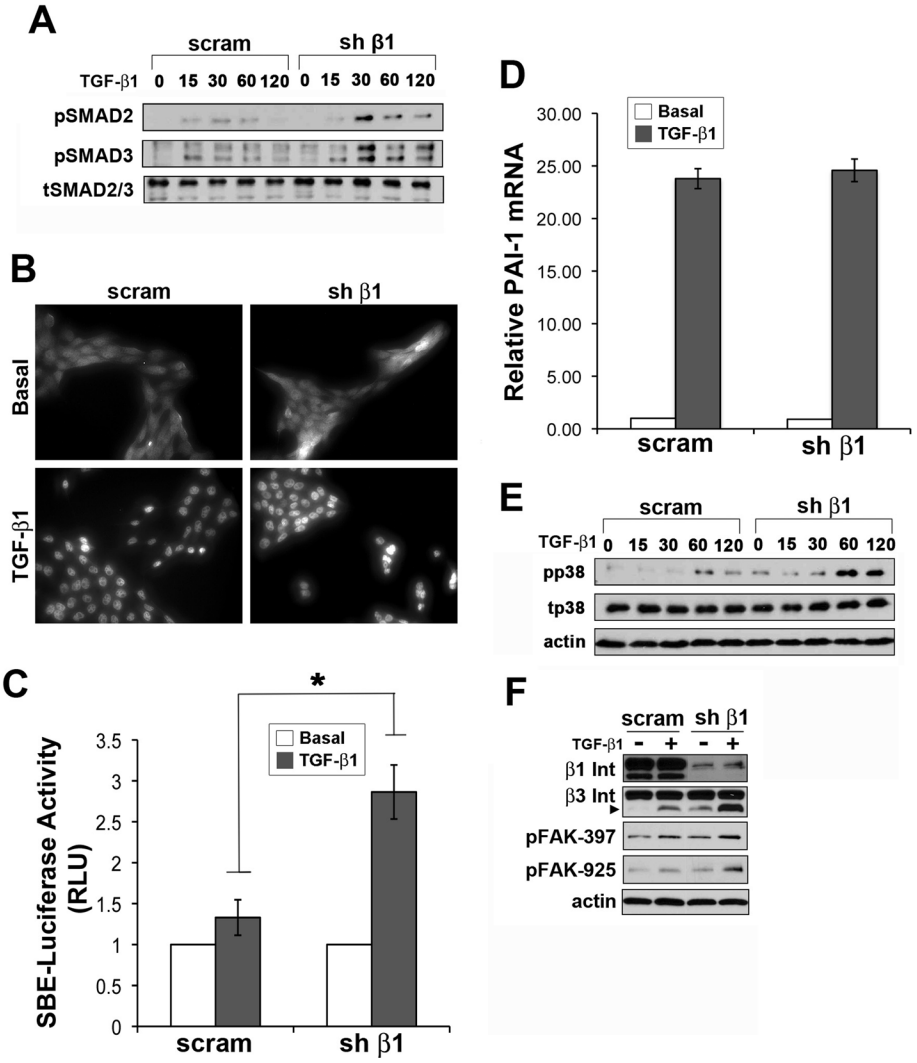

FIGURE 4: Compensatory β3 integrin expression enhances TGF-β signaling in β1 integrin–deficient 4T1 cells. (A) Quiescent parental (scram) and β1 integrin-deficient 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for 0–120 min as indicated, at which point the phosphorylation status of Smad2 and Smad3 was analyzed by immunoblotting. Data are representative of three independent analyses. (B) Smad2/3 immunofluorescence (200×) depicts the subcellular localization of Smad2/3 in basal and TGF-β1 (5 ng/ml; 30 min)–stimulated parental (scram) and β1 integrin–deficient 4T1 cells. Data are representative of three independent experiments. (C) Parental (scram) and β1 integrin–deficient 4T1 cells were transiently transfected with pCMV-β-gal and pSBE-luciferase reporter genes and subsequently stimulated with TGF-β1 (5 ng/ml) for 24 h. Data are mean (±SE) of four independent experiments completed in triplicate. (D) Parental (scram) and β1 integrin–deficient 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for 48 h, at which point alterations in PAI-1 mRNA were analyzed by semiquantitative real-time PCR. Data are mean (±SE) of three independent experiments completed in triplicate. (E, F) Parental (scram) and β1 integrin–deficient 4T1 cells were stimulated for 0–120 min (E) or 24 h (F) with TGF-β1 (5 ng/ml) before monitoring the phosphorylation status and expression levels of p38 MAPK. Data are representative of three (E) or two (F) independent analyses.

basal conditions but readily displayed elongated morphologies and stress fibers in response to TGF-β (Figure 3E). Moreover, depletion of β1 integrin elevated the epithelial features of 4T1 cells, both basally and after stimulation with TGF-β (Figure 3E). Somewhat surprisingly and in light of these morphological features, we observed that β1 integrin deficiency reduced the expression of E-cadherin and cytokeratin-19 in untreated 4T1 derivatives as compared with their parental counterparts, events that were refractory to TGF-β administration (Supplemental Figure S4). As compared with parental 4T1 cells, those engineered to lack β1 integrin expression produced significantly greater quantities of the mesenchymal markers, vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9; Figure 3G and Supplemental Figure S4). Finally, to determine whether the exacerbated expression of VEGF and MMP-9 specifically reflected the compensatory expression of β3 integrin, we transfected parental (scram) and β1 integrin–deficient 4T1 cells with control or β3 integrin–specific small interfering RNA (siRNA), which attenuated the expression of β3 integrin transcripts (Figure 3G, left). In doing so, we found β3 integrin depletion to promote the down-regulation of VEGF and MMP-9 expression in β1 integrin–deficient 4T1 cells (Figure 3G, middle and right). Collectively these findings suggest that compensatory β3 integrin expression was insufficient in rescuing the capacity of 4T1 cells to complete invasive and EMT programs; however, this same cellular condition did promote prosurvival signaling and enhanced VEGF and MMP-9 expression.

Compensatory β3 integrin expression enhances TGF-β signaling in β1 integrin–deficient 4T1 cells

To further characterize the interplay between β1 and β3 integrins in regulating TGF-β signaling, we surveyed the coupling of this cytokine to its canonical and noncanonical effectors in parental and β1 integrin–deficient 4T1 cells. We previously demonstrated that 4T1 cells have diminished Smad3/4 transcriptional activity as compared with their indolent 67NR counterparts, despite the fact that these cell lines harbor similar levels of phosphorylated Smad3 (Wendt *et al.*, 2009). As shown in Figure 4A, parental (scram) and β1 integrin–depleted 4T1 cells similarly activated Smad2/3 by 30 min of TGF-β treatment; however, the magnitude of this response was more robust in β1 integrin–depleted cells than in their parental counterparts. Accordingly, Smad2/3 appeared to localize more readily in the nuclei of β1 integrin–deficient 4T1 cells (Figure 4B), which significantly enhanced their Smad3/4 transcriptional activity as compared with that in parental 4T1 cells (Figure 4C). Of interest, we observed that PAI-1 transcripts were similarly induced by TGF-β in both 4T1 derivatives (Figure 4D), suggesting involvement of noncanonical TGF-β in mediating this response (Song *et al.*, 2012). In support of this supposition, β1 integrin–deficient 4T1 cells possessed elevated activation of p38 MAPK (Figure 4E) and FAK (Figure 4F) in 4T1 cells that harbored compensatory β3 integrin expression. Taken together, these findings indicate that inactivation of β1 integrin significantly enhances TGF-β signaling in metastatic breast cancer cells.

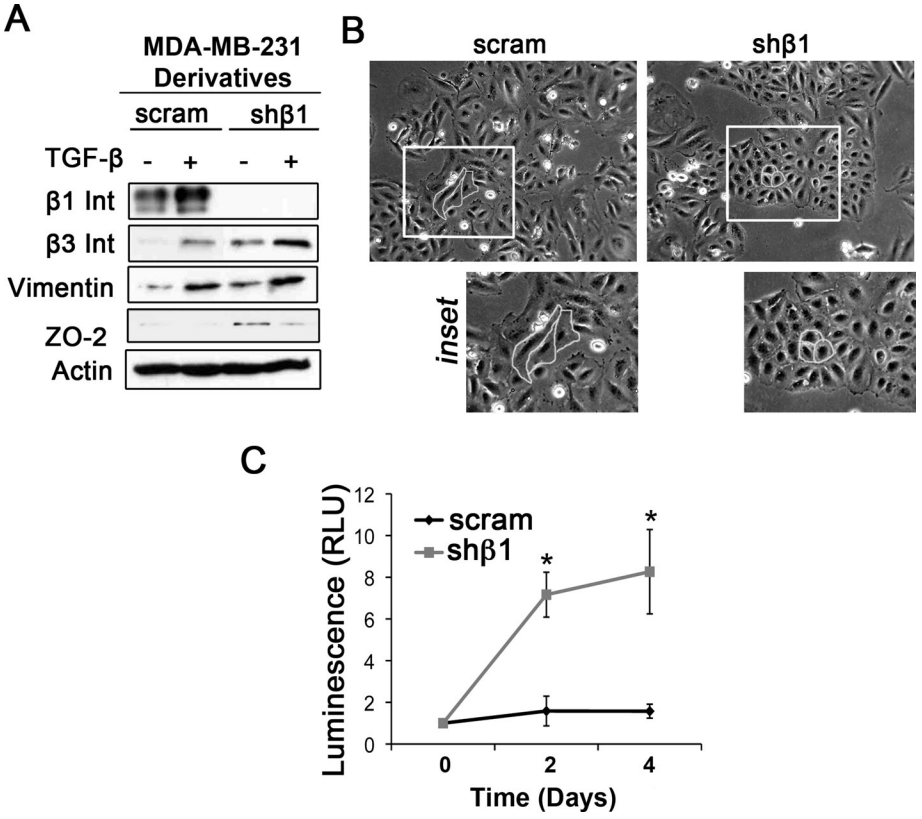

FIGURE 5: Inactivation of β1 integrin elicits compensatory expression of β3 integrin in human MDA-MB-231 cells. Parental (scram) and β1 integrin–deficient (shβ1) MDA-MB-231 cells were stimulated with TGF-β1 (5 ng/ml) for 4 d before monitoring alterations in the extent of β1 integrin deficiency, as well as β3 integrin compensation and the expression of vimentin, ZO-2, and β-actin by immunoblotting (A), and to assess changes in their morphologies by light microscopy (100×; B). Data are representative of at least three independent analyses. (C) Parental (scram) and β1 integrin–deficient MDA-MB-231 cells were propagated in compliant 3D-organotypic cultures for 4 d. The growth of these organoids was monitored by longitudinal bioluminescence. Data are representational (±SE) of three independent experiments completed in triplicate (*$p < 0.025$).

Inactivation of β1 integrin elicits compensatory expression of β3 integrin in human triple-negative breast cancer cells

To evaluate the extent to which β1 integrin inactivation elicits β3 integrin switching in human breast cancer cells, we depleted β1 integrin expression in metastatic human MDA-MB-231 breast cancer cells (Figure 5A). Consistent with what we observed in the 4T1 cells, β1 integrin–deficient MDA-MB-231 cells exhibited compensatory β3 integrin expression and acquired more epithelial-like morphologies and features (Figure 5, A and B). Indeed, compared to their parental (scram) counterparts, β1 integrin–deficient MDA-MB-231 cells simultaneously expressed elevated levels of 1) the mesenchymal marker vimentin, whose expression was further induced by TGF-β, and 2) the epithelial marker ZO-2, whose expression was suppressed by TGF-β (Figure 5A). These findings are consistent with the acquisition of an augmented EMT phenotype in β1 integrin–deficient MDA-MB-231 cells stimulated with TGF-β. Finally, we examined the functional implications of β1 → β3 integrin switching by monitoring the growth of parental and β1 integrin–deficient MDA-MB-231 organoids in three-dimensional (3D) organotypic cultures that mimic the elasticity of normal breast (Paszek *et al.*, 2005) and lung microenvironments (Lopez *et al.*, 2008). Strikingly, β1 integrin–deficient MDA-MB-231 cells exhibited significantly elevated organoid growth rates compared with their parental counterparts in compliant 3D-organotypic cultures (Figure 5C). Collectively these findings demonstrate the relevance of β1 → β3 integrin switching in human breast cancer cells, which enhances their growth in compliant 3D-organotypic microenvironments via compensatory β3 integrin expression.

Compensatory β3 integrin expression is essential in enhancing acinar growth of β1 integrin–deficient 4T1 cells

To determine the functional implications of β1 → β3 integrin switching in our mouse models of triple-negative breast cancer, we first propagated parental and β1 integrin–deficient 4T1 cells in rigid, collagen-rich 3D-organotypic cultures to mimic their growth in primary tumor microenvironments (Butcher *et al.*, 2009; Erler and Weaver, 2009; Taylor *et al.*, 2011). In doing so, we observed that parental (scram) 4T1 cells formed highly branched structures, as opposed to those formed by their β1 integrin–deficient counterparts (Figure 6A), which also grew significantly faster than parental cells in these same rigid microenvironments (Figure 6B). Of interest, the growth dynamics of both 4T1 derivatives were identical upon being propagated in compliant 3D-organotypic cultures (Figure 6C). Of importance, administering neutralizing αvβ3 integrin antibodies (LM609) significantly inhibited the growth of β1 integrin–deficient 4T1 cells in compliant 3D-organotypic microenvironments (Figure 6, C and D). Collectively these findings indicate that inactivation of β1 integrin confers triple-negative breast cancers a selective growth advantage in collagen-rich primary tumor microenvironments, as well as in pulmonary microenvironments in part via compensatory β1 → β3 integrin switching.

Compensatory β3 integrin expression is essential for the growth and metastasis of β1 integrin–deficient 4T1 tumors in mice

The foregoing findings clearly demonstrate the essential role of compensatory β3 integrin expression in rescuing the growth of 4T1 organoids in 3D-organotypic culture systems. As such, we extended these analyses to assess the function of β1 → β3 integrin switching in mediating the growth and metastasis of β1 integrin–deficient 4T1 tumors produced in syngeneic BALB/c mice. In doing so, we observed that both 4T1 derivatives exhibited similar rates of tumor formation and growth upon engraftment in the mammary fat pad (Figure 7, A and B). In accord with Figure 6D, we also observed that parental and β1 integrin–deficient 4T1 cells exhibited similar kinetics and extent of pulmonary metastasis in BALB/c mice (Figure 7C). Thus these findings suggest that compensatory expression of β3 integrin renders β1 integrin–deficient 4T1 tumors competent to undergo metastatic progression. Indeed, ex vivo isolation and propagation of pulmonary metastases derived from β1 integrin–deficient 4T1 tumors confirmed the retention of compensatory β3 integrin expression by these metastatic isolates, as well as showed that these

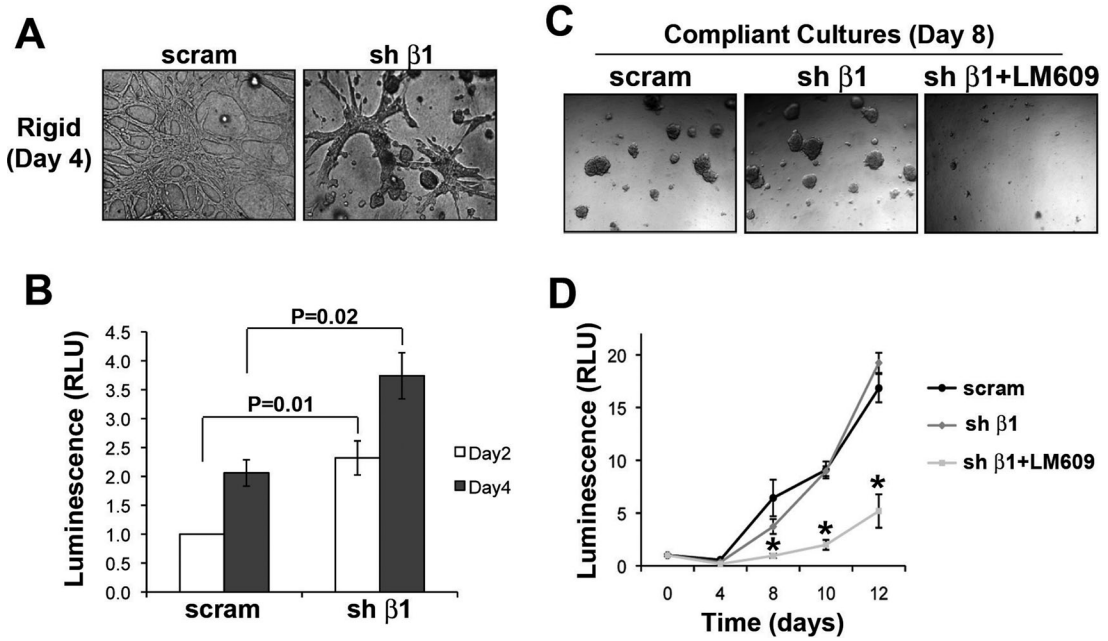

FIGURE 6: Compensatory β3 integrin expression is essential in enhancing acinar growth of β1 integrin–deficient 4T1 cells. (A, B) Parental (scram) and β1 integrin–deficient 4T1 cells were propagated in rigid 3D-organotypic cultures (3 mg/ml type I collagen) for 4 d, at which point differences in organoid growth and morphology were monitored by phase contrast microscopy (50×; A) and longitudinal bioluminescence (B). Data are mean (±SE) of three independent experiments completed in triplicate. (C, D) Parental (scram) and β1 integrin–deficient 4T1 cells were propagated in compliant 3D-organotypic cultures for 12 d in the absence or presence of the neutralizing αvβ3 integrin antibody LM609 (15 μg/ml). The growth and morphology of the resulting organoids were monitored by phase contrast microscopy (50×; C) and longitudinal bioluminescence (D). Data are mean (±SE) of three independent experiments completed in triplicate (*$p < 0.035$).

same cells more robustly up-regulated their expression of β3 integrin in response to TGF-β than their parental counterparts (Supplemental Figure S5). To demonstrate that compensatory β3 integrin expression was indeed responsible for driving the development and metastatic progression of β1 integrin–deficient 4T1 tumors, we engineered dual β1/β3 integrin–deficient 4T1 cells that exhibited reduced capacity to undergo β1 → β3 integrin switching (Supplemental Figure S5). Indeed, compared to parental (scram) 4T1 tumors, those formed by dual β1/β3 integrin–deficient 4T1 cells were significantly smaller (Figure 7, D and E) and possessed significantly reduced capacity to metastasize to the lungs of BALB/c mice (Figure 7F). Thus these findings demonstrate the ability of compensatory β1 → β3 integrin switching to rescue the progression of β1 integrin–deficient triple-negative breast cancers in vivo.

Finally, we explored the necessity of β3 integrin expression in driving 4T1 tumor growth and metastasis by engineering 4T1 cells to overexpress either wild-type β3 integrin or its inactive mutant, D119A-β3 integrin (Diaz-Gonzalez *et al.*, 1996; Galliher and Schiemann, 2006). In complementary analyses, we also rendered 4T1 cells deficient in β3 integrin expression by transducing them with lentiviral particles that encoded shRNA against β3 integrin (Supplemental Figure S5). As shown in Table 1, elevating wild-type β3 integrin expression significantly enhanced 4T1 tumor growth compared with their parental counterparts. Conversely, inactivating β3 integrin function in 4T1 cells either by their expression of D119A-β3 integrin or of shRNA against β3 integrins dramatically reduced their ability to produce tumor relative to their parental counterparts (Table 1). Collectively these findings demonstrate the role of compensatory β3 integrin expression in rescuing mammary tumor development and metastatic progression after inactivation of β1 integrin. They also suggest that β3 integrin function is dominant to that of β1 integrin in mediating the tumorigenicity of triple-negative breast cancers.

DISCUSSION

The acquisition of metastatic phenotypes in breast cancers correlates with elevated levels of TGF-β signaling and include essential inputs derived from β1 and β3 integrins (Taylor *et al.*, 2010; Parvani *et al.*, 2011). The objective of this study was to determine whether inhibiting the oncogenic functions of TGF-β by targeted inactivation of β1 integrin could be circumvented by compensatory expression of β3 integrin. In addressing this question, we were surprised to observe minimal interplay between β1 and β3 integrins in normal, nontransformed MECs. In stark contrast, we identified an inherent β1 → β3 integrin switching mechanism that enabled metastatic breast cancer cells to bypass diminished β1 integrin signaling inputs via their ability to up-regulate β3 integrin expression, which maintains oncogenic TGF-β signaling (Figure 8). The up-regulation of β3 integrin expression by β1 integrin–deficient breast cancer cells depended on the activity of p38 MAPK (Figure 2), which presumably couples to HoxA10, CBP, or FoxC2 to elicit the synthesis of β3 integrin transcripts (Bei *et al.*, 2007; Hayashi *et al.*, 2008). Collectively these events culminate in the continued development and metastatic progression of aggressive triple-negative breast cancers (Figure 7). In addition to p38 MAPK activity, we also observed that elevated MMP-9 expression was associated with β1 → β3 integrin switching (Figure 3), which may account for the increased activation of Smads 2 and 3 (Figure 4) via the release of latent TGF-β and other growth factors from inactive extracellular matrix depots (Figure 8; Egeblad and Werb, 2002; Taylor *et al.*, 2010). Although the mechanisms responsible for mediating these events remain to be fully

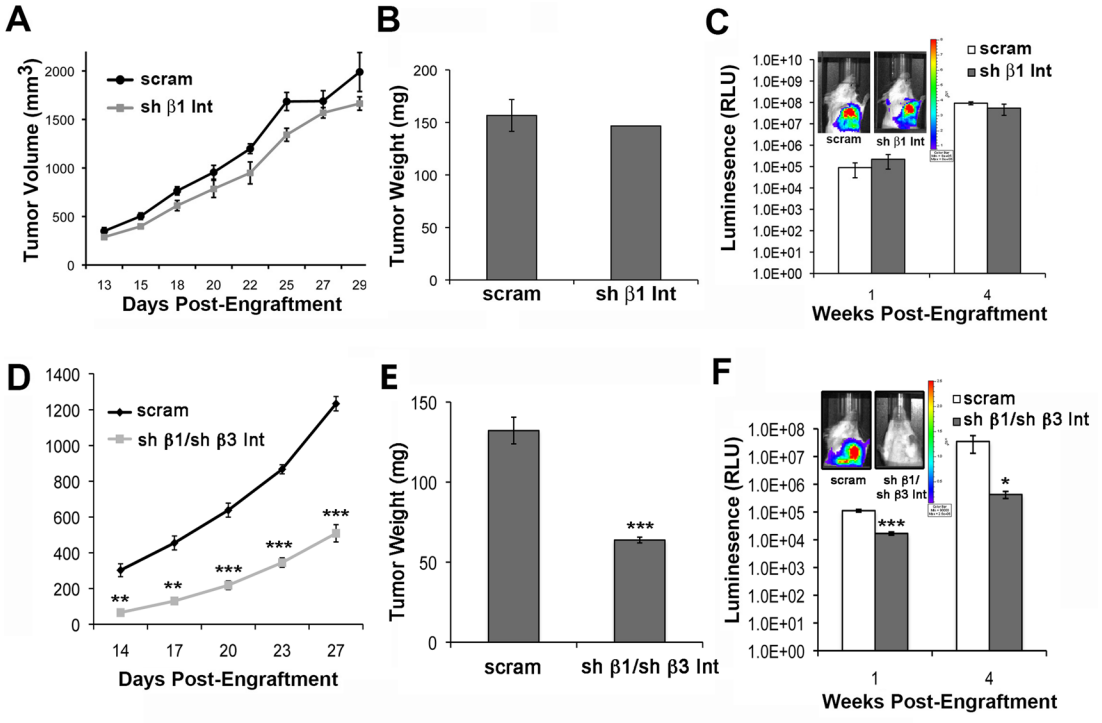

FIGURE 7: Compensatory β3 integrin expression is essential for the growth and metastasis of β1 integrin-–deficient 4T1 tumors in mice. (A) Parental (scram) and β1 integrin–deficient 4T1 cells (12,000 cells/mouse) were engrafted into the fat pads of female BALB/c mice. Tumor growth was monitored using digital calipers on the indicated days postengraftment. Data are mean (±SE; $n = 5$) tumor volumes. (B) Primary tumors from A were excised and weighed at the time of killing. Data are mean tumor weights (±SE; $n = 5$). (C) Bioluminescence imaging of pulmonary metastasis from parental (scram) and β1 integrin–deficient 4T1 tumors from A at weeks 1 and 4 postengraftment. Inset, representative bioluminescence images of parental (scram) and β1 integrin–deficient 4T1 lung metastases. Data are mean (±SE) pulmonary area flux units detected at the indicated time points. (D) Parental (scram) and dual β1/β3 integrin (shβ1/shβ3 Int)–deficient 4T1 cells (10,000 cells/mouse) were engrafted into the fat pads of female BALB/c mice. Tumor growth was monitored using digital calipers on the indicated days postengraftment. Data are mean tumor volumes (±SE; $n = 5$). (E) Primary tumors from D were excised and weighed at the time of killing. Data are mean tumor weights (±SE; $n = 5$). (F) Bioluminescence imaging of pulmonary metastasis from parental (scram) and shβ1/shβ3 integrin–deficient 4T1 tumors from D at weeks 1 and 4 postengraftment. Inset, representative bioluminescence images of parental (scram) and β1 integrin–deficient 4T1 lung metastases. Data are mean (±SE) pulmonary area flux units detected at the indicated time points. *$p < 0.05$, **$p < 0.0005$, ***$p < 0.00005$.

elucidated, we suspect that either 1) inactivation of β1 integrin, which elevates the expression of Dab2 (J.G.P. and W.P.S., unpublished data), enhances TGF-β receptor recycling and Smad2/3 phosphorylation and activation (Penheiter et al., 2010); or 2) β1 integrin deficiency elevates TGF-β signaling by alleviating steric hindrance within β1 integrin:TβR-IIβ3 integrin complexes (Galliher and Schiemann, 2006), thereby enabling TβR-I to more efficiently access and activate Smad2/3. Of interest, we previously demonstrated that maximal coupling of TβR-II to β3 integrin required the latter to be activated by its preferred substrate, vitronectin (Galliher and Schiemann, 2006). Thus it is tempting to speculate that compensatory β3 integrin expression synergizes with vitronectin to enhance oncogenic TGF-β signaling during multiple stages of metastatic progression, including 1) intravasation, 2) survival during dissemination in the circulatory system, and 3) reinitiation of proliferation programs during metastatic outgrowth (Figure 8; Preissner, 1991).

EMT programs stimulated by TGF-β are associated with mammary tumor development and metastatic progression (Taylor *et al.*, 2010). We previously demonstrated the ability of TβR-II to interact physically with both β1 and β3 integrins (Galliher and Schiemann, 2006), suggesting that the physiological output of TGF-β signaling reflects the interplay of TβR-II with available β1 and β3 integrins (Figure 8). Of interest, our previous findings indicated that β3 integrin expression is dominant to that of β1 integrin in determining the function of TGF-β in responsive cells (Galliher and Schiemann, 2006, 2007; Galliher-Beckley and Schiemann, 2008). Our present findings reinforce this idea and show that metastatic breast cancer cells are hard wired to activate β3 integrin–dependent pathways when confronted with a loss of β1 integrin–mediated signaling inputs. Moreover, inactivating β3 integrin function either alone or in combination with that of β1 integrin in 4T1 cells clearly alleviated their tumorigenicity in mice, a reaction that was not rescued by residual or compensatory expression of β1 integrin (Figure 7 and Table 1). Along these lines, it is unclear how β1 and β3 integrins compete for the attention of TGF-β receptors. What is clear is that the formation and stabilization of focal adhesion complexes reflect a dynamic process of assembly and disassembly, which may facilitate β1 and β3 integrin switching during distinct activation states of focal adhesion complexes. This notion is supported by the preferential ability of β3 integrin to drive focal adhesion formation, as determined by vinculin immunofluorescence, which also readily detected vinculin in the nuclei of post-EMT, β3 integrin–expressing NMuMG cells upon completion of the EMT program (Supplemental Figure S2). Of interest, nuclear localization of vinculin was

Experimental condition	Final tumor volume (mm^3)	Final tumor weight (mg)
Experiment 1		
Green fluorescent protein	324.45 (±70.8)	187.4 (±23.2)
WT-β3 integrin	110.4 (±15.8)*	426.7 (±39.6)*
D119A-β3 integrin	59.5 (±11.5)*	148.5 (±29.1)
Experiment 2		
Scram	1233.9 (±40.2)	132.2 (±8.3)
Shβ3 integrin	694.6 (±47.3)**	71.0 (±7.3)***

Parental (GFP) or WT-β3 integrin– or D119A-β3 integrin–expressing 4T1 cells (12,000 cells/mouse; experiment 1) and parental (Scram) or β3 integrin-deficient 4T1 cells (10,000 cells/mouse; experiment 2) were engrafted into the fat pads of syngeneic BALB/c mice. Tumor development was monitored over a span of 4 wk. Data are mean (±SE; $n = 5$) final tumor volumes and weights (*$p < 0.05$; **$p < 0.0005$; ***$p < 0.000005$).

TABLE 1: Functional disruption of β3 integrin inhibits primary tumor growth.

reported in carcinoma cells and linked to their activation of β-catenin (Ben-Ze'ev, 1999), whose activity was dramatically induced in β3 integrin–expressing NMuMG cells (Supplemental Figure S3). Moreover, whereas elevated vinculin expression has been shown to inhibit cell motility in traditional two-dimensional culture systems, this same cellular condition actively promotes cell motility in mechanically rigid 3D culture systems (Mierke, 2009). On the basis of these findings, we propose that compensatory β3 integrin expression enhances the motility and metastasis of breast cancer cells by promoting the formation of focal adhesion complexes and augmenting β-catenin signaling needed to induce EMT programs. Along these lines, future studies need to determine the extent to which nuclear accumulation of vinculin functions as a predictive biomarker for aggressive breast cancers.

In summary, our findings clearly demonstrate that sole targeting of β1 integrin to treat metastatic breast cancers may prove to be temporarily efficacious during initial stages of breast cancer progression; however, the capacity to induce compensatory β3 integrin expression by aggressive mammary tumors provides these evolving carcinomas with a powerful tool to circumvent β1 integrin inactivation, thereby ensuring the metastatic progression of β3 integrin–expressing breast cancer cells. Clinically, our findings suggest that dual β1 and β3 integrin targeting appears to be necessary to alleviate metastatic disease in breast cancer patients.

MATERIALS AND METHODS

Cell lines and reagents

Normal NMuMG, metastatic 4T1 cells, and MDA-MB-231 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured as described previously (Wendt and Schiemann, 2009). 4T1 and MDA-MB-231 cells were engineered to stably express firefly luciferase by transfection with pNifty-CMV-luciferase and selection with Zeocin (500 μg/ml; Invitrogen, Carlsbad, CA). 4T1 cells overexpressing wild-type β3 integrin or its inactive counterpart, D119A-β3 integrin, were generated and characterized previously (Galliher and Schiemann, 2006). The β1 and β3 integrins were functionally disrupted by lentiviral-mediated transduction of verified shRNAs against β1 (pLKO.1-puro; Open Biosystems, Huntsville, AL) or β3 (pGIPZ-GFP; Open Biosystems) integrins as described previously (Taylor *et al.*, 2011). In all cases,

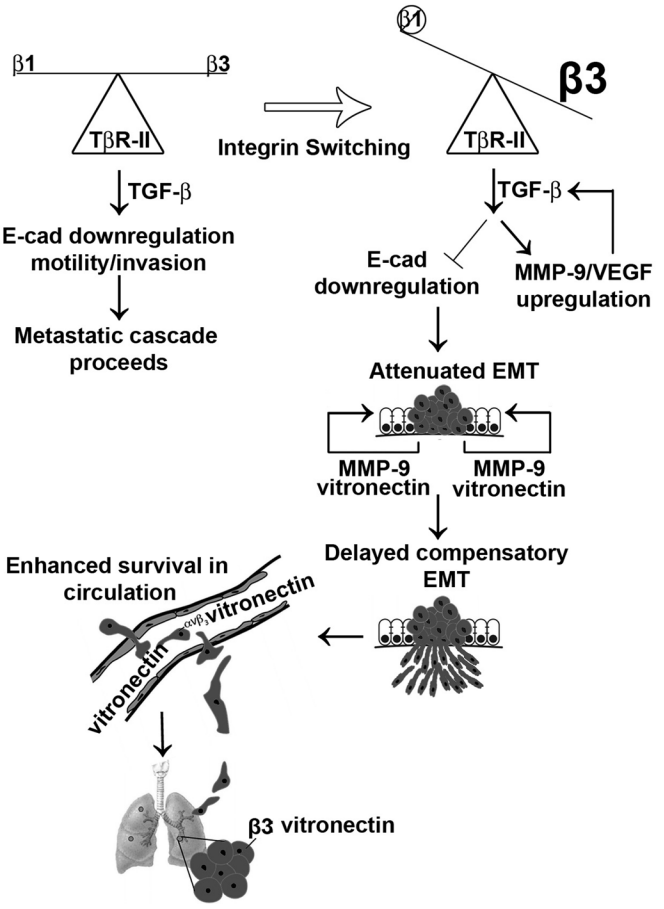

FIGURE 8: Model of the dichotomous roles of β1 and β3 integrins in mediating breast cancer metastasis. Integrin switching between β1 and β3 integrins in metastatic 4T1 cells uncouples TGF-β from down-regulating E-cadherin expression, thereby attenuating the acquisition of EMT and migratory phenotypes. Elevated expression of MMP-9 and VEGF is associated with this integrin switching event and contributes to autocrine TGF-β signaling and activation of compensatory EMT programs. The physiological distribution of vitronectin expression may selectively mediate the pulmonary outgrowth of cells that underwent β1 → β3 integrin switching.

separate cohorts of cells were transduced with scrambled nonsilencing shRNAs to monitor off-target activities. NMuMG, 4T1, or MDA-MB-231 cells that stably expressed either nonsilencing shRNA or those against β1 integrin were selected over a span of 14 d in puromycin (5 μg/ml), whereas those expressing shRNA against β3 integrin were isolated by flow cytometry for green fluorescent protein expression by the Cytometry and Imaging Microscopy Core in the Case Comprehensive Cancer Center. The extent of β1 and β3 integrin deficiency was determined by immunoblotting for these integrins as described.

Western blot analyses

Immunoblotting analyses were performed as previously described (Taylor *et al.*, 2011). Briefly, parental (scram) and integrin-manipulated NMuMG, 4T1, and MDA-MB-231 cells were seeded into six-well plates (500,000 cells/well) and allowed to adhere overnight, at which point they were incubated in the absence or presence of TGF-β1 (5 ng/ml) for 0–24 h as indicated. Afterward, detergent-solubilized whole cell extracts (WCEs) were prepared by lysing the cells in buffer H (50 mM β-glycerophosphate, 1.5 mM

ethylene glycol tetraacetic acid, 1 mM dithiothreitol, 0.2 mM sodium orthovanadate, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.3), and 30 μg/lane of clarified WCE was fractionated through 10% SDS–PAGE gels, transferred electrophoretically to nitrocellulose, and immunoblotted with following primary antibodies: 1) anti–β1 integrin (1:1000; Cell Signaling, Danvers, MA); 2) anti–β3 integrin (1:1000; Cell Signaling); 3) anti–αv integrin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); 4) anti–phospho-Y397-FAK (1:1000; Cell Signaling); 5) anti–phospho-Y925-FAK (1:1000; Cell Signaling); 6) anti-FAK (1:1000; Santa Cruz Biotechnology); 7) anti–E-cadherin (1:1000; BD Biosciences, San Jose, CA); 8) anti–phospho-p38 MAPK (1:500; Cell Signaling); 9) anti–p38 MAPK (1:1000; Santa Cruz); 10) anti–phospho-Smad2 (1:500; Cell Signaling); 11) anti–phospho-Smad3 (1:500; Cell Signaling); 12) anti-Smad2/3 (1:1000; Cell Signaling); 13) anti–β-actin (1:1000; Santa Cruz Biotechnology); 14) anti–ZO-2 (1:1000; Cell Signaling); and 15) anti-vimentin (1:1000; BD Biosciences). For FAK analyses, cells were maintained in serum-reduced conditions (1% fetal bovine serum) and treated with TGF-β1 for 0–24 h as indicated.

Immunofluorescence analyses

Immunofluorescence studies were performed as described previously (Wendt and Schiemann, 2009). Briefly, NMuMG or 4T1 cells (25,000 cells/well) were allowed to adhere overnight onto glass chamber slides and stimulated with TGF-β1 (5 ng/ml) for 24 h. Afterward, the cells were washed with PBS, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton-X 100, and stained with Alexa Flour 488–phalloidin (25 μM; Invitrogen), anti-vinculin antibodies (1:250; BD Biosciences), or anti-Smad2/3 antibodies (1:250; BD Biosciences) according to manufacturer's instructions. Subsequently, cells stained for vinculin and Smad2/3 were treated with biotin-labeled donkey anti-mouse secondary antibodies (1:500; Jackson ImmunoResearch, West Grove, PA), followed by treatment with streptavidin–fluorescein isothiocyanate (1:1000; Vector Laboratories, Burlingame, CA). Slides were mounted with 25 μl of Prolong solution with 4′,6-diamidino-2-phenylindole (Invitrogen) to visualize nuclear staining.

Mammosphere assays

Mammosphere assays were executed as described previously (Dontu *et al.*, 2003). Briefly, single-cell suspensions of NMuMG cells were prepared and plated (50 cells/well) in 96-well, low-attachment plates. The cultures were fed every 3–4 d with serum-free DMEM (Invitrogen) supplemented with basic fibroblast growth factor (20 ng/ml; Invitrogen), epidermal growth factor (20 ng/ml; Invitrogen), B27 (Gibco, Life Technologies, Carlsbad, CA), and heparin (4 μg/ml; Sigma-Aldrich, St. Louis, MO), and the resulting mammospheres were enumerated on day 8 by light microscopy.

Reporter gene assays

The β-catenin and Smad3/4-dependent reporter gene assays were performed as described previously (Taylor *et al.*, 2011). Briefly, NMuMG and 4T1 derivatives (40,000 cells/well) were seeded onto 24-well plates and allowed to adhere overnight. The next morning, the cells were transiently transfected with LT1 transfection reagent (Mirus, Madison, WI), which contained 450 ng/well of total DNA that consisted of 400 ng of either pTopFlash or pSBE-luciferase plasmids, together with 50 ng of pCMV-β-gal. Twenty-four hours later, the transfectants were washed and placed for an additional 24 h in serum-free media supplemented with TGF-β1 (5 ng/ml) or the TβR-I antagonist SB431452 (10 μM; Calbiochem, San Diego, CA). Afterward, luciferase and β-gal activities present in detergent-solubilized extracts were determined.

Apoptosis assay

4T1 derivatives were seeded onto 96-well plates (10,000 cells/well) and allowed to adhere overnight. The next morning, the cells were washed and incubated in 50 μl of serum-free media supplemented with diluent or TGF-β1 (5 ng/ml) for 48 h, at which point the extent of caspase 3/7 activity was quantified using the Caspase-Glo 3/7 luminescence assay system according to the manufacturer's recommendations (Promega, Madison, WI).

Semiquantitative real-time PCR analyses

Real-time PCR studies were performed as described previously (Wendt and Schiemann, 2009; Taylor *et al.*, 2011). Briefly, NMuMG or 4T1 derivatives (500,000 cells/well) were seeded overnight onto six-well plates, transfected with a control siRNA or one that specifically targets β3 integrin (5′-GCUCAUCUGGAAGCUACUCAUCAC; IDT, Coralville, IA), and subsequently stimulated with TGF-β1 (5 ng/ml) for 24 h. Afterward, total RNA was isolated using the RNeasy Plus Kit (Qiagen, Valencia, CA) and reverse transcribed using the iScript cDNA Synthesis System (Bio-Rad, Hercules, CA). Semiquantitative real-time PCR was conducted using iQ-SYBR Green (Bio-Rad) according to manufacturer's recommendations. In all cases, differences in RNA concentration for individual genes were normalized to their corresponding glyceraldehyde-3-phosphate dehydrogenase RNA signals. The oligonucleotide primer pairs used are provided in Supplemental Table S1.

3D-organotypic cultures

The 3D-organotypic cultures using the "on-top" method were performed as described (Taylor *et al.*, 2011). Briefly, NMuMG, 4T1, or MDA-MB-231 derivatives (2000 cells/well) were cultured in eight-well chamber slides on 100-μl Cultrex cushions (Trevigen, Gaithersburg, MD) in complete media supplemented with 5% Cultrex. Where indicated, the Cultrex cushions were rendered biomechanically rigid by inclusion of type I collagen (3 mg/ml; BD Biosciences), at which point organoid growth was monitored by bright-field microscopy or bioluminescence growth assays where indicated using luciferin substrate (Paszek *et al.*, 2005; Wendt *et al.*, 2011).

Cell motility and invasion assays

Cell migration and invasion assays were performed as described previously (Wendt and Schiemann, 2009). Briefly, confluent NMuMG cell cultures were wounded with a micropipette tip (200 μl) and immediately placed in 1% serum-containing medium supplemented with TGF-β1 (5 ng/ml), the p38 MAPK inhibitor SB203580 (10 μM; BD Biosciences), or neutralizing β1 integrin antibodies (5 μg/ml; Millipore, Billerica, MA) as indicated. Bright-field images of wounded monolayers were obtained immediately after wounding and at various time points thereafter. Wound closure was measured by SlideBook Imaging Software (Intelligent Imaging Innovations, Denver, CO). The ability of 4T1 cells (50,000 cells/well) to invade reconstituted basement membranes was measured using modified Boyden chambers as previously described (Wendt and Schiemann, 2009) in the presence or absence of an MMP-9 inhibitor (10 μM; Calbiochem).

Tumor growth and bioluminescence imaging

4T1 derivatives harboring altered expression of β1, β3, or both integrins were engineered to stably express firefly luciferase and

subsequently were injected (10,000 or 12,000 cells/mouse as indicated) into mammary fat pads of female BALB/c mice. Afterward, primary tumor growth and their pulmonary metastasis were monitored and determined as described previously (Wendt and Schiemann, 2009). All animal studies were performed in accordance with the Institutional Animal Care and Use Committee for Case Western Reserve University.

Statistical analyses

Statistical values were defined using an unpaired Student's *t* test with $p < 0.05$ considered significant.

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