Matrix rigidity regulates a switch between TGF-β1–induced apoptosis and epithelial–mesenchymal transition

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ABSTRACT **The transforming growth factor-β (TGF-β) signaling pathway is often misregulated during cancer progression. In early stages of tumorigenesis, TGF-β acts as a tumor suppressor by inhibiting proliferation and inducing apoptosis. However, as the disease progresses, TGF-β switches to promote tumorigenic cell functions, such as epithelial–mesenchymal transition (EMT) and increased cell motility. Dramatic changes in the cellular microenvironment are also correlated with tumor progression, including an increase in tissue stiffness. However, it is unknown whether these changes in tissue stiffness can regulate the effects of TGF-β. To this end, we examined normal murine mammary gland cells and Madin–Darby canine kidney epithelial cells cultured on polyacrylamide gels with varying rigidity and treated with TGF-β1. Varying matrix rigidity switched the functional response to TGF-β1. Decreasing rigidity increased TGF-β1–induced apoptosis, whereas increasing rigidity resulted in EMT. Matrix rigidity did not change Smad signaling, but instead regulated the PI3K/Akt signaling pathway. Direct genetic and pharmacologic manipulations further demonstrated a role for PI3K/Akt signaling in the apoptotic and EMT responses. These findings demonstrate that matrix rigidity regulates a previously undescribed switch in TGF-β–induced cell functions and provide insight into how changes in tissue mechanics during disease might contribute to the cellular response to TGF-β.**

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INTRODUCTION

Transforming growth factor-β (TGF-β) is a pleiotropic cytokine essential for many physiological processes, including embryonic development, immune function, and wound healing (Wu and Hill, 2009). Misregulation of TGF-β signaling can contribute to the progression of disease states such as organ fibrosis and cancer, and a key to treating these diseases will be a better understanding of the TGF-β signal transduction machinery (Massague, 2008). However, due to its widespread effects, the role of TGF-β is not well understood. This is perhaps best illustrated in the context of tumor progression, although analogous situations can be found in other settings. During early stages of tumorigenesis, TGF-β acts as a tumor suppressor. TGF-β induces growth arrest and apoptosis in most normal epithelial cells in vitro (Pietenpol *et al.*, 1990; Hannon and Beach, 1994; Siegel and Massague, 2003). Mice in which the *TGFB1* or *SMAD* genes are disrupted are prone to the development of cancer (Zhu *et al.*, 1998; Engle *et al.*, 1999; Go *et al.*, 1999). Retrospective studies of various human tumor types have also found frequent down-regulation or mutations inactivating the TGF-β signaling pathway (Kaklamani *et al.*, 2005; Stuelten *et al.*, 2006; Bacman *et al.*, 2007). In later stages of cancer progression, however, TGF-β is believed to switch roles and promote tumor progression and metastasis (Derynck *et al.*, 2001; Wakefield and Roberts, 2002; Tang *et al.*, 2003). Within the tumor, TGF-β enhances migration, invasion, survival, and epithelial–mesenchymal transition (EMT) (Massague, 2008). High levels of TGF-β in clinical settings are associated with a poor prognosis (Friess *et al.*, 1993; Wikstrom *et al.*, 1998; Fukai *et al.*, 2003), and treatment with TGF-β in animal models results in larger, more metastatic tumors (Wikstrom *et al.*, 1998; Fukai *et al.*, 2003; Muraoka *et al.*, 2003). TGF-β also plays an active role in

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Abbreviations used: α-SMA, α-smooth muscle actin; coll I, collagen type I; EMT, epithelial–mesenchymal transition; FN, fibronectin; MDCK, Madin–Darby canine kidney cells; NMuMG, normal murine mammary gland cells; PA, polyacrylamide; rBM, reconstituted basement membrane; TGF-β, transforming growth factor-β; ZO-1, zonula occludens-1.

remodeling of the tumor microenvironment, promoting activation of fibroblasts, increasing angiogenesis, and suppressing immune surveillance (Bierie and Moses, 2006). Although the switch in TGF-β from a tumor suppressor to promoter during disease progression is well documented, it is still unclear how this switch occurs. One possibility is that changes in the cellular microenvironmental context guide the cellular response to TGF-β.

Although many aspects of the cellular microenvironment change during disease, including soluble factors, cell–cell interactions, and cell–extracellular matrix (ECM) adhesion, changes in the mechanical properties of the microenvironment may also modulate the response to the TGF-β. The mechanical stiffness of tissue microenvironments varies widely, as adipose tissue is less rigid than muscle, which is less rigid than bone, and tissue stiffness can also change within the same type of tissue during disease states (Butcher *et al.*, 2009). In the context of cancer progression, as well as tissue fibrosis, increased tissue stiffness is well documented and is due to a number of factors, including extracellular matrix remodeling, deposition, and cross-linking (Ebihara *et al.*, 2000; Levental *et al.*, 2009). Several recent studies have shown that such changes in matrix rigidity can regulate many cellular functions, including focal adhesion maturation, cell spreading, actin stress fiber formation, and cell motility (Pelham and Wang, 1997; Lo *et al.*, 2000; Yeung *et al.*, 2005). Several cell types cultured on compliant substrates decrease proliferation and increase apoptosis as compared with cells on rigid substrates (Wang *et al.*, 2000; Klein *et al.*, 2009; Tilghman *et al.*, 2010; Mih *et al.*, 2011). Differentiation of many cell types can also be regulated by matrix rigidity, including human mesenchymal stem cells, portal fibroblasts, mammary epithelial cells, and endothelial cells (Vailhe *et al.*, 1997; Paszek *et al.*, 2005; Engler *et al.*, 2006; Li *et al.*, 2007; Alcaraz *et al.*, 2008). Because matrix rigidity can regulate a number of cell functions also regulated by TGF-β, such as proliferation, apoptosis, and differentiation, and tissues become stiffer during disease progression, we hypothesized that changes in matrix rigidity could regulate TGF-β–induced cellular functions.

In this study, we examined whether matrix rigidity regulates TGF-β–induced cell function. We examined two cell functions—apoptosis and EMT—as representative responses to TGF-β classically associated with tumor suppression or promotion, respectively (Massague, 2008). In most nontransformed epithelial cells, TGF-β induces programmed cell death, or apoptosis; this is one way TGF-β suppresses tumorigenesis during early stages of the disease (Rahimi and Leof, 2007). In contrast, EMT is a key step during metastasis, which occurs during later stages of disease, and is characterized by dissolution of epithelial cell–cell junctions, remodeling of cell–matrix adhesion, and increased motility (Lee *et al.*, 2006). In studies presented here, we found a novel PI3K/Akt-mediated switch in which substrate rigidity controlled TGF-β1–induced cell functions—epithelial cells cultured on compliant substrates underwent apoptosis when treated with TGF-β1, whereas on more rigid substrates, TGF-β1 induced EMT. These findings suggest that matrix mechanics plays a key role in regulating the opposing functional effects of TGF-β1.

RESULTS

Matrix rigidity regulates TGF-β1–induced cell fate

To explore whether matrix rigidity influences cellular responses to TGF-β1 in a noncancerous genetic background, we used normal murine mammary gland epithelial cells (NMuMG) and Madin–Darby canine kidney epithelial cells (MDCK), both well established in vitro model systems of EMT (Miettinen *et al.*, 1994). We first examined NMuMG cells that were cultured on fibronectin-conjugated polyacrylamide (PA) gels with a range of elastic modulus (E) from 0.4 to 60 kPa and then treated with TGF-β1. NMuMG cells cultured on PA gels exhibited differences in morphology as a function of substrate compliance (Figure 1A). Cells on the most rigid gels (E > 14 kPa) appeared cuboidal and formed a monolayer on the surface identical to cells on tissue culture plastic. In contrast, cells on compliant gels (E < 1 kPa) were rounded and formed spherical clusters. On rigid PA gels (E > 5 kPa) or on tissue culture plastic, TGF- β1 treatment induced an elongated morphology and scattering of cells, characteristic of an EMT (Figure 1A). Examination of known EMT markers confirmed this response, as evidenced by delocalization of the epithelial junctional markers zonula occludens-1 (ZO-1) and E-cadherin and increased expression of mesenchymal markers N-cadherin, α-smooth muscle actin (α-SMA), and the EMT-associated transcription factor Snail (Figure 1, B, C, and E). Although E-cadherin was displaced from adherens junctions, no significant decrease was observed in E-cadherin protein expression, similar to observations from other groups using the NMuMG cell line (Figure 1B; Bakin *et al.*, 2000; Shintani *et al.*, 2006; Bailey and Liu, 2008). Although TGF-β1 did not appear to induce EMT on compliant gels, as indicated by decreased N-cadherin, α-SMA, and Snail expression as compared with rigid gels, phase and immunofluorescence imaging revealed a dramatic increase in TGF-β1–induced apoptosis on compliant gels (Figure 1, A and D, and Supplemental Figure S1B). Apoptosis was confirmed by nuclear fragmentation and caspase activity (Figure 1, D and E). On compliant gels, ~28% of cells were positive for cleaved caspase-3 by immunofluorescence after 24 h of TGF-β1 treatment, whereas 13% of cells were positive on rigid gels (Supplemental Figure S1C). Increased apoptosis on compliant gels was observed across three orders of magnitude of TGF-β1 concentration, from 0.1 to 10 ng/ml (Supplemental Figure S1D). TGF-β1 treatment was necessary for the observed increase in apoptosis, as basal levels of apoptosis were similar across substrates of different stiffness (Figure 1, D and E, and Supplemental Figure S1C). On substrates with modulus ranging from 1 to 8 kPa, there was a decrease in apoptosis, as well as a concomitant increase in Snail expression (Figure 1E); this range of modulus is also associated with measurements of excised tumor tissue (Paszek *et al.*, 2005). Snail, a transcription factor induced by TGF-β and responsible for the down-regulation of E-cadherin, is a critical factor regulating EMT and was used here as an early marker of EMT, as the rapid onset of apoptosis on compliant gels prevented reliable measurement of later-stage markers (Supplemental Figure S1, A and B; Cano *et al.*, 2000; Peinado *et al.*, 2003; Cho *et al.*, 2007). These data suggest that matrix rigidity regulates a functional switch between apoptosis and EMT in response to TGF-β1.

A similar switch in cell fate between apoptosis and EMT was also observed in MDCK epithelial cells, suggesting that this control mechanism is not restricted to mammary epithelia. MDCK cells cultured on rigid gels underwent a classic EMT with TGF-β1 treatment, as indicated by an elongated morphology, actin stress fibers, and delocalization of the epithelial cell–cell adhesion markers E-cadherin and ZO-1 (Figure 2A). Compliant substrates, conversely, inhibited this transition, and MDCK cells retained a rounded morphology, cortical actin, and epithelial adherens and tight junctions with TGF-β1 treatment. Similar to the NMuMGs, increased apoptosis was also observed in MDCK cells cultured on compliant substrates (Figure 2B).

ECM proteins promote cell adhesion but also provide biochemical and biophysical cues that can regulate cell function and signaling (Hynes, 2009). Thus it was not clear whether the rigidity-regulated switch between EMT and apoptosis was specific to the fibronectin (FN) conjugated to the PA gels or occurred irrespective of the ECM protein to which cells attached. To address this question, NMuMG

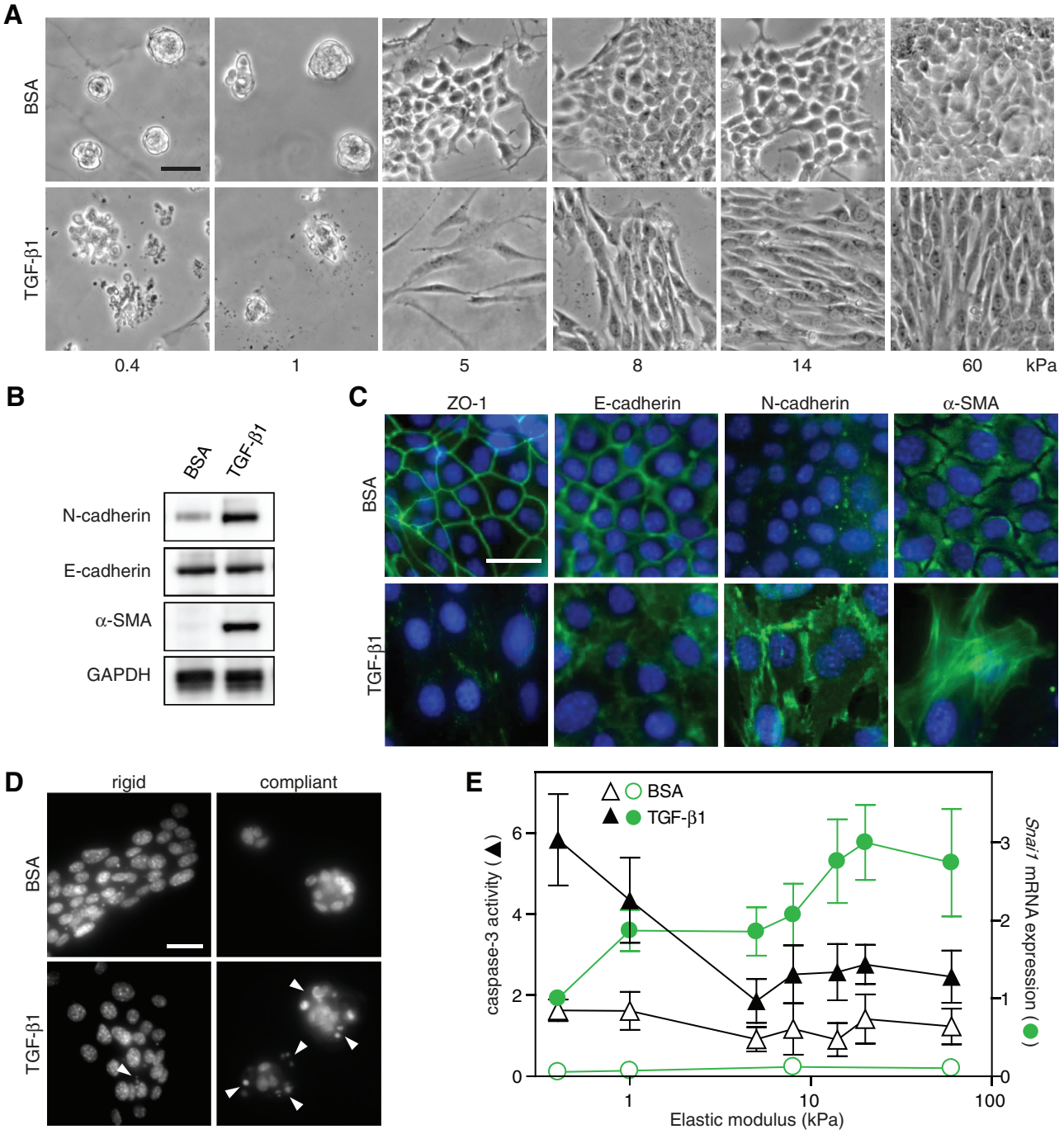

FIGURE 1: Matrix rigidity regulates TGF-β1–induced EMT and apoptosis in NMuMG cells. (A) Phase contrast images of cells cultured on PA gels with elastic modulus ranging from 0.4 to 60 kPa and treated with TGF-β1 or BSA control. (B) Western blot of N-cadherin (135 kDa), E-cadherin (120 kDa), α-SMA (42 kDa), and GAPDH control (38 kDa) in cells cultured on rigid (8 kPa) PA gels. (C) Immunofluorescence images of cells cultured on rigid PA gels. (D) Hoechst-stained nuclei of cells cultured on rigid (8 kPa) and compliant (0.4 kPa) gels. Fragmented nuclei indicated by white triangles. (E) Caspase-3 activity (▲, Δ) and Snai1 mRNA expression (●, ○) in cells cultured on PA gels. n = 5 ± SEM. Bars, 50 μm.

cells were cultured on polyacrylamide gels conjugated with FN, reconstituted basement membrane (rBM; commercially known as Matrigel), or collagen I (coll I) and treated with TGF-β1. NMuMGs cultured on substrates conjugated with FN or rBM were morphologically similar; however on coll I substrates, cell spreading was increased (Figure 3A). Substrate compliance inhibited TGF-β1–induced EMT regardless of ECM type, as indicated by decreased Snail expression (Figure 3B). Compliant substrates conjugated with FN or rBM also increased TGF-β1–induced apoptosis (Figure 3C). However, cells cultured on coll I substrates had dramatically reduced levels of caspase-3 activity in all conditions, although increased compliance still enhanced levels of apoptosis. Because cell spreading can regulate apoptosis and coll I increased cell spreading on compliant substrates, it was unclear whether the decrease in apoptosis was due to increased cell spreading or more specifically to coll I signaling (Chen *et al.*, 1997). To address this question, each

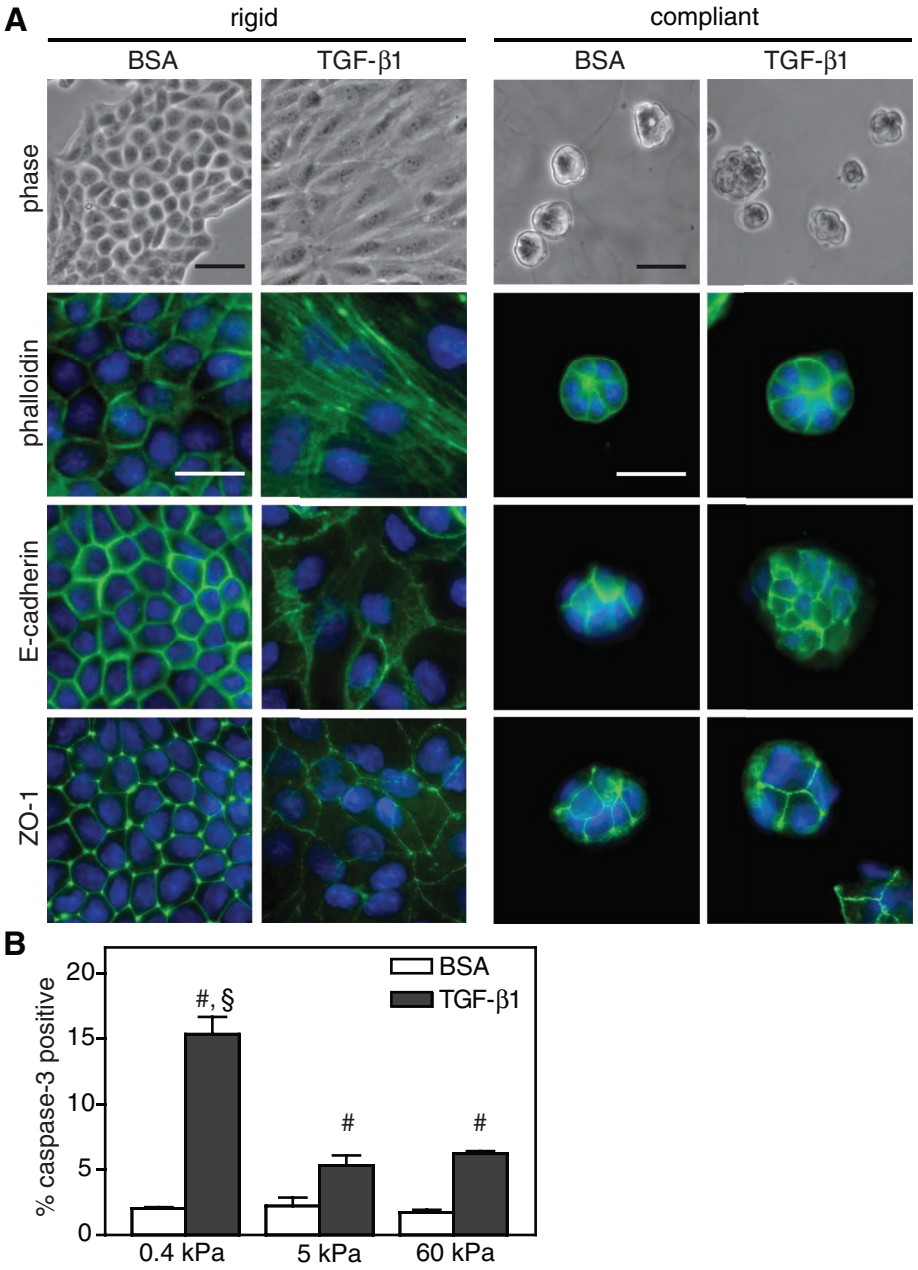

FIGURE 2: TGF-β1–induced EMT and apoptosis in MDCK cells cultured on polyacrylamide gels. (A) Immunostaining on rigid (5 kPa) and compliant (0.4 kPa) PA gels in cells treated with TGF-β1 or BSA control. (B) Graph of percentage of cells positive for cleaved caspase-3 immunofluorescence. n = 3 ± SEM. #p < 0.01 as compared with BSA conditions; §p < 0.01 as compared with 5- and 60-kPa TGF-β1 conditions. Bars, 50 μm.

ECM was microcontact printed onto polydimethylsiloxane (PDMS)–coated coverslips to restrict cell spreading (area of 289 μm^2) or to allow cells to fully spread (Figure 3D). Restricting cell spreading, similar to compliant substrates, increased TGF-β1–induced apoptosis compared with fully spread cells for all ECM types (Figure 3D). Whereas increased apoptosis was observed in unspread cells on coll I substrates as compared with fully spread cells, apoptosis on coll I substrates was significantly less than for cells cultured on FN or rBM, suggesting that coll I specifically inhibits apoptosis on compliant substrates in addition to regulating cell spreading.

Given that the apoptotic response occurred within hours, whereas a full EMT required at least 48 h of TGF-β1 treatment, it was not clear whether the decreased EMT on compliant gels was a result of TGF-β1–induced cell death or compliance directly regulated EMT independent of its effects on cell survival. To address this, we blocked the apoptotic response by either overexpressing the survival factor, Bcl-xL, or treating with a pan-caspase inhibitor, ZVAD-FMK, and observed whether EMT on compliant gels would be rescued (Boise *et al.*, 1993). As a control, both reagents decreased caspase-3 activity and prevented nuclear fragmentation (Supplemental Figure S2). When apoptosis was inhibited, NMuMGs cultured on compliant gels still failed to undergo EMT. E-cadherin remained localized to junctions, N-cadherin and α-SMA failed to express, and cells did not transition to an elongated phenotype (Figure 4, A and B). Together these data suggest that substrate stiffness regulates a switch in the response of cells to TGF-β1 between EMT and apoptosis and that these two responses are independently regulated.

Previous studies showed that cell density can regulate TGF-β–induced cell functions and that cells grown to confluence do not undergo EMT (Petridou *et al.*, 2000; Nelson *et al.*, 2008). Specifically, less TGF-β bound to TGF-β receptors and Smad translocation was reduced in confluent cells (Petridou *et al.*, 2000). To investigate whether matrix rigidity regulates TGF-β signaling through a similar mechanism, NMuMGs were plated at 60% confluence (1×10^5 cells/cm^2) and treated with TGF-β1. As early as 2 h after TGF-β1 treatment, Smad4 translocated to the nucleus in NMuMGs to similar degrees on both rigid and compliant substrates (Figure 5A). Furthermore, use of a Smad-responsive 3TP-luciferase reporter plasmid also showed no difference in Smad transcriptional activity on rigid versus compliant substrates (Figure 5B; Wrana *et al.*, 1992; Yingling *et al.*, 1997). Together these results suggest that TGF-β receptor/Smad signaling functions at similar levels on compliant and rigid substrates and is not responsible for the matrix rigidity–induced switch in TGF-β function.

Matrix rigidity could modulate TGF-β1 signaling at numerous levels in addition to the Smad signaling pathway. Previous work showed that increased matrix rigidity and treatment with TGF-β1 each can promote actin stress fiber and focal adhesion formation (Pelham and Wang, 1997; Yeung *et al.*, 2005). Similar stress fiber and focal adhesion responses are seen upon treatment with TGF-β1 (Miettinen *et al.*, 1994; Edlund *et al.*, 2002). In addition, focal adhesion kinase (FAK), one of the main signaling components within focal adhesions, can also be regulated by matrix rigidity and TGF-β1 and is associated with cell survival and EMT (Ilic *et al.*, 1998; Wang *et al.*, 2004; Paszek *et al.*, 2005; Cicchini *et al.*, 2008; Zouq *et al.*, 2009). To investigate whether FAK may be involved in this system, we first

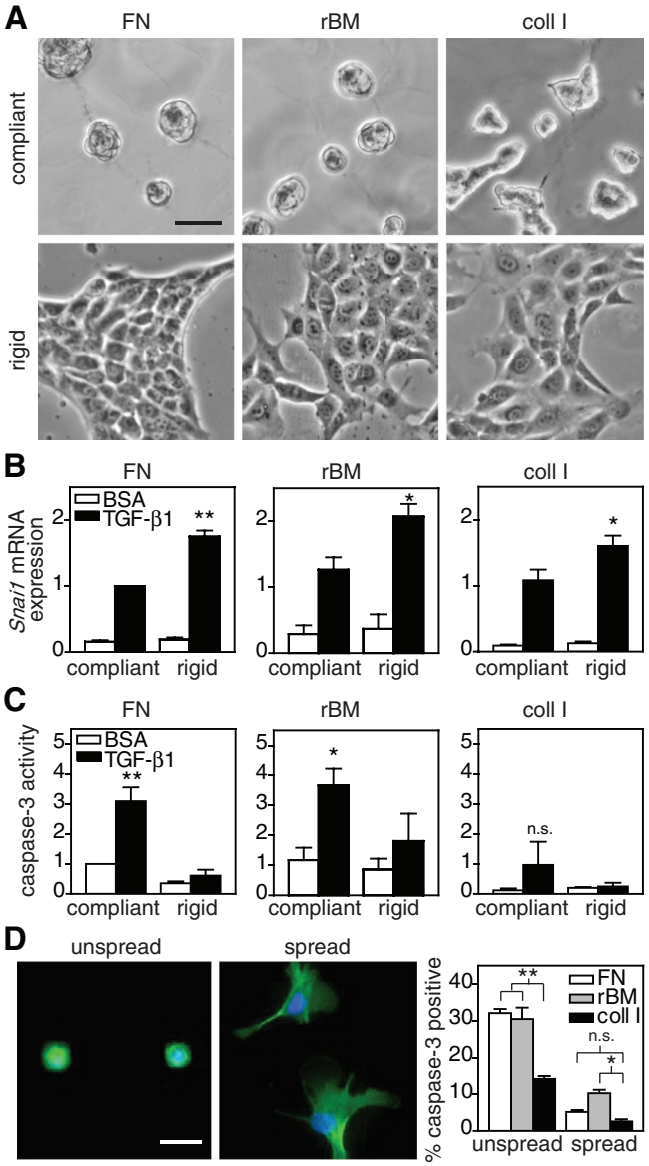

FIGURE 3: TGF-β1–induced EMT and apoptosis in NMuMG cells cultured on polyacrylamide gels conjugated with ECM. (A) Phase contrast images of cells cultured on FN, rBM, or coll I conjugated PA gels. (B) Snai1 mRNA expression in cells on compliant and rigid gels treated with TGF-β1. (C) Caspase-3 activity in cells on compliant and rigid gels treated with TGF-β1. (D) Phalloidin (green)- and Hoechst (blue)-stained cells on 289 μm^2 islands (unspread) or large areas (spread) of microcontact-printed FN. Graph of percentage of cells treated with TGF-β1 positive for cleaved caspase-3 immunofluorescence. n = 3 ± SEM. *p < 0.05; **p < 0.01. Bars, 50 μm.

examined whether both matrix compliance and TGF-β1 modulated focal adhesion formation and FAK phosphorylation. Prominent focal adhesions, as indicated by punctate immunofluorescence staining for vinculin, and actin stress fibers were observed in NMuMGs cultured on rigid substrates (Figure 6A). On compliant substrates, focal adhesion markers were diffuse, and cortical actin was observed (Figure 6B). Treatment with TGF-β1 qualitatively increased focal adhesion size on rigid substrates, but no effect was observed on compliant gels. We also observed increased phospho-FAK localization to focal adhesions in a manner that directly correlated with vinculin localization (Figure 6A). Western blot analysis confirmed this observation, showing increased levels of phospho-FAK (Figure 6C). However, specific activity of FAK (phospho-FAK normalized to total FAK) showed no significant difference between compliant and rigid gels, as FAK protein levels were greatly decreased on compliant gels. In a recent study, FAK protein expression was found to be critical for the mesenchymal phenotype and Snail expression in mouse embryonic fibroblasts, so we hypothesized that overexpressing FAK in cells on compliant gels may rescue Snail expression and EMT (Li *et al.*, 2011). To test this possibility, we overexpressed FAK using an adenoviral vector (Supplemental Figure S3A). Overexpression of FAK did not rescue Snail mRNA expression on compliant gels; however a decrease in apoptosis was observed (Figure 6, D and E). Previous reports showed decreased FAK expression on collagen gels due to FAK degradation by calpain (Wang *et al.*, 2003). In this system, however, we did not observe lower–molecular weight bands associated with FAK degradation by Western blot, and treatment with a calpain inhibitor, ALLN, did not increase FAK expression or inhibit apoptosis on compliant gels (data not shown).

Whereas overexpression of wild-type FAK rescued cell survival on compliant gels, expression of CD2-FAK, an activated FAK allele (Frisch *et al.*, 1996), failed to inhibit apoptosis on compliant gels (Supplemental Figure S3E). Further supporting these data, pharmacological inhibition of FAK activity with the small-molecule inhibitor PF 573228 reduced Y397 FAK phosphorylation but did not affect EMT or apoptosis (Supplemental Figure S3, B and C). Expression of the dominant-negative FRNK and the phosphorylation mutant FAK Y397F, both at physiological levels and highly overexpressed, did not reduce FAK phosphorylation at Y397 and did not affect EMT or apoptosis (Supplemental Figure S3E). These data suggest that matrix rigidity is regulating FAK signaling by modulating FAK protein levels and that FAK levels in turn regulate compliance-induced apoptosis but not EMT. Other important regulators of stress fiber and focal adhesion formation, the RhoGTPases, were also investigated using the pharmacological inhibitors Y27632—a ROCK (Rho kinase) inhibitor—and NSC 23766—a Rac1 inhibitor. Neither inhibitor significantly affected apoptosis or EMT on compliant or rigid gels (Supplemental Figure S4, A and B).

Matrix rigidity regulates apoptosis and EMT through PI3K and Akt

Similar to FAK, the PI3K/Akt pathway has been shown to regulate both EMT and survival in a variety of settings and can be regulated by matrix stiffness (Chen *et al.*, 1998; Bakin *et al.*, 2000; Levental *et al.*, 2009). To investigate whether substrate rigidity regulates the PI3K/Akt signaling pathway, we first measured Akt phosphorylation at serine 473. Because insulin is an essential component of the growth media of NMuMGs and insulin is known to stimulate Akt activity, exposure to insulin was included as a background control (Burgering and Coffer, 1995). In all cases, NMuMGs cultured on compliant gels showed decreased Akt activation compared with cells on rigid gels (Figure 7A). Inhibition of PI3K or Akt activity with pharmacological inhibitors LY294002 and two Akt inhibitors increased TGF-β1–induced apoptosis (Figure 7, B and C). Inhibition of PI3K decreased Snail mRNA expression on rigid gels; however, inhibition of Akt did not (Figure 7D). Although these studies suggest that PI3K is necessary for survival and EMT following TGF-β1 treatment, it was not clear whether it was also sufficient. We increased PI3K activity by adenoviral expression of p110-CAAX, a membrane-localized subunit of PI3K, and observed suppression of apoptosis on compliant gels to similar levels observed on rigid gels (Figure 7, E and F). p110-CAAX expression, however, did not rescue Snail mRNA

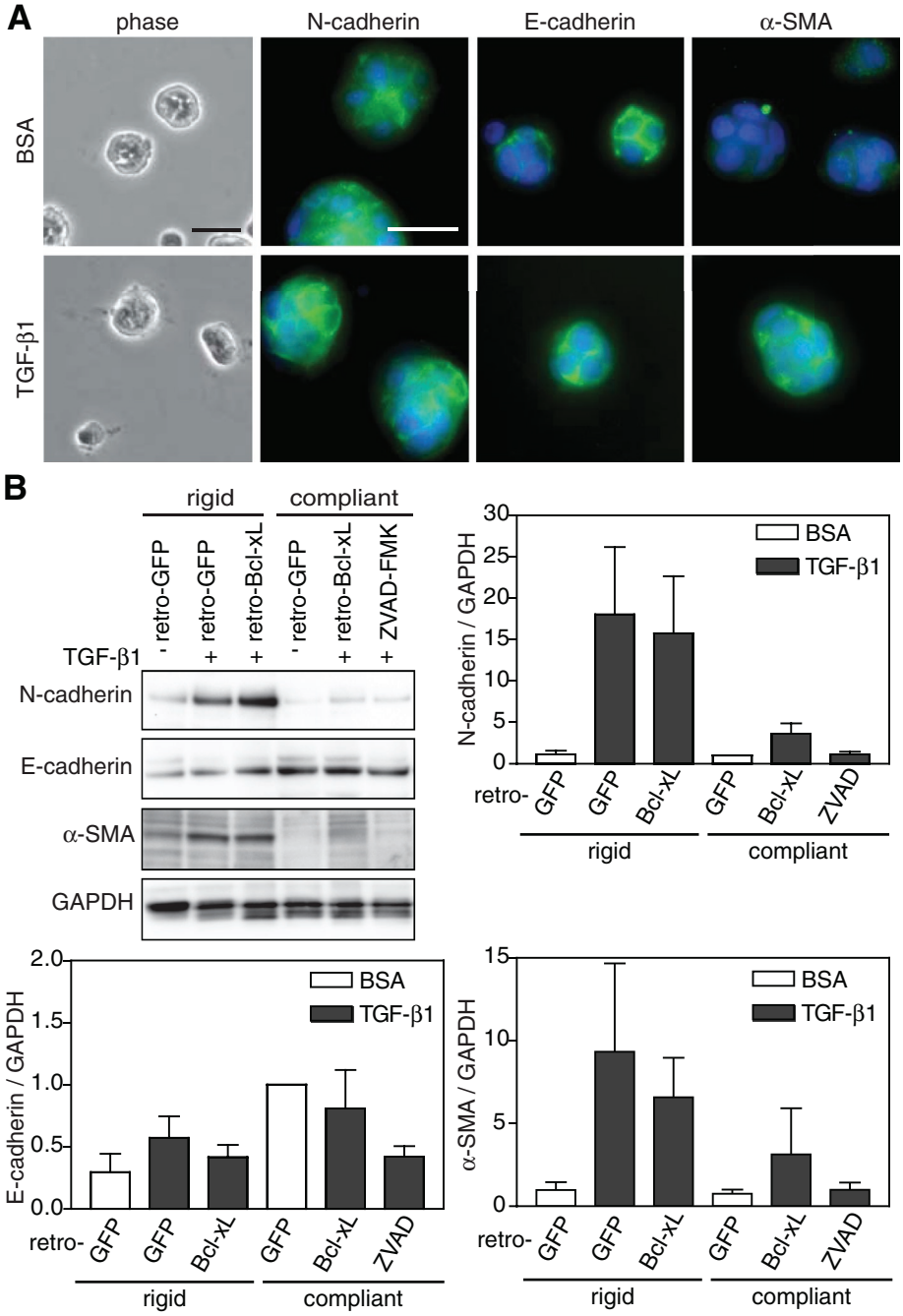

FIGURE 4: Decreased matrix rigidity inhibits EMT independent of apoptosis. (A) Phase contrast and immunostaining for N-cadherin, E-cadherin, α-SMA, and nuclei of NMuMG cells infected with retro-Bcl-xL on compliant gels. (B) Western blot and quantification of N-cadherin, E-cadherin, α-SMA, and GAPDH in NMuMG cells infected with retro-GFP or retro-Bcl-xL or treated with 400 μM ZVAD-FMK, plated on rigid and compliant gels, and treated with TGF-β1. n = 4 ± SEM. Bars, 50 μm.

expression (Figure 7G). Together these data demonstrate a role for PI3K and Akt in transducing substrate compliance and regulating the compliance-induced switch in cellular response to TGF-β1.

DISCUSSION

We find that decreasing matrix rigidity inhibits PI3K/Akt activity and through this action impinges on both survival and EMT. Numerous previous studies demonstrated the importance of the PI3K/Akt signaling pathways for cell survival (Dudek *et al.*, 1997; Khwaja *et al.*, 1997) and EMT (Bakin *et al.*, 2000; Kattla *et al.*, 2008). Although we found inhibition of Akt activity by two pharmacological inhibitors increased apoptosis on rigid substrates, EMT was unaffected. This could be explained by demonstration of distinct regulatory roles for the Akt isoforms (Irie *et al.*, 2005), and here pharmacological inhibition would not differentiate between the isoforms. It is perhaps not surprising that upregulation of PI3K failed to rescue EMT on low-rigidity substrates. Given the many disparate processes that are necessary to drive EMT, it is likely that additional points of regulation are affected by rigidity (Cannito *et al.*, 2010). In mesenchymal cells (as opposed to epithelial cells), one component of EMT has been reported to exhibit remnants of this control mechanism. TGF-β–induced smooth muscle actin expression in fibroblasts and trabecular meshwork cells (associated with myofibroblast differentiation) appears to be suppressed with decreased matrix rigidity (Arora *et al.*, 1999; Li *et al.*, 2007; Chen *et al.*, 2011; Han *et al.*, 2011). Moreover, inhibitors of PI3K/Akt signaling can block α-SMA expression (Han *et al.*, 2011). Although it would be inappropriate to suggest that mechanical regulation of EMT is equivalent to α-SMA expression, since EMT involves many additional regulatory steps, including loss of epithelial markers, cell–cell adhesions, and polarity, these studies do suggest some conserved mechanisms. More starkly, the stiffness-induced switch between apoptosis and EMT that we report here in two epithelial cell systems is absent in fibroblastic cells, suggesting a new function of matrix rigidity to regulate a switch *between* TGF-β–induced functions. Further elucidation of these mechanisms is likely forthcoming, as recent studies are beginning to uncover the wide array of signaling pathways affected by rigidity, including integrin activation, focal adhesion assembly, and numerous signaling pathways, including Rho GTPases, mitogen-activated protein kinases, FAK, and phosphoinositide kinase-3 (Fringer and Grinnell, 2001; Wozniak *et al.*, 2003; Paszek *et al.*, 2005; Friedland *et al.*, 2009; Klein *et al.*, 2009; Levental *et al.*, 2009).

The results presented here also highlight the complex interplay among matrix rigidity, cell spreading, and ECM subtypes. Here we found that TGF-β–induced EMT is inhibited on compliant substrates independent of ECM subtypes, but that the compliance-induced apoptosis was more dramatic when cells engaged FN or rBM as compared with coll I. Of interest, we observed that cell spreading was enhanced on coll I, and it was previously reported that cell spreading can antagonize apoptosis (Chen *et al.*, 1997). Indeed, the coll I–induced reduction in apoptosis appears to be due in part to the increased cell spreading, since controlling cell

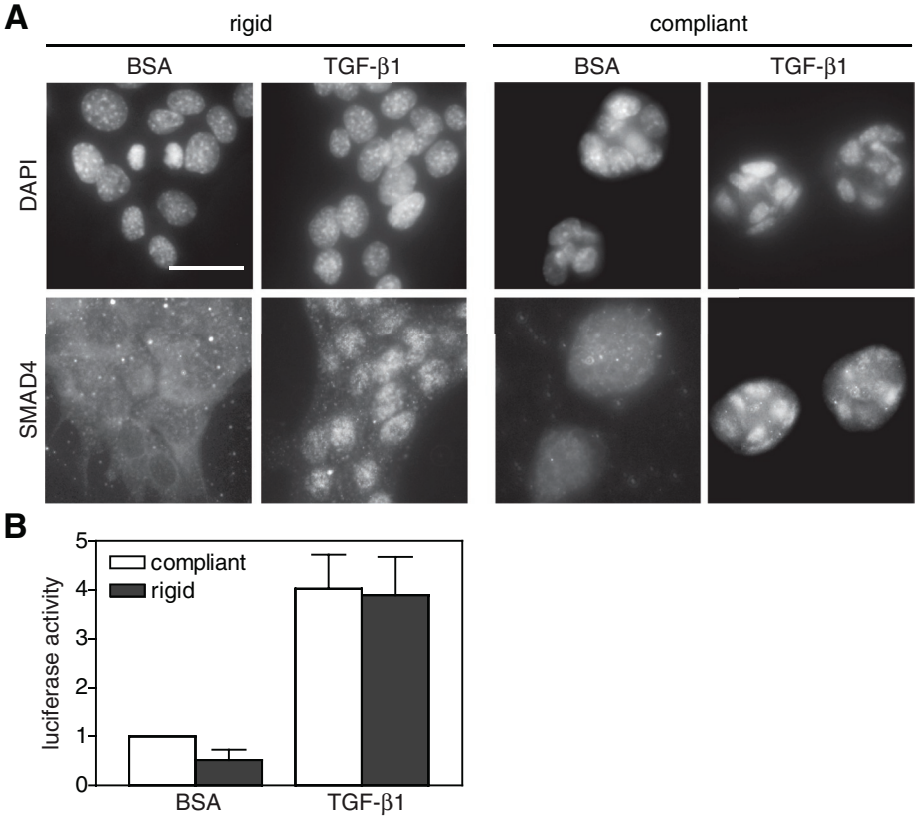

FIGURE 5: Smad signaling in NMuMG cells on compliant and rigid gels. (A) Immunostaining for Smad4 and nuclei in NMuMG cells cultured on rigid and compliant PA gels treated with TGF-β1. (B) Luciferase activity in cells transfected with 3TP-luciferase reporter plasmid on compliant and rigid gels. n = 4 ± SEM. Bars, 50 μm.

area through microcontact printing partially accounted for this difference. However, coll I was still partially able to inhibit cell death even when cell spreading was restricted. Thus binding this ECM appears to have an additional benefit, possibly through specific collagen receptors such as α2β1 integrin or the discoidin domain receptors 1/2 (Ongusaha *et al.*, 2003). In other cell types, such as fibroblasts and endothelial cells, adhesion to coll I reduced cell spreading (Yeung *et al.*, 2005), and in a melanoma cell line coll I did not affect cell spreading but increased cell stiffness and adhesion strength (Byfield *et al.*, 2009). Thus, although there is a widely demonstrated link between substrate stiffness and cell spreading (Pelham and Wang, 1997; Yeung *et al.*, 2005; Fu *et al.*, 2010), how specific ECMs can impact this response may depend on the cell type. These results highlight that regulation of cell function by matrix rigidity can be affected by other cell–matrix adhesion inputs, such as cell spreading and ECM subtype.

TGF-β regulates a diverse array of cellular functions, including proliferation, motility, and differentiation, and these effects are distinct in many cell types. How TGF-β regulates often divergent functions even in a single cell type, particularly in disease contexts such as tumorigenesis, is not well understood. It has been reported that some cell types spontaneously undergo apoptosis on compliant substrates (Wang *et al.*, 2000, 2007), but in the work presented here TGF-β was a required trigger for death. Here we found that on compliant substrates, with a modulus similar to that of native breast tissue, TGF-β induces apoptosis, whereas on rigid substrates, with a modulus similar to that of tumor or fibrotic tissue, TGF-β induces EMT. These results provide a possible explanation for the switch in TGF-β's action from tumor suppressor to promoter during tumorigenesis. Furthermore, these studies highlight the central role for matrix mechanics in regulating cell signaling and fate.

MATERIALS AND METHODS

Cell culture and reagents

NMuMG and MDCK cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured according to their recommendations. Reagents were obtained as follows. Monoclonal antibodies: α-smooth muscle actin (1A4), Smad4 (DCS-46), and vinculin (hVIN-1) (Sigma-Aldrich, St. Louis, MO); glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 6C5; Applied Biosystems/Ambion, Austin, TX); and E-cadherin (36), N-cadherin (32), and FAK (77; BD Biosciences, San Diego, CA). Polyclonal antibodies: ZO-1 (Zymed Laboratories, San Francisco, CA); pY397 FAK (Invitrogen, Carlsbad, CA); and pAkt, Akt, Bcl-xL, cleaved caspase-3, and FAK (Cell Signaling Technology, Beverly, MA). ECMs (FN, coll I, and rBM), all from BD Biosciences.

Cells were plated at a density of 0.1×10^6 cells/cm^2 on FN-functionalized polyacrylamide gels for 16 h in growth medium. Compliant gels referred to in the text indicate gels with an elastic modulus of 0.4 kPa, and rigid gels have E > 5 kPa. The cells were rinsed in sterile phosphate-buffered saline (PBS) and then growth factor starved in high-glucose DMEM for 2 h. Cells were treated with 10 μg/ml insulin (Sigma-Aldrich) and 2 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN) for 2 h (RNA isolation, FAK, and Akt Western blotting), 4 h (caspase activity, focal adhesion staining, luciferase assays), 24 h (nuclei fragmentation, cleaved caspase-3 staining), or 48 h (for EMT staining and Western blotting). For inhibitor studies, cells were treated 1 h prior to TGF-β treatment with ZVAD-FMK (400 μM; Enzo Life Sciences, Plymouth, PA), Y-276322 (10 μM) and PF 573228 (1 μM; Tocris Biosciences, Ellisville, MO), and NSC 23766 (10 μM), LY294002 (10 μM), Akt Inhibitor VIII (1 μM), and Akt Inhibitor V (10 μM) (Calbiochem, La Jolla, CA).

Polyacrylamide gel preparation

Polyacrylamide gels were prepared as previously described (Winer *et al.*, 2009). Mechanical properties of the polyacrylamide gels were controlled by varying the percentage of acrylamide and bis-acrylamide as follows: elastic modulus (% acrylamide; % bis-acrylamide), 0.4 kPa (3; 0.05), 1 kPa (3; 0.1), 5 kPa (5.5; 0.15), 8 kPa (5; 0.3), 14.5 kPa (7.5; 0.15), 20 kPa (8; 0.264), and 60 kPa (10; 0.5). Gels were functionalized with 20 μg/ml FN, 20 μg/ml coll I, or 140 μg/ml rBM in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 8, for 1 h at room temperature (RT; 2 h on ice for rBM), rinsed in double-distilled H_2O (ddH_2O), incubated with 1% (vol/vol) ethanolamine in 50 mM HEPES, pH 8, for 30 min, and rinsed with ddH_2O. The gels were sterilized in 5% (vol/vol) isopropanol in PBS for 1 h at RT and rinsed two times with sterile PBS before plating with cells.

Preparation of micropatterned substrates

Micropatterned substrates were prepared as described (Pirone *et al.*, 2006). Briefly, micropatterned stamps were fabricated by

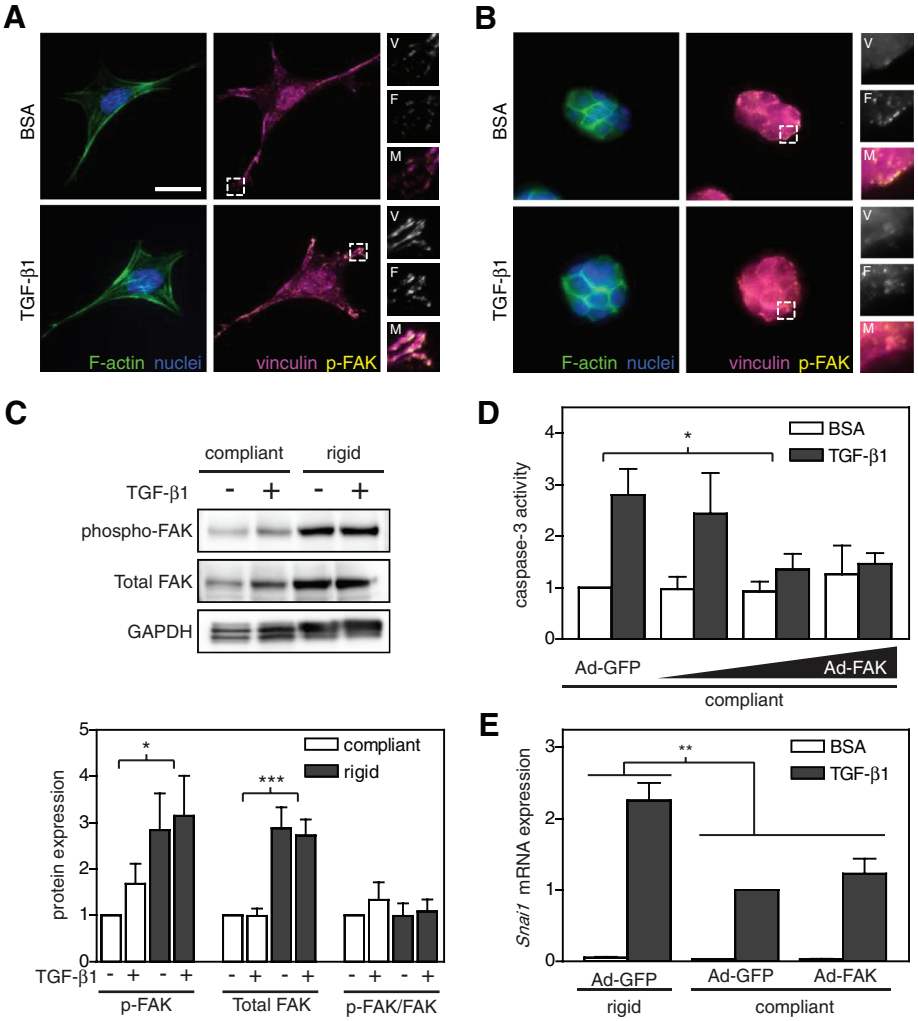

FIGURE 6: Effect of matrix rigidity and TGF-β1 on the actin cytoskeleton and focal adhesion formation in NMuMG cells. (A,B) Immunofluorescence images of F-actin (green), nuclei (blue), vinculin (magenta), and phospho-FAK (yellow) on rigid (8 kPa) (A) and compliant (0.4 kPa) (B) gels. Inset shows magnification of vinculin (V), phospho-FAK (F), and merged (M) images. (C) Western blot and quantification of phospho-FAK (125 kDa), total FAK (125 kDa), and GAPDH (38 kDa). Caspase-3 activity (D) and Snai1 mRNA expression (E) in NMuMG cells infected with Ad-GFP or Ad-FAK cultured on rigid and compliant gels treated with TGF-β1. n = 4 ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. Bars, 25 μm.

casting PDMS (Sylgard 184; Dow Corning, Midland, MI) on a photolithographically generated master. Stamps were immersed for 1 h in 20 μg/ml fibronectin or 20 μg/ml collagen I or 2 h on ice in 140 μg/ml rBM, washed two times in water, and thoroughly dried with nitrogen. Protein was transferred to surface-oxidized PDMS-coated glass coverslips. Stamped coverslips were immersed in 0.2% Pluronic F127 (BASF, Florham Park, NJ) in PBS for 1 h and rinsed in PBS before cell seeding.

Adenovirus production

FAK, FRNK, FAK-Y397F, and green fluorescent protein (GFP) recombinant adenoviruses were constructed as described previously (Pirone *et al.*, 2006) using the AdEasy XL system (Stratagene, Santa Clara, CA) according to manufacturer's instructions. The CD2-FAK adenovirus was generated by C. Henke (University of Minnesota, Minneapolis, MN) and p110-CAAX by L. Romer (Johns Hopkins University, Baltimore, MD). Expression was optimized and verified by Western blot.

Retrovirus production

Retrovirus was produced as described (Ory *et al.*, 1996). Bcl-xL plasmid was obtained from Addgene (Cambridge, MA; plasmid 8790; Cheng *et al.*, 2001).

Caspase-3 activity assays

Caspase-3 activity was determined by EnzChek Caspase-3 Assay Kit #1 (Invitrogen) according to the manufacturer's instructions. Caspase activity was normalized to total DNA content as determined by CyQUANT Cell Proliferation Assay (Invitrogen).

Western blotting

Cells were rinsed in PBS, lysed in ice-cold modified RIPA buffer (25 mM HEPES, 75 mM NaCl, 1% NP-40, 0.25% deoxycholate, 1 mM EDTA, 1 mM NaF, 1× Halt protease and phosphatase inhibitor cocktail [Thermo Scientific, Waltham, MA]), and centrifuged at 14,000 RPM for 10 min at 4°C. Protein concentration was determined by Precision Red Advanced Protein Assay (Cytoskeleton, Denver, CO). A 25-μg amount of protein was separated by denaturing SDS–PAGE, electroblotted onto polyvinylidene fluoride blocked with 5% bovine serum albumin (BSA) or milk in 0.3% Tween-20 in Tris-buffered saline (TBS), immunoblotted with specific antibodies (1:1000), and detected using horseradish peroxidase–conjugated secondary antibodies (1:5000; Jackson ImmunoResearch Laboratories, West Grove, PA) and SuperSignal West Dura (Pierce, Thermo Fisher Scientific, Rockford, IL) as a chemiluminescent substrate. Densitometric analysis was performed using a VersaDoc imaging system with QuantityOne software (Bio-Rad Laboratories, Hercules, CA).

Microscopy, immunofluorescence, and image acquisition

Samples were rinsed in PBS and fixed in 4% paraformaldehyde at RT for 10 min, or, for E-cadherin and ZO-1 staining, cells were fixed in 1:1 acetone/methanol on ice for 20 min. After fixation, samples were permeabilized with 0.5% Triton-X, blocked in 10% goat serum for 1 h at RT, incubated with primary antibodies (1:200) for 1 h at RT, rinsed with PBS, then incubated with Alexa Fluor 488, 555, or 647 secondary antibodies (1:200), Alexa Fluor 488 phalloidin (1:200; Invitrogen), and Hoechst 33342 (1:1000; Invitrogen) for 1 h at RT. Samples were rinsed in PBS, then mounted with Fluormount-G (Electron Microscopy Sciences, Hatfield, PA). Images were acquired at RT using an epifluorescence microscope (model TE200; Nikon, Melville, NY) equipped with Plan Fluor 10×, 0.3 numerical aperture (NA), and Plan Apo 60×, 1.4 NA, oil immersion lenses, Spot camera, and software (Diagnostic Instruments, Sterling Heights, MI). Some image levels were adjusted using Photoshop (Adobe, San Jose, CA).

For pY397 FAK and vinculin immunofluorescence samples were rinsed with ice-cold cytoskeleton extraction buffer (10 mM 1,4-piperazinediethanesulfonic acid, 50 mM NaCl, 150 mM sucrose, 3 mM

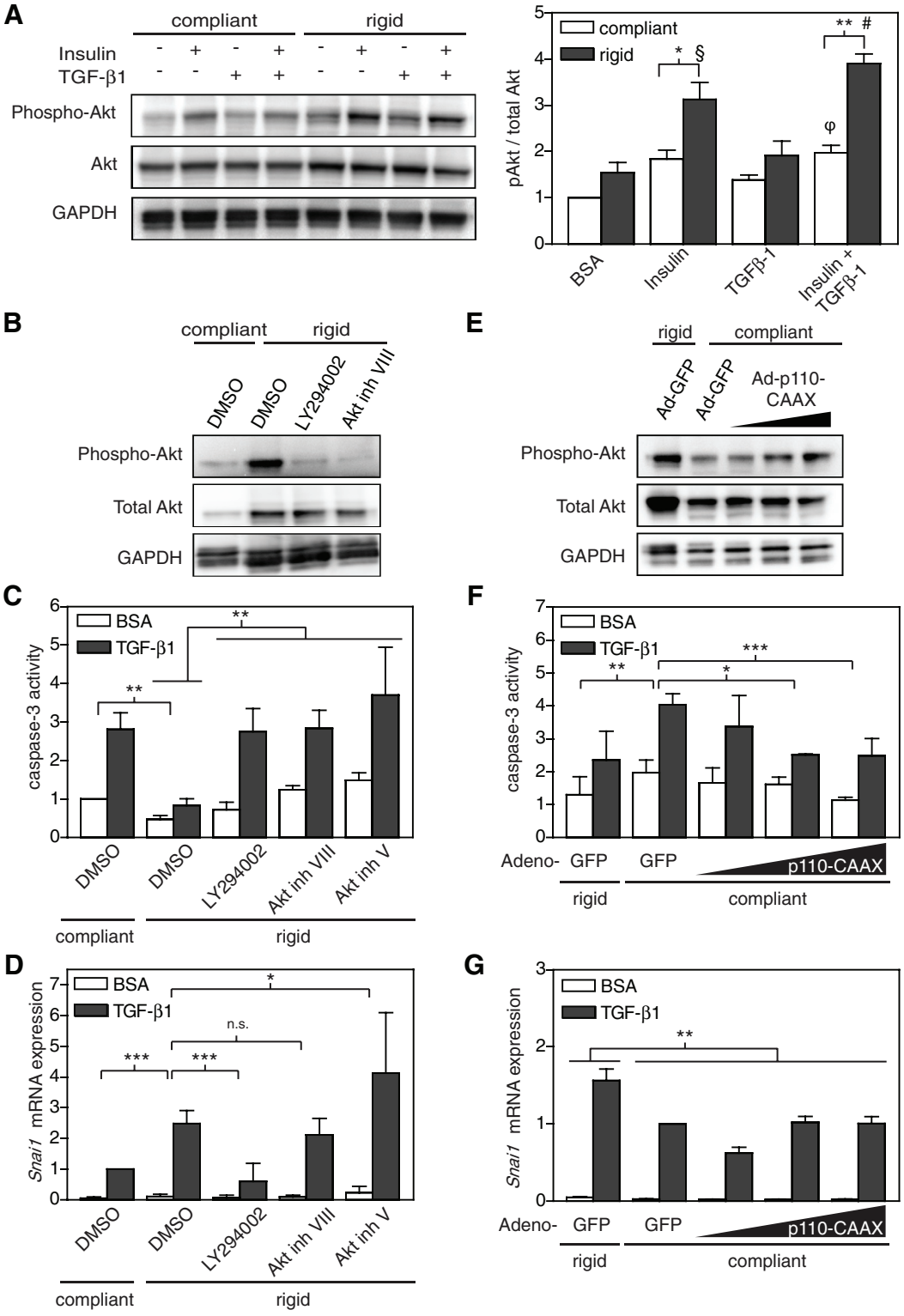

FIGURE 7: Regulation of Akt activity by matrix rigidity in NMuMG cells. (A) Western blot and quantification of phospho-Akt (60 kDa), total Akt (60 kDa), and GAPDH (38 kDa) in cells plated on compliant and rigid polyacrylamide gels. (B–D) Caspase-3 activity and Snai1 mRNA expression in cells treated with DMSO control, 10 μM LY294002, 1 μM Akt inhibitor VIII, or 10 μM Akt inhibitor V. (E–G) Caspase-3 activity and Snai1 mRNA expression in cells infected with Ad-GFP or Ad-p110-CAAX. n = 3 ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; §p < 0.01 rigid + insulin compared with BSA and TGF-β1 conditions; #p < 0.01 rigid + insulin/TGF-β1 compared with all conditions; ϕp < 0.01 compliant + insulin/TGF-β1 compared with compliant BSA condition.

$MgCl_2$, 1× Halt protease, and phosphatase inhibitor cocktail) for 1 min on ice, followed by two 30-s incubations with cytoskeleton buffer plus 0.5% Triton, one rinse with cytoskeleton buffer, and fixation with 4% paraformaldehyde for 10 min at RT. Staining was completed as described. Images were acquired at RT using an epifluorescence microscope (Axiovert 200M; Carl Zeiss MicroImaging,

Jena, Germany) equipped with 63× Plan-Apochromat, 1.4 NA, oil immersion objective, an AxioCam camera, and AxioVision software.

Real-time RT-PCR

Total RNA was isolated using an RNeasy Mini or Micro Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) with 0.5 μg of total RNA per reaction. Quantitative PCR was performed in an ABI 7300 system (Applied BioSystems) using TaqMan gene expression assays according to the manufacturer's instructions. Results were analyzed using the relative quantitation method, and all mRNA expression data were normalized to 18S expression in the corresponding sample and then to the control sample. TaqMan gene expression assays used were as follows: Snai1 (Mm00441533_g1), 18S (Hs99999901_s1).

Luciferase assays

Cells were transfected with p3TP-lux (plasmid 11767; Addgene; Wrana *et al.*, 1992) using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions 24 h before plating. Transfected cells were treated with TGF-β1 for 6 h and then lysed and analyzed using the dual-luciferase reporter assay (Promega, Madison, WI). Luminescence was measured with GloMax 20/20 Luminometer (Promega). Luciferase values were normalized to DNA content as described for caspase-3 activity assays.

Statistical analysis

Data were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA) to perform two-way analysis of variance with Bonferroni posttests to test for significance ($p < 0.05$) between conditions.

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