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.

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 TGFβ1 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 (Derynick 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
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 responses 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
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
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 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.

**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. ECM was microcontact printed onto polydimethylsiloxane (PDMS)–coated coverslips to restrict cell spreading (area of 289 μm²) 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 × 10⁶ cells/cm²) 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...
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. 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 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).

**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 islands (unspread) or large areas (spread) of microcontact-printed FN. Graph of percentage of cells positive for cleaved caspase-3 immunofluorescence on compliant and rigid gels (Supplemental Figure S4, A and B).
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 up-regulation 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

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**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.
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 × 10^4 cells/cm² 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-27632 (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 BSA in 50 mM 8-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), pH 7.5, for 1 h at room temperature (RT; 2 h on ice for BSA), rinsed in double-distilled H2O (ddH2O), incubated with 1% (vol/vol) ethanolamine in 50 mM HEPES, pH 7.5, for 30 min, and rinsed with ddH2O. The gels were sterilized in 5% (vol/vol) isopropanol in PBS for 1 h at RT and rinsed twice 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
casting PDMS (Sylgard 184; Dow Corning, Midland, MI) on a photo-lithographically 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 use.

**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 (Innogen) according to the manufacturer’s instructions. Caspase activity was normalized to total DNA content as determined by CyQUANT Cell Proliferation Assay (Innogen).

**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, 1x 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 secondary antibodies (1:5000; Jackson Immunoresearch Laboratories, West Grove, PA) and detected using horseradish peroxidase–conjugated secondary antibodies (1:5000; Jackson Immunoresearch Laboratories, West Grove, PA). Alexa Fluor 488, 555, or 647 secondary antibodies (1:200), Alexa fluor phosphatase inhibitor cocktail [Thermo Scientific, Waltham, MA]) as a chemiluminescent substrate. Densitometric analysis was performed using a VersaDoc imaging system with QuantityOne software (Bio-Rad Laboratories, Hercules, CA).

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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 10x, 0.3 numerical aperture (NA), and Plan Apo 60x, 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 cytokeratin 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.

MgCl2, 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,
Luciferase assays

Cells were transfected with p3TP-lux (plasmid 11767; Addgene; Wrana et al., 1992) using Lipofectamine 2000 (Invitrogen) according to the 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.

ACKNOWLEDGMENTS

We thank C. Henke, D. Pirone, and L. Romer for generously providing reagents and R. Asoian, R. Wells, and C. Sarkar for helpful discussions. This work was supported in part by grants from the National Institutes of Health (EB00262, HL73305, GM74048) and the Center for Engineering Cells and Regeneration of the University of Pennsylvania. J.L.L. was supported by the National Science Foundation and M.A.W. by a Ruth L. Kirschstein National Research Service Award (F32 AR054219-01).

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Matrix rigidity regulates apoptosis–EMT switch

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Abstract

Tumour cells may utilise changes in the mechanical properties of their microenvironment to promote their survival, growth, and metastatic potential. The extracellular matrix (ECM) is a complex fibrillar network that provides both physical cues and growth factors to cells. The ECM is also a major component of tumours, and its mechanical properties have been shown to influence tumour cell behaviour. In this review, we discuss the role of the ECM in regulating the expression of genes involved in cell proliferation, apoptosis, and EMT. We also consider the potential implications of these findings for the development of new therapeutic strategies.

Introduction

The ECM is a dynamic and heterogeneous network of proteins and carbohydrates that provides structural support and regulates cell function in healthy tissues. In contrast, the ECM of tumours is remodelled and exhibits altered mechanical properties, which can influence tumour cell behaviour and promote metastasis.

Mechanical Cues from the ECM:

1. Matrix rigidity: The rigidity of the ECM can influence cell behaviour, with studies demonstrating that increased matrix rigidity correlates with increased cell proliferation and decreased apoptosis.

2. Fibronectin: Fibronectin is a major ECM component that plays a role in cell adhesion and migration. It has been shown to promote cell survival, proliferation, and metastasis.

3. Laminin: Laminin is another major ECM component that is involved in cell adhesion and migration. It has been shown to promote cell survival, proliferation, and metastasis.

Cell Adhesion and Migration:

The ECM provides a physical framework for cell adhesion and migration, with studies showing that altered ECM properties can lead to changes in cell adhesion and migration patterns. For example, increased matrix rigidity can lead to increased cell adhesion and decreased cell migration.

Stress Fibres:

Cell adhesion and migration are regulated by stress fibres, which are actin stress fibres that provide structural support and allow cells to move within the ECM. Studies have shown that altered ECM properties can lead to changes in stress fibre organization and function.

Matrix Metalloproteinases (MMPs):

MMPs are a family of enzymes that degrade the ECM, allowing cells to move within the ECM and invade surrounding tissues. Studies have shown that altered ECM properties can lead to changes in MMP expression and activity, which can influence cell migration and invasion.

Conclusions

The ECM is a dynamic and heterogeneous network of proteins and carbohydrates that provides both physical cues and growth factors to cells. The ECM is also a major component of tumours, and its mechanical properties have been shown to influence tumour cell behaviour. In this review, we discuss the role of the ECM in regulating the expression of genes involved in cell proliferation, apoptosis, and EMT. We also consider the potential implications of these findings for the development of new therapeutic strategies.