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Regulation of TGF-B1-Induced Apoptosis and Epithelial-Mesenchymal Transition by Matrix Rigidity

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Abstract
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REGULATION OF TGF-β1-INDUCED APOPTOSIS AND EPITHELIAL-MESENCHYMAL TRANSITION BY MATRIX RIGIDITY

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ABSTRACT

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Transforming growth factor-β (TGF-β) plays an important role in several diseases that characteristically involve changes in tissue rigidity, such as cancer and tissue fibrosis. To determine whether matrix rigidity regulates the effects of TGF-β, we examined NMuMG and MDCK epithelial cells cultured on polyacrylamide gels with varying rigidity and treated with TGF-β1. Decreasing matrix rigidity reduced cell spreading and increased TGF-β1-induced apoptosis, while increasing matrix rigidity resulted in epithelial-mesenchymal transition (EMT). To more carefully control cell spreading, microcontact printing was used to restrict ECM area and revealed that reducing cell spreading also increased apoptosis. Apoptosis on compliant substrates was associated with decreased FAK expression, and FAK overexpression rescued cell survival but not EMT. Further investigation revealed manipulations of FAK activity, using pharmacological inhibitors or expression of FAK mutants, did not affect apoptosis or EMT, suggesting that FAK regulates apoptosis through expression but not activity. Additional investigation into the signaling pathways regulated by rigidity revealed a role for PI3K/Akt. We observed increased Akt activity with increasing rigidity, and that PI3K/Akt activity was necessary for cell survival and EMT on rigid substrates. These

findings demonstrate that matrix rigidity regulates a switch in TGF-β-induced cell functions through rigidity-dependent regulation of FAK and PI3K, and suggest that changes in tissue mechanics during disease contribute to the cellular response to TGF-β.

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Chapter 1

Introduction

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 often complicated. This is perhaps best illustrated in the context of tumor progression, though analogous situations can be found in other settings. During early stages of tumorigenesis, TGF-β acts a tumor suppressor. *In vitro*, TGF-β induces growth arrest and apoptosis in most normal epithelial cells (Hannon and Beach, 1994; Pietenpol et al., 1990; Siegel and Massague, 2003). Mice in which the *TGFB1* or *SMAD* genes are disrupted are prone to the development of cancer (Engle et al., 1999; Go et al., 1999; Zhu et al., 1998). Retrospective studies of various human tumor types have also found frequent downregulation or mutations inactivating the TGF-β signaling pathway (Bacman et al., 2007; Kaklamani et al., 2005; Stuelten et al., 2006). In later stages of cancer

progression, however, TGF-β is thought to switch roles and become a tumor promoter through tumor cell autonomous mechanisms and by regulating the tumor stroma. 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; Fukai et al., 2003; Wikstrom et al., 1998), and treatment with TGF-β in animal models results in larger, more metastatic tumors (Fukai et al., 2003; Muraoka et al., 2003; Wikstrom et al., 1998). 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.

While many aspects of the cellular microenvironment change during disease, including soluble factors, cell-cell interactions, and cell-ECM adhesion, changes in the mechanical properties of the microenvironment likely 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 (Dechene et al., 2010; Ebihara et al., 2000; Samani et al., 2007). 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 crosslinking (Ebihara et al., 2000; Levental et al., 2009). *In vitro* studies have revealed that such

changes in matrix rigidity can regulate a number of cellular functions. Cells cultured on compliant substrates decrease proliferation and increase apoptosis as compared to cells on rigid substrates (Klein et al., 2009; Wang et al., 2000). Matrix rigidity modulates focal adhesion maturation, cell spreading, actin stress fiber formation, and cell motility (Lo et al., 2000; Pelham and Wang, 1997; Yeung et al., 2005). 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 (Alcaraz et al., 2008; Engler et al., 2006; Li et al., 2007; Paszek et al., 2005; Vailhe et al., 1997). Because matrix rigidity can regulate a number of cell functions, including functions 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 non-transformed epithelial cells, TGF-β induces programmed cell death, or apoptosis, and induction of apoptosis 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 and is characterized by dissolution of epithelial cell-cell junctions, remodeling of cell-matrix adhesion, and increased motility (Lee et al., 2006). Normal murine mammary gland epithelial cells (NMuMG) and Madin-Darby canine kidney epithelial cells (MDCK), well established *in vitro* model systems of EMT (Miettinen et al., 1994), were used to determine whether these effects

exist even in a non-cancerous genetic background. We found that substrate rigidity controlled TGF-β1-induced cell functions - epithelial cells cultured on compliant substrates underwent apoptosis when treated with TGF-β1, while on more rigid substrates, TGF-β1 induced EMT. Mechanistic studies revealed cells cultured on compliant gels had decreased Akt activity, and modulation of the PI3K/Akt pathway could regulate the switch between EMT and apoptosis.

Chapter 2

Background

2.1 TGF-β

TGF-β was first identified as a cytokine that induces anchorage independent growth in fibroblasts (Assoian et al., 1984; de Larco and Todaro, 1978). The three mammalian TGF-β isoforms, TGF-β1, 2, and 3, are a part of the larger TGF-β superfamily which has over 30 members, including the TGF-βs, bone morphogenetic proteins (BMP), growth and differentiation factors (GDF), Activins and Nodal. The TGF-β superfamily regulates a myriad of biological processes from embryonic development to tissue homeostasis, and misregulation of the TGF-β signaling pathway results in a number of diseases. TGF-β1 is upregulated during tumorigenesis to greater extent than other isoforms, and thus has been the focus of the most cancer research to date (Derynck et al., 1987; Dickson et al., 1987). This work focuses on the microenvironmental regulation of TGF-β1-induced cell functions, motivated by disease states associated with changes in tissue stiffness, such as cancer and fibrosis.

2.1.1 Dual role of TGF-β during cancer progression

TGF-β has been shown to both suppress and promote cancer progression (Bierie and Moses, 2006). This dual role is likely due to the myriad of cell functions that TGF-

β regulates. TGF-β acts a tumor suppressor through inhibition of proliferation and induction of apoptosis. TGF-β is a potent inhibitor of cell proliferation in many cell types, including epithelial, endothelial, neural, hematopoietic, and some mesenchymal cells. Proliferation is inhibited by TGF-β in several ways, including transcriptional upregulation of the cyclin-dependent kinase inhibitors $p21^{CIP1/WAF1}$ and $p15^{INK4b}$, downregulation of cdc25A, transcriptional repression of c-myc, and induced protein phosphatase 2A (PP2A) association with and inactivation of p70 S6K (Datto et al., 1995; Hannon and Beach, 1994; Iavarone and Massague, 1997; Petritsch et al., 2000; Pietenpol et al., 1990). TGF-β also suppresses tumor formation through induction of apoptosis. TGF-β induces apoptosis through a variety of mechanisms, including activation of caspase-8, transcriptional upregulation of death associated protein kinase, downregulation of Bcl-xL, upregulation of Bim and Bmf, and activation of JNK and p38 (Chipuk et al., 2001; Kim et al., 2004; Perlman et al., 2001; Ramjaun et al., 2007; Yamashita et al., 2008). These anti-tumorigenic effects are also observed *in vivo*. Mice in which the *TGFB1* or *SMAD* genes are disrupted are prone to the development of cancer (Engle et al., 1999; Zhu et al., 1998). Expression of dominant-negative TβRII increased propensity of lung, mammary, and skin tumors (Bottinger et al., 1997; Go et al., 1999). TβRII has also been found to be mutated in both sporadic and inherited colon cancer, and restoration of the receptor can reverse malignant transformation (Markowitz et al., 1995; Wang et al., 1995).

While TGF-β can act as a tumor suppressor through inhibition of proliferation and induction of apoptosis, TGF-β also regulates several functions that promote cancer

progression including EMT, ECM production, and cell motility (Massague, 2008). EMT is characterized by a loss of epithelial polarity, disassembly of epithelial cell-cell adhesions, cytoskeletal reorganization, ECM remodeling, and increased migration (Thiery et al., 2009). While EMT is a normal physiological process necessary for development, inappropriate induction of EMT is associated with tumor progression and fibrosis (Lee et al., 2006). The ability of TGF-β to enhance cell migration is especially important for metastasis, and several *in vitro* studies have shown that TGF-β treatment stimulates motility and invasiveness in non-tumorigenic cell lines transfected with the oncogene ErbB2 (Seton-Rogers et al., 2004; Ueda et al., 2004). Many *in vivo* studies also support a role for TGF-β as a tumor promoter. Increased levels of TGF-β1, through exogenous administration or by selection for cells overexpressing TGF-β1, facilitated tumor formation of MCF7 breast cancer cells implanted in nude mice (Arteaga et al., 1993). Overexpression of activated TβRI or active TGF-β1 accelerated metastases from neu-induced primary mammary tumors in transgenic mice (Muraoka et al., 2003; Siegel et al., 2003).

Several studies have also captured both the tumor suppressor and promoter functions of TGF-β. In a report by Cui and colleagues in 1996, TGF-β1 expression in keratinocytes in transgenic mice showed a biphasic action during long term chemical carcinogenesis treatment, with TGF-β1 inhibiting benign tumor outgrowth but enhancing malignant conversion (Cui et al., 1996). Other reports have shown a similar biphasic action, with an exogenous tumor stimulant such as a chemical carcinogen or oncogene cooperating with TGF-β to increase tumor invasion and metastasis (Ao et al., 2006; Bandyopadhyay et al., 1999; Hojo et al., 1999; Oft et al., 1998; Oft et al., 1996).

It is not well understood how TGF-β can switch functions from tumor suppressor to tumor promoter during cancer progression. The main hypothesis revolves around evasion of TGF-β's cytostatic effects through mutations or deletions in the TGF-β signaling pathway (Massague, 2008). However, because TGF-β can control so many functions, and often in the same genetic background, we postulate that the cellular microenvironment provides another level of regulation for TGF-β-induced cell functions.

2.1.2 TGF-β signaling pathways

TGF-β is secreted from cells in a large latent complex with a C-terminal latency TGF-β binding protein and a non-covalently bound latency associated peptide (Annes et al., 2003). The latency TGF-β binding protein allows TGF-β to bind to extracellular matrix (ECM) components such as fibrillin and fibronectin (FN) (Unsold et al., 2001). This large latent complex can be activated in several ways including proteolysis, interaction with other proteins, and even mechanical force (Annes et al., 2002; Ge and Greenspan, 2006; Wipff et al., 2007). Activated TGF-β binds to TGF-β receptor II (TβRII) which recruits and phosphorylates TβRI (Wrana, J.L. et al., 1994). The TβR1 kinase then phosphorylates Smad2 and Smad3 which associate with Smad4, translocate to the nucleus, and initiate transcription (Lagna et al., 1996; Nakao et al., 1997; Yingling et al., 1997) (Fig. 2.1).

The Smad pathway is considered the canonical TGF-β signaling cascade, however several other Smad-independent signaling pathways have also been implicated in the action of TGF-β, including ERK, p38, JNK, PI3K, and RhoGTPases (Zhang,

2009). TGF-β activation of ERK has been reported in a number of cell types, including epithelial cells and fibroblasts (Hartsough and Mulder, 1995; Mucsi et al., 1996). More recent studies have shown direct binding and phosphorylation of Shc to TβRI and II (Galliher and Schiemann, 2007; Lee et al., 2007), which allows recruitment of Grb2/Sos, activation of Ras and, further downstream, activation of Erk (Fig. 2.1). Two other MAPKs, JNK and p38, have also been shown to be directly activated by TGF-β (Bhowmick et al., 2001b; Frey and Mulder, 1997; Hocevar et al., 1999; Yu et al., 2002). TGF-β stimulation of JNK and p38 is mediated by TRAF6 interacting directly with TβRI and II (Sorrentino et al., 2008; Yamashita et al., 2008). Binding of TRAF6 to the receptor complex promotes association and activation of TAK1 which leads to downstream activation of JNK and p38 (Fig. 2.1). Several other pathways in addition to MAPKs have also been connected to TGF-β signal transduction, including the RhoGTPases: RhoA (Bhowmick et al., 2001a; Edlund et al., 2002), Cdc42 (Barrios-Rodiles et al., 2005; Wilkes et al., 2003), and Rac (Hubchak et al., 2009), although it is still unclear exactly how TGF-β receptor complex regulates these pathways. Finally, the PI3K/Akt pathway can also be regulated by TGF-β (Bakin et al., 2000; Shin et al., 2001; Vinals and Pouyssegur, 2001; Wilkes et al., 2005). Further studies revealed an indirect interaction of p85, the regulatory subunit of PI3K, with TβRI and II, and that activity of TβRI was necessary for PI3K activation (Yi et al., 2005) (Fig. 2.1).

2.1.3 Regulation of apoptosis by TGF-β

TGF-β has long been recognized as potent stimulus of apoptosis, or programmed cell

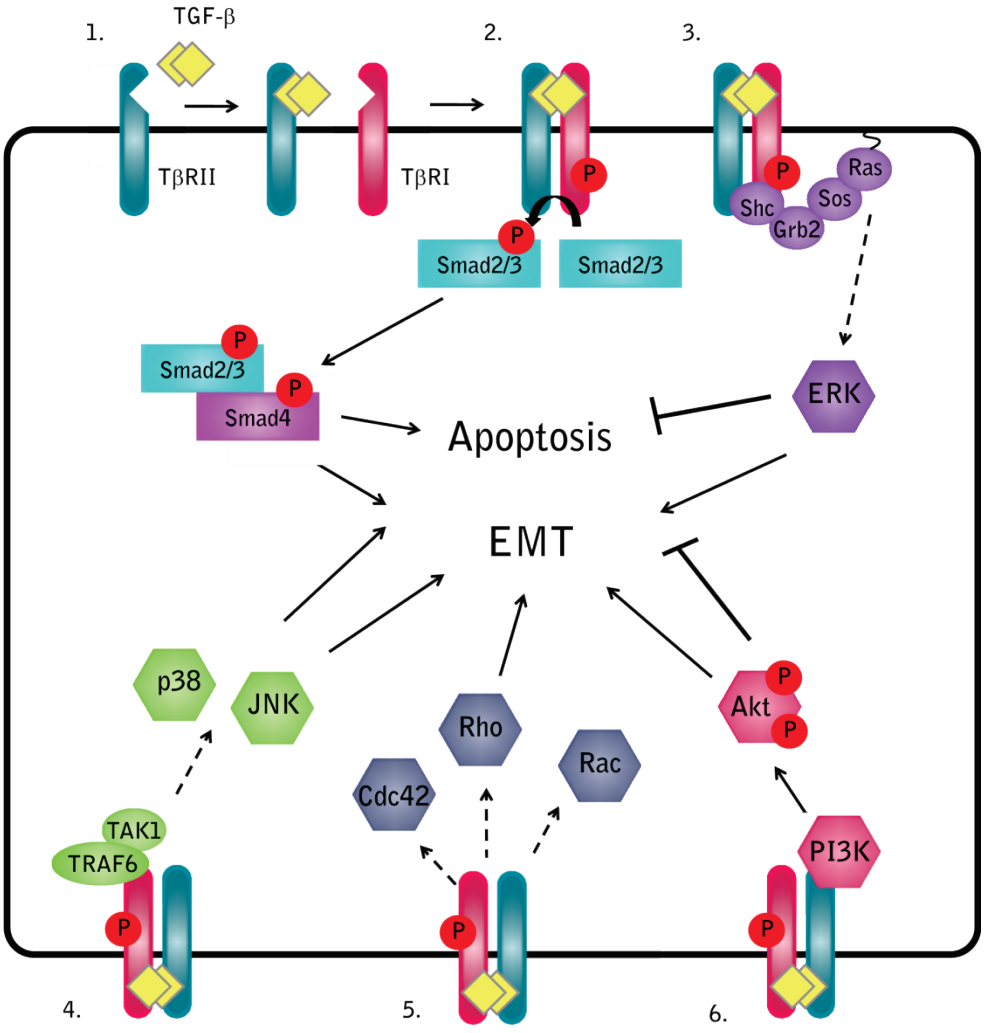

Figure 2.1 Illustration of TGF-β1 signaling.

1. TGF-β binds to TβRII, and facilitates TβRI binding and activation. 2. The activated receptor complex phosphorylates Smad2/3, which allows complex formation with Smad4 and nuclear translocation to induce apoptosis and EMT. 3. Shc binds the receptor complex, allowing binding of Grb2, Sos, activation of Ras, and further downstream Erk. Erk promotes EMT and inhibits apoptosis. 4. TRAF6 binds activated TβRI, allowing TAK1 binding and activation, leading to downstream activation of JNK and p38. JNK and p38 activity are important for induction of EMT and apoptosis. 5. The activated receptor complex activates the RhoGTPases, through still undiscovered mechanisms, and influence EMT. 6. The p85 subunit interacts indirectly with the TβRII, facilitating activation of PI3K, then Akt. PI3K/Akt activity is important for EMT and inhibits apoptosis.

death, in a number of cell types (Lin and Chou, 1992; Oberhammer et al., 1992; Rotello et al., 1991; Yanagihara and Tsumuraya, 1992). Induction of apoptosis occurs both through Smad dependent and independent signaling mechanisms (Fig. 2.1). TGF-β activation of the Smad pathway can transcriptionally upregulate several apoptotic related factors, such as Fas, AP1, and death associated protein kinase (Herzer et al., 2008; Jang et al., 2002; Kim et al., 2004; Yamamura et al., 2000). Upstream of transcription, several studies have shown that sequestering of Smad3 by direct binding of Akt prevents Smad nuclear translocation and apoptosis (Conery et al., 2004; Remy et al., 2004). TGF-β/Smad signaling also regulates expression of Bcl-2 family members, including upregulation of pro-apoptototic proteins such as Bax, Bim, and Bmf (Motyl et al., 1998; Ramjaun et al., 2007; Yano et al., 2006), as well as downregulation of anti-apoptotic factors Bcl-2 and Bcl-xL (Chipuk et al., 2001; Francis et al., 2000; Motyl et al., 1998). While the Smad proteins play an important role in TGF-β-induced apoptosis, there is also evidence that Smads are not an absolute requirement for apoptosis. As mentioned earlier, TGF-β can activate JNK and p38 to regulate apoptosis independent of Smad signaling (Perlman et al., 2001; Yamashita et al., 2008). Additionally, a novel septin related protein was discovered, apoptosis-related protein in the TGF-β signaling pathway (ARTS), that also participates in TGF-β mediated apoptosis (Gottfried et al., 2004; Larisch-Bloch et al., 2000). The many studies summarized here highlight the variety of ways TGF-β stimulates apoptosis, and motivated the work in Chapter 6 to more carefully dissect how matrix rigidity might regulate TGF-β-induced apoptosis.

2.1.4 Regulation of EMT by TGF-β

Epithelial-mesenchymal transition is the process whereby epithelial cells lose their epithelial characteristics and transdifferentiate to a mesenchymal phenotype. This transition includes loss of apico-basal polarity, disruption of cell-cell and cell-matrix adhesions, degradation of the surrounding ECM, and cytoskeletal reorganization resulting in a more migratory phenotype and cell scattering. EMT is described by a set of characteristics, but there is no standard definition that clearly demarcates EMT. These characteristics include: a change in morphology from cuboidal to more elongated and fibroblastic, a switch from cortical actin to pronounced actin stress fibers, a decrease in epithelial markers, such as E-cadherin and ZO-1, a gain of mesenchymal markers like N-cadherin, α-sma, vimentin, and Snail, and an increase in motility. EMT is a critical process during many developmental steps, including gastrulation, neural crest formation, palatal growth, and heart valve formation (Mercado-Pimentel and Runyan, 2007; Nawshad et al., 2004; Solnica-Krezel, 2005; Tucker, 2004). After development is complete, epithelia serve specialized functions and do not typically differentiate, except during wound healing or in disease contexts such as fibrosis or cancer (Thiery et al., 2009). EMT is hypothesized to contribute to tumor cell invasion and metastasis, and gene expression associated with EMT is often associated with poor prognosis in a wide array of cancers, including thyroid, breast, pancreatic, and colorectal cancer (Thiery et al., 2009). Additionally, recent studies have observed morphological evidence of EMT at the invasive fronts of colorectal and mammary tumors (Prall, 2007; Wyckoff et al., 2007). Elucidating the mechanisms regulating EMT

and the associated cell functions will be an important step in understanding tumor progression and metastasis.

A number of signaling mechanisms have been described to regulate EMT, but the literature can be confusing and contradictory, likely due to the various disparate events characteristic of EMT. A number of growth factors have been associated with EMT induction, including EGF, TGF-α, FGF, and HGF, however it remains controversial whether these induce a true EMT or just cell scattering. TGF-β, conversely, has been convincingly demonstrated to control EMT during development and in several in vitro models (Boyer et al., 2000), and we will focus on the signaling downstream of TGF-β here (Fig. 2.1). As mentioned earlier, one of the ways TGF-β signals are transduced to the nucleus and initiate transcription is through the Smad pathway, and, not surprisingly, the Smad pathway has been found to be important for TGF-β-induced EMT (Piek et al., 1999). For example, when TβRI is mutated to prevent Smad binding but still retain MAPK signaling, TGF-β fails to induce EMT (Itoh et al., 2003; Yu et al., 2002). Similarly, disruption of Smad2 or Smad3 blocks EMT in both *in vitro* and *in vivo* studies (Saika et al., 2004; Sato et al., 2003; Valcourt et al., 2005), and later studies uncovered that Smad 3 upregulates transcription of Snail, a transcription factor that represses E-cadherin and promotes EMT (Cho et al., 2007; Vincent et al., 2009). While a number of studies have found Smad signaling to be indispensible for TGF-β-induced EMT, contradictory evidence also exists that EMT can occur independently of the Smad pathway. For example, in one study, siRNA knockdown of Smad4 expression did not affect EMT, and other studies have shown upregulation of

Snail is independent of Smad4 (Levy and Hill, 2005; Medici et al., 2006; Peinado et al., 2003).

As highlighted before, TGF-β also regulates several Smad-independent pathways, and many of these pathways were discovered because of their role in regulating EMT. Several *in vitro* studies found TGF-β-induced RhoA activity was necessary for EMT (Bhowmick et al., 2001a; Cho and Yoo, 2007; Masszi et al., 2003), as well as *in vivo* during embryonic chick heart development (Tavares et al., 2006). In another report, TGF-β downregulation of RhoA at tight junctions was necessary to facilitate their disassembly, and suggests that TGF-β may spatially regulate Rho during EMT (Ozdamar et al., 2005). In addition to Rho, a number of studies have demonstrated a synergistic effect on EMT between TGF-β and MAPK signaling (Grande et al., 2002; Janda et al., 2002; Uttamsingh et al., 2008), and that blocking Erk activity inhibits EMT (Xie et al., 2004; Zuo and Chen, 2009). Interestingly, JNK and p38 MAPKs, which are also important for TGF-β-induced apoptosis, have been implicated during EMT (Alcorn et al., 2008; Bakin et al., 2002; Liu et al., 2008; van der Velden et al., 2010; Yu et al., 2002). Finally, TGF-β stimulates the PI3K/Akt pathway, and PI3K/Akt activity was found to be necessary for EMT in both mammary and renal epithelial cell systems (Bakin et al., 2000; Bhowmick et al., 2001b; Kattla et al., 2008). TGF-β activation of Akt is often observed concurrently with activation of ERK (Bakin et al., 2000; Medici et al., 2006; Peinado et al., 2003), and this activity is important for Snail expression (Peinado et al., 2003). In squamous carcinoma cells, expression of a constitutively active Akt stimulated EMT (Grille et al., 2003) while inhibition of Akt activity induced reversion of EMT, or MET, mesenchymal-epithelial transition (Hong et al., 2009).

Whether these manipulations will also apply to non-tumorigenic cells or other cancers is still unknown, however Akt is often dysregulated during cancer and delineating its role during EMT will likely yield vital insights into the role of TGF-β and EMT during tumor progression.

2.2 The regulation of cell function by matrix rigidity

Mechanical properties, by definition, describe the properties of a material under stress, and these properties of a tissue or cell culture substrate can be measured by a variety of methods, including rheology, confined compression, tensile testing, microindentation, and atomic force microscopy. The reported values for a material's mechanical properties are influenced by a number of factors, including sample preparation (hydration, anisotropy, polymerization conditions) and test parameters (temperature, dynamic vs static, boundary conditions, length scale, etc), thus measurements made by different methods and different labs often do not completely agree. While not numerically equivalent, these measurements are usually in relative agreement, for example that adipose tissue is less rigid than muscle which is less rigid than bone (Fig. 2.2). Additionally, tissue stiffness can also change within the same type of tissue during disease states, such as fibrosis (Dechene et al., 2010; Ebihara et al., 2000), liver cirrhosis (Yeh et al., 2002), cancer (Lyshchik et al., 2005; Samani et al., 2007), and atheroscelrosis (Wang et al., 2008). In this work, references to a modulus or rigidity, refer to the Young's modulus (E), or the elastic tensile modulus.

2.2.1 Changes in tissue rigidity during tumorigenesis

A common theme from the literature relating to TGF-β's role in cancer is that the oncogenic potential of TGF-β increases during tumor progression, indicating a

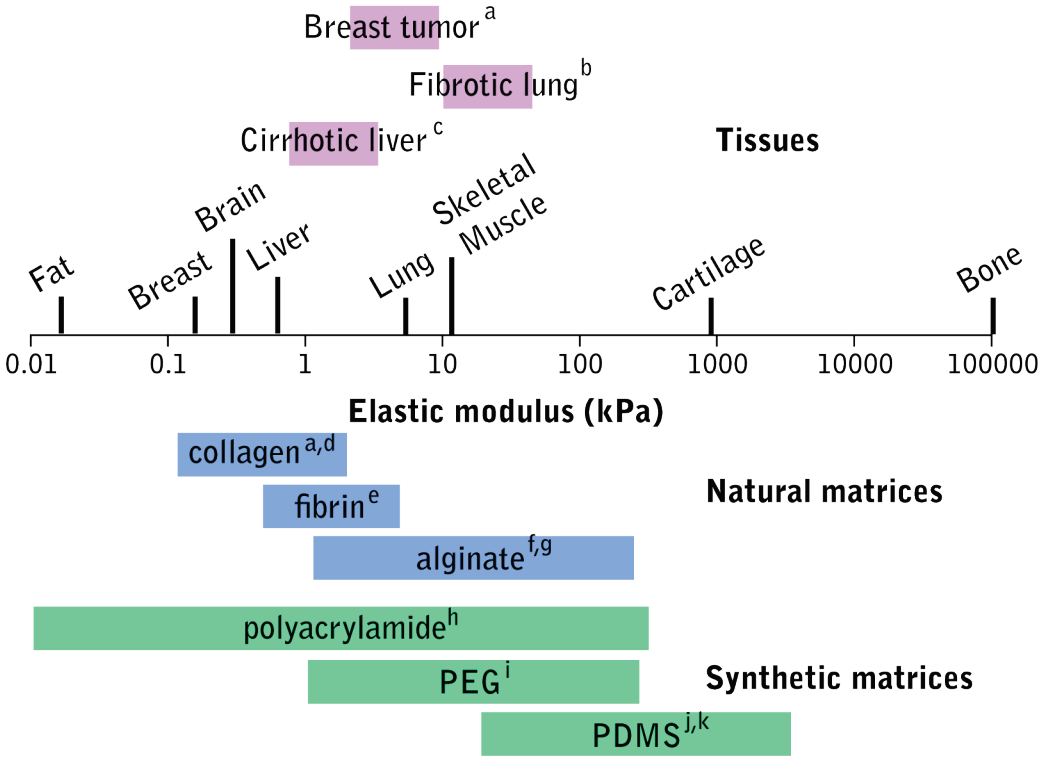

Figure 2.2 Rigidity of tissues, natural, and synthetic matrices.

Tissue values adapted from (Levental et al., 2007). Disease tissue values: a (Paszek et al., 2005), b (Ebihara et al., 2000), c (Yeh et al., 2002). Natural matrices: d (Gehler et al., 2009), e (Georges et al., 2006), f (Wang et al., 2003a), g (West et al., 2007). Synthetic matrices: h (Yeung et al., 2005), i (Nemir and West, 2010), j (Fuard et al., 2008), k (Teixeira et al., 2009).

switch occurs during tumorigenesis that allows cells to escape inhibition of proliferation and apoptosis and become more migratory and metastatic. Accumulation of genetic mutations in tumor cells is one component contributing to disease progression, however there are also dramatic changes occurring in the stromal compartment surrounding these cells. In addition to unchecked proliferation of tumor cells, there is an influx of activated fibroblasts, immune cells, and endothelial cells (Tlsty, 2001). This increase in cells leads to hypoxia and an altered and often elevated secretion of various growth factors (Hockel and Vaupel, 2001). There is also increased deposition and reorganization of the ECM (Burns-Cox et al., 2001; Kauppila et al., 1998; Strongin, 2006; Zhu et al., 1995). Leaky blood and lymphatic vessels lead to increased interstitial pressure (Padera et al., 2004). The combination of these processes increases overall tissue rigidity, a hallmark of cancer which has long been used to detect cancer through physical palpation and several imaging modalities (Khaled et al., 2004; Manduca et al., 2001; Parker and Lerner, 1992). It is well accepted that tumors become stiffer during disease progression, however how these changes in the mechanical properties of a tissue regulate tumorigenesis and TGF-β signaling remains unclear.

2.2.2 Regulation of cell function by matrix rigidity

Numerous recent studies in the past decade have demonstrated that matrix rigidity regulates cell function, including proliferation, apoptosis, and differentiation. These studies investigated a number of different cell systems and utilized both natural ECM components and synthetic substrates to vary matrix rigidity. For example, differentiation of human mesenchymal stem cells can be directed by substrate rigidity, whereby more compliant substrates promoter neuronal or adipogenic fate, and rigid

substrates promote osteogenesis (Engler et al., 2006; Fu et al., 2010). Substrate stiffness also regulates neuronal function, including differentiation of neural progenitors (Seidlits et al., 2010; Teixeira et al., 2009) , neurite extension (Balgude et al., 2001), and selective growth of neurons and astrocytes in a mixed population (Georges et al., 2006). Several studies have shown a role for matrix rigidity in regulation of capillary network formation using fibrin, poly(ethylene glycol), and self assembling peptide gels (Ghajar et al., 2008; Miller et al., 2010; Sieminski et al., 2007; Vailhe et al., 1997). Fibroblast biology has been extensively investigated. Compliant substrates decrease proliferation and increase apoptosis as compared to cells on rigid substrates (Klein et al., 2009; Park et al., 2010; Wang et al., 2000; Wang et al., 2007). Additionally, matrix rigidity also modulates focal adhesion maturation, cell spreading, actin stress fibers formation, and cell motility in fibroblasts (Fringer and Grinnell, 2001; Lo et al., 2000; Pelham and Wang, 1997; Yeung et al., 2005). Epithelial cell proliferation (Klein et al., 2009), apoptosis (Wang et al., 2007), and morphogenesis are regulated by matrix rigidity (Alcaraz et al., 2008; Paszek et al., 2005; Wozniak et al., 2003). Additionally a number of other cell systems are responsive to matrix rigidity, including chondrocytes (Genes et al., 2004; Klein et al., 2010), hepatocytes (Godoy et al., 2009; Li et al., 2007), and myoblasts (Boontheekul et al., 2007; Engler et al., 2004). A common theme among this literature is that mimicking tissue rigidity, either the native or disease states, often reveals important insights into the regulation of cell function.

2.3 Methods to control rigidity

Tissues within the body have different mechanical properties. Intuitively one would surmise that these properties might regulate cell function, and, the studies

summarized above demonstrate that cell function is indeed regulated by matrix rigidity. In order to study the regulation of cell function by matrix rigidity, researchers have developed a number of ways to modulate the mechanical properties of the microenvironment. These methods include natural matrices, such as collagen and fibrin, as well as synthetic materials like polyacrylamide and poly(ethylene glycol). Each method has pros and cons, and observations using each method must be interpreted in light of these. Here we highlight some of the most common methods and the advantages and drawbacks associated with each method, with a focus on the polyacrylamide system used in this work.

2.3.1 Natural matrices

A number of different natural matrices have been utilized to study the effects of substrate rigidity on cell function. One of the most widely used is type I collagen, which is commercially available and relatively inexpensive. Additionally, collagen I is the most abundant protein in vertebrate animals, well tolerated for in vivo studies, and highly adhesive for many cell types. Collagen I is commonly isolated from rat tail and solubilized in acetic acid, and a gel can be formed by increasing the pH and temperature of the collagen solution. Polymerization conditions, such as pH, temperature, collagen concentration, and fibril alignment, can affect the mechanical properties of the gel formed (Barocas et al., 1998; Roeder et al., 2002; Roeder et al., 2009). Changing the concentration of collagen from 1 to 5 mg/ml changes the elastic modulus of the gel from 100 to 800 Pa as measured by rheology (Gehler et al., 2009) (Fig. 2.2). The collagen matrix can also be stiffened after polymerization by glycation or enzymatic processes (Elbjeirami et al., 2003; Girton et al., 2000). In addition to changing the

properties of the matrix itself, the boundary conditions of the collagen gel can be manipulated to change the rigidity that the cells "feel". In 2D, cells can be cultured on a thick layer of collagen gel, for a compliant matrix, or on collagen adsorbed to glass from a very dilute solution, to simulate a rigid matrix (Godoy et al., 2009; Wang et al., 2003b). Finally, the collagen gel can also be released from the culture dish, reducing the isometric tension within the gel (Grinnell, 2000; Wozniak et al., 2003).

Fibrin gels are also a popular natural matrix used for in vitro rigidity studies, including for neuronal growth and angiogenesis assays (Georges et al., 2006; Ghajar et al., 2008; Vailhe et al., 1997). Fibrin gels use the natural clotting matrix, whereby fibrinogen monomers polymerize with the addition of thrombin. Fibrin gel architecture and rigidity, similar to collagen, can also be controlled by gelation conditions and monomer concentration, with a modulus range from 0.6 to 6 kPa (Blomback and Bark, 2004; Georges et al., 2006) (Fig. 2.2). Fibrin and collagen are not linearly elastic materials, and stiffen with increasing strain (Winer et al., 2009b). Culturing cells at high densities in these materials can change their mechanical properties, and should be taken into account during experimental design and interpretation of data.

Alginates are polysaccharides originally isolated from brown algae that can be ionically or chemically crosslinked to form hydrogels (Augst et al., 2006). Alginate is naturally protein resistant (Smetana, 1993), which can be an advantage but also requires covalently coupling adhesive peptides to the alginate to facilitate cell adhesion (Rowley et al., 1999). The rigidity of alginate can be controlled in variety of ways, including the molecular weight and composition of the polysaccharides, the stoichiometry of the alginate with the chelating cation, and the gelling temperature (Augst et al., 2006),

resulting in a modulus range from 1 to over 200 kPa (Wang et al., 2003a; West et al., 2007) (Fig. 2.2). Alginate gels have been used to study stiffness in a variety of settings, including in vitro follicle development, myoblast phenotype, stem cell proliferation, and chondrocyte adhesion (Boontheekul et al., 2007; Genes et al., 2004; Hsiong et al., 2008; West et al., 2007).

2.3.2 Synthetic matrices

Purified, biologically derived materials, such as collagen and fibrin, have an intrinsic amount of biochemical and biophysical variability due to the inherent variability between animals and preparations. This variability leads to inconsistencies between experiments, as well as a high degree of heterogeneity within single gels. Additionally, the dynamic range of elastic moduli that can be reasonably achieved with these systems is limited by biochemical and biophysical constraints of these unique macromolecules. Therefore, although these materials have proven to be useful for clarifying the general influence of matrix on cell and tissue phenotypes, they are not as tractable for defining precise molecular mechanisms mediating mechanotransduction. To more carefully elucidate these mechanisms, synthetic materials have been developed that isolate changes in rigidity from other confounding factors such changes in ligand density and matrix remodeling.

First introduced by Pelham and Wang in 1997, polyacrylamide (PA) hydrogels have been widely adopted to study the effect of substrate rigidity on cell function for several reasons. First, the materials to fabricate PA gels are commercially available, inexpensive, and familiar to biologists, as polyacrylamide has long been utilized for protein separation by gel electrophoresis. PA gel rigidity is well characterized and

easily controlled by varying the concentration of the bis-acrylamide and acrylamide monomers, with an elastic modulus range from 0.1 to 150 kPa (Pelham and Wang, 1997; Yeung et al., 2005). Additionally, PA is clear and nonfluorescent, making it an ideal substrate for fixed and live microscopic imaging. Limitations of the PA gel system include the inability to embed cells or implant the material *in vivo* due to the toxicity of acrylamide. PA is also resistant to protein adsorption, so proteins must be chemically conjugated to the surface, which allows for careful control of ligand density separate from rigidity, but the protein conjugation process can be time intensive and problematic, as described below.

Several methods have been developed to chemically couple proteins to the surface of PA. The original method by Pelham and Wang, described protein conjugation to the PA gels using sulfosuccinimidyl-6-(40-azido-20-nitrophenylamino) hexanoate, known as sulfo-SANPAH. Polymerization of the acrylamide and bis-acrylamide monomers is initiated using a standard ammonium persulfate/TEMED free radical-dependent polymerization reaction, and the solution is dropped onto a coverslip activated with aminopropyltrimethoxysilane and gluteraldehyde. After polymerization, the sulfo-SANPAH crosslinker is layered on top of the gel and crosslinked to the gel using exposure to UV light. This photoactivation step is usually repeated twice, and then the gels are incubated with a protein solution to allow the sulfosuccinimidyl group at the end of sulfo-SANPAH to react with the primary amines in the protein. The sulfo-SANPAH method is still used by a number of labs, although limitations with the crosslinker, including limited solubility and stability, short shelf life, and expense, can make it difficult to work with. Methods have been also been developed that utilize other

crosslinking reagents. For example, in carbodiimide-mediated cross-linking, EDC, (1-ethyl-3-(3-dimethylamino-propyl))carbodiimide-HCl, is mixed into the acrylamide solution, and after polymerization incubated overnight to conjugate the protein to the surface (Beningo et al., 2002). N-succinimidyl ester of acrylaminohexanoic acid (N6) can also be mixed into the acrylamide mixture before polymerization, and one end of the molecule reacts with the acrylamide and the other with primary amines, similar to sulfo-SANPAH (Johnson et al., 2007; Reinhart-King et al., 2005). N6 is commercially available but sold in milligram quantities and prohibitively expensive, so it must be synthesized in the lab, a lengthy two to three day process that while not expensive does require an extensive chemistry background (Pless et al., 1983). A similar crosslinking reagent to the N6, N-hydroxysuccinimide ester (NHS ester) also incorporates the crosslinking reagent during polymerization but is commercially available and inexpensive. Unlike the N6 and EDC methods mentioned above, the acrylamide solution is overlaid with an immiscible toluene solution containing the NHS ester during polymerization (Fig. 2.3). The NHS ester copolymerizes with the surface of the PA gel, and then is reactive with the primary amines, allowing protein conjugation. While the NHS ester method is less expensive and time intensive than the sulfo-SANPAH method mentioned above, addition of the toluene layer can affect gel polymerization and requires practice to produce homogeneous surfaces on very compliant (< 1 kPa) gels. In this work, we have used the NHS ester to conjugate proteins to the surface of the PA gels.

Similar to PA gels, poly(ethylene glycol) (PEG) hydrogels resist protein adsorption and allow for independent control of matrix rigidity and ligand density.

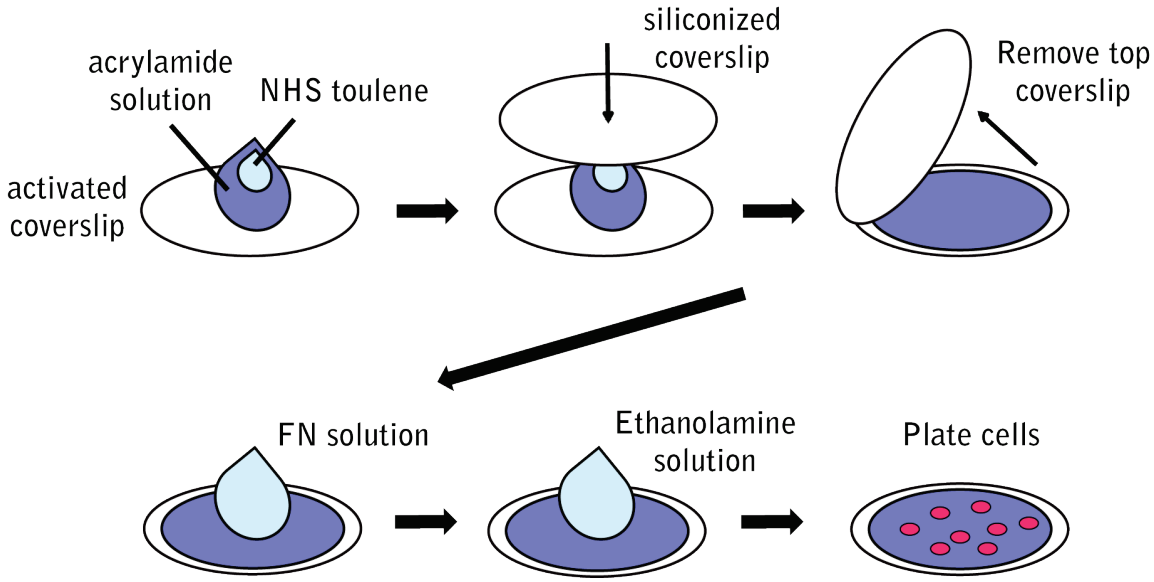

Figure 2.3 Polyacrylamide gel preparation.

The polyacrylamide solution is placed on an activated coverslip and overlaid with the NHS crosslinker dissolved in toluene. A siliconized coverslip is placed on top of the acrylamide and toluene solutions. After the acrylamide solution is fully polymerized, the siliconized coverslip is removed. The polyacrylamide gel is then incubated with the protein solution, then the ethanolamine solution, and finally plated with cells.

However, PEG gels permit 3D encapsulation of cells and *in vivo* implantation. Additionally, different peptide sequences can be covalently incorporated to control a number of parameters including cell adhesion and degradability (Nuttelman et al., 2008). The rigidity of PEG hydrogels can be controlled by varying the molecular weight and concentration of the macromer. PEG gels are not as compliant as natural matrices or PA gels, with a lower limit for the elastic modulus around 1 kPa (Nemir and West, 2010) (Fig. 2.2). Substrate rigidity has been studied using PEG hydrogels in a number of cell systems, including muscle stems cells, neurite extension, angiogenesis assays, and smooth muscle cells (Gilbert et al., 2010; Gunn et al., 2005; Miller et al., 2010; Peyton et al., 2006). PEG gels provide an attractive alternative to PA gels and will likely gain in popularity over the coming years due to the ability to study rigidity in 3D and *in vivo*.

Poly(dimethyl siloxane) (PDMS), a silicone elastomer used widely for soft lithography, has also been used as to study the effects of substrate rigidity (Fuard et al., 2008; Gray et al., 2003; Park et al., 2010; Teixeira et al., 2009). Although hydrophobic, PDMS can be used as cell culture substrate if plasma treated to render the surface hydrophilic. PDMS rigidity is controlled by varying the crosslinker concentration, and the temperature and time of baking (Fuard et al., 2008). PDMS can also be molded into different shapes, and work by our lab has recently shown that varying the length of PDMS micropillars can simulate changes in rigidity and has similar effects on cell morphology and stem cell differentiation (Fu et al., 2010).

We have focused on the most common materials used today, but many other materials have been utilized to study material properties on cell function, including

agarose (Balgude et al., 2001; Ulrich et al., 2010), hyaluronic acid (Seidlits et al., 2010), ionic self assembling peptide gels (Sieminski et al., 2007) and polyelectrolyte multilayers (Schneider et al., 2007) to name just a few. While the development of synthetic materials has allowed the careful control of matrix rigidity independent of other confounding factors, these systems can also lose some of the important biochemical and biophysical information in natural matrices. For example, many synthetic materials incorporate RGD peptides to facilitate cell adhesion. However, RGD is a very small region of fibronectin, and many studies have demonstrated the importance of the other domains of fibronectin, including for integrin binding, (Friedland et al., 2009), growth factor binding (Rahman et al., 2005), and interactions with other ECM molecules (Hynes, 2009). A number of ECM components in addition to fibronectin support cell adhesion, bind to different integrin heterodimers, and regulate cell function. Also, most synthetic materials are not affected by enzyme degradation or ECM deposition, so cells cannot remodel their matrix as occurs *in vivo*. Thus, while valuable insights can be gained by synthetic materials or purified biological materials, a reductionist approach might miss important regulatory actions of the ECM and matrix rigidity on cell function.

2.4 Summary

The cellular microenvironment is a critical regulator of cell function, however many questions still remain regarding how crosstalk between the microenvironment and growth factor signaling regulates cell function. In the following chapters we investigate how the microenvironment regulates TGF-β-induced cell signaling and cell function. In Chapter 4, we examine the regulation of TGF-β-induced apoptosis and EMT by the

adhesive microenvironment through control of matrix rigidity, cell seeding density, and microcontact printing. We next explored the role of matrix rigidity in regulating focal adhesion signaling (Chapter 5) and the PI3K/Akt pathway (Chapter 6) to control the switch between apoptosis and EMT. Finally, in Chapter 7, we discuss the significance and future directions motivated by the work presented here.

Chapter 3

Materials and methods

3.1 Cell culture and reagents

NMuMG and MDCK cells were obtained from American Type Culture Collection and cultured according to their recommendations. Reagents were obtained as follows. Monoclonal antibodies: α-smooth muscle actin (1A4; Sigma-Aldrich), Smad4 (DCS-46; Sigma-Aldrich), vinculin (hVIN-1; Sigma-Aldrich), GAPDH (6C5; Ambion), E-cadherin (36; BD Biosciences), N-cadherin (32; BD Biosciences), FAK (77; BD Biosciences). Polyclonal antibodies: ZO-1 (Zymed Laboratories), pY397 FAK (Invitrogen), pAkt (cell signaling), Akt (cell signaling), Bcl-xL (cell signaling), cleaved caspase-3 (cell signaling), FAK (Cell Signaling Technology**).**

3.2 Experimental set up

Cells were cultured on FN-functionalized polyacrylamide gels, microcontact printed substrates, or at the indicated density for 16 hr in growth medium. The cells were rinsed in sterile PBS, and then growth factor starved in HGDMEM for 2 hr. Cells were treated in the absence of serum with 10 μg/ml insulin (Sigma-Aldrich) and 10 ng/ml TGF-β1 (R&D Systems) for 2 hr (RNA isolation, FAK and Akt western blotting), 4 hr (caspase activity, focal adhesion immunofluorescence, luciferase

activity), or 48 hr (for EMT immunofluorescence and western blotting). For inhibitor studies, cells were treated 1 hr prior to TGF-β1 treatment with ZVAD-FMK (400 μM; Enzo Life Sciences), IETD-CHO (10 and 100 μM; Calbiochem), LEHD-CHO (10 and 100 μM; Calbiochem), PF 573228 (1 μM; Tocris Biosciences), LY294002 (10 μM; Calbiochem), or Akt Inhibitor VIII (1 μM; Calbiochem).

3.3 Polyacrylamide gel preparation

Polyacrylamide gels were prepared as described previously with minor modifications (Winer et al., 2009a; Yeung et al., 2005). 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), 60 kPa (10; 0.5). Acrylamide polymerization was initiated by 0.1% (v/v) TEMED and 0.1% (w/v) ammonium persulfate. 25 μL of acrylamide solution was pipetted directly onto activated 18 mm coverslips (400 μL for 50 mm coverslips). 10 μl of 20 μg/ml acrylic acid *N*-hydroxysuccinimide (NHS) ester (Sigma Aldrich) dissolved in toluene (45 μl for 50 mm) was pipetted directly on to the acrylamide solution, and a Rain-X coated coverslip placed on top of both solutions. After 10 min of polymerization, the top coverslip was removed, and the gels were rinsed in ddH_2O. Gels were functionalized with 20 μg/ml FN in 50 mM HEPES pH8 for 1 hr at RT, and then rinsed in ddH_2O. The NHS ester was quenched by incubation with 1% (v/v) ethanolamine in 50 mM HEPES pH 8 for 30 min at RT. After rinsing with ddH_2O, the polyacrylamide gels were transferred to a sterile dish and sterilized in 5% (v/v)

isopropanol in PBS for 1 hr at RT. Before plating with cells, the gels were rinsed two times with sterile PBS.

3.4 Preparation of micropatterened substrates

Micropatterned substrates were prepared as described (Pirone et al., 2006). Briefly, micropatterned stamps were fabricated by casting poly(dimethoxysilane) (PDMS) (Sylgard 184, Dow Corning)on a photolithographically-generated master. Stamps were immersed for 1 hr in 20 μg/ml fibronectin, 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) in PBS for 1 hr and rinsed in PBS before cell seeding.

3.5 Adenovirus production

FAK, FRNK, FAK-Y397F, p110-CAAX (Upstate Biotechnology), and GFP recombinant adenoviruses were constructed as described previously (Pirone et al., 2006) using the AdEasy XL system (Stratagene) according to manufacturer's instructions. The CD2-FAK adenovirus was generated by C. Henke (University of Minnesota) and CA PI3K by L. Romer (Johns Hopkins University). Expression was optimized and verified by western blot.

3.6 Retrovirus production

Retrovirus was produced as described (Ory et al., 1996) with 293GPG cells. Bcl-xL plasmid was obtained from Addgene (Plasmid 8790, (Cheng et al., 2001)) and myr-Akt plasmid from M. Birnbaum (University of Pennsylvania). Briefly, 293 GPG cells were cultured in 90% HGDMEM, 10% FBS, 1 μg/ml tetracycline, 2 μg/ml

puromycin, 300 μg/ml G418 and passaged at 80% confluence at a 1:5 dilution. For retroviral production, $9x10^6$ 293 GPG cells were plated in a 10 cm dish in 90% HGDMEM and 10 % FBS (virus production medium) with all antibiotics removed. The next day, the medium was replaced with 6.5 mLs of 1% HEPES, 10% FBS, 89% HGDMEM, and the cells were transfected with 12 μg retroviral DNA and 48 μl TransIT-LT1 transfection reagent (Mirus) per 10 cm dish. The next day, medium was discarded and replaced with virus production medium. Medium was collected at 24, 48, and 72 hrs later, and stored at 4°C until final collection. Collected medium was centrifuged to remove cells at 1000 RPM for 5 minutes and viral supernatant was sterile filtered, aliquotted, and stored at -80°C. For retroviral infection, viral supernatant was added to recipient cells plus 8 μg/ml polybrene. Expression was optimized and verified by western blot.

3.7 Caspase-3 activity assays

Caspase-3 activity was determined by EnzChek Caspase-3 Assay (Invitrogen). Briefly, cells were lysed in provided lysis buffer and incubated at -80°C for 10 minutes to complete lysis. Samples were thawed at RT and centrifuged for 5 minutes at 7000 RPM. 50 μl of cell lysate (or lysis buffer for blank well) was mixed with 50 μl of reaction buffer plus the Z-DEVD-AMC caspase substrate in a 96-well plate and incubated for 30 min at RT. Fluorescence was measured at 350 nm excitation/ 485 nm emission. Caspase activity was normalized to total DNA content as determined by CyQUANT Cell Proliferation Assay (Invitrogen). In a separate well, 10 μl of lysate (or 10 μl of lysis buffer for blank), 1 μl of CyQUANT dye, and 90 μl of CyQUANT lysis

buffer were mixed, incubated for 5 minutes at RT, and fluorescence was measured at 485 nm excitation/ 530 nm emission. Blank values were subtracted from all wells.

3.8 Western blotting

Cells were rinsed in PBS and 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)), and centrifuged at 14000 RPM for 10 minutes at 4°C. Protein concentration was determined by Precision Red Advanced Protein Assay (Cytoskeleton). 25 μg of protein were separated by denaturing SDS-PAGE, electroblotted onto PVDF blocked with 5% BSA or milk in TBS-0.3% Tween-20, immunoblotted with specific antibodies, and detected using horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and SuperSignal West Dura (Pierce Chemical Co.) as a chemiluminescent substrate. Densitometric analysis was performed using a VersaDoc imaging system with QuantityOne software (Bio-Rad Laboratories).

3.9 Microscopy, immunofluorescence, and image acquisition

Samples were rinsed in PBS, and fixed in 4% paraformaldehyde at RT for 10 minutes, or, for E-cadherin staining, cells were fixed in 1:1 acetone/methanol on ice for 20 minutes. After fixation, all samples were rinsed two times with PBS, permeabilized with 0.5% Triton-x, and blocked in 10% goat serum for 1 hr at RT. Samples were incubated with primary antibodies (1:200) for 1hr at RT, rinsed three times with PBS, then incubated with Alexa Fluor 488, 555, or 647 secondary antibodies (1:200; Invitrogen), Alexa Fluor 488 Phalloidin (1:200, Invitrogen), and Hoechst 33342 (1:1000; Invitrogen) for 1 hr at RT. Samples were rinsed three times in PBS, then

mounted with Fluormount G (Electron Microscopy Sciences). Images were acquired using an epifluorescence microscope (model TE200; Nikon), equipped with Plan Fluor 10×, 0.3 NA, and Plan Apo 60×, 1.4 NA, oil immersion lenses, Spot camera and software (Diagnostic Instruments). Some image levels were adjusted using Photoshop (Adobe).

For pY397 and vinculin immunofluorescence, samples were rinsed with ice cold cytoskeleton extraction buffer (10 mM PIPES, 50 mM NaCl, 150 mM sucrose, 3 mM MgCl2, 1X Halt protease and phosphatase inhibitor cocktail) for 1 min on ice, followed by two 30 second incubations with cytoskeleton buffer plus 0.5% triton, one rinse with cytoskeleton buffer, and fixation with 4% paraformaldehyde for 10 minutes at RT. Staining was completed as above. Images were acquired using an epifluorescence microscope (Axiovert 200M; Carl Zeiss MicroImaging, Inc.) equipped with 63x Plan-Apochromat, 1.4 NA, oil immersion objective, an Axiocam camera, and Axiovision software.

3.10 Real-time RT-PCR

Total RNA was isolated using an RNeasy Mini or Micro kit (Qiagen) according to the manufacturer's instructions. Complementary DNA (cDNA) was transcribed with high-capacity cDNA reverse transcription kit (Applied Biosystems) with 0.5 ug of total RNA per reaction. Quantitative polymerase chain reaction (PCR) was performed in an ABI 7,300 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).

3.11 Luciferase assays

Cells were transfected with p3TP-lux Addgene plasmid 11767 (Wrana et al., 1992) using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. The next day, transfected cells were seeded on polyacrylamide gels overnight. Transfected cells were treated with TGF-β1 for 4 hours, then lysed and analyzed using the dual-luciferase reporter assay (Promega). Luminescence was measured with GloMax 20/20 Luminometer (Promega). Luciferase values were normalized to DNA content as described in caspase-3 activity assays.

3.12 siRNA transfection

0.37 x 10^6 cells per well in a 6 well plate were plated, and the next day transfected according to manufacturer's instructions with Lipofectamine 2000 control (Invitrogen), 5 nM scrambled siRNA, and 5-100 nM FAK siRNA #1 and #2 (Ambion). 24 hr after transfection, cells were replated for experiments.

Chapter 4

Regulation of apoptosis and EMT by the cellular microenvironment

4.1 Introduction

The adhesive cellular microenvironment is a key regulator of cell function, and adhesion to the ECM is necessary for survival in many cell types (Frisch and Francis, 1994; Meredith et al., 1993). In addition to survival, manipulation of cell-ECM adhesion by a variety of methods has revealed that adhesion also regulates numerous other cell functions, including proliferation, apoptosis, and differentiation (Chen et al., 1997; McBeath et al., 2004). A classic method to modulate cell-ECM and cell-cell adhesion is through cell seeding density. In many cell types, increased seeding density "crowds" cells together and reduces cell spread area and ECM contact while increasing cell-cell adhesion (Liu et al., 2006; Nelson et al., 2008). Because seeding density affects more just than just cell-ECM adhesion, many methods have been developed to more carefully dissect how cells interact with the ECM, including varying adhesive ligand density, microcontact printing, and nanopatterned surfaces (Arnold et al., 2004; Tan et al., 2004).

In this chapter, we examine the effects of the cellular microenvironment on TGF-β-induced cell function. We examined two cell functions, apoptosis and epithelial-mesenchymal transition (EMT), as representative responses to TGF-β classically associated with tumor suppression or promotion, respectively (Massague, 2008). By modulating matrix rigidity using polyacrylamide gels, we found that epithelial cells cultured on compliant substrates underwent apoptosis when treated with TGF-β1, while on more rigid substrates, TGF-β1-induced EMT. By inhibiting apoptosis on compliant substrates, we found that NMuMG cells still failed to undergo a complete EMT. To further investigate how changes in the cellular microenvironment affect cell function, we seeded cells at different densities and assessed TGF-β1-induced apoptosis and EMT. Interestingly, we found increasing cell density inhibited both apoptosis and EMT. Because changing matrix rigidity or cell seeding density affects both cell-ECM and cell-cell adhesion, we isolated the effect of changing cell-ECM adhesion by micropatterning islands of fibronectin to control spreading of single cells. Cells in which spreading was restricted were found to have increased apoptosis in response to TGF-β1. Taken together, these findings reveal that the cellular microenvironment regulates TGF-β1-induced cell functions, and suggest that during diseases such as cancer and fibrosis, changes in the microenvironment likely play an important the role in the regulation of TGF-β1-induced cell fates.

4.2 Results

4.2.1 Matrix rigidity regulates TGF-β1-induced EMT and apoptosis

To explore whether matrix rigidity influences cellular responses to TGF-β1, we first examined normal murine mammary gland (NMuMG) epithelial cells cultured on fibronectin-conjugated polyacrylamide (PA) gels with a range of elastic moduli from 0.4 to 60 kPa and treated with TGF-β1. Before addition of TGF-β1, NMuMG cells cultured on PA gels exhibited differences in morphology as a function of substrate compliance (Fig. 4.1). 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 more rounded and formed spherical clusters. On more 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 epithelial-to-mesenchymal transition (EMT) (Fig. 4.1). Examination of known EMT markers confirmed this response, as evidenced by delocalization of the epithelial junctional markers, ZO-1 and E-cadherin, and increased expression of mesenchymal markers N-cadherin and α-smooth muscle actin (α-SMA) (Fig. 4.1). In contrast, cells did not appear to undergo EMT on compliant PA gels, as exemplified by loss of expression of the EMT associated transcription factor, Snail, with decreasing substrate rigidity (Fig. 4.2). Interestingly, compliant substrates (E < 1 kPa) not only suppressed EMT, but also induced a rapid and dramatic increase in apoptosis in response to TGF-β1 treatment, as evidenced by TGF-β1-induced caspase activity on compliant gels but not rigid gels (Fig. 4.2). This was observed across a range of TGF-β1 concentrations

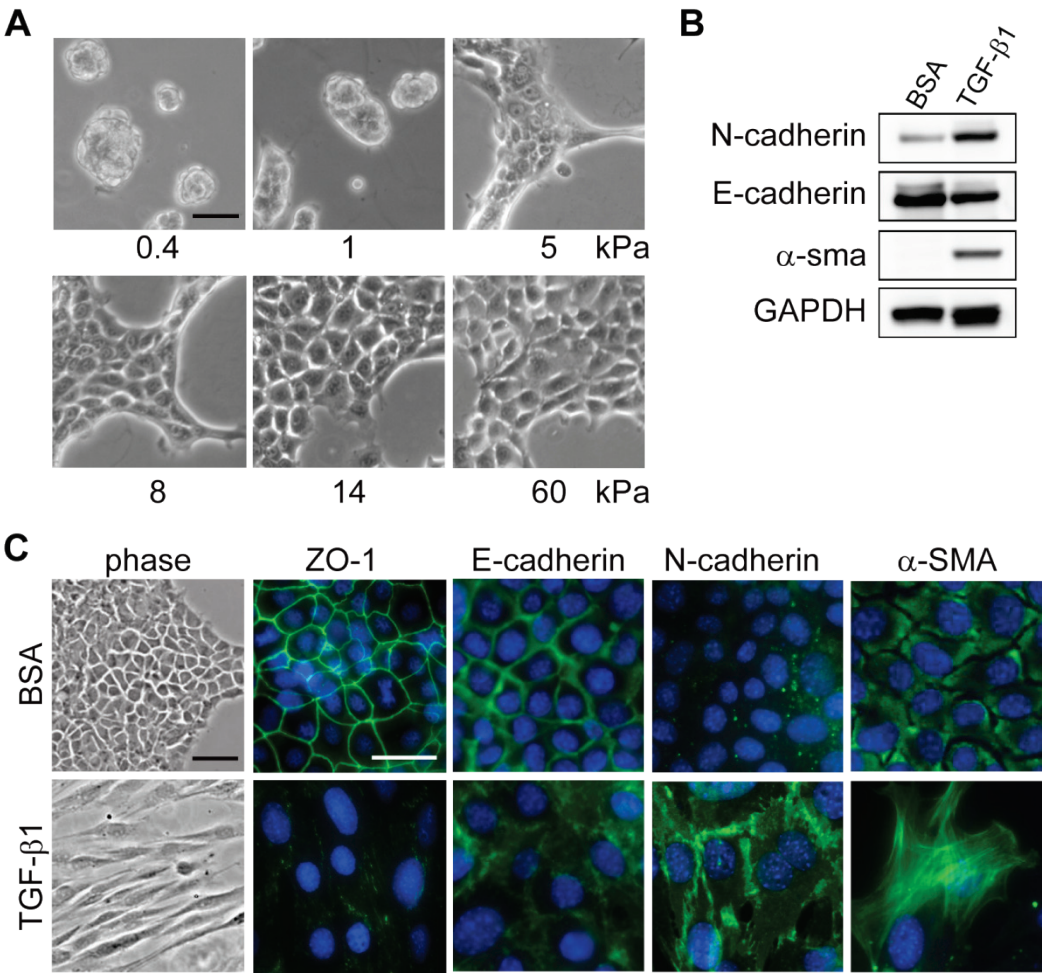

Figure 4.1 TGF-β1 induces EMT in NMuMG cells.

(A) Phase contrast images of NMuMG cells cultured on PA gels with elastic moduli ranging from 0.4 to 60 kPa for 24 h. (B) Western blot of N-cadherin (135 kD), E-cadherin (120 kD), α-sma (42 kD), and GAPDH control (38 kD) in NMuMG cells cultured on rigid PA gels treated with TGF-β1 or BSA control for 48 hr. (C) Phase contrast images and immunostaining of NMuMG cells cultured on rigid (E=8 kPa) PA gels and treated with TGF-β1. Bars, 50 μm.

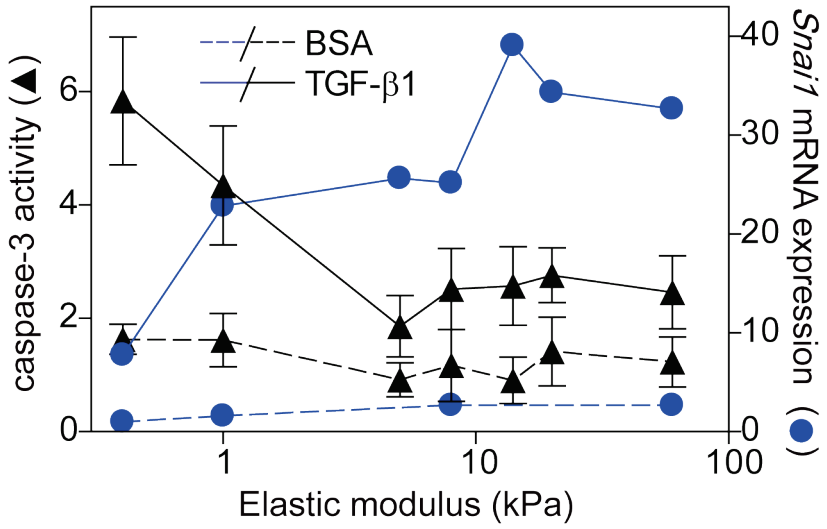

Figure 4.2 Matrix rigidity regulates TGF-β1-induced EMT and apoptosis.

Graph of caspase-3 activity (▲) and Snai1 mRNA expression (●) (representative of n=5) in NMuMG cells plated on PA gels treated with TGF-β1 (solid lines) or BSA control (dashed lines). Error bars indicate the SEM of 5 independent experiments.

and by 24 hrs most cells cultured on compliant gels treated with TGF-β1 exhibited significant nuclear fragmentation (Fig. 4.3). A similar switch in TGF-β1-induced cell fate was also observed in MDCK epithelial cells, with increased apoptosis on compliant gels and induction of EMT on rigid gels (Fig. 4.3 and 4.4), suggesting that this control mechanism is not restricted to mammary epithelia.

One way matrix rigidity may regulate the switch between EMT and apoptosis is by blocking TGF-β/Smad signaling on compliant substrates. In the canonical TGF-β signaling pathway, ligand binding activates the TGF-β receptor complex which phosphorylates Smad2 and Smad3, allowing Smad2/3 and Smad4 complex formation and translocation to the nucleus to regulate transcription. To investigate the effects of matrix rigidity on TGF-β/Smad signaling, we first observed the nuclear translocation of Smad4. As early as 2 hrs after TGF-β1 treatment, Smad4 translocated to the nucleus in NMuMGs to similar degrees on both rigid and compliant substrates (Fig. 4.5). In addition, use of a Smad-responsive 3TP-luciferase reporter plasmid also showed no difference in Smad transcriptional activity on rigid versus compliant substrates (Fig. 4.5) (Wrana et al., 1992; Yingling et al., 1997). These results suggest that Smad signaling is not involved in matrix rigidity regulation of TGF-β1-induced cellular functions.

Given that the apoptotic response occurred within hours while EMT occurred after several days, it was not clear if the decreased EMT on compliant gels was a result of TGF-β1-induced cell death, or if compliance directly regulated EMT independent of its effects on cell survival. To address this, we blocked the apoptotic response by either

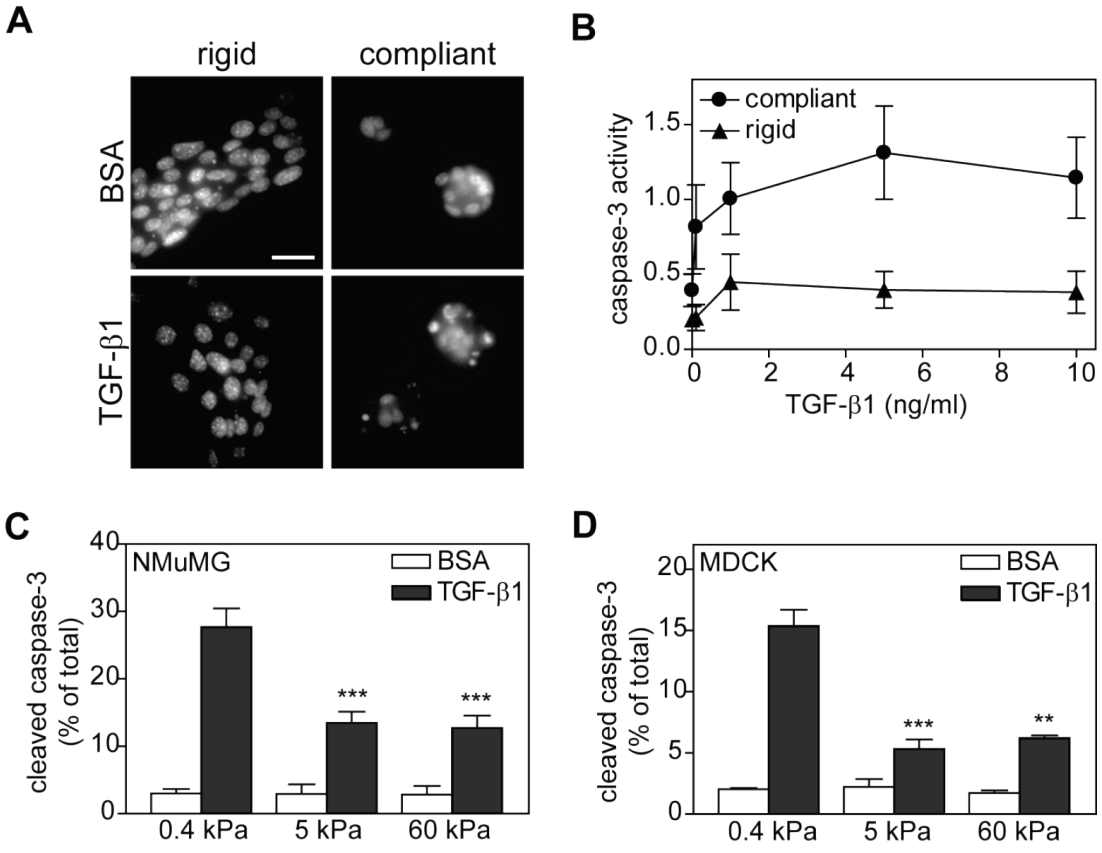

Figure 4.3 Matrix rigidity regulates TGF-β1-induced apoptosis.

(A) Hoechst stained nuclei of NMuMG cells cultured on rigid (E=5 kPa) and compliant (E=0.4 kPa) gels. (B) Graph of caspase-3 activity in NMuMG cells treated with 0.1 to 10 ng/ml TGF-β1. (C, D) Graph of percentage of positive staining for cleaved-caspase-3 immunofluorescence in NMuMG (C) or MDCK cells (D) treated with TGF-β1 for 24 hr. Error bars indicate SEM of three independent experiments. **, $P<0.01$; ***, $P<0.001$, calculated by *t* test compared to 0.4 kPa +TGF-β1. Bar, 50 μm.

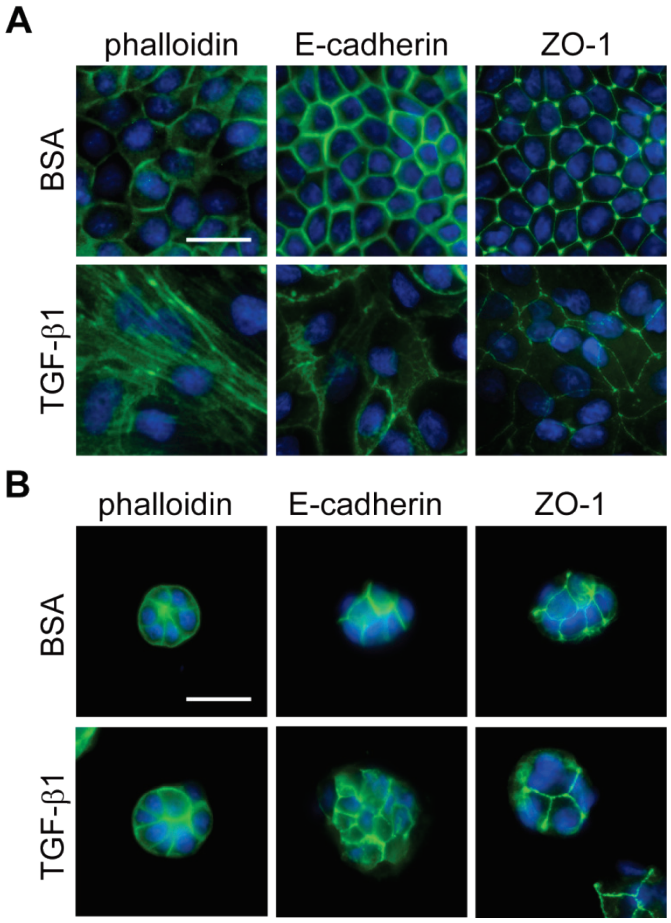

Figure 4.4 Matrix rigidity regulates TGF-β1-induced EMT in MDCK cells.

(A, B) Immunostaining of MDCK cells cultured on rigid (E=5 kPa) (A) and compliant (E=0.4 kPA). (B) PA gels and treated with TGF-β1 or BSA control for 48 hr. Bars, 50 μm.

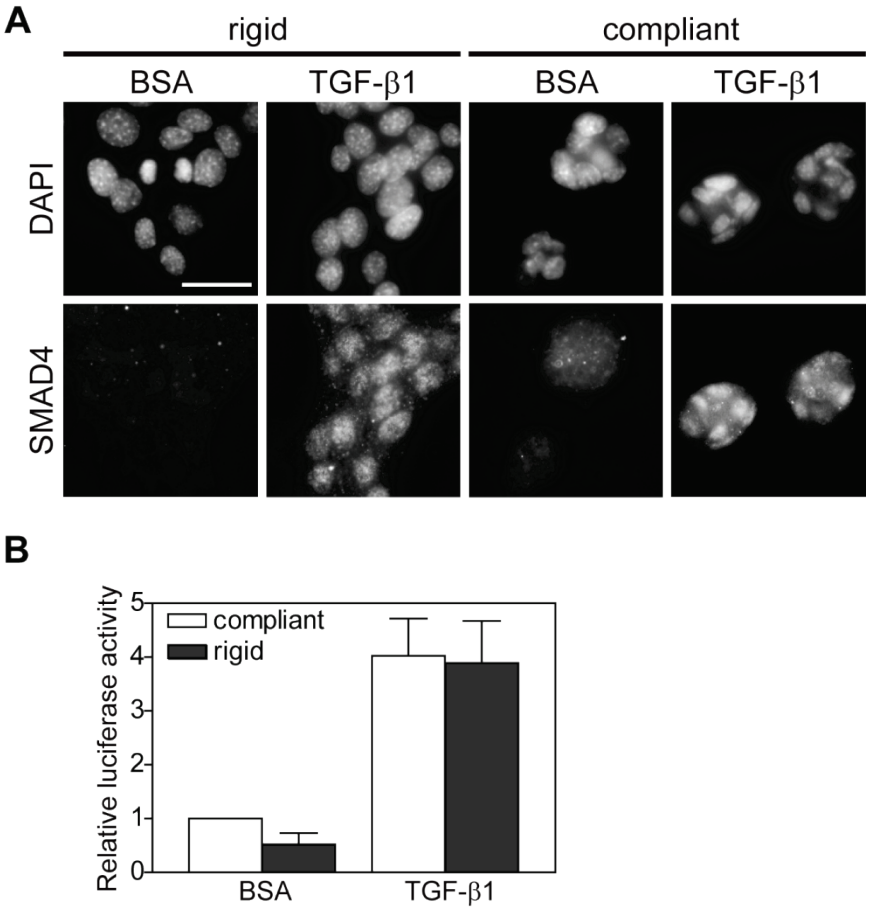

Figure 4.5 Smad signaling is unaffected by matrix rigidity.

(A) Immunostaining for Smad4 and nuclei in NMuMG cells cultured on rigid (E=8 kPa) and compliant (E=0.4 kPA) PA gels, and treated with TGF-β1 or BSA control for 8 hr. (B) Graph of luciferase activity in NMuMG cells transfected with p3TP-lux, plated on PA gels, and treated with TGF-β1. Error bars indicate the SEM of three independent experiments.

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. Both reagents decreased caspase-3 activity and prevented nuclear fragmentation (Fig. 4.6). 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 (Fig. 4.6 and 4.7). 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.

4.2.2 Cell density regulates TGF-β1-induced EMT and apoptosis

Previous studies have shown that cell density can regulate TGF-β-induced cell functions, and that cells grown to confluence do not undergo EMT (Nelson et al., 2008; Petridou et al., 2000). To investigate if cell density regulates the switch between apoptosis and EMT, NMuMG and MDCK cells were seeded at different densities and treated with TGF-β1 (Fig. 4.8). Similar to published reports, confluent cells did not undergo EMT, as indicated by retention of a cuboidal epithelial phenotype and lack of N-cadherin expression (Fig. 4.8 and 4.9) (Petridou et al., 2000). E-cadherin expression did not change significantly by varying seeding density. Interestingly, increasing seeding density also inhibited TGF-β1-induced apoptosis (Fig. 4.8). Although matrix rigidity did not affect TGF-β/Smad signaling, increasing cell density resulted in decreased TGF-β1-induced 3TP luciferase activity (Fig. 4.10). Together these results suggest that matrix rigidity and cell density regulate apoptosis and possibly EMT in different ways.

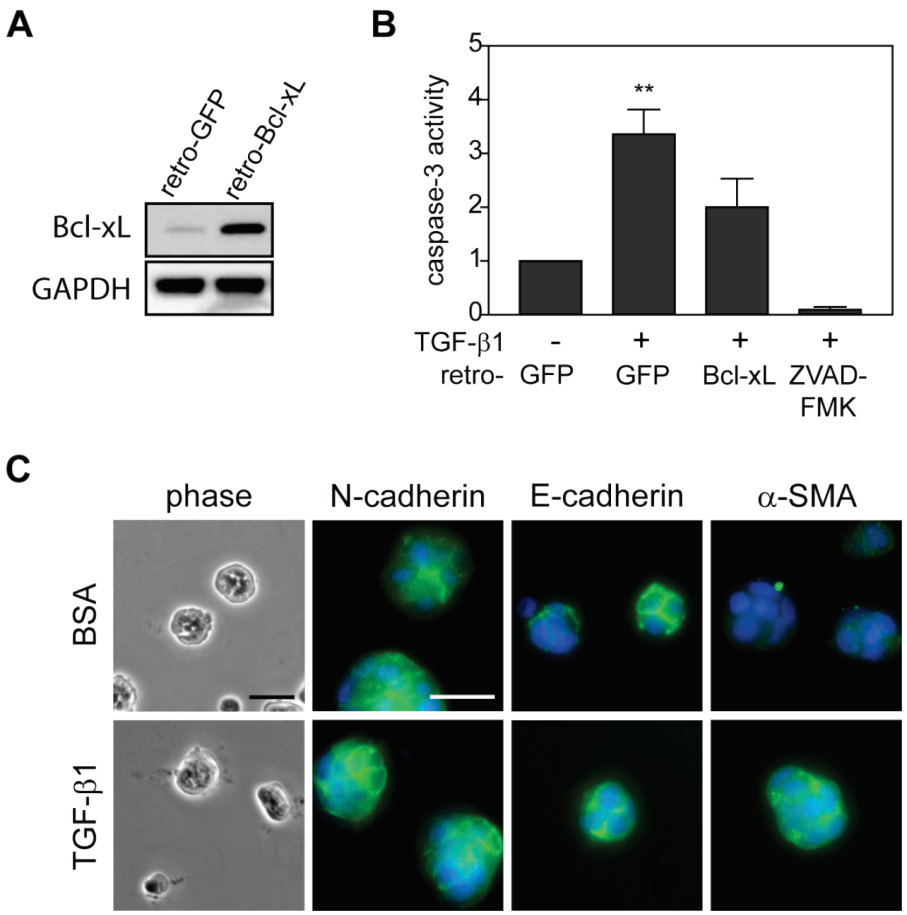

Figure 4.6 Decreased matrix rigidity inhibits EMT independent of apoptosis.

(A, B) Western (A) and graph of caspase-3 activity (B) in NMuMG cells infected with retro-GFP, retro-Bcl-xL, or treated with 400 μM ZVAD-FMK, plated on rigid (E=8 kPA) and compliant (E=0.4 kPA) gels, and treated with TGF-β1 or BSA control. (C) Immunostaining for N-cadherin, E-cadherin, α-sma, and nuclei of NMuMG cells infected with retro-Bcl-xL on compliant gels and treated with TGF-β1. Error bars indicate SEM of three independent experiments. **, P<0.01, calculated by *t* test. Error bars indicate SEM of 3 independent experiments. Bars, 50 μm.

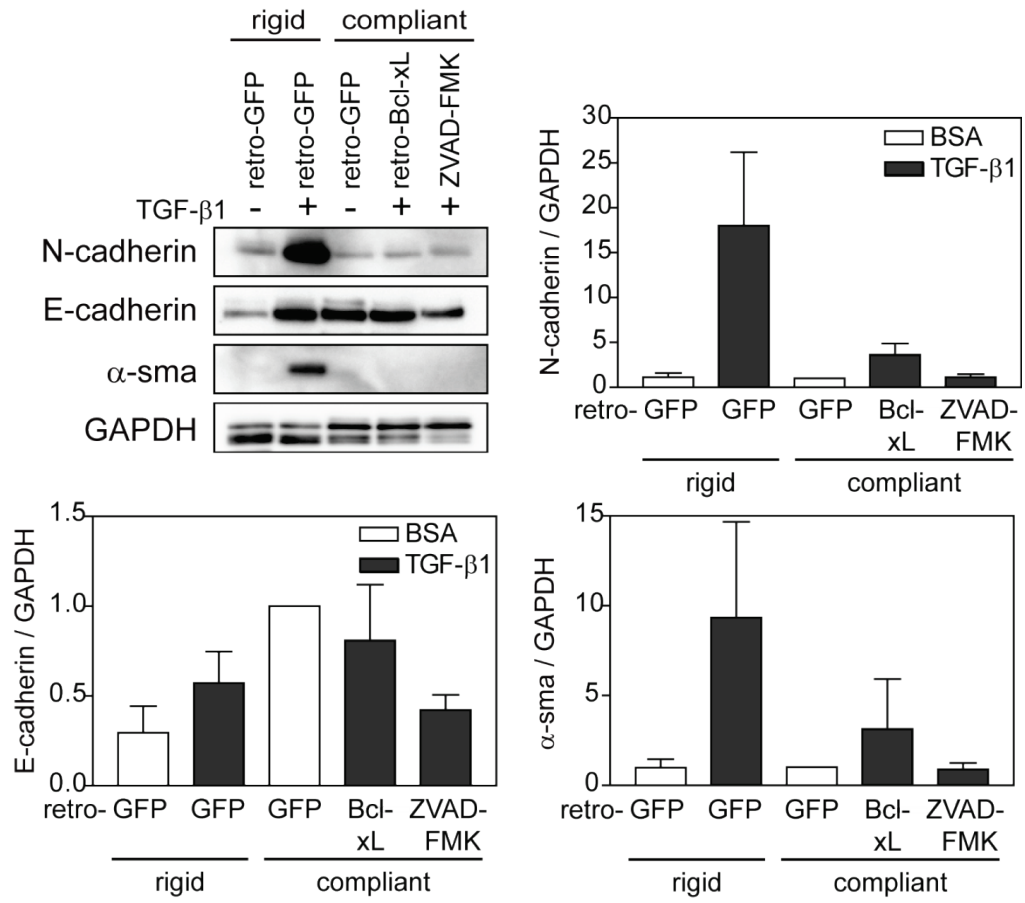

Figure 4.7 Decreased matrix rigidity inhibits EMT independent of apoptosis.

Western blot and quantification of N-cadherin (135 kD), E-cadherin (120 kD), α-sma (42 kD), and GAPDH control (38 kD) in NMuMG cells infected with retro-GFP, retro-Bcl-xL, or treated with 400 μM ZVAD-FMK, plated on rigid (E=8 kPa) and compliant (E=0.4kPa) gels, and treated with TGF-β1 or BSA control. Error bars indicate SEM of 4 independent experiments.

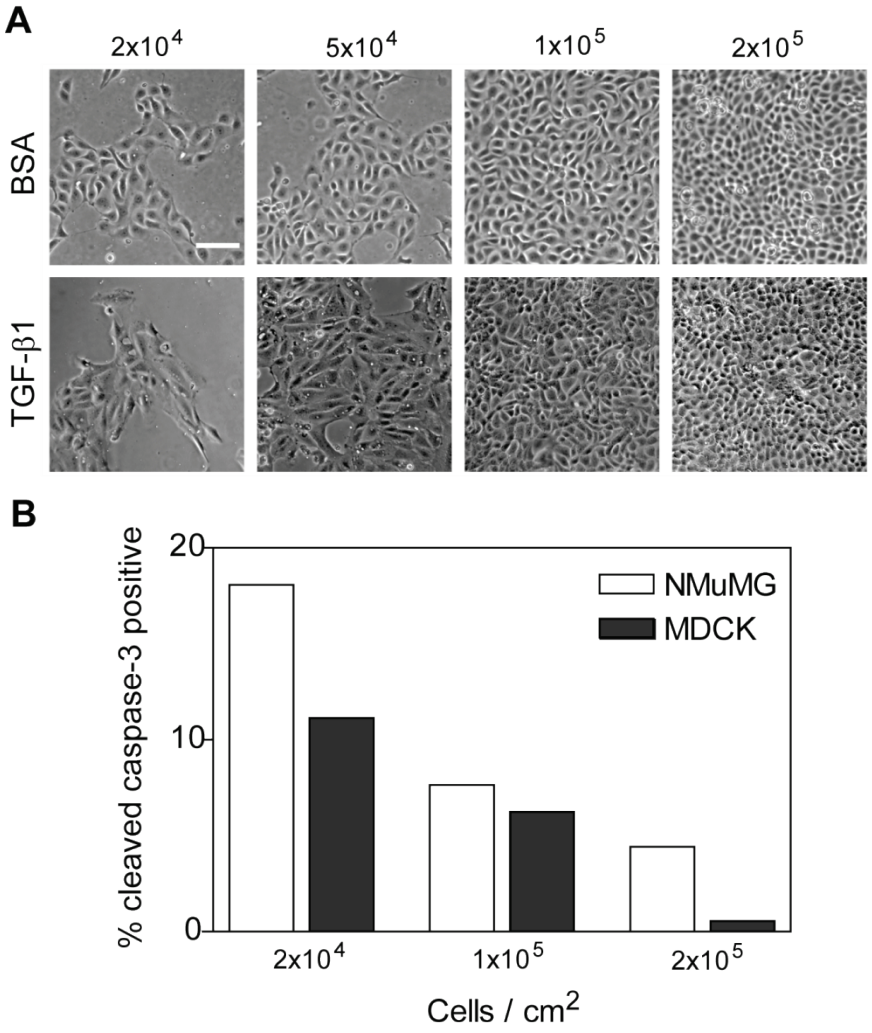

Figure 4.8 Cell seeding density regulates TGF-β1-induced apoptosis.

(A) MDCK cells seeded at increasing density on tissue culture plastic, treated with TGF-β1 for 48 hr. (B) Graph of cleaved caspase-3 positive cells plated at increasing density and treated with TGF-β1 for 24 hr. n=1. Bar, 50 μM.

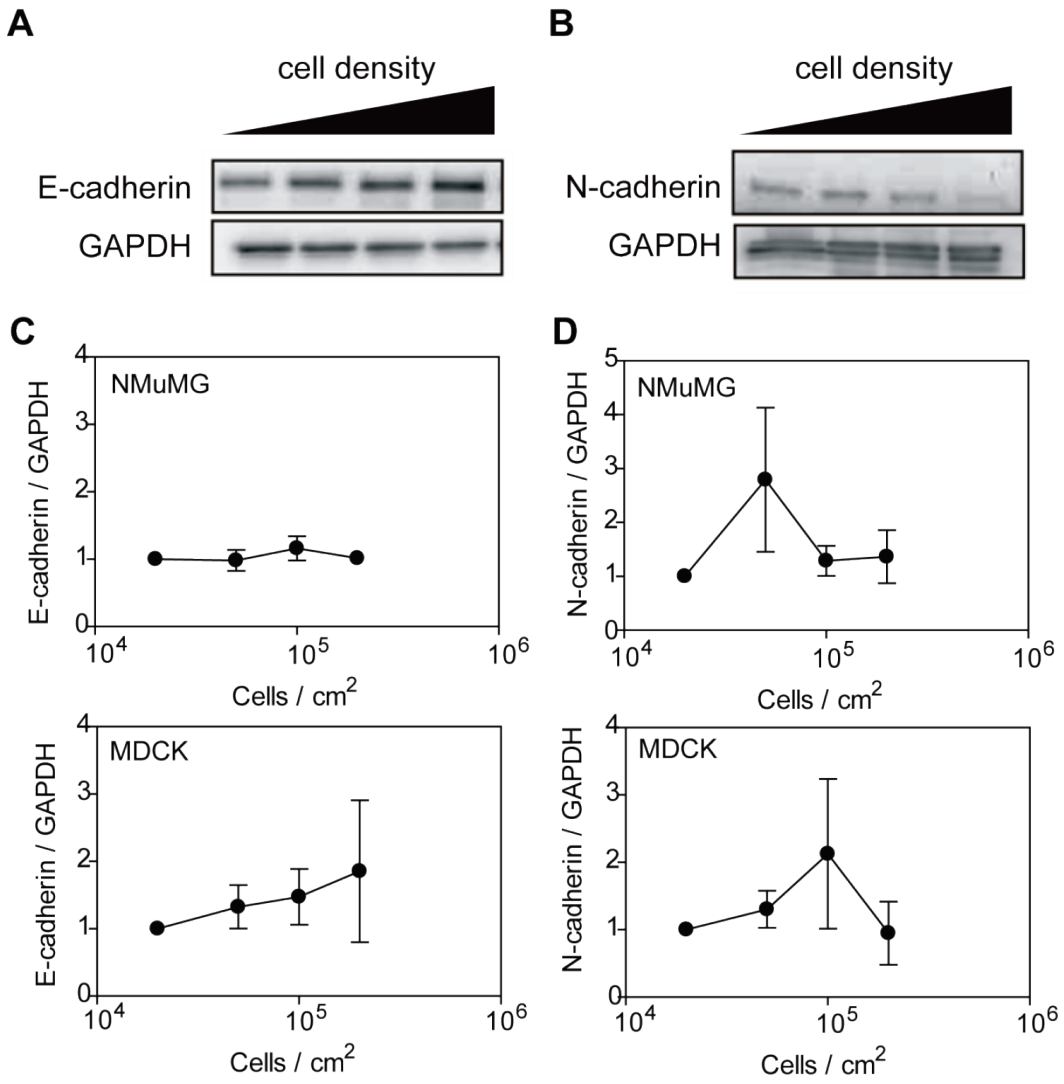

Figure 4.9 Cell seeding density regulates TGF-β1-induced N-cadherin expression. (A, B) Western blot of E-cadherin (120 kD) (A), N-cadherin (135 kD) (B), and GAPDH (38 kD) expression in MDCK cells seeded at increasing density, treated with TGF-β1 for 48 hr. (C, D) Quantification of E-cadherin (C) and N-cadherin (D) expression in NMuMG and MDCK cells plated at increasing density and treated with TGF-β1 for 48 hr. Error bars indicate SEM of four independent experiments.

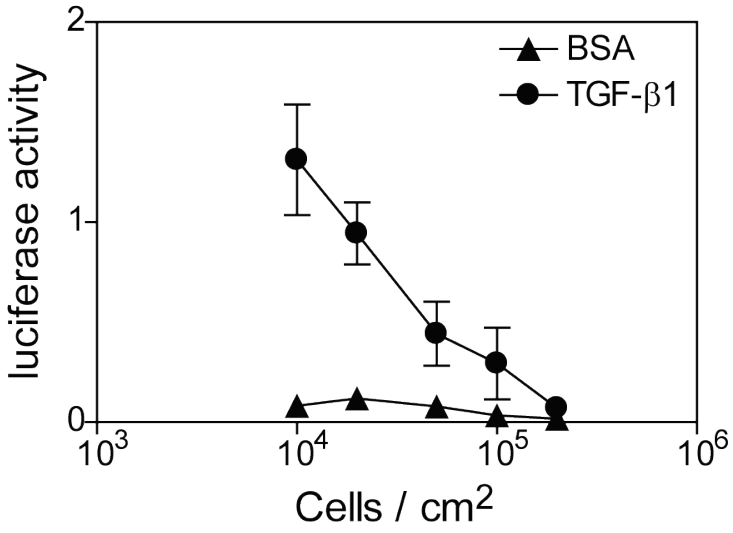

Figure 4.10 Cell seeding density regulates TGF-β1-induced 3TP luciferase activity. 3TP luciferase activity in MDCK cells seeded at increasing density, treated with TGF-β1 for 24 hr. Error bars indicate standard deviation of two independent experiments.

4.2.3 Cell spreading regulates TGF-β1-induced apoptosis

Matrix rigidity and cell density could regulate TGF-β1-induced apoptosis and EMT at a number of levels, including through regulation of cell-ECM adhesion. Previous work has shown that inhibition of cell spreading inhibits MMP-induced EMT, but the effect on apoptosis was not studied (Nelson et al., 2008). Here we used microcontact printing to limit cell spreading to 225 μm^2 islands of FN or allowed the cells to fully spread on FN printed with flat stamps (Fig. 4.11). Cells in which spreading was restricted underwent significantly more apoptosis than cells that were fully spread (Fig. 4.11). These results indicate that one way matrix rigidity regulates apoptosis may be through regulation of cell-ECM adhesion and cell spreading.

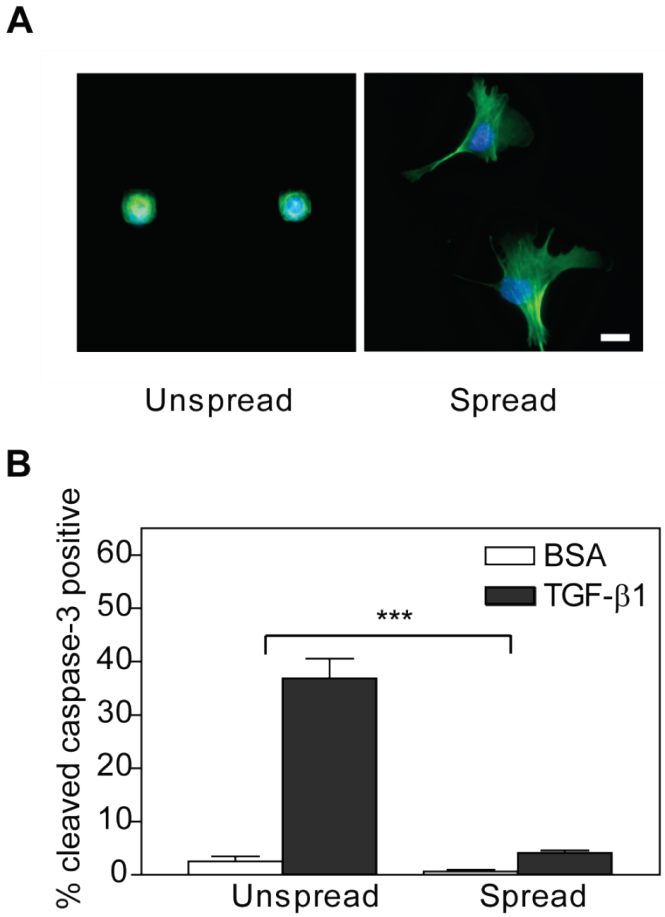

Figure 4.11 Cell spreading regulates TGF-β1-induced apoptosis.

(A) NMuMG cells seeded on 225 μm^2 micropatterned islands (unspread) or flat stamps (spread) of fibronectin. (B) Graph of percent positive cleaved caspase-3 cells treated with TGF-β1 for 24 hr. Error bars indicate SEM of three independent experiments. ***, P<0.001, calculated by two-way ANOVA. Bar, 25 μm.

4.3 Discussion

In this chapter, we provide evidence that the extracellular microenvironment regulates TGF-β1-induced EMT and apoptosis. Inhibition of cell spreading or seeding on compliant substrates promotes apoptosis with TGF-β1 treatment. Compliant substrates also inhibit EMT, and it will be interesting in future experiments to explore the role of cell spreading and EMT. Previous work indicates MMP but not TGF-β-induced EMT is inhibited by restricting cell spreading (Nelson et al., 2008), but it is unclear whether cell spreading is important for TGF-β-induced EMT in the system presented here because of the use of different cell types and due to the results presented earlier that matrix rigidity does inhibit EMT.

Increasing cell density to confluence, similar to compliant substrates, inhibited TGF-β-induced EMT. Unlike compliant substrates, however, confluence also inhibited apoptosis. The inhibition of TGF-β-induced functions by confluence is likely due to the down regulation of the TGF-β receptors, as reported previously (Petridou et al., 2000), and as supported by data presented here that increasing cell density decreased TGF-β/Smad responsive luciferase reporter activity. Compliant substrates, conversely, did not affect luciferase activity, indicating that matrix rigidity likely regulates TGF-β-induced cell functions by a different mechanism than cell seeding density. One intriguing possibility is that in addition to restricting cell spreading, confluence induces apico-basal polarity (Balcarova-Stander et al., 1984; Rodriguez-Boulan and Nelson, 1989). Polarity has previously been shown to confer apoptosis resistance to mammary epithelial cells in 3D through a different mechanism than regulation of receptor

expression level (Weaver et al., 2002), but this work raises two interesting possibilities. One, are confluent, polarized monolayers in 2D resistant to apoptotic stimuli other than TGF-β? And, two, if cells plated on compliant substrates were allowed to polarize before addition of TGF-β, would apoptosis be inhibited through receptor down regulation or some other mechanism? Additionally, it is still unclear how cells sense matrix rigidity, possibilities include changes in F-actin dynamics, cellular contractility, integrin binding, cell-cell adhesion, and focal adhesion formation (Chan and Odde, 2008; Fouchard et al., 2011; Huebsch et al., 2010; Liu et al., 2010; Yeung et al., 2005). It is also likely that several mechanisms are involved and that different mechanisms are important for different cell functions. Future studies to explore these questions will be invaluable towards the understanding of microenviromental regulation of TGF-β-induced functions both *in vitro* and *in vivo*.

Chapter 5

Role of FAK in matrix rigidity-regulated apoptosis

5.1 Introduction

Matrix rigidity and TGF-β, independently, have been shown to regulate actin stress fiber and focal adhesion formation (Edlund et al., 2002; Miettinen et al., 1994; Pelham and Wang, 1997; Yeung et al., 2005). Additionally, focal adhesion kinase (FAK), one of the main signaling components within focal adhesions, can also be regulated by matrix rigidity and TGF-β1 (Cicchini et al., 2008; Paszek et al., 2005; Walsh et al., 2008; Wang et al., 2004). Several studies have demonstrated that FAK is necessary for EMT in hepatocytes and renal tubular epithelial cells (Cicchini et al., 2008; Deng et al., 2010), and that FAK plays a prominent role in survival of several cell types, including epithelial cells and fibroblasts (Frisch et al., 1996; Ilic et al., 1998; Sonoda et al., 2000; Xia et al., 2004; Zouq et al., 2009). It is unknown, however, if cross talk between matrix rigidity and TGF-β regulates focal adhesion signaling to control apoptosis and EMT.

FAK activity and localization is regulated by cell-ECM adhesion. Cells adhere to the ECM through integrins, transmembrane receptors that consist of selectively

paired α and β subunits which bind to different ECM molecules. Binding to the ECM induces integrin clustering, and this aggregation recruits intracellular scaffolding and signaling components to form focal adhesions and connections to the cytoskeleton. Over 50 proteins have been identified that localize to focal adhesions, including i) integrin-binding proteins, ii) adaptors and scaffolding enzymes, and iii) enzymes (Zaidel-Bar et al., 2007). These protein complexes transmit and receive mechanochemical information from the inside of the cell, and this signaling controls numerous downstream cellular functions, including proliferation, survival, motility, and transcription. Tyrosine phosphorylation by recruited kinases is one of the main signaling events that take place at focal adhesions. There are many kinases localized to focal adhesions, and FAK is one of the best characterized. FAK interacts with β integrin tails and localizes to focal adhesions (Chen et al., 2000; Schaller et al., 1992; Schaller et al., 1995). FAK is initially activated by integrin engagement which induces autophosphorylation at Y397 (Schaller et al., 1994). After phosphorylation at Y397, Src, another tyrosine kinase present in focal adhesions, binds to FAK and increases FAK activity by phosphorylating it at Y567 and Y577 (Calalb et al., 1995). Src also phosphorylates FAK at Y861 and Y925 to activate docking sites for other signaling components (Calalb et al., 1996; Schlaepfer et al., 1994; Schlaepfer et al., 1998) and activation of a number of downstream signaling pathways, including MAPKs (Schlaepfer et al., 1994), RhoGTPases (Medley et al., 2003; Zhai et al., 2003), and PI3K (Chen et al., 1996).

In the following chapter, we characterize the effects of matrix rigidity and TGF-β on the actin cytoskeleton, focal adhesion formation, and FAK. FAK's role in TGF-β-induced-EMT and apoptosis will also be explored.

5.2 Results

5.2.1 FAK is important for rigidity-regulated apoptosis but not EMT

To investigate whether FAK may be involved in this system, we first examined whether matrix compliance and TGF-β1 modulated focal adhesion formation and FAK phosphorylation. Prominent focal adhesions, as indicated by punctate immuno-fluorescence staining for vinculin, and actin stress fibers were observed in NMuMGs cultured on rigid substrates (Fig. 5.1 A). On compliant substrates, focal adhesion markers were diffuse and cortical actin was observed (Fig. 5.1 B). 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 by immunofluorescence, in a manner that directly correlated with vinculin localization (Fig. 5.1 A). Western blot analysis confirmed this observation, showing increased levels of phosphorylated Y397-FAK (Fig. 5.1 C and D). Interestingly, however, specific activity of FAK (pFAK normalized to total FAK) showed no significant difference between compliant and rigid gels, as FAK protein levels were greatly decreased on compliant gels. Decreased FAK expression and a lack of change in FAK specific activity on compliant gels suggest that compliance regulates FAK signaling primarily at the level of protein expression rather than its phosphorylation. To test this possibility, we overexpressed FAK using an adenoviral vector, and observed a decrease in TGF-β1-induced apoptosis on compliant gels, but

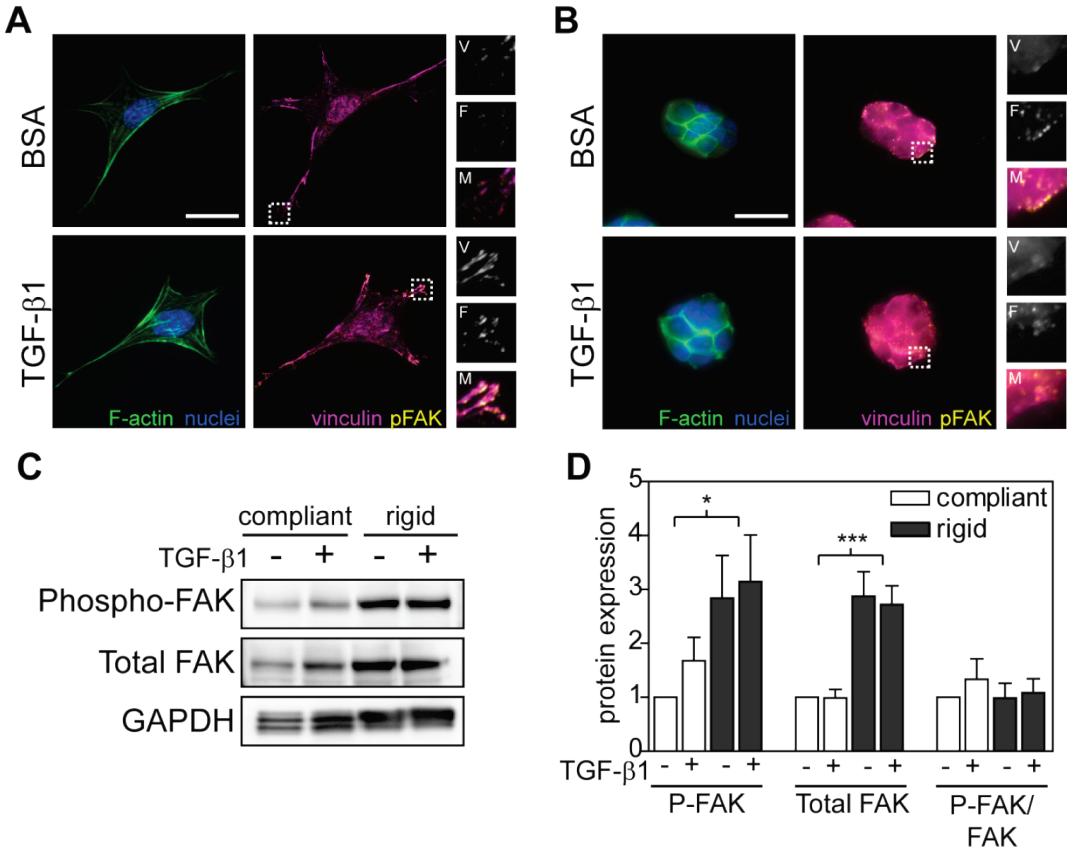

Figure 5.1 FAK expression is regulated by matrix rigidity.

(A,B) NMuMG cells immunostained for F-actin (green), nuclei (blue), vinculin (magenta), and phospho-FAK (yellow) on rigid (E=8 kPA) (A) and compliant (E=0.4 kPA) (B) gels. Inset shows magnification of vinculin (V), phospho-FAK (F), and merged (M) images. (C, D) Western blot and quantification of phospho-FAK (125 kD), total FAK (125 kD), and GAPDH (38 kD). Error bars indicate the SEM of at least three independent experiments. *, $P < 0.05$; ***, $P<0.001$, calculated by two-way ANOVA. Bars, 25 μm.

not a rescue of Snail mRNA expression (Fig. 5.2). Previous reports have shown decreased FAK expression on collagen gels due to FAK degradation by calpain (Wang et al., 2003b). 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). A caveat to this observation is that the inhibitor was used at 10 μM, the concentration used in previous studies, and a higher concentration of 20 μM caused death on compliant substrates, even before addition of TGF-β1.

5.2.2 Manipulation of FAK activity does not affect apoptosis or EMT

While 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 (Fig. 5.3 D and E). Further supporting this model, pharmacological inhibition of FAK activity with the small molecule inhibitor, PF 573228, reduced Y397 FAK phosphorylation, but did not affect EMT or apoptosis (Fig. 5.3 A, 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 TGF-β1-induced EMT or apoptosis (Fig. 5.3 D and E). 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.

5.2.3 Knockdown of FAK expression does not affect apoptosis

While FAK overexpression inhibited TGF-β1-induced apoptosis on compliant substrates, it was not clear if FAK expression was necessary for NMuMG survival on

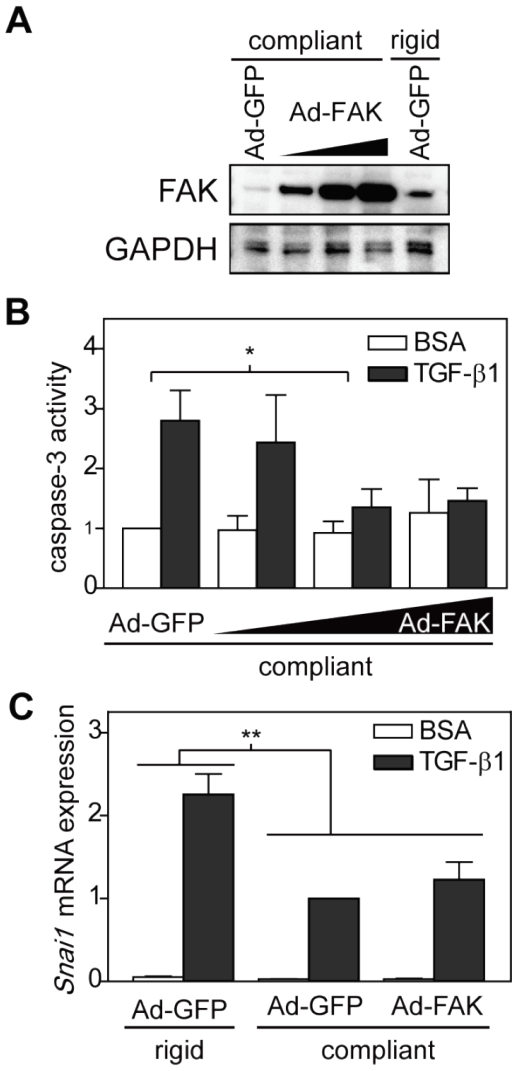

Figure 5.2 FAK expression rescues survival on compliant gels but not EMT.

(A) Western blot of NMuMG cells infected with Ad-GFP or Ad-FAK. (B,C) Caspase-3 activity (B) and *Snai1* mRNA expression (C) in NMuMG cells infected with Ad-GFP or Ad-FAK cultured on rigid and compliant gels treated with TGF-β1. Error bars indicate the SEM of at least three independent experiments. *, $P < 0.05$; **, $P<0.01$, calculated by two-way ANOVA.

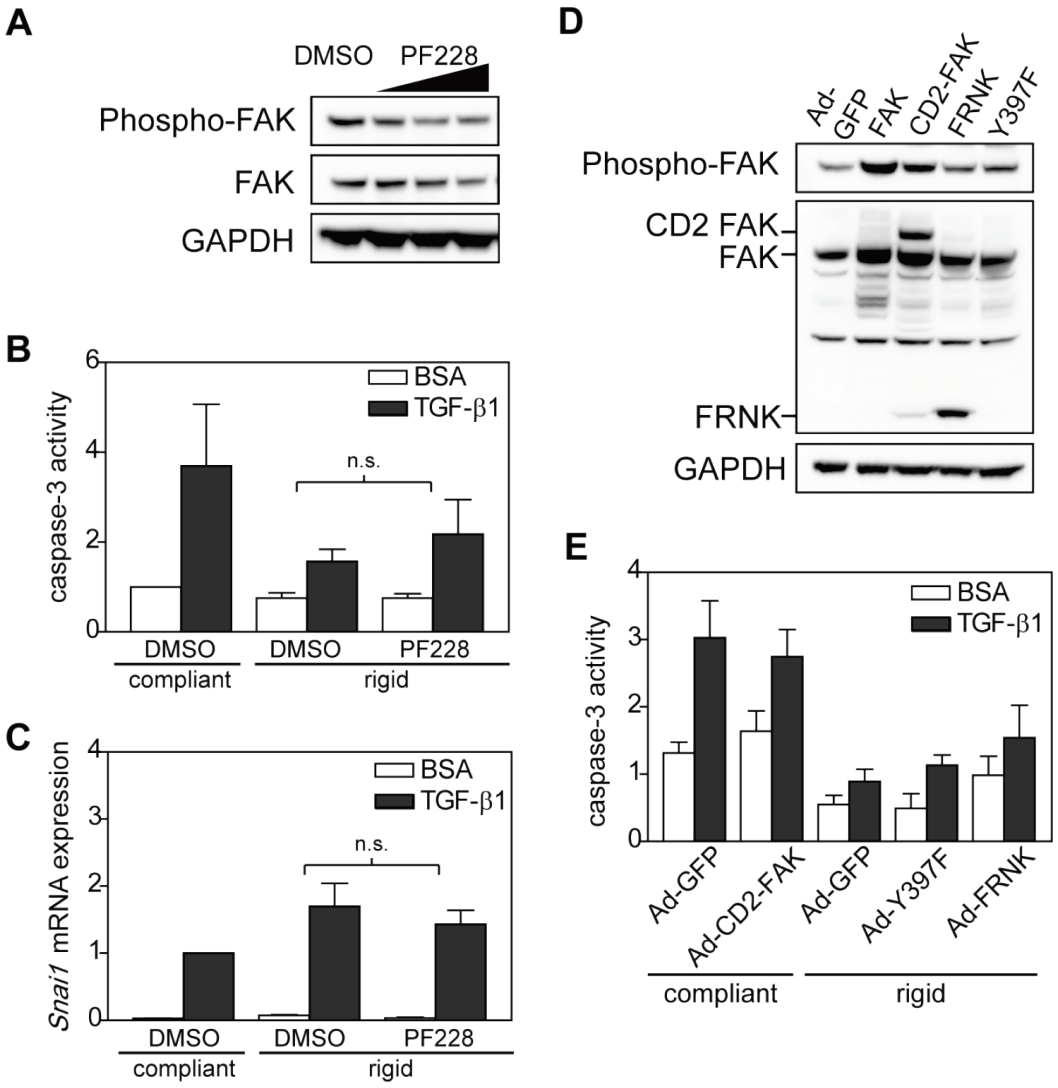

Figure 5.3 Manipulation of FAK activity does not affect EMT or apoptosis.

(A) Western blot of FAK (125 kD) and GAPDH control (38 kD) in NMuMG cells treated with 1, 5, and 10 μM PF 573228 in NMuMG cells. (B, C) Graphs of caspase-3 activity (B) and *Snai1* mRNA expression (C) in NMuMG cells treated with 1 μM PF 573228. (D, E) Western blot (D) and graph of caspase-3 activity (E) in NMuMG cells infected with Ad-GFP, Ad-FAK, Ad-CD2 FAK, Ad-FRNK, and Ad-Y397F FAK, plated on PA gels, and treated with TGF-β1. Error bars indicate the SEM of at least three independent experiments.

rigid substrates. Knockdown of FAK expression with short interfering RNA (siRNA) reduced FAK expression by approximately 80% as compared to lipofectamine or scrambled siRNA controls (Fig. 5.4). Despite decreased FAK expression, apoptosis was not affected with or without TGF-β1 treatment (Fig. 5.4). We also did not observe increased death after siRNA transfection when comparing lipofectamine control or FAK siRNA conditions.

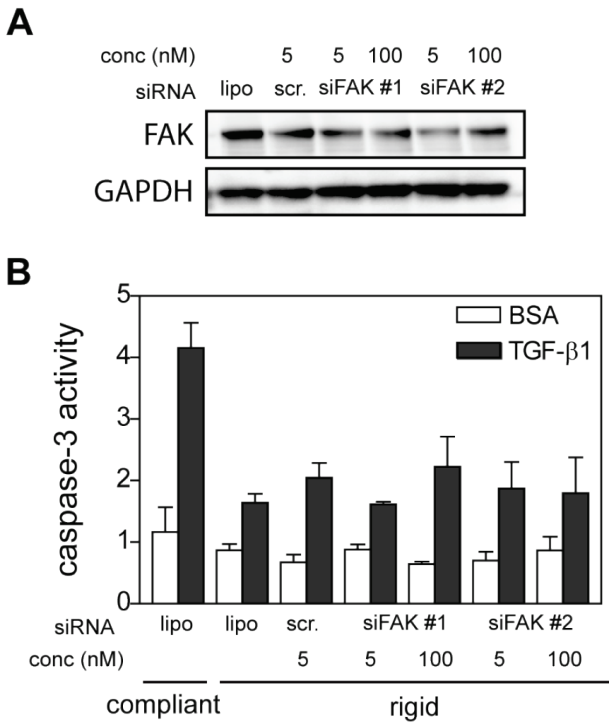

Figure 5.4 FAK knockdown does not increase apoptosis.

(A, B) Western blot (A) and graph of caspase-3 (B) activity in NMuMG cells transfected with lipofectamine control, scrambled siRNA control (scr.), FAK siRNA #1 and #2 (5 and 100 nM), and plated on compliant and rigid gels. Error bars represent SEM of four independent experiments.

5.3 Discussion

In this chapter, we investigated the role of matrix rigidity and TGF-β in regulating focal adhesion formation and FAK signaling. Similar to previous studies, compliant substrates inhibited stress fiber and focal adhesion formation (Pelham and Wang, 1997; Yeung et al., 2005). Here we show that while TGF-β can increase stress fiber and focal adhesion formation on rigid substrates, it was not sufficient to overcome inhibition by compliant substrates. Further investigation revealed compliance modulates FAK activity by protein expression and not phosphorylation. The decrease in FAK expression on compliant substrates agrees with a previous report (Wang et al., 2003b), and this work as well as others demonstrated a role for calpain in the proteolytic degradation of FAK (Carragher et al., 1999; Chan et al., 2010). However, inhibition of calpain with the pharmacological inhibitor ALLN did not rescue FAK expression or cell survival on compliant substrates in our system, although higher concentrations of ALLN could not be investigated due to increased cell death. In addition, most reports of FAK degradation by calpain used a collagen matrix (Bhadriraju et al., 2009; Wang et al., 2003b), and because we used fibronectin as our adhesive matrix, FAK degradation might be regulated by a different mechanism. Future studies are needed to delineate the mechanism regulating FAK expression, such as investigation of FAK mRNA expression or degradation by other enzymes, such as caspase-6 or 7 (Gervais et al., 1998; Wen et al., 1997). Rescue of FAK protein levels by adenoviral overexpression promoted cell survival on compliant substrates. However decreasing FAK expression using siRNA did not increase death, which may be due to insufficient knockdown, compensation by other factors such as Pyk2 (Lim et al., 2010), or that overexpression of

FAK inhibits apoptosis in a way that does not connect to the TGF-β1-induced apoptosis observed on compliant substrates (Lim et al., 2008; Sonoda et al., 2000).

Contrary to previous reports, we did not find a role for FAK in regulating EMT (Cicchini et al., 2008; Deng et al., 2010). A more careful characterization of focal adhesion components, such as Src, zxyin, and paxillin, might reveal a role for focal adhesion signaling in the regulation of EMT (Mori et al., 2009; Tumbarello et al., 2005). Matrix rigidity might regulate EMT through other mechanisms related to cell-ECM adhesion as well, such as Rho activity or contractility (Bhowmick et al., 2001a; Cho and Yoo, 2007; Gomez et al., 2010). Investigation of these pathways using activated or dominant negative Rho mutants and contractility inhibitors, like ML-7 or blebbistatin, might reveal another level of control whereby matrix rigidity regulates EMT.

Chapter 6

Role of PI3K/Akt in matrix rigidity regulated EMT and apoptosis

6.1 Introduction

Because modulation of FAK expression regulated TGF-β1-induced apoptosis but not EMT, we investigated whether other pathways might be regulated by rigidity and prove important for both survival and EMT. Akt is a prominent player in cell survival, proliferation, growth control, and metabolism and is often hijacked during tumor progression. Akt promotes cell survival through many mechanisms including inhibition of proapoptotic proteins such as BAD, caspase-9, and the forkhead transcription factor family, activation of NF-κB, and antagonizing p53 (Brunet et al., 1999; Cardone et al., 1998; Datta et al., 1997; Kane et al., 1999; Mayo and Donner, 2001). Relevant to this work, TGF-β1 can also induce phosphorylation of Akt and activation of the PI3K/Akt pathway is required for EMT in murine mammary epithelial cells and rat kidney epithelial cells (Bakin et al., 2000; Kattla et al., 2008). In addition, TGF-β1-induced Akt activity also contributes to cell survival (Chen et al., 1998; Conery et al., 2004; Remy et al., 2004; Shin et al., 2001). Matrix adhesion is also a known

regulator of PI3K and Akt (Armulik et al., 2004; Levental et al., 2009; Matter and Ruoslahti, 2001).

The Akt/protein kinase B (PKB) family of serine-threonine kinases consists of three members, Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ. Activity of Akt is regulated by upstream signaling by phosphoinositide-3 kinase (PI3K) that upon activation by growth factors phosphorylates PIP2 to become PIP3. PIP3 interacts with the Pleckstrin homology (PH) domain of Akt promoting translocation to the plasma membrane and subsequent phosphorylation of Ser473 and Thr308. PDK1 phosphorylates Thr308, however the kinase that phosphorylates Ser473 has not been fully characterized, although some evidence exists for autophosphorylation or phosphorylation by integrin linked kinase (Persad et al., 2001; Stephens et al., 1998; Toker and Newton, 2000). Akt activity can be downregulated by PTEN phosphatase, which removes a phosphate from PIP3, and thereby induces Akt translocation away from the plasma membrane.

In this chapter, we examined the hypothesis that signals from matrix rigidity and TGF-β converge on PI3K/Akt to regulate the switch between EMT and apoptosis. We observed that both matrix rigidity and TGF-β1 treatment stimulated Akt activity, and pharmacological inhibition of PI3K/Akt increased cell death and inhibited EMT. Increasing PI3K activity rescued cell survival but not EMT on compliant gels. Additional studies explore the regulation of other downstream members of the apoptotic response, including Bcl-2 family members and initiator caspases.

6.2 Results

6.2.1 Matrix rigidity regulates apoptosis and EMT through PI3K and Akt

To investigate if 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. In all cases, NMuMGs cultured on compliant gels showed decreased Akt activation compared to cells on rigid gels (Fig. 6.1). Treatment with TGF-β1 induced more apoptosis on compliant gels as compared to rigid gels with or without insulin treatment (Fig. 6.1 C). Inhibition of PI3K or Akt activity with pharmacological inhibitors increased TGF-β1-induced apoptosis in NMuMGs on rigid gels (Fig. 6.2 A and B). Inhibition of PI3K decreased Snail mRNA expression on rigid gels, however inhibition of Akt did not (Fig. 6.2 C). Although these studies suggest that PI3K is necessary for survival and EMT following TGF-β1 treatment, it was not clear if it was also sufficient. We increased PI3K activity by adenoviral expression of a constitutively active p110-CAAX, a membrane localized subunit of PI3K, and observed suppression of apoptosis on compliant gels to similar levels observed on rigid gels (Fig. 6.3 A and B). p110-CAAX expression, however, did not rescue Snail mRNA expression on compliant gels (Fig. 6.3 C).

While increasing PI3K activity rescued cell survival, retroviral expression of a constitutively active Akt1 modified with the Src myristoylation sequence (myr-Akt1) did not affect apoptosis or EMT (Fig. 6.4). Expression was verified but no downstream targets were investigated. Akt may not be the key regulatory element in this system or

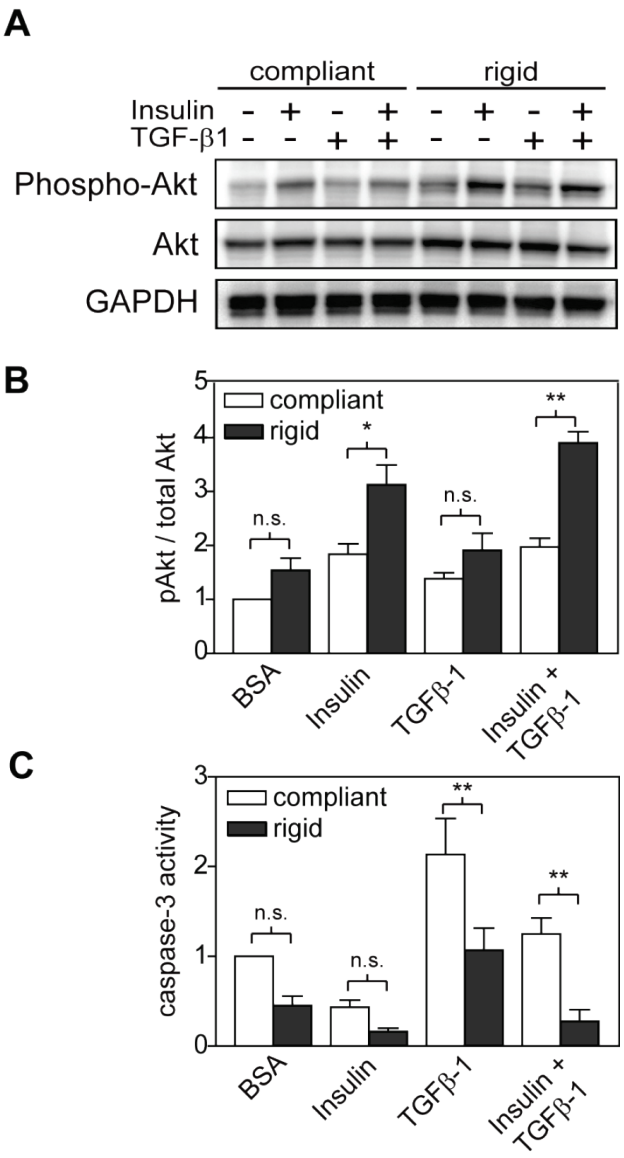

Figure 6.1 Akt activity is regulated by matrix rigidity

(A,B) Western blot (A) and quantification (B) of pAkt (60 kD), total Akt (60 kD), and GAPDH control (38 kD) in NMuMG cells plated on compliant (E=0.4 kPa) and rigid (E=8 kPa) polyacrylamide gels. (C) Caspase-3 activity in NMuMG cells. Error bars indicate the SEM of at least five independent experiments. *, $P < 0.05$; **, $P<0.01$, calculated by two-way ANOVA with Bonferroni posttests.

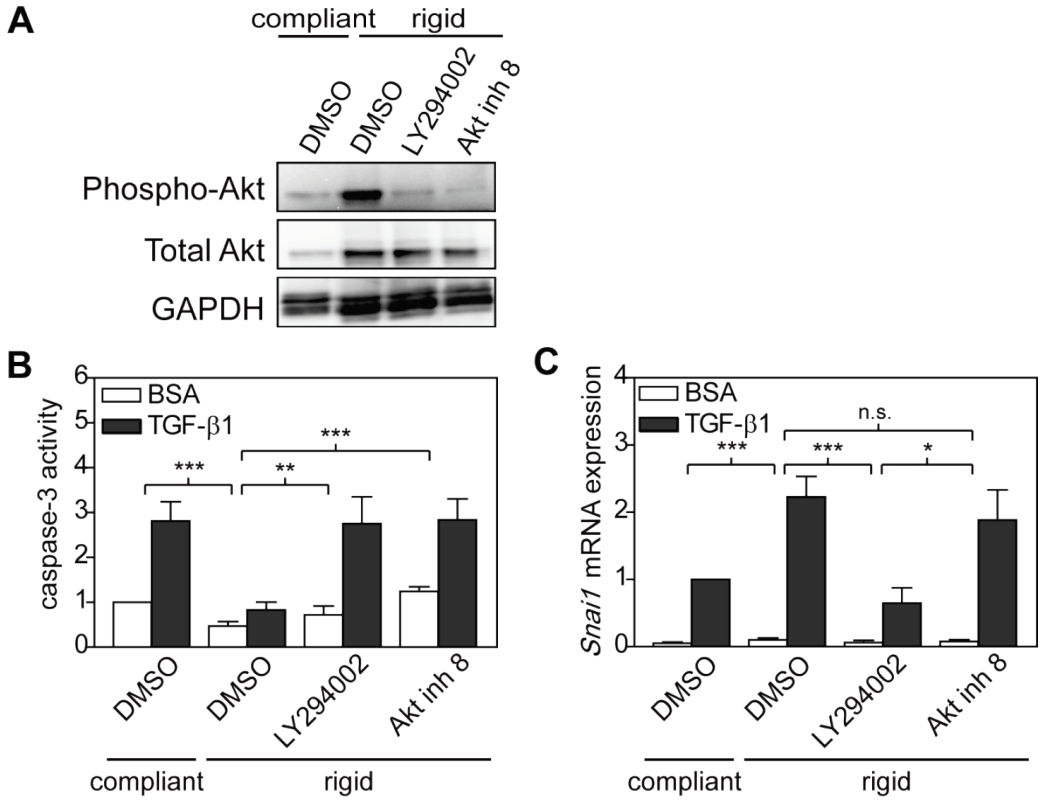

Figure 6.2 PI3K/Akt activity is necessary for EMT and cell survival

(A) Western blot of phospho-Akt, total Akt, and GAPDH in NMuMG cells treated with DMSO control, 10 μM LY294002, or 1 μM Akt inhibitor 8. (B,C) Graphs of caspase-3 activity (B) and *Snai1* mRNA expression (C) in NMuMG cells treated with DMSO, LY294002, or Akt inhibitor 8. Error bars indicate the SEM of at least three independent experiments. *, $P < 0.05$; **, $P<0.01$; ***, $P<0.001$, calculated by two-way ANOVA.

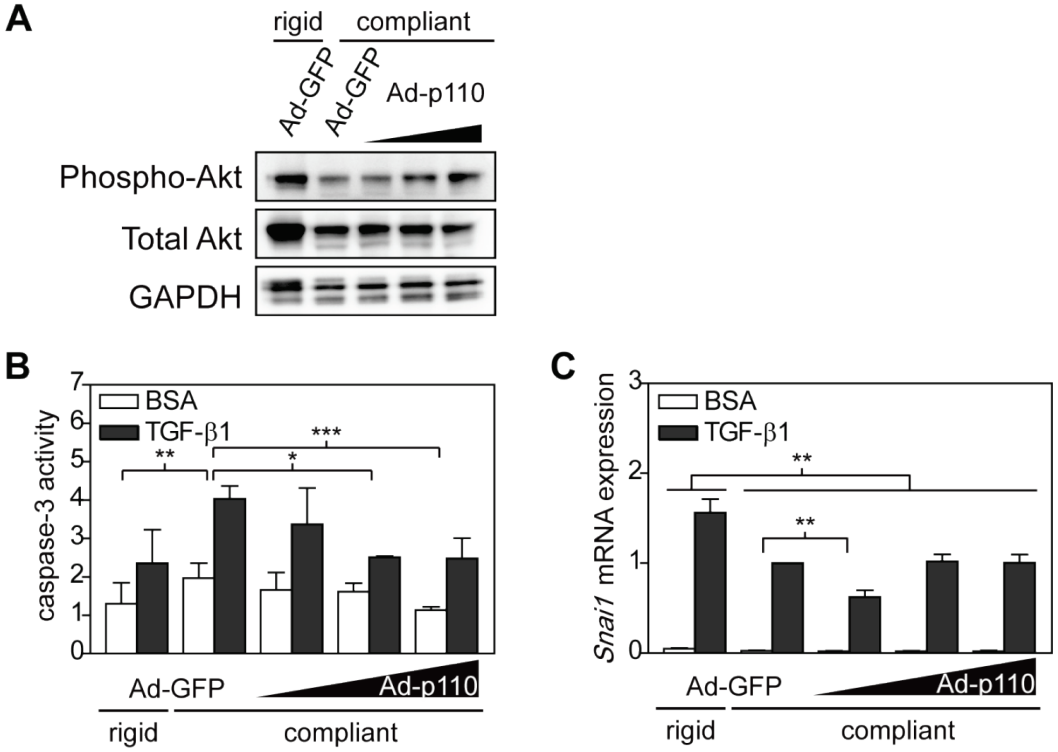

Figure 6.3 Increasing PI3K activity inhibits apoptosis but does not rescue EMT. (A) Western blot of phosphor-Akt, Akt, and GAPDH in NMuMG cells infected with Ad-GFP or Ad-p110. (B, C) Graphs of caspase-3 activity (B) and *Snai1* mRNA expression (C) in NMuMG cells infected with Ad-p110. Error bars indicate the SEM of at least three independent experiments. *, $P < 0.05$; **, $P<0.01$; ***, $P<0.001$, calculated by two-way ANOVA.

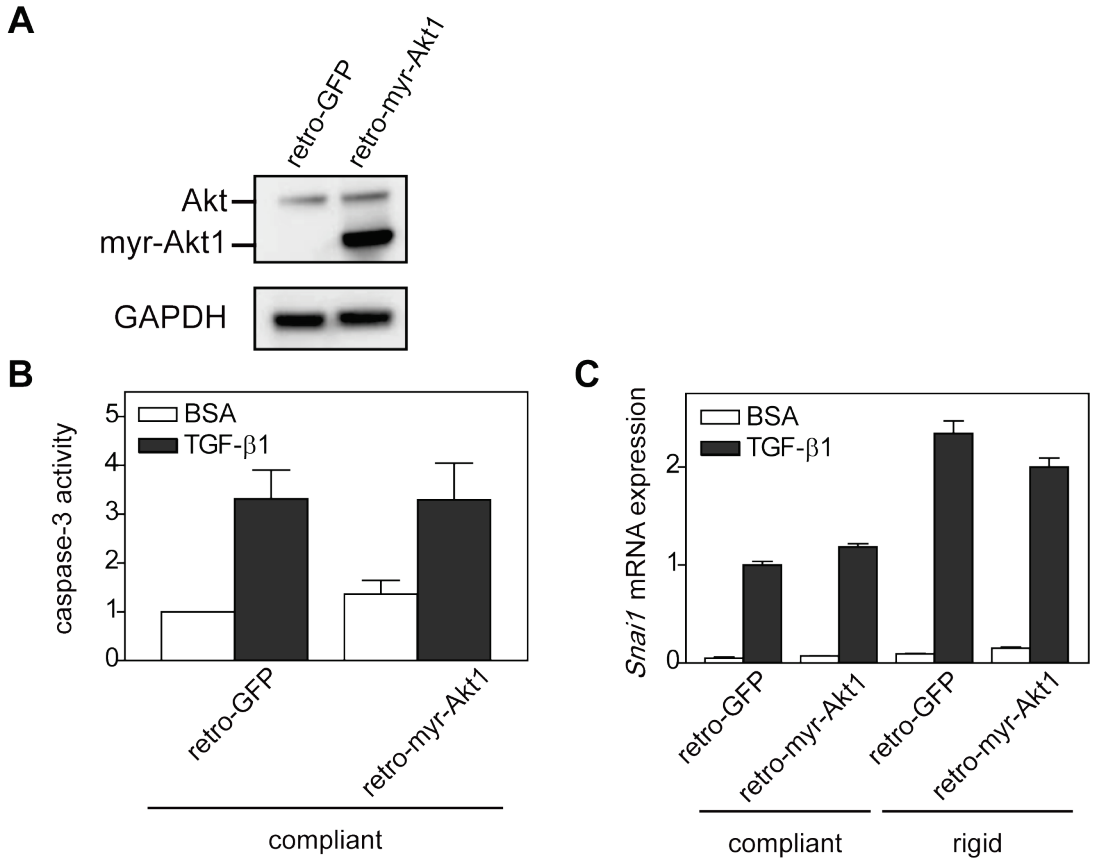

Figure 6.4 Myr-Akt1 does not affect apoptosis or EMT

(A) Western blot of Akt, myr-Akt1, and GAPDH in NMuMG cells. (B,C) Graphs of caspase-3 activity (B) and *Snai1* mRNA expression (C) in NMuMG cells infected with retro-GFP or retro-myr-Akt, plated on compliant and rigid PA gels, and treated with TGF-β1. Error bars indicate SEM of at least three independent experiments.

the construct may not act as a constitutively active Akt, tethering it to the membrane with the myristoylation sequence might somehow interfere with the function of Akt. Despite the lack of effect of the myr-Akt1 construct, data described above demonstrate a role for PI3K and Akt in transducing substrate compliance and regulating the compliance-induced switch in cellular response to TGF-β1.

6.2.2 FAK manipulations do not regulate Akt activity

Previous work has shown that FAK plays an important role in cell survival through several mechanisms, including through regulation of Akt activity (Chen et al., 1996; Sonoda et al., 2000). Data from this work showed that FAK expression was decreased on compliant gels, and overexpression inhibited TGF-β1-induced apoptosis on compliant gels. In this system, however, Akt activity was not affected by FAK manipulations. FAK overexpression did not affect Akt phosphorylation or expression, and siRNA knockdown of FAK also did not affect Akt phosphorylation (Fig. 6.5 A and B).

6.2.3 Matrix rigidity regulates expression of Bcl-2 and Bcl-xL

Previous studies have demonstrated that one way TGF-β induces apoptosis is by regulating Bcl-2 family members, including decreasing expression of the anti-apoptotic Bcl-2 and Bcl-xL (Chipuk et al., 2001; Motyl et al., 1998) and upregulating pro-apoptotic members such as Bax, Bim, and Bmf (Ramjaun et al., 2007; Yano et al., 2006). ECM adhesion has also been demonstrated to regulate Bcl-2 expression. α5β1 integrin binding to fibronectin activates the PI3K/Akt pathway which upregulates Bcl-2 expression and enhances cell survival (Matter and Ruoslahti, 2001; Zhang et al., 1995). To investigate if Bcl-2 and Bcl-xL were regulated by matrix rigidity and TGF-β in this

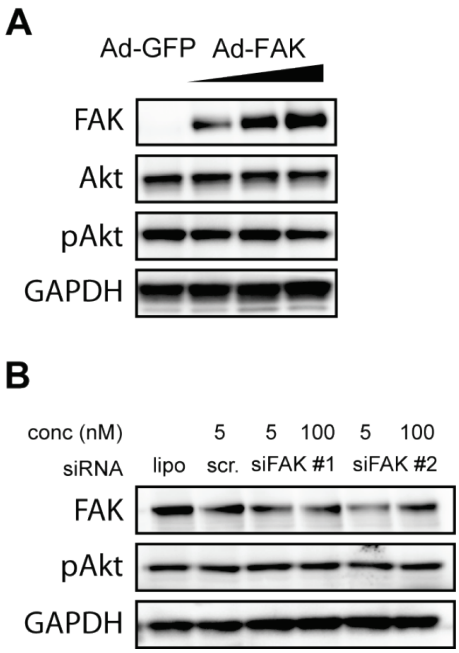

Figure 6.5 FAK expression does not affect pAkt.

(A) Western blot of FAK, pAkt, and GAPDH in NMuMG cells overexpressing FAK. (B) Western blot of FAK, pAkt, and GAPDH in NMuMG cells transfected with lipofectamine control, scrambled siRNA control, FAK siRNA #1 and #2 (5 and 100 nM).

system, we analyzed protein expression in NMuMGs plated on compliant and rigid gels, in growth medium or treated with TGF-β1. In all culture conditions, cells on compliant gels had reduced Bcl-2 expression as compared to rigid gels (Fig. 6.6). A slight but not significant reduction was also observed for Bcl-xL expression.

6.2.4 Role of initiator caspases in TGF-β1-induced apoptosis

Proapoptotic caspases, a family of cysteine proteases responsible for the execution of apoptotic cell death, are subdivided into two groups, initiator caspases, caspase-2, 8, 9, and 10, and effector caspases, caspase-3,6 and 7. Caspases are expressed in an inactive proenzyme form and are activated by proteolytic processing. The effector, or executioner, caspases are responsible for DNA degradation, nuclear condensation, and plasma membrane blebbing that are characteristic of apoptosis. The effector caspases become active after cleavage by the initiator caspases. Initiator caspases can be activated by two pathways, the extrinisic and intrinisic pathways. In the extrinisic pathway, ligand binding to a death receptor initiates apoptosis through the death inducing complex (DISC) and subsequent activation of caspase-8. The intrinsic, or mitochondrial, pathway is initiated by internal stressors such as DNA damage or hypoxia, and results in activation of caspase-9 and mitochondrial permeabilization.

Information about which pathway, extrinsic or intrinsic, is initiated after TGF-β treatment on compliant gels would aid in understanding how matrix rigidity regulates apoptosis (Frisch, 1999). NMuMG cells plated on compliant PA gels were treated with inhibitors to caspase-8 and -9, IETD-CHO and LEHD-CHO respectively, prior to TGF-β1 treatment. Caspase-3 activity was reduced by the caspase-8 inhibitor at 10 μM and by the caspase-9 inhibitor at 100 μM (Figure 6.7). Because the inhibitors might affect

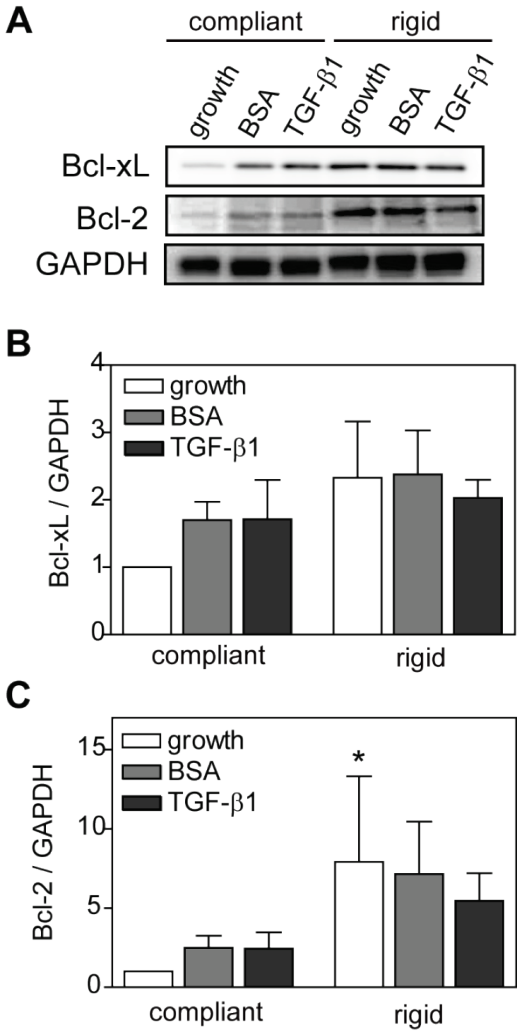

Figure 6.6 Matrix rigidity regulates Bcl-2 expression.

(A, B) Western blot (A) and quantification (B) of Bcl-xL (27 kD), Bcl-2 (26 kD), and GAPDH (38 kD) expression in NMuMG cells in growth media or treated with TGF-β1, and plated on compliant and rigid gels. Error bars indicate SEM of four independent experiments. *, $P<0.05$ calculated by *t* test.

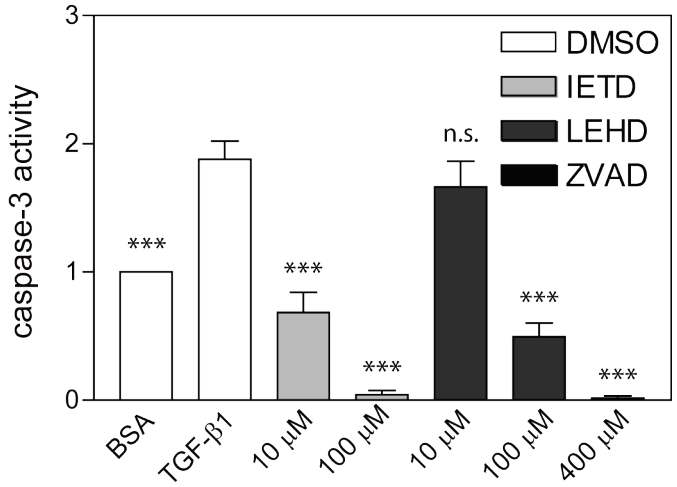

Figure 6.7 Inhibition of caspase-8 and caspase-9 inhibits apoptosis.

Graph of caspase 3 activity in NMuMG cells plated on compliant PA gels, treated with DMSO control, IETD (caspase-8), LEHD (caspase-9), and ZVAD (caspase-3) pharmacological inhibitor prior to TGF-β1 treatment. Error bars indicate SEM of four independent experiments. ***, $P<0.001$ as compared to DMSO TGF-β1 condition, calculated by one-way ANOVA.

other caspases, particularly at higher concentrations, more studies will need to be done, such as caspase-8 and caspase-9 activity assays, and further work to characterize apoptosis, such as nuclear fragmentation assays.

6.3 Discussion

Numerous previous studies have demonstrated the importance of the PI3K/Akt signaling pathways for cell survival (Dudek et al., 1997; Khwaja et al., 1997; Shin et al., 2001) and EMT (Bakin, A.V. et al., 2000, Kattla, J.J. et al., 2008). Here, we find that decreasing matrix rigidity inhibits PI3K/Akt activity, and through this action impinges on both survival and EMT. Increasing PI3K activity by adenoviral expression of a constitutively active p110 subunit of PI3K rescued cell survival but not EMT. While previous literature stated expression of p110 induced EMT in the absence of TGF-β1, EMT in that study was only characterized by a loss of tight junctions, and there was no evidence of a morphology change or upregulation of mesenchymal markers (Bakin et al., 2000). Myr-Akt1 was also not sufficient to rescue EMT in our studies, although these results are not entirely surprising, given other work where downregulation of the Akt1 isoform induced EMT, and the Akt2 isoform was necessary for this transformation (Irie et al., 2005). Studies isolating each isoform, Akt1, 2, and 3, through overexpression and siRNA manipulations would likely help elucidate the role of matrix rigidity in regulating Akt and EMT induction in this system. Another important question that arises from the results in this chapter is how does matrix rigidity regulate PI3K/Akt? In this system, it does not appear that FAK is regulating PI3K/Akt activity, but there are other possibilities. Matrix rigidity could regulate integrin linked kinase (ILK), which has been postulated as the elusive second kinase responsible for

phosphorylation of Ser473 (Delcommenne et al., 1998; Persad et al., 2001). Additionally, rigidity could modulate PTEN, a lipid phosphatase that antagonizes PI3K signaling, or traditional growth factor signaling upstream of PI3K.

Finally, work presented at the end of this chapter begins to dissect the apoptotic mechanism regulated by matrix rigidity and TGF-β1. Matrix compliance downregulates Bcl-2 and Bcl-xL expression, and overexpression of Bcl-xL rescues cell survival, suggesting that apoptosis occurs through the intrinsic mitochondrial pathway. Pharmacological inhibition of the initiator caspase-8 and 9 reduced caspase-3 activity, however more work is needed to confirm the involvement of these caspases and the specificity of the inhibitors, such as caspase activity assays with the substrate specific to each caspase and, to avoid non-specific inhibition of caspase-3 activity, apoptosis assays such as nuclear fragmentation or TUNEL. Additionally, exploration of the role of FAK and Akt in the regulation of Bcl-2 family members and caspase activation will also yield valuable information on the regulation of apoptosis by both of these pathways.

Chapter 7

Conclusions and Future Directions

7.1 Conclusions

TGF-β regulates a diverse array of cellular functions, including proliferation, motility, and differentiation. How TGF-β regulates often divergent functions, particularly in disease contexts such as fibrosis and tumorigenesis, is not well understood. Here we demonstrate that matrix rigidity regulates a switch between TGF-β-induced apoptosis and EMT, and we investigated the signaling mechanisms involved in this switch.

In Chapter 4, we explored how varying cell adhesion to the ECM modulated the cellular response to TGF-β. Decreasing substrate rigidity increased TGF-β-induced apoptosis and inhibited EMT. Modulating cell-ECM adhesion by changing cell seeding density revealed that at confluence, a condition with decreased cell spreading and increased cell-cell contact, both apoptosis and EMT are inhibited. Finally, limiting ECM adhesion of single cells by microcontact printing, increased TGF-β-induced apoptosis, similar to compliant substrates, suggesting that limiting cell adhesion may be the mechanism whereby rigidity controls apoptosis.

The role of focal adhesion formation and signaling in regulating apoptosis and EMT was investigated in Chapter 5. Compliant matrices inhibited stress fiber and focal

adhesion formation even with TGF-β1 treatment. Further investigation revealed FAK was regulated at the level of protein expression and not activity. Compliant matrices decreased expression of FAK, and overexpression of FAK rescued cell survival. Modulation of FAK activity, by a pharmacological inhibitor or various adenoviral expressed mutants, did not affect apoptosis or EMT.

Further mechanistic studies in Chapter 6 demonstrated that matrix rigidity regulates PI3K/Akt activity to control the switch in TGF-β1-induced apoptosis and EMT. Decreasing matrix rigidity inhibits PI3K/Akt activity, and through this action impinges on both survival and EMT. Increasing PI3K activity rescued survival but not EMT on compliant substrates. It is perhaps not surprising that upregulation of FAK or PI3K failed to rescue EMT on low rigidity substrates, given the many disparate processes that are collectively coordinated to drive EMT.

7.2 Future Directions

7.2.1 Further exploration of the cellular microenvironment

In this thesis, we have explored the role of the cellular microenvironment in regulating TGF-β-induced cell functions. Our results demonstrate that modulating the microenvironment, by changing substrate rigidity, cell seeding density, or cell spreading, can regulate TGF-β-induced apoptosis and EMT. Through the use of microcontact printing, we demonstrated that restricting cell spreading alone increases TGF-β1-induced apoptosis. From other work in the lab, we have also observed that increasing cell-cell contact did not affect TGF-β1-induced apoptosis, suggesting that

matrix rigidity is regulating apoptosis through changes in cell spreading and not cell-cell adhesion (unpublished data). Previous studies have demonstrated a role for cell spreading, cell-cell adhesion, and contractility for induction of EMT, but whether these observations are true in the system used here remains to be seen (Gomez et al., 2010; Nelson et al., 2008). Although cell-cell adhesion did not appear to play a role at early time points, it would be interesting to observe if polarized multicellular structures are resistant to TGF-β-induced apoptosis or EMT (Weaver et al., 2002).

We have used fibronectin as the adhesive ligand in the studies presented here, but other matrices including collagen I and basement membrane proteins, such as laminins and collagen IV, would elucidate if certain integrins are important for the matrix rigidity regulated switch between apoptosis and EMT. These studies would also give extra insight into regulation of cell function during disease progression. In normal tissues, epithelial cells are in contact with a basement membrane, and during tumor progression and metastasis, tumor cells encounter different ECM components, such as collagen I. Additionally, the results presented here were limited to 2D studies, and it will be important to determine how the effects observed in this work translate to a 3D environment using methods discussed earlier, such as collagen and PEG gels.

7.2.2 Further elucidating molecular mechanisms regulated by matrix rigidity and TGF-β

The studies presented in this work demonstrate a role for FAK and Akt in regulating apoptosis, however there are several other mechanisms that warrant further study and will elucidate how matrix rigidity regulates TGF-β1 induced apoptosis. As discussed in Chapter 2, a number of pathways can be activated downstream of TGF-β

that are also important for EMT and apoptosis, and it is likely that these pathways may also be involved in this switch, including MAPKs and RhoGTPases.

7.2.2.1 The role of EMT in cell survival

In Chapter 4, we explored the possibility that compliant substrates inhibit EMT by increasing TGF-β-induced apoptosis thus preventing EMT. Apoptosis occurred within hours of TGF-β treatment, however EMT could only be fully characterized after several days, leading us to ask whether cells on compliant substrates would undergo EMT at longer time points. To answer this question, we inhibited apoptosis and demonstrated that cells on compliant substrates, even with inhibition of apoptosis, were not able to fully undergo EMT. The reverse question could also be posed, though: does induction of EMT protect cells from apoptosis? Indeed, several reports from the literature show that after induction of EMT, cells become immune to TGF-β-induced apoptosis (Del Castillo et al., 2006; Gal et al., 2008; Robson et al., 2006; Valdes et al., 2002). Additionally, Snail, a transcription factor both necessary and sufficient for EMT, and the closely related Slug are also a survival factors (Franco et al., 2010; Leroy and Mostov, 2007; Vega et al., 2004). It would be interesting to overexpress Snail on compliant substrates and observe whether there is inhibition of apoptosis and induction of EMT. And, conversely, does siRNA knockdown of Snail on rigid substrates increase apoptosis and inhibit EMT? A more clear understanding of the role of Snail in EMT and apoptosis would help to delineate the interplay between these two functions and the regulation by matrix rigidity.

7.2.2.2 Further regulation of EMT and apoptosis by focal adhesions

We demonstrated that compliant substrates decreased FAK expression, and increasing FAK expression rescued cell survival. However, we did not identify the mechanism regulating FAK expression. We did not find a role for calpain mediated degradation as previous literature had reported, however previous studies used a collagen matrix and found a role for the discoidin domain receptors (DDR1 and 2) in FAK degradation (Bhadriraju et al., 2009; Wang et al., 2003b). Because we used fibronectin as our adhesive matrix, FAK degradation is likely regulated by a different mechanism. Future studies are needed to delineate the mechanism regulating FAK expression, such as investigation of FAK mRNA expression or degradation by other enzymes, such as caspase-6 or 7 (Gervais et al., 1998; Wen et al., 1997). Rescue of FAK protein levels by adenoviral overexpression promoted cell survival on compliant substrates. However decreasing FAK expression using siRNA did not increase death, which may be due to insufficient knockdown, or compensation by other factors such as Pyk2 (Lim et al., 2010). Observation of apoptosis during siRNA transfection might reveal that FAK is necessary for survival in these cell lines, and explain why increased siRNA concentration did not decrease FAK expression. Finally, investigation of Pyk2 expression and activity would reveal if Pyk2 compensation prevents increased apoptosis by FAK knockdown.

Contrary to previous reports, we did not find a role for FAK in regulating EMT (Cicchini et al., 2008; Deng et al., 2010). A previous study has reported compliance induced degradation of focal adhesion components in addition to FAK, including talin, paxillin, and p130Cas (Wang et al., 2003b). A more careful characterization of focal

adhesion components, such as Src, zxyin, and paxillin, in the system presented in this work might reveal a role for focal adhesion signaling in the regulation of EMT (Mori et al., 2009; Tumbarello et al., 2005). Additionally, if degradation of focal adhesion components was inhibited with a protease inhibitor, is induction of EMT restored and apoptosis prevented on compliant substrates? Further investigation into the role of focal adhesion regulation of EMT and apoptosis will further elucidate how matrix rigidity controls these processes.

7.2.2.3 Role of Rho-mediated contractility in matrix rigidity regulation of EMT and apoptosis

Matrix rigidity might regulate EMT and apoptosis through other mechanisms related to cell-ECM adhesion as well, such as Rho activity and contractility. Matrix rigidity has been shown to regulate Rho activity and actin stress fiber formation in a number of previous studies (Krndija et al., 2010; Paszek et al., 2005; Provenzano et al., 2009; Wozniak et al., 2003). TGF-β1 also increases Rho activity and stress fiber formation (Bhowmick et al., 2001a; Cho and Yoo, 2007; Gomez et al., 2010). In this work, we observed stress fiber formation on rigid gels, which was increased with the addition of TGF-β1, however we did not measure or manipulate Rho activity. Investigation of these pathways using activated or dominant negative Rho mutants and contractility inhibitors, like ML-7 or blebbistatin, would likely reveal another level of control whereby matrix rigidity regulates EMT.

7.3 Concluding Remarks

This thesis explores the role of matrix rigidity in regulating cell function. We demonstrated that varying matrix rigidity regulates a switch between TGF-β-induced apoptosis and EMT. We found that on compliant substrates, with a modulus similar to native breast tissue, TGF-β induces apoptosis, whereas on rigid substrates, with a modulus similar to tumor or fibrotic tissue, TGF-β induces EMT. Further investigation revealed matrix rigidity downregulates FAK expression and inhibits PI3K/Akt activity, and through this action impinges on both survival and EMT. As discussed in the future directions section, further studies are needed to fully understand the complex interplay between the cellular microenvironment, TGF-β, and the underlying signaling mechanisms. The work presented here provides a possible explanation for the switch in TGF-β's action from tumor suppressor to promoter during tumorigenesis, and likely extends to other disease contexts such as atherosclerosis and fibrotic diseases, during which there is tissue stiffening and TGF-β is a major contributing factor. Furthermore, these studies highlight the central role for matrix mechanics in regulating cell signaling and fate, and stress the importance of considering physical factors in biological systems.

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