SUBSTRATE RIGIDITY REGULATES FIBRONECTIN MATRIX ASSEMBLY
IN FIBROBLASTS

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A DISSERTATION
PRESENTED TO THE FACULTY
OF PRINCETON UNIVERSITY
IN CANDIDACY FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

RECOMMENDED FOR ACCEPTANCE
BY THE DEPARTMENT OF MOLECULAR BIOLOGY
[Adviser: Jean Schwarzbauer]

April 2013
Abstract

Cells sense and respond to the mechanical properties of their microenvironment. We investigated whether these properties affect the ability of cells to assemble a fibrillar fibronectin (FN) matrix. Analysis of matrix assembled by cells grown on FN-coated polyacrylamide gels of varying stiffnesses showed that rigid substrates stimulate FN matrix assembly, whereas assembly is inhibited on softer substrates. Stimulating integrins with Mn$^{2+}$ treatment increased FN assembly on softer gels, suggesting that integrin binding is deficient on soft substrates. Guanidine hydrochloride-induced extension of the substrate-bound FN rescued assembly on soft substrates to a similar degree as Mn$^{2+}$ treatment and also increased the initiation of assembly at FN matrix assembly sites. In contrast, increasing actin-mediated cell contractility did not rescue FN matrix assembly on soft substrates. Thus, rigidity-dependent FN matrix assembly is determined by extracellular events, namely, the engagement of FN by cells and the induction of FN conformational changes. Extensibility of FN in response to substrate stiffness may serve as a mechanosensing mechanism whereby cells use pericellular FN to probe the stiffness of their environment.
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To my mother, who left this world in better shape than she found it.
Acknowledgements

I would like to thank …

Dr. Jean Schwarzbauer, for her valuable guidance, mentorship, and insightful ideas.

Dr. Hilary Coller and Dr. Celeste Nelson, for their continued support and guidance.

Dr. Yibin Kang, for his time and assistance with my thesis.

Elena Chiarchiaro, for all her help and enthusiasm.

The current and past members of the Schwarzbauer Lab, with a special thanks to Dr. Adam Engler, for his technical instruction.

My friends and family, for their encouragement and unwavering love and support.
Chapter 1

Introduction

The ECM regulates cell behavior

The extracellular matrix (ECM) is a network of proteins and proteoglycans that encompasses cells, providing structural support and organizing them into functionally distinct tissues (Hynes and Yamada, 2011). In addition to its structural functions, ECM also plays a regulatory role in cell behavior. The ECM interacts with cell surface receptors, providing cells with information about their environment and regulating crucial cellular processes including proliferation, differentiation, migration, and apoptosis. These cellular processes mediate global biological processes, such as embryonic development and the maintenance of tissues.

Cells deposit the components of the ECM and arrange them into a complex network. In multicellular organisms, there are two structurally distinct forms of ECM (Hynes and Yamada, 2011). Stroma is composed of a mesh-like arrangement of fibrils that encompass fibroblasts in a three-dimensional matrix. In contrast, the basement membrane is a dense, sheet-like structure that excludes cells, serving as a barrier between tissues. Many cell types produce ECM, including fibroblasts, which constantly secrete and assemble components of the stroma, making them valuable tools for studying the creation and maintenance of ECM.

It is critical for an organism’s ECM to be properly maintained. Aberrant expression, deposition, or rearrangement of ECM components can dramatically alter its mechanical and biochemical characteristics. Changing the properties of the ECM affects how cells interact with it, thus, sending cells different signals and altering cell behavior. This, in turn, can lead to pathological cell behavior and disease. For example, fibrosis is characterized by a dramatic increase in the deposition of several ECM components and a lack of organization (Cox and Erler, 2011).

The molecular composition of the ECM is one aspect that determines its structural and functional role. The proteins and proteoglycans that comprise the ECM include
fibronectin, collagen, and heparan sulfate proteoglycans, which often interact with each other and affect each other’s activities. For example, fibronectin seeds collagen assembly, and collagen I is not incorporated into the ECM in the absence of any fibronectin matrix in vitro (Sottile and Hocking, 2002).

**Fibronectin is an important part of the ECM**

Fibronectin (FN) is a large glycoprotein that is a major component of the ECM. It is found in all vertebrates, and its wide expression in vivo suggests multiple functions in a variety of processes, including embryonic development (Darribere and Schwarzbauer, 2000; George et al., 1993), tissue repair (Midwood et al., 2006), and immune responses (Coito et al., 2000). Knocking down FN in mouse embryos results in early embryonic lethality, confirming that FN is essential during development (George et al., 1993). Altered FN expression and matrix organization have been associated with numerous pathologies, including cancer and tissue fibrosis (Hynes, 1990b; Williams et al., 2008).

**Interactions with fibronectin**

Fibronectin is a dimer composed of two subunits, approximately 250 kDa each, joined near their carboxyl termini by two disulfide bonds (Figure 1.1). Each subunit is comprised of repeats of three types of structurally distinct domains, classified as type I, II and III modules. Both the type I and type II modules contain internal disulfide bonds that maintain their structures, but type III repeats lack disulfide bonds, making them more flexible and able to unfold (Hynes, 1990a). The modules are arranged together at an angle, allowing FN to be stretched by straightening out the angles between the modules.

Fibronectin is multifunctional due to the affinity of its domains for various cell surface receptors and other ECM proteins, including other FN molecules. Modules III₀ and III₁₀ consist of the cell-binding domain, by which cells bind to FN via integrin receptors. Integrins are heterodimeric transmembrane cellular receptors that bind ECM proteins and mediate cell adhesion and ECM assembly (Barczyk et al., 2009). They are composed of an alpha and beta subunit, which determine their binding specificities. Fibronectin binds several different integrins, but the α₅β₁ integrin is the primary receptor
Figure 1.1 – Domain structure of fibronectin. The FN molecule has a modular structure consisting of three types of repeats: types I, II, and III. A pair of disulfide bonds at the C-terminus connects the dimers. The first five type I modules make up the assembly domain and are part of the 70-kDa fragment. The III9–10 domain comprises the cell-binding domain. FN binding sites are present along the length of the molecule, as well as binding sites for other ECM molecules. (modified from Singh et al. 2010)
involved in FN matrix assembly (Ruoslhti, 1991; Schwarzbauer and DeSimone, 2011; Wu et al., 1993). Module III\textsubscript{10} contains the cell-binding tripeptide, Arg-Gly-Asp (RGD), which mediates cell adhesion and is essential for cells to bind certain integrins, including integrin $\alpha_5\beta_1$. Cell attachment and spreading can be promoted on solid surfaces by coupling short peptides containing the RGD sequence (Rajagopalan et al., 2004), and soluble sequences that contain the RGD sequence compete with FN for cell binding, inhibiting cell attachment to the FN matrix (Gehlsen et al., 1988). Module III\textsubscript{9} contains the synergy sequence Pro-His-Ser-Arg-Asp (PHSRN), which cooperates with the RGD sequence to enhance cell adhesion and assembly of FN into the ECM (Aota et al., 1994; Sechler et al., 1997). The cell-binding domain is necessary for the initiation of FN matrix assembly, though FN lacking this domain can be incorporated into the matrix after assembly has commenced (Ichihara-Tanaka et al., 1992).

Fibronectin self-association sites serve different functions in FN matrix assembly. The intramolecular interaction between III\textsubscript{12-15} and III\textsubscript{2-3} is thought to keep FN in its soluble, compact conformation (Johnson et al., 1999), which prevents FN from forming fibrils in the absence of cells. Other FN-binding sites promote FN matrix assembly. The 70-kDa fragment of FN contains the first five type I modules (I\textsubscript{1-5}), termed the ‘assembly domain’, which interact with all other FN-binding sites and is essential for FN to be incorporated into the ECM (Schwarzbauer, 1991). Addition of the 70-kDa fragment to cells effectively inhibits FN fibril formation, as it competes with full-length FN for FN-binding (McKeown-Longo and Mosher, 1985).

Other modules along the length of the molecule bind FN and are thought to be involved in FN matrix assembly. In particular, the III\textsubscript{1-2} domain contains FN binding sites that are involved in the assembly of a mature FN matrix. FN lacking this domain does not form a mature FN matrix (Sechler et al., 2001).

**Extensibility of fibronectin**

Fibronectin molecules are in a compact conformation when they are secreted by cells, but FN can be structurally extended to varying degrees. Intramolecular interactions maintain FN in a compact, globular conformation (Johnson et al., 1999), likely by “folding up” the FN dimer. Once these interactions are broken, the FN molecule assumes
an extended conformation, whereby the FN dimer assumes a string, or bead-like
conformation (Engel et al., 1981; Erickson and Carrell, 1983). This extended
conformation of FN can be further unfolded in two different ways (Figure 1.2). The
modules of FN are arranged together at an angle (Leahy et al., 1996; Sharma et al., 1999),
enabling FN to be stretched by straightening out the angles between the modules.
Alternatively, the lack of internal disulfide bonds within the FN type III repeats allows
them to be unfolded (Erickson, 1994). FN could also be unfolded via a combination of
the two. A recent study concluded that only six of the 15 type III repeats within a FN
monomer are unfolded in vivo, with only two of the six being unfolded solely in fibrillar
FN (Lemmon et al., 2011). Thus, it appears that a few FN type III domains are forcefully
unfolded, but domain unfolding isn’t the sole method of FN extension.

Although some molecular-recognition sites are present in the regions between
modules, several cryptic binding sites have been identified that are buried within
individual FN modules in the folded state. These sites require mechanical, chemical, or
thermal unfolding of the FN molecule in order to be exposed. For example, the III
module of FN contains a cryptic binding site for the 70 kDa fragment of FN (Hocking et
al., 1994), and the III_{10} module binds for the III_{1} module upon heat denaturation (Hocking
et al., 1996). The III_{4-5} domain of FN contains cryptic binding sites for both the 70-kDa
fragment and other III_{4-5} domains (Maqueda et al., 2007). Variation in the unfolding of
FN within fibrils may change the biochemical cues along a fibril.

Forces can induce the unfolding of FN modules, which results in alterations in
molecular-recognition sites, or the exposure of peptide sequences that are otherwise
hidden in the unfolded modules (Hocking et al., 1994; Moyano et al., 1999; Vogel, 2006).
Cells can generate an intracellular force that can then be transmitted to the extracellular
environment through transmembrane linkages with the cytoskeleton. Actin molecules
within the cell become organized into filaments, which then associate with myosin
motors (Burridge and Chrzanowska-Wodnicka, 1996). These myosin motors enable cells
to contract by moving the actin filaments, which are connected to the intracellular
surfaces of cells. Cell contractility is sufficient to partially unfold FN in matrix fibers
(Baneyx et al., 2002).
Figure 1.2 – Fibronectin unfolding. The type III FN modules are arranged at an angle, as shown in A. FN molecules can be unfolded by either straightening the angles between modules (B), or unfolding type III modules (C). (from Erickson 1994)
Fibronectin matrix assembly involves multiple steps

Soluble FN molecules are assembled into an insoluble fibrillar matrix in a complex cell-mediated process that involves several steps (Singh, Carraher et al. 2010; Schwarzbauer and DeSimone 2011) (Figure 1.3). 1) Fibronectin matrix assembly begins when soluble, compact FN dimers bind to α5β1-integrin receptors on the cell surface. 2) Integrin binding leads to an increase in cell contractility, mediated by the recruitment of intracellular molecules that connect the integrins to the cytoskeleton. This allows cells to stretch their FN ligands from a compact to an extended form, exposing cryptic FN-binding sites along the length of the molecule. 3) Exposure of these FN-binding sites promotes intermolecular interactions and formation of fibrils. Short, detergent-soluble FN fibrils begin to form between adjacent cells and the substrate. As matrix assembly proceeds, these soluble fibrils are irreversibly converted into stable, detergent-insoluble fibrils that comprise the mature ECM. The mechanism by which cells convert soluble FN into insoluble FN remains unclear. However, it is believed that strong forces exerted by the cells partially unfold FN, allowing FN molecules to form strong non-covalent interactions (Chen and Mosher, 1996; Singh et al., 2010).

The first step of FN matrix assembly is dependent upon integrins and the availability of FN. The α5β1 integrin is the primary receptor involved in FN matrix assembly (Ruoslahti, 1991; Wu et al., 1993) and is the only integrin able to bind soluble FN (Huveneers et al., 2008). Integrins on the cell surface bind FN molecules through the cell-binding domain (McDonald et al., 1987), and function-blocking antibodies against either α5 or β1 integrins block initial FN fibril formation (Darribere et al., 1990; Roman et al., 1989). Binding of both the III9 and III10 modules are required for the initiation of FN matrix assembly (Aota et al., 1994; Garcia et al., 2002; Sechler et al., 1997). Cell-adhesion to FN requires only the III10 module, containing the RGD cell-binding sequence; however, this bond is weak (Friedland et al., 2009) and will not support FN matrix assembly by itself. The integrin must also bind the synergy site in the III9 module, which strengthens the FN-integrin bond (Friedland et al., 2009). Recombinant FN with a
Figure 1.3 – **Major steps of FN matrix assembly.** A. Soluble compact FN molecules bind α5β1 integrins on the cell surface. B. FN-binding results in the re-organization of the cytoskeleton and a change in the conformation of the bound FN that exposes FN binding sites. C. FN molecules associate through the newly uncovered FN binding sites, and fibrils are formed. (modified from Singh et al. 2010)
mutation in the synergy site is not assembled into matrix unless the integrins are artificially stimulated (Sechler et al., 1997).

Following integrin-FN binding, intracellular proteins are recruited to the cytoplasmic domains of the integrins. These proteins stimulate cell signaling and connect the FN-bound integrins to the actin cytoskeleton. FN matrix assembly requires an intact actin cytoskeleton (Hynes, 1990a; Wu et al., 1995; Zhong et al., 1998). Stimulation of actin-mediated cell contractility increases FN matrix assembly on rigid glass or plastic substrates (Zhang et al., 1994), and inhibition of cell contractility reduces FN fibril formation and FN binding to cell monolayers (Zhong et al., 1998). The actin cytoskeleton is the presumptive source for the force that unfolds FN (Zhong et al., 1998). This increase in cell contractility is thought to change the conformation of the integrin-bound FN and expose previously buried FN binding sites.

The extracellular integrin domain binds FN, while the intracellular domain is connected to the cytoskeleton via focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996). Focal adhesions consist of numerous signaling and actin-binding molecules that are activated and recruited to the cytoplasmic tail of integrins, following integrin binding to an ECM ligand. Focal adhesion kinase (FAK) and talin are both focal adhesion proteins that are important for FN matrix assembly (Green et al., 2009; Ilic et al., 2004). FAK is a protein kinase that becomes phosphorylated in response to integrin-ECM binding (Schaller, 2001), and FAK-null cell do not assemble a FN matrix (Ilic et al., 2004). Talin is an actin-binding protein that links integrins to the actin cytoskeleton (Moser et al., 2009), and down-regulation of talin-integrin binding inhibits FN matrix assembly (Green et al., 2009). Following ECM binding, the integrins begin to cluster (Roca-Cusachs et al., 2009). The local concentrations of the integrin-associated signaling and actin-binding molecules increase, and a focal adhesion complex is formed.

Formation of an adhesion complex leads to the re-organization of the cytoskeleton and an eventual increase in cell contractility. The linkage to the cytoskeleton is important, as inhibition of this linkage disperses integrins and focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996). This internal stress can be transmitted to the extracellular ligand through its association with the integrin adhesion complex. The
cytoskeleton pulls on the FN ligand via integrin attachments, and the ligand, in turn, resists deformation by this stress. If the stress applied to the FN ligand is greater than the strength of the molecular bonds holding the protein together, then the FN molecule should become partially unfolded. Partially unfolding FN would reveal FN binding sites that were previously unavailable (Zhong et al., 1998).

In a current model of FN matrix assembly, FN fibrils are formed when FN binding sites are exposed via integrin-mediated stretching, allowing nearby FN molecules to associate (Singh et al., 2010). FN molecules initially interact with each other through their N-terminal assembly domains (Dzamba and Peters, 1991), but as assembly progresses, other FN-binding sites along the length of the molecule may participate as well, likely dependant upon the conformation of the FN molecule and their degree of exposure. These FN-FN interactions enable the soluble cell-associated fibrils to stabilize into detergent-insoluble FN matrix. Conversion of detergent-soluble FN into detergent-insoluble FN is thought to involve β-strand exchange interactions between type III modules of FN (Litvinovich et al., 1998; Singh et al., 2010), which would likely require a strong integrin-FN connection in order to transmit an unfolding force large enough to partially disrupt the structure of type III modules.

Substrate stiffness could affect FN matrix assembly at different points during the cell-mediated process. This thesis examines which steps are affected during stiffness-dependent FN matrix assembly.

**Stiffness has a biological role**

It has long been known that the molecular composition of the pericellular environment regulates cell behaviors, but the mechanical properties also mediate cell responses. Cells sense and respond to differences in substrate rigidity, which has been shown to influence behaviors such as growth (Wang et al., 2000), migration (Isenberg et al., 2009; Pelham and Wang, 1997), and differentiation (Engler et al., 2004b; Engler et al., 2006; Engler et al., 2007b), although details of the mechanochemical mechanisms have not been fully elucidated.

The stiffness of an object is characterized by its ability to resist deformation, and is defined by its Young’s modulus, E (Chen, 2008). A rigid substrate resists deformation
more readily than a soft substrate and thus possesses a larger E. The E of a substrate can be determined by applying force to it (stress), and then measuring the resulting change in length (strain). The ratio of stress to strain determines the elastic modulus of a material. The Young’s modulus is quantified in Pascal (Pa) units, where Pa = Newtons/meter$^2$. Biological tissues are often measured in kilo-Pascal (kPa) units. The stiffness of tissues within the body varies greatly, with brain tissue being among the softest (several hundred Pa) and mineralized bone among the most rigid (several million kPa) (Figure 1.4) (Levental et al., 2007).

The two main techniques used to determine E for a bio-compatible material are micro-indentation and rheology. Micro-indentation techniques, such as atomic force microscopy (AFM), directly measure the elastic modulus by vertically poking the sample with a controlled force (stress) to a measured depth (strain) (Chen, 2008). Rheology measures the stiffness of a sample by applying a known force (stress) horizontal to the surface of the sample and measuring the deformation of a sample (strain) (Janmey and Schlwa, 2008).

In multicellular organisms, cells function within dynamic tissues whose mechanical properties can change during development (Mammoto and Ingber, 2010), or with the progression of diseases including cancer (Paszek et al., 2005), fibrosis (Yin et al., 2007), and atherosclerosis (Matsumoto et al., 2002). Diseases often result in the stiffening of tissue, with tumor formation and tissue fibrosis markedly increasing tissue stiffness. For example, healthy adipose tissue ranges from 0.2-2 kPa, but as a breast tumor develops, the surrounding tissues increase in stiffness to 4-12 kPa (Gefen and Dilmoney, 2007; Paszek et al., 2005; Samani et al., 2003). An increase in tissue stiffness can also correlate with the degree of disease progression. For example, Yin et al. reported a correlation between the stiffness of liver tissue and the progression of fibrosis, starting with measurements of 2 kPa for normal liver up to 12 kPa for the most advanced stages of fibrosis (Yin et al., 2007).

Tissue rigidity plays an important role in cancer development, progression and metastasis, with increased rigidity promoting an oncogenic phenotype (Levental et al., 2009; Paszek et al., 2005). Tumors often result in an increase in tissue stiffness, a
Figure 1.4 – Tissues have a range of stiffnesses. Soft tissues within the human body range from 0.1 kPa up to 100 kPa. Brain tissue is one of the softest tissues, and bone is among the most rigid. Pathologies such as fibrosis often lead to an increase in tissue stiffness (from Huang et al. 2012).
characteristic that is commonly used to detect their presence (Khaled et al., 2004; Sinkus et al., 2000). Increased tumor rigidity has been found to correlate with an increase in metastasis (Akiri et al., 2003; Colpaert et al., 2001) and a decrease in successful treatment (Netti et al., 2000). The stiffening of tumorogenic tissue is largely due to an increase in the deposition and crosslinking of collagen (Levental et al., 2009; Martin and Boyd, 2008; Ramaswamy et al., 2003); however, the amount of other ECM proteins such as FN have also been found to dramatically increase in breast tumors (Christensen, 1992; Koukoulis et al., 1993), and FN has been implicated in the progression of other cancers, including prostate cancer, melanoma, and lung carcinoma (Han et al., 2006; Schamhart and Kurth, 1997; van Muijen et al., 1995). Current research supports a mechanism of increased tissue stiffness promoting an oncogenic phenotype through stiffness-dependent regulation of integrins. Indeed, integrin levels and integrin-mediated signaling is altered within rigid tumors (Guo and Giancotti, 2004; Koukoulis et al., 1993; Liu et al., 2004), and an oncogenic phenotype can be controlled by modulation of integrin levels, activity and signaling (Liu et al., 2004; Paszek et al., 2005; White et al., 2004). For example, Paszek et al. found that a stiffer matrix induced integrin clustering and signaling and enhanced breast cancer malignancy, but this phenotype could be reversed by inhibiting integrin signaling (Paszek et al., 2005). These results are also supported by the work of Levental et al., who reported that invasion of cancer cells into the surrounding matrix could be enhanced by stiffening the ECM or by forcing integrin clustering, but inhibiting integrin signaling prevented stiffness-induced invasion (Levental et al., 2009). Thus, integrin-mediated oncogenic behavior seems to be regulated by the rigidity of the pericellular environment.

Diseases that lead to an increase in tissue stiffness are often also characterized by an increase in the deposition of ECM proteins. FN is a major component of the ECM, and an excessive and disordered FN matrix is present in fibrotic diseases (Muro et al., 2008) and hypertrophic scars (Kischer and Hendrix, 1983). It is known that FN matrix assembly is mediated by integrin receptors and regulated by intracellular signals, cytoskeletal organization, and availability of FN (Singh et al., 2010). In an early study, Halliday and Tomasek (Halliday and Tomasek, 1995) reported that cells form a FN
matrix on tensioned collagen gels attached to plastic, but not on relaxed, free-floating gels, indicating an effect of substrate mechanical properties on FN matrix assembly. However, little is known about how the mechanical properties of the pericellular environment affect FN matrix assembly and what mechanisms are involved.

**Cells can sense stiffness: Mechanosensing**

A cell’s ability to properly sense and respond to environmental cues is crucial to its survival and function. Cells sense the stiffness of their environment by pulling on their substrate and feeling the resistance (Discher et al., 2005). A rigid substrate provides the cells with greater external resistance than a soft substrate. The cell is able to sense this resistance and responds by adjusting the force with which it pulls on the substrate to match the resisting force. This cellular tug-of-war allows cells to sense the stiffness of their environment. Indeed, cells have been shown to increase the force with which they pull on a FN-coated bead as the resisting force is increased (Choquet et al., 1997). This also led to an increase in the strength of the integrin-cytoskeletal linkage, allowing a stronger force to be exerted on the integrin and, therefore, on the FN ligand.

Cells are capable of actively responding to physical cues in their environment through mechanosensing, which is the ability of a cell to detect and respond to forces as a result of the link between the cytoskeleton and ECM. Cells are then able to translate these external mechanical signals into intracellular biochemical signals through a process termed mechanotransduction (Janmey and Weitz, 2004).

Integrins are the main cellular receptors that transmit mechanical stresses across the plasma membrane (Katsumi et al., 2004), and mechanotransduction likely occurs through integrin-mediated cellular adhesions. Cells adhere to their ECM through focal adhesions, in which cytosolic proteins are recruited to the intracellular domains of integrins. Focal adhesions form when a cell can exert large forces against the ECM, with an increased size on substrates of increasing stiffness (Pelham and Wang, 1997). These proteins form a membrane-bound complex that anchors the integrin to the actin cytoskeleton. This gives rise to multiprotein signaling complexes that are likely relayed to the nucleus to affect various cellular functions.
Several different mechanisms of mechanotransduction have been proposed. One possibility is that cells sense the force in the integrin-cytoskeleton linkage through changes in the conformation of specific intracellular focal adhesions proteins in response to internally or externally applied force. The rigidity of the substrate provides resistance to force-induced displacements, thus more rigid substrates would be more likely to induce conformational changes in focal adhesion proteins. For example, the focal adhesion protein talin has been shown to change conformation upon the application of force, exposing cryptic binding sites for the actin-binding protein vinculin (del Rio et al., 2009). Talin directly connects the integrin cytoplasmic domain to the actin cytoskeleton and vinculin-binding reinforces this connection (Galbraith et al., 2002). Together, talin and vinculin have been reported to transmit forces between the cytoskeleton and ECM (Hu et al., 2007; Jiang et al., 2003). The resisting force of a rigid substrate could be transmitted to talin via its integrin link, and this force could unfold talin, exposing the vinculin-binding site. Vinculin binding would strengthen the linkage, allowing the cytoskeleton to increase the force applied to the integrin. In this way, the force-induced unfolding of a focal adhesion protein could mediate mechanotransduction.

Cellular response to substrate rigidity can also vary depending on the adhesive ligand. For example, human mesenchymal stem cells favor myogenic differentiation on 25 kPa substrates coated with FN, but when the ligand is changed to collagen-coated, differentiation is favored on substrates over three times that stiffness, at 80 kPa (Rowlands et al., 2008). Cells plated on different ligands also exhibit differences in the molecules required for mechanosensing. Filamin A, an actin crosslinking protein, is necessary for cell response to stiffness on collagen, but not on FN (Byfield et al., 2009). Differing cell responses to different ligands could result from the difference in receptors for each ligand. Cells bind FN, but not collagen, via α5β1 integrins, and this integrin has been shown to react according to the rigidity of the substrate (Friedland et al., 2009). Different integrins also require different molecules for activation and signal transduction. Also, the ligands themselves react differently to applied forces. Unlike collagen, the conformation of FN molecules vary depending on the applied cellular forces. Thus, the identity of the adhesive ligand seems to play a part in the ability of a cell to sense the rigidity of its substrate.
Thesis objectives

Here we use polyacrylamide gels of different stiffnesses to determine the effects of substrate rigidity on fibroblast assembly of FN matrix. Measurements of detergent-insoluble matrix and analyses of fibril formation were used to identify the steps of assembly that vary with gel stiffness. We observed that FN matrix assembly is upregulated on rigid substrates and propose that this is primarily due to a deficiency in cell-mediated FN conformational changes on softer substrates. These findings establish a mechanism for stiffness-dependent FN matrix assembly, identifying factors that may contribute to the progression of diseases.
Chapter 2
Materials and Methods

Cell culture

NIH 3T3 fibroblasts were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) with 10% bovine calf serum (Hyclone) and antibiotic/antimycotic cocktail (Invitrogen). HT1080 fibroblasts were cultured in DMEM with 10% fetal bovine serum (Hyclone) and antibiotic/antimycotics, and 0.1 μM dexamethasone was added whenever FN matrix assembly was desired. CHOα5-17 cells were cultured in DMEM with 10% fetal clone II serum (Hyclone), antibiotic/antimycotics, 2 mM glutamine (Gibco), 1% nonessential amino acids (Gibco), and 100 μg/ml Geneticin (Invitrogen). Cells were cultured for no more than 30 passages. Cells were passaged by rinsing with phosphate-buffered saline (PBS), detached with trypsin in versene (Gibco), and replated in fresh growth media. For experiments, cells were rinsed with PBS, detached with trypsin, counted on a hemocytometer, and diluted in fresh growth media to the desired final cell concentration.

Fibronectin

Plasma FN was purified from fresh frozen rat plasma or spent human plasma by gelatin-Sepharose affinity chromatography (Wilson and Schwarzbauer, 1992). The recombinant 70-kDa fragment of FN was generated using the baculovirus insect cell expression system (Aguirre et al., 1994). Fibronectin and 70-kDa were biotinylated with Sulfo-NHS-Biotin (Pierce). The FN or 70-kDa was dialyzed into 50 mM sodium bicarbonate pH 8.5 and then incubated with a 50-fold excess of Sulfo-NHS-Biotin for 2 hours on ice. The proteins were then dialyzed into either 20 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) pH 11, 150 mM NaCl or 50 mM 3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid (EPPS) pH 8.5, 150 mM NaCl. Fluorescently-labeled 70-kDa was made using a similar procedure with Sulfo-NHS-
Alexa488 (Pierce). All incubation and dialysis steps were carried out in low-light conditions or in the dark. The recombinant III<sub>1-6</sub> fragment of FN was expressed in E. coli as a fusion with maltose binding protein (MBP) (Aguirre et al., 1994).

**Preparation of polyacrylamide substrates**

Polyacrylamide substrates were made as previously described (Moshayedi et al., 2010; Pelham and Wang, 1997). First, approximately 170 μL of 0.1M NaOH was evaporated onto 12 mm circular glass coverslips, which were then incubated with 130 μL (3-Aminopropyl)trimethoxysilane (Sigma) for 4 minutes. Both sides of the coverslips were thoroughly rinsed with deionized water (dI H<sub>2</sub>O). They were then put in a sterile 10 cm petri dish filled with dI H<sub>2</sub>O and placed on a slow shaker. The dI H<sub>2</sub>O was changed twice at five-minute intervals. The coverslips were then incubated in a 0.5% gluteraldehyde solution in sterile PBS for 30 minutes on a slow shaker. The coverslips were rinsed with dI H<sub>2</sub>O three times, each at 5 minute intervals, as before. They were then removed from the petri dish and air-dried. Polyacrylamide mixtures were prepared for each gel stiffness, from stocks of 40% acrylamide (GE Healthcare), 2% crosslinker N,N’-methylene-bis-acrylamide (GE Healthcare), and sterile PBS, and the polyacrylamide mixtures were degassed for 20 minutes in a vacuum chamber. Recipes for each gel stiffness and their acrylamide and bis-acrylamide ratios are shown in Figure 2.1. The recipes for the gels made according to Engler et al. are in Figure 2.1A (Engler et al., 2007a), and the recipes for the gels made according to Moshayedi et al. are in Figure 2.1B (Moshayedi et al., 2010). A 1% v/v of ammonium persulfate and 0.3% v/v of tetramethylethylenediamine were added to the polyacrylamide mixture, and following a brief vortex, 15 μL aliquots were pipetted onto 22 x 60 mm coverglasses. The coverslips were then placed on top of the polyacrylamide droplets, treated-side down, and allowed to dry for approximately 10-15 minutes, or until the unused acrylamide mixture polymerized. The coverglasses were then immersed in petri dishes filled with sterile PBS, to prevent the gels from drying out. The gels attached to the coverslips were gently removed from the coverglasses and placed gel-side-up in non-tissue culture-treated 24-well polystyrene plates filled with sterile 20 nM EPPS pH 7.5 (Sigma). The gels were rinsed twice with sterile 20 mM EPPS pH 7.5 and sterilized in the tissue culture hood by
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Figure 2.1 – Polyacrylamide recipes. A. The ratios (%) of acrylamide (AA) to crosslinker N,N’-methylene-bis-acrylamide (Bis-A) that yield gels with Young’s Moduli (E) of 1 kPa, 3 kPa, 8 kPa, 20 kPa, and 34 kPa, as determined by Engler et al. 2007, are shown. The volumes (mL) of 40% acrylamide, 2% bis-acrylamide, and PBS are listed that make up 10 mL of polyacrylamide mixture for each gel stiffness. B. The ratios (%) of acrylamide (AA) to bis-acrylamide (Bis-A) that yield gels ranging from 0.3 kPa to 91 kPa, as determined by Moshayedi et al. 2010, are shown. The volumes (mL) of 40% acrylamide, 2% bis-acrylamide, and PBS are listed that make up 10 mL of polyacrylamide mixture for each gel stiffness.
exposing them to UV light for 20-30 minutes. Sterile gels were stored at 4°C for up to 2 weeks.

**Crosslinking FN to the surfaces of polyacrylamide substrates**

Human plasma fibronectin in 50 mM EPPS pH 8.5, 150 mM NaCl (Sigma-Aldrich) was covalently attached to the gel surfaces using a photo-activated bifunctional crosslinker N-Sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH) (Pierce). A fresh 2 mM solution of sulfo-SANPAH was made in sterile 20 mM EPPS pH 7.5. 350 µL was added to each well containing a gel and they were incubated under a 365 nm UV lamp for 10 minutes, changing the orientation of the plate every 2 minutes to ensure equal exposure. The gels were rinsed once with sterile 20 mM EPPS pH 7.5 and once with sterile 50 mM EPPS pH 8.5, 150 mM NaCl and then removed from the plate. A solution of human plasma FN in sterile 50 mM EPPS pH 8.5, 150 mM NaCl was incubated on the surface of each gel for 2-3 hours at room temperature. All FN was dialyzed into 50 mM EPPS pH 8.5 prior to crosslinking to polyacrylamide gels. In some gel preparations, FN was pretreated with guanidine hydrochloride (GdnHCl) prior to crosslinking to the gel surface. FN was diluted into a solution of 1 M GdnHCl in 50 mM EPPS pH 8.5, 150 mM NaCl and then crosslinked to the gels as above. The FN solution was drained from the gels using a kimwipe, and the gels were rinsed twice with sterile PBS and then placed into a sterile non-tissue culture-treated 24-well polystyrene plate and then rinsed three times with sterile PBS. Gels were stored in sterile PBS overnight at 4°C.

**Rheometry measurements of polyacrylamide gels**

Rheological measurements of the gels were performed on a Physica MCR 501 rheometer (Anton Paar, Austria). Measurements were done with a parallel plate (10 mm) and at 25°C in an environmental chamber with PBS to keep the gels hydrated. The complex shear modulus was determined using an oscillatory shear strain (0.01-0.1% strain, 0.75 Hz), and the values were adjusted to account for sample thickness. The
Young’s modulus (E) was calculated from the measured storage modulus (G’), using \( E = 2G'(1+v) \) and assuming the Poisson’s ratio of polyacrylamide is 0.48 (Boudou et al., 2006). Polyacrylamide gels were made as previously described, but they were made on 25 mm circular glass coverslips with 66 \( \mu \)L of the polyacrylamide mixture.

**Cell culture, fluorescence staining, and microscopy on polyacrylamide substrates**

Polyacrylamide gels were incubated at 37°C first in sterile 2% BSA in PBS for 1 hour and then in cell media for 1 hour, prior to use in tissue culture. Cells were plated onto polyacrylamide gels in a 24-well dish at a density of 4x10^5 cells per well. Cells were allowed to attach and spread for 2 hours, after which 10 \( \mu \)g/ml rat plasma FN was added. 10 \( \mu \)M LPA, 5 nM Calyculin A, or 1 mM MnCl_2 was also added at this time. After an additional 4 hours or 10 hours, cells were washed with PBS containing 0.5 mM MgCl_2, fixed with 4% paraformaldehyde in PBS/0.5 mM MgCl_2 and stained with either rat-specific anti-FN monoclonal antibody IC3 (Sechler et al., 1996) at 1:1000 dilution or polyclonal antiserum R457 against the N-terminal 70-kDa fragment of FN (Aguirre et al., 1994) at 1:100 dilution in 2% ovalbumin in PBS followed by secondary antibodies AlexaFluor-conjugated goat anti-mouse or anti-rabbit IgG (Invitrogen) at 1:600. Matrix was visualized using a Nikon Eclipse Ti microscope equipped with a Hamamatsu ORCA R2 Camera. Images were acquired and normalized using iVision software (BioVision technologies). Fields were randomly chosen, using DAPI staining to visualize cell nuclei.

A similar procedure was used to visualize actin filaments, except cells were plated at a density of 1x10^5 cells per well, and after 6 hours, cells were fixed, permeabilized with 0.5% Triton-X, and stained with rhodamine-phalloidin (Invitrogen).

**Detection and quantification of FN matrix assembly sites**

Polyacrylamide gels were prepared for tissue culture, as previously described. Cells were plated onto polyacrylamide gels in a 24-well dish at a density of 5x10^4 cells per well. NIH 3T3 cells were allowed to attach and spread for 2 hours, after which 20 \( \mu \)g/ml biotinylated 70-kDa and 10 \( \mu \)g/ml rat plasma FN were added. After an additional
30 minutes, cells were fixed with 4% paraformaldehyde in PBS/0.5 mM MgCl₂ and bound 70-kDa was detected with AlexaFluor488-conjugated streptavidin (Invitrogen) used at 1 µg/ml. Alternatively, CHOα5-17 cells were plated with 20 µg/ml rat plasma FN and 40 µg/ml AlexaFluor-conjugated 70-kDa. After 2 hours, cells were fixed with 4% paraformaldehyde in PBS, 0.5 mM MgCl₂.

Images of bound 70-kDa were normalized using iVision software. For quantification of FN matrix assembly sites, cells were outlined and the area and average intensity per pixel were measured. Only cells within the area range of 450 square pixels (the smallest cell on substrates crosslinked with GdnHCl-treated FN) to 1412 square pixels (the largest cell on substrates with untreated FN) were used.

**Isolation of DOC-soluble and DOC-insoluble fractions**

Cells were plated onto polyacrylamide gels as described for matrix immunofluorescence. At the desired time, gels were transferred to new wells filled with cold PBS. The PBS was carefully aspirated and 150 µl DOC lysis buffer (2% DOC, 20 mM Tris-HCl, pH 8.8, 2 mM EDTA, and protease inhibitor cocktail (Roche) (Sechler et al., 1996) were added. Cells were gently removed from the gels with a rubber scraper, and after transferring the lysates to a microcentrifuge tube, an additional 50 µL DOC lysis buffer was used to rinse the gel and scraper and was then added to the first lysate. The lysates were subsequently passed through a 26-gauge, 3/8 inch needle approximately 5 times. They were then centrifuged for 20 minutes at 14,000 rpm at 4°C. The supernatants consisting of the DOC-soluble fractions were removed and placed in new tubes. An additional 150 µL DOC lysis buffer was used to rinse the pellets and the samples were centrifuged again. The remaining supernatants were discarded, and the DOC-insoluble pellets were resuspended in 60 µL SDS solubilization buffer: 1% SDS, 20 mM Tris-HCl pH 8.8, 2 mM EDTA and protease inhibitors. The DOC-insoluble samples were vortexed and boiled for 5 minutes.
Detection and quantification of FN by immunoblotting

Total protein concentrations were determined for the DOC-soluble samples using a BCA assay (Pierce), and equal amounts of total DOC-soluble protein or the equivalent proportions of DOC-insoluble samples were analyzed by SDS-PAGE using 5% polyacrylamide gels. Samples were transferred to nitrocellulose membranes and blocked overnight in buffer A: 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20. Membranes were immunoblotted with either IC3 ascites diluted 1:10,000, R457 antiserum diluted 1:2000, or anti-GAPDH (14C10) (Cell Signaling) at 1:2000 in buffer A for 1 hour at room temperature. DOC-soluble samples were immunoblotted in parallel with antibodies against GAPDH to ensure equal sample loading. Goat-anti-mouse or goat-anti-rabbit conjugated to horse radish peroxidase was used at a dilution of 1:10,000 as a secondary antibody. Immunoblots were developed with SuperSignal West Pico Chemiluminescent substrate (Pierce). Band intensities were measured with Quantity One software (Bio-Rad). Each blot was exposed for at least three different times, and the band intensities were quantified from the exposures that yielded signals within the linear range. Quantities for each band were normalized to GAPDH and then to either the 12 h soft band intensity or the 6 h rigid untreated band intensity before being averaged over multiple experiments.

ELISA

Polyacrylamide gel substrates were blocked with 2% BSA in PBS for 1 hour and then incubated with either 10 µg/ml anti-human FN MAB1890 (Millipore) or 1:100 anti-human III1-2 monoclonal antibody 5E6 (Karuri et al., 2009) diluted in 1%BSA/PBS for 1 hour at room temperature followed by a 1 hour incubation with biotinylated goat-anti-mouse or goat-anti-rabbit IgG (GIBCO, 1:2000 in 1% BSA/PBS) at 4°C. The gel substrates were then incubated with streptavidin-β-galactosidase (Invitrogen, 1:500 in 1% BSA-PBS, 1.5 mM MgCl2, and 2 mM 2-mercaptoethanol) for 1 hour at 4°C. After transferring the substrates to a new plate, they were incubated with p-nitrophenyl-β-D-galactopyranoside (Sigma, 1 mg/ml) in 50 mM sodium phosphate pH 7.2, 1.5 mM MgCl2 at room temperature for 50 min. The reaction was stopped by the addition of 0.5 M Na2CO3, and samples were quantified by absorbance at 405 nm on an ELx800 Plate
Reader (BioTek Instruments). Absorbance values were normalized to the amount of FN crosslinked to the surfaces of the gels or coverslips, and the values were averaged between experiments.

A similar procedure was used to detect the amount of FN or III_{1-6} fragment crosslinked to the surface of the polyacrylamide gels. The recombinant III_{1-6} fragment was detected using the polyclonal antiserum R184 against the III_{1-6} domain of FN at a 1:100 dilution. Biotinylated FN crosslinked to the surface of gels was detected with the same procedure, minus the incubation with primary antibody and biotinylated IgG.

**Cell adhesion assay**

Polyacrylamide gels were incubated at 37°C first in sterile 2% BSA in PBS for 1 hour and then in DMEM for 1 hour, prior to plating cells. Cells were harvested by removing them from the tissue culture plate with 0.1 mg/ml TPCK-trypsin in versene at room temperature. The trypsin was then inactivated with 0.5 mg/ml soybean trypsin inhibitor (SBTI) in PBS. The cells were spun down and resuspended in fresh SBTI before being spun down again. The cells were then resuspended in DMEM without serum, counted on a hemocytometer, and diluted in DMEM to the final cell concentration of 5x10^4 cells/ml. 1 mL of cells was added to each gel in a 24-well dish. Cells were allowed to attach for 1 hour at 37°C and then gently washed one time. Cell adhesion was quantified by counting the number of cells that remained attached in random field images.

**Immunoprecipitation**

On ice, 1 µg/ml MAB 1890 (Millipore) or IST-5 (Sirius Biotech) was added to 1 mL of either 10 µg/ml human plasma FN in PBS or conditioned media from WI38-2A fibroblasts. The samples were incubated for 1 hour at room temperature under gentle rotation. 100 µl protein G-coupled Sepharose beads in PBS were added, and the samples were incubated for an additional 1 hour at room temperature under gentle rotation. The tubes were then centrifuged for 10 minutes, the supernatant was removed, and the beads
were washed with PBS three times, each time centrifuging and removing the supernatant. The beads were resuspended in 100 µl ESB (2% SDS, 10% glycerol, and 80 mM Tris-HCl pH 6.8) and boiled for 5 minutes to elute the protein. The beads were spun out for 10 minutes, and 0.1M DTT was added to the supernatant prior to running on an SDS gel. FN in samples were detected using a similar protocol for detecting FN in DOC-soluble and –insoluble samples.
Chapter 3
Characterization and Optimization of Polyacrylamide Gels

Introduction

The stiffness of most connective tissues ranges from 0.1 kPa – 100 kPa, yet cells are typically studied on plastic or glass materials with elastic moduli several orders of magnitude higher (GPa). Polyacrylamide gels developed by Pelham and Wang were among the first to allow detailed investigations into the role of substrate stiffness on cell behavior (Pelham and Wang, 1997). These substrates can be created over a wide range of stiffnesses, by polymerizing varied amounts of acrylamide and the crosslinker, bis-acrylamide, atop activated glass coverslips (Aplin and Hughes, 1981; Wang and Pelham, 1998). Proteins, such as collagen and FN, are then conjugated to the otherwise inert surfaces of these thin, transparent gels in order to induce cell attachment and spreading (Wang and Pelham, 1998).

One of the advantages of polyacrylamide is that it is relatively inactive, not interacting with or binding to bioactive molecules such as proteins or nucleic acids. It is this lack of protein-binding that makes polyacrylamide suitable for other systems, such as SDS-PAGE. This inert quality is advantageous because it prevents nonspecific cell adhesion and allows one to control what is bound to the surface and what the cells interact with. Fibronectin was crosslinked to the surfaces of the gels used in the following experiments, so the cells interact only with that FN. Other polymer materials have been used to study the effects of stiffness on cell behavior in vitro, including biological gels composed of collagen or hyaluronic acid. But cells and proteins interact with the components of these gels, making it difficult to differentiate between the specific contributions of the attached protein versus the effect of the gel component itself, or an interaction between the attached protein and the gel component. Polyacrylamide gels solve this problem by allowing the mechanical properties of the gel to be changed, while
keeping the molecular composition constant.

Substrates of varying stiffnesses were made by attaching polyacrylamide gels to glass coverslips, and FN was covalently crosslinked to the surfaces of the gels in order to make them cell-adhesive (Figure 3.1A). The concentrations of acrylamide and crosslinker bis-acrylamide directly influence the resulting stiffness of the gels. A lower concentration of both constituents will result in a more compliant gel, whereas a higher concentration will result in a stiffer gel. By varying the amount of bis-acrylamide, polyacrylamide gels of different stiffnesses are made. Figure 3.1B shows that a solution of 5% total acrylamide can be used to make gels of three different stiffnesses, merely by adding different amounts of bis-acrylamide linker.

The inertness of polyacrylamide makes it challenging to covalently conjugate proteins to its surface. Fibronectin was crosslinked to the surfaces of the gels using the heterobifunctional crosslinker sulfo succinimidyl-6-(4’-Azido-2’-Nitrophenyl-Amino) hexanoate (sulfo-SANPAH). Upon exposure to UV light, one end of the sulfo-SANPAH molecule reacts nonspecifically with polyacrylamide, while the other end of the molecule preferentially reacts with primary amines often found on proteins (Wang and Pelham, 1998). It is effective in conjugating protein to polyacrylamide, but it has several drawbacks, such as high cost and limited stability. Thus, the necessity of crosslinking was considered, and other techniques for crosslinking were investigated.

Unless otherwise specified, NIH 3T3 cells were used for all cell-based experiments. NIH 3T3 is a fibroblast cell line derived from mouse embryos and is considered a standard fibroblast cell line (Todaro and Green, 1963). NIH 3T3 cells are beneficial for studying FN matrix assembly, as they readily assemble considerably large amounts of FN matrix with no need for stimulation. They also exhibit the characteristic of “contact inhibition”, whereby cells stop proliferating when they reach the cell density that forms a confluent monolayer. HT1080 fibroblasts were used in one experiment. The HT1080 fibrosarcoma cell line is unable to assemble FN matrix without stimulation from the glucocorticoid dexamethasone, which increases the rate of FN synthesis and enables cell-mediated FN matrix assembly Brenner et al. (2000). However, dexamethasone-treated HT1080 cells still assemble less FN matrix than NIH 3T3 cells, and the full range of effects from dexamethasone treatment are still unknown. HT1080 cells also lack contact
Figure 3.1 – Preparation of polyacrylamide gel substrates. A. Substrates are made by attaching polyacrylamide gels to glass coverslips. Fibronectin is then crosslinked to the surface of the gels so that fibroblasts can attach. B. By varying the amount of bis-acrylamide, polyacrylamide gels of different stiffnesses are made. A solution of 5% total acrylamide can yield a 1 kPa, 3 kPa, or 8 kPa polyacrylamide gel, depending on the concentration of bis-acrylamide used. (from Engler et al. 2007)
inhibition, so once cells have formed a confluent monolayer, they will begin to grow on top of one another. Cells were grown and most experiments were performed in the presence of serum. Serum contains many proteins and growth factors that promote cell growth and homeostasis (Alberts et al., 2008).

In order to separate the effects of stiffness, substrates must be used that differ only in their stiffness. The focuses of this chapter are 1) characterizing the polyacrylamide system used in my studies and how they affect pertinent cell behaviors, and 2) optimizing components of the system so they can most efficiently be used to study the effects of stiffness on FN matrix assembly.
Results

Stiffnesses of the polyacrylamide gels

To study the effect of substrate stiffness on FN matrix assembly, we measured assembly on polyacrylamide gel substrates of varying stiffness. Polyacrylamide gels were prepared according to the procedure of either 1) Engler et al. (Engler et al., 2007a) or 2) Moshayedi et al. (Moshayedi et al., 2010). The recipes from Engler et al. yielded polyacrylamide gels with Young’s moduli (E) of 1 kPa, 3 kPa, 8 kPa, 20 kPa and 34 kPa, as measured via AFM (Figure 3.2A) (Engler et al., 2007a). The recipes from Moshayedi et al. yielded polyacrylamide gels with Young’s moduli (E) of 0.3 kPa, 0.9 kPa, 3.6 kPa, 9.4 kPa, 30 kPa, and 90 kPa, as measured using rheology and confirmed with AFM (Moshayedi et al., 2010) (Figure 3.2B). These recipes were optimized by the authors to maximize the homogeneity of the gel’s surface topology and its mechanical properties.

Variability in the techniques used to measure the polyacrylamide gel stiffnesses can result in variability in the reported values of a gel. By comparing the ratios of acrylamide to bis-acrylamide for the two sets of gels, we can infer which gels have comparable stiffnesses. The 1 kPa and 3 kPa gels from the first set of gels have almost the same recipes as the 0.3 kPa and 0.9 kPa gels from the second set, respectively. These four gels have been classified as “soft” (Figure 3.2A, B). Likewise, the 20 kPa and 34 kPa gels from the first set of gels have very similar recipes as the 9.4 kPa and 30 kPa gels from the second set, respectively. Along with the stiffest 90 kPa gel, these gels were classified as “rigid”. The 8 kPa gels from the first set of gels and the 3.6 kPa gel from the second set fall in between the soft and rigid gels and were classified as having an “intermediate” stiffness. Experiments in this and the following chapter support this classification system, showing that cells plated on soft, intermediate, and rigid gels exhibit distinct cell behaviors.

In order to compare whether we were making gels similar to theirs, the stiffnesses of three of the gels, 0.9 kPa (soft), 3.6 kPa (intermediate), 9.4 kPa (rigid), were measured via rheology (Figure 3.3). Our experiment determined the gels to be 1.6 kPa, 4.3 kPa, and 7.4 kPa, respectively. Our measurements yielded a smaller range than the reported stiffnesses, but they still reported that the three gels had three distinct stiffnesses. Taking
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Figure 3.2 – Polyacrylamide ratios and stiffnesses. A. Ratios of acrylamide (AA) to crosslinker N,N’-methylene-bis-acrylamide (Bis-A) that yield gels with Young’s Moduli (E) of 1 kPa, 3 kPa, 8 kPa, 20 kPa, and 34 kPa, as determined by Engler et al. 2007. B. Ratios of acrylamide (AA) to bis-acrylamide (Bis-A) that yield gels ranging from 0.3 kPa to 91 kPa, as determined by Moshayedi et al. 2010. Young’s Moduli (E) were calculated using the shear moduli (G’) using the equation E = 2G’(1+v).
Figure 3.3 – Rheology measurements of polyacrylamide gels. The storage moduli ($G'$) were measured for three polyacrylamide gel substrates using rheology and averages of at least two tests on separate gels are shown. The measured values are compared with the values published in Moshayedi et al. 2010. The average storage moduli ($G'$) were used to calculate the corresponding Young’s moduli (E). Values are the mean +/- S.E.M. for at least two experiments.
standard error into account, the differences between our measured values and the
published values are only 0.5 kPa for the soft and 1.2 kPa for the rigid. The measurement
for the intermediate gel fell within the range of error and, thus, can be considered the
same.

**Crosslinking protein to the gel surface is necessary for cell spreading**

To determine whether polyacrylamide gels adsorbed with FN would support cell
attachment and growth, NIH 3T3 cells were plated on soft and rigid gels either
crosslinked or adsorbed with 15 µg/ml FN (Figure 3.4). Cells adhered, but did not
spread on gels adsorbed with FN. Increasing the concentration of adsorbed FN to 50
µg/ml still did not enable cell spreading on FN.

**Ligand density has an impact on cell adhesion and cell spreading**

To analyze the impact ligand density has on cell behavior, NIH 3T3 cells were
plated on soft, intermediate and rigid polyacrylamide gels crosslinked with 5 µg/ml, 15
µg/ml or 50 µg/ml FN solutions of FN, and cells were visualized after 12 hours (Figure
3.5A). Varying the concentration of crosslinked FN led to a difference in cell density,
with cell density increasing with an increase in ligand density. This increase in cell
density is most likely due to an increase in cell attachment, so we measured cell adhesion
on soft polyacrylamide gels crosslinked with 5 µg/ml, 15 µg/ml or 45 µg/ml FN solutions
of FN (Figure 3.5B). HT1080 cells were plated on these substrates in cell media with
dexamethasone, but lacking serum and were imaged after 1 hour. Cell adhesion was
quantified by counting the number of cells that remained attached. Increasing the
concentration of crosslinked FN from 15 µg/ml to 45 µg/ml almost doubled the number
of adherent cells.

To examine the impact ligand density has on cell spreading, NIH 3T3 cells were
plated on soft, intermediate and rigid polyacrylamide gels crosslinked with low
concentration (15 µg/ml, 10 µg/ml, and 7 µg/ml, respectively) and high concentration
(100 µg/ml) solutions of FN (Figure 3.6A). Increasing the concentration of crosslinked
Figure 3.4 – Crosslinking is necessary for cell spreading. NIH 3T3 cells were plated on soft (3 kPa) and rigid (34 kPa) gels crosslinked with 15 μg/ml FN or adsorbed with either 15 μg/ml FN or 50 μg/ml FN. Cells were imaged 12 hours later.
Figure 3.5 – Ligand density affects cell adhesion. A, NIH 3T3 cells were plated in media plus 10% serum on soft (1kPa) and intermediate (8 kPa) gels crosslinked with either 5 µg/ml, 15 µg/ml or 50 µg/ml solutions of FN, and cells were observed after 12 hours. B, HT1080 cells were plated in media with dexamethasone and without serum on soft (1 kPa) gels crosslinked with either 5 µg/ml, 15 µg/ml or 45 µg/ml solutions of FN. Cells were counted from images taken after 1 hour (mean +/- S.E.M., n=4)
Figure 3.6 – Ligand density affects cell spreading.  A, Soft (1 kPa), intermediate (8 kPa), and rigid (20 kPa) polyacrylamide gels were crosslinked with low concentration (7-15 µg/ml) and high concentration (100 µg/ml) solutions of FN. NIH 3T3 cells in growth media plus 10% serum were plated on the gels, and the cells were imaged 1 hour later.  B, Soft (1 kPa), intermediate (8 kPa), and rigid (20 kPa) polyacrylamide gels were crosslinked with low concentration (7-15 µg/ml) and high concentration (100 µg/ml) solutions of biotinylated FN, and the amount of FN crosslinked to the surfaces was determined by ELISA at 405 nm.
FN changed the morphologies of the cells on the different stiffnesses, with higher concentrations of FN increasing cell spreading. The amount of FN crosslinked to the surfaces of these polyacrylamide gels was quantified by crosslinking equivalent amounts of biotinylated FN to the gels and detecting it via ELISA (Figure 3.6B). Using high concentrations solutions of FN led to an obvious increase (roughly 2-fold) in the amount of FN attached to the surfaces of the gels; however, the increase might be higher, since the large amounts of biotinylated FN could have saturated the assay.

**Measurements of fibronectin crosslinked to gels**

Since ligand density impacts cell spreading and confluence, it was important to make sure the same amount of FN was crosslinked to each polyacrylamide gel, regardless of stiffness. Biotinylated FN was crosslinked to the surfaces of the gels, and an ELISA with labeled streptavidin was used to measure the amounts attached. We found that higher levels of FN were crosslinked to stiffer substrates (Figure 3.7A), resulting in a substrate with both variable stiffness and variable ligand density.

In order to produce gels of different stiffnesses, with the same amount of FN ligand, FN concentrations used for crosslinking were adjusted to yield equal amounts of surface-bound FN on all gel stiffnesses (Figure 3.7 B, C). Lower concentrations were used when crosslinking FN to stiffer substrates, and the concentration of FN needed in order to crosslink the same amount of FN to each stiffness was determined. Thus, similar amounts of FN were crosslinked to polyacrylamide gels, regardless of stiffness.

**Cell spreading is enhanced on stiff substrate**

In order to observe the effects of substrate stiffness on cell spreading, cells were plated on soft, intermediate, and rigid polyacrylamide gels. Cells spread more on rigid substrates than on softer substrates (Figure 3.8). This also led to cells on rigid substrates reaching confluence more quickly than cells plated on softer substrates (Figure 3.9, top). When cells were plated at low density (5 x 10^4 cells/cm^2), cells on a rigid substrate were confluent after 6 hours, while cells on soft and intermediate substrates were subconfluent. In order to ensure that any difference in matrix assembly is not due to a difference in cell
Figure 3.7 – Equivalent amounts of FN are crosslinked to gel surfaces. Biotinylated FN was detected at a wavelength of 405 nm. A, Greater amounts of FN crosslink to more rigid gels. A 15 µg/ml solution of biotinylated FN was incubated with soft (1 kPa), intermediate (8 kPa), and rigid (34 kPa) gels during crosslinking. Detection of FN by ELISA with labeled streptavidin shows different amounts of FN crosslinked to the gels. B, Soft (1 kPa), intermediate (8 kPa), and rigid (34 kPa) gels were incubated with 20 µg/ml, 15 µg/ml, and 9 µg/ml FN, respectively. An ELISA showed that equivalent amounts of FN were crosslinked to the gels. C, Equivalent amounts of FN were crosslinked to 0.1 kPa, 0.9 kPa 3.6 kPa, 9.4 kPa, 30 kPa, and 91 kPa gels by incubating them with 20 µg/ml, 18.5 µg/ml, 15.5 µg/ml, 10 µg/ml, 11 µg/ml, and 9 µg/ml, respectively. (mean +/- S.E.M, n = 2).
Figure 3.8 – Cells spreading increased on rigid substrates. NIH 3T3 cells were plated on soft, intermediate, and rigid gels, and cells were photographed after 2 hours of attachment. A, 1 kPa, 3 kPa, 8 kPa, 20 kPa, and 34 kPa gels were crosslinked with a FN solution of 15 µg/ml. B, 0.1 kPa, 0.9 kPa 3.6 kPa, 9.4 kPa, 30 kPa, and 91 kPa gels were crosslinked with a FN solution of 20 µg/ml, 18.5 µg/ml, 15.5 µg/ml, 10 µg/ml, 11 µg/ml, and 9 µg/ml, respectively.
Figure 3.9 – Cells plated at high density. NIH 3T3 cells were plated on soft (0.9 kPa), intermediate (3.6 kPa), and rigid (9.4 kPa) gels at low density (5 x 10⁴ cells/ cm²) and high density (2 x 10⁵ cells/ cm²). After 6 hours, cells plated at high density are confluent on all three stiffnesses.
spreading or cell density, cells were plated at a high enough density \((2 \times 10^5 \text{ cells/cm}^2)\), so that cells were confluent on all stiffnesses ([Figure 3.9](#), bottom).
**Discussion**

In order to study the effect of microenvironmental stiffness has on the ability of cells to assemble a FN matrix, a system must be used that is able to separate the mechanical effects from the molecular effects. The polyacrylamide gel system successfully accomplishes this task. Polyacrylamide gel substrates of different stiffnesses were made, and the cells reacted to both the stiffness of the substrate and to its FN ligand. The FN had to be covalently crosslinked to the gels in order to support cell spreading, showing that the cells were specifically interacting with the crosslinked FN. Cell behavior was not a result of non-specific interactions with the substrate, or due to any interactions with molecules in the serum. Even though more FN was crosslinked to the surfaces of stiffer substrates, the amounts of FN could be made equivalent by varying the concentration of FN during crosslinking. This created gels that differed only in stiffness.

The stiffnesses of the gels used in experiments fall within the range of the tissue stiffnesses found within the body. The stiffness of some of the gels correlate with the stiffnesses of tissues in both the healthy and diseased state. Since diseases often result in the stiffening of tissue, the rigid substrates resembled the physiological stiffnesses of scar or fibrotic tissue, while the softer substrates paralleled the stiffnesses of healthy tissue (Yin et al., 2007).

Ligand density has been shown to influence rigidity responsive cell behaviors, including cell spreading (Engler et al., 2004a). It may be possible that differences in stiffness-dependent cell behavior are merely due to a disparity in the amount of adhesive ligand crosslinked to substrates of different stiffnesses. Thus, the effect of ligand density on cell behavior was examined, and the preparation of the polyacrylamide gels was optimized to produce gels of varying stiffness with the same amount of crosslinked FN.

Cell shape is important, as it is associated with cell behavior and function. For example, a change in cell shape can be an indicator of malignancy or a transition to a mobile state, and it can also determine cell survival (Chen et al., 1998). Cell morphology has been shown to be affected by the compliance of the substrate (Pelham and Wang, 1997). Variations in cell spreading on gels of different stiffnesses could affect FN matrix assembly because cells must be confluent in order to assemble a stable FN matrix. Cells
that spread more form a confluent monolayer sooner than cells that spread less, and would thus begin assembling a FN matrix sooner than the subconfluent cells. The appropriate cell density was determined so that cell confluence and shape were similar on all substrates.

Cell spreading varied according to the stiffness of their substrates, and this behavior served as way to characterize the substrates and divide them into categories of “soft”, “intermediate” and “rigid”. Also, variations in the preparation of polyacrylamide gels and in the techniques used to measure their stiffnesses can lead to variability in their calculated Young’s moduli. Two sets of gels, prepared using different recipes, were used in the experiments in this thesis, but their Young’s moduli were calculated separately. Cell spreading in response to substrate stiffness served as an independent variable to compare the two sets of gels. Cells on the softest substrates (1 kPa and 0.3 kPa) reacted similarly in that they attached to the gel but did not spread. Cells plated on the second set of soft substrates (3 kPa and 0.9 kPa) were able to both attach and spread. These four gels were categorized as “soft”. Cells plated on the 8 kPa and 3.6 kPa gels spread more than cells on the soft substrates, but less than cells on the rigid substrates, so they were both termed “intermediate”. Cell spreading on the stiffest substrates (20 kPa and 34 kPa; 9.4 kPa, 30 kPa and 91 kPa) was similar, and they were all considered “rigid”.

Interestingly, cell spreading on the rigid substrates was analogous, despite the almost 10-fold difference in stiffness (9.4 kPa versus 91 kPa). This might indicate that cells may behave similarly above a certain stiffness threshold. This phenomenon was observed by Solon et al., who reported that cell spreading does not significantly increase on gels greater than 10 kPa, or even on glass (Solon et al., 2007).

Thus, these polyacrylamide gel serve as an efficient system to study the effects of substrate stiffness on cell-mediated FN matrix assembly.
Chapter 4

Fibronectin matrix assembly is increased on stiffer substrates

Introduction

During FN matrix assembly, cells transform compact FN dimers into linear and branched arrangements of fibrils that are gradually and irreversibly converted into a mature detergent-insoluble form (McKeown-Longo and Mosher, 1983) through formation of strong protein-protein interactions (Chen and Mosher, 1996; Singh et al., 2010). FN matrix assembly is detected largely by two methods: Immunofluorescence (IF) and detergent deoxycholate (DOC) solubility assays. IF with anti-FN antibodies allows for the visualization of FN matrix assembled at different timepoints, on substrates of different stiffnesses, and following different treatments. DOC solubility assays are used to characterize and quantify assembled FN matrix from cell lysates (McKeown-Longo and Mosher, 1983). The FN is first separated according to its solubility in DOC detergent. The DOC-soluble fraction is composed of intracellular, cell-bound, and nascent fibrillar FN, as well as various intracellular proteins. DOC-soluble FN fibrils consist of multiple FN molecules that reversibly bind through relatively weak interactions (McKeown-Longo and Mosher, 1983). In contrast, the DOC-insoluble fraction consists primarily of fibrillar FN whose multiple FN molecules are held together through strong, non-covalent interactions (Chen and Mosher, 1996; Ohashi and Erickson, 2009). The conversion of FN fibrils from DOC-soluble to DOC-insoluble is irreversible, and a measure of DOC-insoluble FN determines the amount of stable, mature FN matrix.

Two cellular events precede FN fibril formation: 1) The binding of soluble FN dimers to $\alpha 5\beta 1$ integrin receptors on the cell surface, and 2) An increase in cell contractility, which forcefully unfolds FN and exposes FN-binding sites. Inhibition of
either or both of these steps would impede assembly and result in reduced amounts of FN matrix.

To investigate the mechanism responsible for stiffness-dependent FN matrix assembly, we first focused on cell contractility. The actin cytoskeleton plays an integral role in FN matrix assembly by modulating cell contractility (Hynes, 1990a; Wu et al., 1995; Zhong et al., 1998). Actin-mediated cell contractility can be stimulated by treating cells with the phospholipid LPA (lysophosphatidic acid) (Zhang et al., 1994), which activates the small GTPase Rho (Hall, 2005). Stimulation of Rho has been shown to increase FN matrix assembly (Yoneda et al., 2007; Zhang et al., 1994; Zhang et al., 1999), and inhibition of Rho decreases FN assembly, resulting in a loss of cell contractility (Zhong et al., 1998).

Rho mediates cell contractility by activating Rho-associated protein kinase (ROCK), which in turn activates myosin light-chain kinase (MLCK) (Hall, 2005). Active MLCK phosphorylates the myosin II light chain (MLC), which allows myosin to bind actin filaments and enables actin-mediated cell contraction. The resulting intracellular forces induce actin reorganization into stress fibers.

Cell contractility can also be stimulated with Calyculin A, an inhibitor of myosin light chain (MLC) phosphatase (Henson et al., 2003). MLC phosphatase dephosphorylates MLC, preventing actin-myosin II binding and terminating cell contraction. Calyculin A inhibits this reaction, promoting cell contraction. We use LPA and Calyculin A to stimulate cell contractility and determine its effect on stiffness-dependent FN matrix assembly.

Cells assemble FN matrix primarily through the use of $\alpha5\beta1$-integrin receptors, and FN matrix assembly is inhibited in $\alpha5\beta1$-null cells or by $\alpha5\beta1$-inhibiting antibodies (Fogerty et al., 1990; Huveneers et al., 2008). Cells interact with extracellular FN via integrins, and integrins connect the ECM to the cytoskeleton. The intracellular domains of the integrins are associated with the cytoskeleton, enabling cells to transmit internal forces to the extracellular environment (Wiesner et al., 2006). Cells bind to FN via $\alpha5\beta1$ integrins and forcefully unfold the FN, exposing cryptic FN-FN binding sites and allowing FN molecules to self-associate and form fibrils (Singh et al., 2010).
The initiation of FN matrix assembly depends on the strong binding of FN to \(\alpha_5\beta_1\) integrins (Singh et al., 2010). The strength of integrin binding can be manipulated by activating the integrins with divalent cations such as Mn\(^{2+}\) to increase ligand binding (Elices et al., 1991; Gailit and Ruoslahti, 1988; Mould et al., 1995). We tested how modulating integrin-FN binding would affect stiffness-dependent matrix assembly.

The availability of FN can also affect this initial step in matrix assembly, since enough FN molecules must be present for cells to bind. As expected, FN-null cells do not assemble a FN matrix in media lacking FN, but they readily assemble exogenously added FN (Sottile et al., 1998). The concentration of FN also affects FN matrix assembly, with higher concentrations and greater expression resulting in an increase in the amount of assembled matrix (Hynes, 1990a). Substrate stiffness might affect gene expression or protein secretion (Williams et al., 2008), which could contribute to differences in assembly. Thus, it is important to control for situations in which FN expression can be altered.

In this chapter, we determine whether FN matrix assembly is affected by substrate stiffness. We also investigate the cellular mechanisms responsible for the stimulation of FN matrix assembly on rigid substrate, testing whether cell contractility or integrin activity could be involved.

* Portions of the results presented in this chapter have been previously reported (Carraher, CL and Schwarzbauer, JE (2011). Effects of Substrate Rigidity on Fibronectin Matrix Assembly. Mol. Biol. Cell 22 (suppl), Abstract No. 359.), or have been submitted for publication (Carraher CL, Schwarzbauer JE. (2013) Regulation of Matrix Assembly through Rigidity-dependent Fibronectin Conformational Changes. J. Biol. Chem., in revision.).
Results

Fibronectin matrix assembly is increased on stiffer substrates

To determine whether substrate stiffness affects cell-mediated assembly of FN into a matrix, we monitored formation of FN matrix on soft (0.9 kPa), intermediate (3.6 kPa), and rigid (9.4 kPa) substrates. These three stiffnesses were chosen because differences in matrix assembly could be detected after 6 hours (Figure 4.1). Immunofluorescence of total NIH 3T3-derived FN was detected with R457, a polyclonal anti-FN antiserum. Equivalent amounts of matrix were detected on the rigid (9.4 kPa) substrate and the stiffer gels (30 kPa and 91 kPa), and essentially no matrix was present on the softest (0.3 kPa) gel.

NIH 3T3 cells were plated on soft, intermediate, and rigid substrates at high density, so that cell confluence and shape were similar on all three substrates. After 6 and 12 hours, assembled matrix was examined by immunofluorescence of matrix fibrils. More FN matrix fibrils were assembled on the rigid substrate than on soft or intermediate substrates at both time points (Figure 4.2A). Cells on intermediate substrates also have more fibrils at 12 hours than cells on the soft substrate. These results show that cells assemble FN matrix relative to the stiffness of their substrate.

In order to control for differences in FN expression or secretion, FN levels in the medium were normalized by addition of exogenous rat FN at 10 µg/ml. Assembly was analyzed at 6 and 12 hours after plating, and exogenous FN in the matrix was detected with IC3, a rat FN-specific monoclonal antibody (Figure 4.2B). Assembly of exogenous FN showed the same correlation between amount of matrix and substrate rigidity as observed for NIH 3T3 cell-derived FN in Figure 4.2A. On the rigid substrate, cells incorporated a higher level of rat FN, and fibrils between adjacent cells appear longer and denser than cells on soft and intermediate substrates at both time points (Figure 4.2B). Cells on the intermediate substrate also had more fibrils than cells on the soft substrate. In fact, very little staining is visible on the soft substrate at either time point.

Immunofluorescence of total (3T3-derived and exogenous rat) FN detected with R457, exhibited the same trend (Figure 4.2C). These results demonstrate that more FN matrix is assembled on stiffer substrates than on softer substrates. This progression is
Figure 4.1 – Matrix assembly on substrates of different stiffness. NIH 3T3 cells were plated on substrates ranging between 0.3 kPa and 91 kPa. FN matrix assembled after 6 hours was detected by staining with R457 antiserum to detect total NIH 3T3 cell-derived FN matrix. Scale bar, 50 µm.
Figure 4.2 – FN matrix assembly is increased on the rigid substrate. A, NIH 3T3 cells were plated on soft (0.9 kPa), intermediate, (3.6 kPa), and rigid (9.4 kPa) substrates and, after 6 and 12 hours, were fixed and stained with R457 to detect total NIH 3T3 cell-derived FN matrix. B, NIH 3T3 cells were allowed to spread on gels for 2 hours, then rat plasma FN was added at 10 µg/ml and, after an additional 4 hours, cells were fixed and stained with IC3, a rat-specific anti-FN antibody. C, NIH 3T3 cells were treated as in (B) and then fixed and stained with R457 to detect total FN matrix. Scale bars, 25 µm.
detected regardless of the source of FN or the presence or absence of exogenous FN. Thus, FN matrix assembly is enhanced by increasing rigidity of the substrate.

**Cells assemble a greater amount of stable FN matrix on rigid substrates**

The amount of stable DOC-insoluble FN matrix was used to compare assembly on different stiffnesses. Cells were plated on soft, intermediate, and rigid substrates, and assembled matrix was examined after 6 and 12 hours by quantification of stable DOC-insoluble matrix (Figure 4.3A). DOC-insoluble FN matrix was detectable in immunoblots of lysates from cells on the rigid substrate at 6 hours and was significantly higher at 12 hours. No FN was detected from cells on the soft gel and very little was seen in lysates from cells on the intermediate stiffness at 6 hours, although DOC-insoluble exogenous FN matrix was detected on the soft and intermediate substrates at 12 hours. These results show that increased substrate rigidity up-regulates the assembly of stable FN matrix. DOC-soluble FN was present on all substrates at both time points, indicating that while cells are able to bind to FN, conversion of bound FN to DOC-insoluble fibrils is enhanced by substrate stiffness.

DOC-insoluble FN band intensities from 12 h cell lysates were quantified and then normalized to the 12 h soft band intensity. Average fold differences in Figure 4.3B show that the level of matrix assembly on the rigid substrate is about 5-fold higher than matrix on soft and intermediate substrates. Cells on the soft and intermediate substrates assembled similar amounts of FN matrix at 12 h as determined by quantification of rat FN matrix. These results show that substrate stiffness affects cell-mediated FN matrix assembly, with rigid substrates enhancing assembly and softer substrates limiting the amount of FN matrix assembled.

**Cells on rigid substrates have more stress fibers**

Since the actin cytoskeleton is important in FN matrix assembly, we looked at the organization of actin in cells on different stiffnesses. NIH 3T3 cells were plated on soft and rigid substrates, and they were fixed, permeabilized and stained for actin 6 hours later. We observed that cells on more rigid substrates exhibited an increase in
Figure 4.3 – Analysis of DOC-insoluble FN matrix.  A, Cells were lysed in DOC buffer at 6 and 12 hours and rat FN in DOC-soluble and DOC-insoluble fractions was detected with IC3 monoclonal antibody.  B, Quantification of rat FN in DOC-insoluble matrix at 12 hours.  The band intensities were measured in the linear range, normalized to GAPDH, and then normalized to the 12 hour soft sample (mean +/- S.E.M, n = 3).
cytoskeletal organization into stress fibers (Figure 4.4). The appearance of actin stress fibers increased with increasing stiffness.

**Increasing cell contractility does not significantly increase stable FN matrix assembly on soft substrates**

To determine the potential involvement of cell contractility in stiffness-dependent FN matrix assembly, we examined whether cells on the soft substrate could be induced to assemble matrix by stimulating cell contractility with LPA. Cells were plated on soft, intermediate, and rigid substrates, and exogenous rat FN and 10 μM LPA were added after two hours. Six hours after plating, the cells were fixed and stained for exogenous FN. Addition of LPA led to a noticeable increase in fibrils on the rigid substrate, but it only slightly increased the amount of fibrils on the soft and intermediate substrates (Figure 4.5A). Similar results were obtained when cells were treated with 5 nM Calyculin A instead of LPA. With addition of Calyculin A, we observed an obvious increase in the density of FN fibrils on the rigid substrate but a very small, if any, increase on the soft or intermediate substrates (Figure 4.5B).

The amount of stable DOC-insoluble FN matrix was used to compare assembly with or without stimulating cell contractility. NIH 3T3 cells were plated and treated as with IF, but 6 hours after plating, they were lysed in DOC buffer for analysis of DOC-insoluble matrix by immunoblotting. While the amount of FN in the DOC-insoluble material was increased on the rigid substrates with LPA or Calyculin A treatment, no DOC-insoluble FN was detected on the soft or intermediate substrates, regardless of treatment (Figure 4.6A).

Quantification of the relative amounts of DOC-insoluble FN revealed that cells on the rigid substrate showed an approximately 4.5-fold and 6.7-fold increase in DOC-insoluble FN matrix with LPA and Calyculin A, respectively (Figure 4.6B). These results show that stimulation of cell contractility is not effective at rescuing FN matrix assembly on soft substrates.
Figure 4.4 – Cytoskeletal organization on substrates of different stiffness. NIH 3T3 cells were plated on soft (1 kPa) or rigid (34 kPa) FN-coated gels. After 6 hours, cells were fixed, permeabilized and stained with rhodamine-phalloidin to visualize actin filaments. Scale bar, 50 µm.
Figure 4.5 – LPA and Calyculin A do not significantly increase FN matrix assembly on soft substrates. A, NIH 3T3 cells were allowed to spread on soft (0.9 kPa), intermediate (3.6 kPa), and rigid (9.4 kPa) gels for 2 hours at which time rat FN at 10 µg/ml and 10 µM LPA were added. At 6 hours after plating, cells were fixed and stained for rat FN using IC3 antibody. Scale bar, 50 µm. B, Cells were plated as in (A) but 5 nM Calyculin A was added instead of LPA. Scale bar, 50 µm.
Figure 4.6 – LPA and Calyculin A do not increase assembly of stable FN matrix on soft substrates. A, NIH 3T3 cells were allowed to spread on soft (0.9 kPa), intermediate (3.6 kPa), and rigid (9.4 kPa) gels for 2 hours at which time rat FN at 10 µg/ml and either 10 µM LPA or 5 nM Calyculin A were added. Cells were lysed after 6 hours, and DOC-insoluble FN was analyzed by immunoblotting with IC3 antibody. B, Quantification of rat FN in DOC-insoluble matrix following LPA and Calyculin A treatment. The band intensities were measured in the linear range, normalized to GAPDH, and then normalized to the untreated rigid sample (mean +/- S.E.M, n = 2).
Integrin stimulation increases stable FN matrix assembly on soft substrates

We tested how modulating integrin-FN binding would affect matrix assembly on softer substrates. FN matrix assembly was measured in the absence or presence of integrin-activating Mn$^{2+}$. Cells on soft, intermediate, and rigid substrates were treated with 1 mM MnCl$_2$, and six hours after plating, they were fixed and stained for exogenous FN or lysed and DOC-insoluble matrix was detected by immunoblotting. Addition of Mn$^{2+}$ significantly increased the amount of FN matrix on all substrates, as detected by immunostaining (Figure 4.7). Mn$^{2+}$ treatment led to a noticeable increase in the apparent number and length of fibrils. The difference was most apparent on soft and intermediate substrates.

Treatment with Mn$^{2+}$ significantly increased the amounts of DOC-insoluble FN on all stiffnesses. No DOC-insoluble FN was detected on untreated soft or intermediate substrates, but Mn$^{2+}$ treatment resulted in obvious bands, similar in intensity to the signal on an untreated rigid substrate (Figure 4.8A). In addition, the amount of DOC-insoluble FN on the rigid substrate became significantly higher with Mn$^{2+}$ treatment.

Quantification of band intensities shows that, with Mn$^{2+}$ treatment, DOC-insoluble FN increased on soft and intermediate substrates from undetectable levels to levels similar to untreated cells on the rigid substrate (0.7-fold and 0.9-fold, respectively) (Figure 4.8B). Mn$^{2+}$ treatment also led to a greater than 2-fold increase in DOC-insoluble FN on rigid substrates. Therefore, integrin-stimulation increases FN matrix assembly on all substrates, including softer ones.
Figure 4.7 – Mn\(^{2+}\) treatment increases FN matrix assembly on soft substrates. NIH 3T3 cells were allowed to spread on soft (0.9 kPa), intermediate (3.6 kPa), and rigid (9.4 kPa) gels for 2 hours at which time 10 \(\mu\)g/ml rat FN and of 1 mM MnCl\(_2\) were added. Cells were fixed and stained for rat FN using IC3 antibody. Scale bar, 25 \(\mu\)m.
**Figure 4.8 – Mn$^{2+}$ treatment increases assembly of stable FN matrix on soft substrates.** A, NIH 3T3 cells were allowed to spread on soft (0.9 kPa), intermediate (3.6 kPa), and rigid (9.4 kPa) gels for 2 hours at which time 10 µg/ml rat FN and of 1 mM MnCl$_2$ were added. Cells were lysed 6 hours after plating and DOC-insoluble FN was analyzed by immunoblotting with IC3 antibody. B, Band intensities from immunoblots of DOC-insoluble FN were quantified with and without Mn$^{2+}$ treatment. The band intensities were normalized to the signal for the untreated rigid sample. Values are the mean +/- S.E.M for at least two experiments.
Discussion

FN matrix assembly is increased on rigid substrate, as determined by IF, and analysis of DOC-insoluble FN shows that increased substrate rigidity up-regulates the assembly of stable FN matrix. DOC-soluble FN was present on all substrates after both 6 hours and 12 hours, indicating that while cells are able to bind to FN, conversion of bound FN to DOC-insoluble fibrils is enhanced by substrate stiffness.

LPA is a well-established stimulant of cell contractility, but it is also present in serum at concentrations of 1-5 µM (Moolenaar, 1995). My experiments were performed in cell media containing 10% serum, so even the untreated samples were exposed to as much as 0.5 µM LPA. The inability of LPA to induce FN matrix assembly on soft and intermediate substrate could be attributed to the presence of LPA in the media. The LPA in the media could be significantly stimulating the untreated cells on soft and intermediate substrates, so its effects might not be visible after adding an additional 10 µM LPA. To eliminate these complicating factors, cell contractility was stimulated by a different mechanism with Calyculin A. Calyculin A is a molecule isolated from the sea sponge *Discodermia calyx*, so it is neither expressed by NIH 3T3 cells nor present in cell media (Fagerholm et al., 2010). The results from Calyculin A treatment resembled the results from LPA treatment, verifying that stimulating cell contractility does not substantially increase FN matrix assembly on softer substrates.

It had been previously shown that stimulation of cell contractility increases FN matrix assembly on rigid glass or plastic substrates (Zhang et al., 1994). Likewise, stimulation of cell contractility in our experiments was effective at increasing FN matrix on the rigid substrate. In contrast, modulation of Rho GTPase or MLC phosphatase activity did not stimulate FN matrix assembly on a soft substrate. The slight increase in FN matrix assembly on soft and intermediate substrates observed by IF could be from an increase in DOC-soluble FN. Stimulating cell contractility could increase the formation of nascent FN fibrils, yet still be unable to support the stabilization of FN bonds that are required to make FN fibrils DOC-insoluble. Indeed, LPA has been shown to increase the binding of FN to the cell surface (Zhang et al., 1994), which would aid in the formation of FN fibrils. But other factors, including integrin binding and signaling, also play an
important role in the assembly of a stable FN matrix and modulation of Rho GTPase or MLC phosphatase activity would not be able to compensate for a deficit in these other pathways. Taken together, these results indicate that the difference in FN matrix assembly between the soft and rigid substrates is not due to a deficit in cell contractility on soft substrate.

Stimulating integrins with Mn$^{2+}$ led to a prominent increase in FN matrix assembly on all samples, including the soft and intermediate substrates. Quantification of DOC-insoluble FN showed that Mn$^{2+}$ treatment increased the assembly of stable FN matrix to the same extent as cells on untreated rigid substrate. These results suggest that, in a soft environment, integrin activity and the strength of FN-integrin binding are affected such that less matrix is assembled.

Stimulating integrins with Mn$^{2+}$ could increase FN matrix in several ways. Softer substrates could inhibit integrin activation, reducing integrin-FN binding, and Mn$^{2+}$ treatment would activate these integrins, leading to an increase in FN-integrin binding. However, no clear connection between integrin activation and substrate stiffness has been established. Paszek et al. found that integrin β1 activation was the same on soft and stiff substrates, in both fibroblasts and mammary epithelial cells (Paszek et al., 2005). They also reported that expression of a constitutively active β1 integrin did not increase FAK phosphorylation or cell spreading of fibroblasts on soft gels. A recent study with bone marrow mesenchymal stem cells has shown that β1 activation is actually increased on soft substrate (Du et al., 2011). Alternatively, softer substrates may not support the formation of strong integrin-FN bonds, which are necessary for FN matrix assembly. FN-integrin bonds are strengthened by a resisting force (Choquet et al., 1997), which soft substrates would not provide; thus, the FN-integrin bonds would remain weak. Mn$^{2+}$ treatment could strengthen FN-integrin binding, allowing cells to apply a greater force to the ligand, which would partially unfold the FN and expose FN binding sites. Friedland et al. reported that the total number of FN-integrin bonds were the same on soft and rigid substrate, but the number of strong FN-α5β1 bonds increased on rigid substrate (Friedland et al., 2009). Thus, the increase in FN matrix following Mn$^{2+}$ treatment is more likely a result of an increase in the strength of FN-α5β1 and not an increase in the total number of FN-α5β1 bonds.
The strength of integrin binding to FN contributes to matrix assembly (Danen et al., 1995; Friedland et al., 2009; Sechler et al., 1997). FN-α5β1 integrin binding depends on both the RGD cell-binding domain in module III10 and the synergy site in III9 (Aota et al., 1994; Garcia et al., 2002). Strong FN-integrin bonds require the synergy site, and the number of strong FN-α5β1 bonds correlates with the stiffness of the substrate (Friedland et al., 2009). If FN matrix assembly is inhibited on soft substrate due to a lack in strong integrin-FN bonds, then Mn$^{2+}$ treatment could increase FN matrix assembly on softer substrates by increasing the number of strong integrin-FN bonds. In effect, FN matrix assembly would be inhibited on soft substrate in the same way that recombinant FN with a mutation at the synergy site is not assembled into matrix (Sechler et al., 1997). In both instances, FN matrix assembly is restored upon stimulation of α5β1 integrin by treatment with Mn$^{2+}$ (Sechler et al., 1997).
Chapter 5

Extension of substrate fibronectin stimulates fibronectin matrix assembly

Introduction

The conformation of FN is important in determining its function as it modulates the interactions between FN and cells or other molecules in the ECM. FN in solution is in a compact conformation that is maintained by intramolecular interactions (Johnson et al., 1999). During the process of FN matrix assembly, cells unfold FN molecules, opening the conformation of the FN molecule and exposing previously unavailable FN-binding sites (Singh et al., 2010). Integrin binding to FN promotes conformational changes that extend FN dimers and expose new FN-binding sites that participate in fibril formation (Singh et al., 2010). In fact, FN molecules within fibrils have been shown to exhibit varying degrees of extension (Baneyx et al., 2001, 2002). Additionally, FRET studies suggest that FN is more extended in fibrils assembled on a rigid substrate than on a soft substrate (Antia et al., 2008). Based on this evidence, we decided to examine how the conformation of substrate-bound FN affects FN matrix assembly.

To do this, we pre-extended FN with 1M guanidine-HCl (GdnHCl) prior to crosslinking it to the polyacrylamide gel substrate. GdnHCl-treatment is a well-established method for partially denaturing fibronectin, as has been verified by various biochemical and biophysical techniques, including spectroscopy (Khan et al., 1990) and sedimentation analysis (Erickson and Carrell, 1983). Fluorescent resonance energy transfer (FRET) studies have shown that fibronectin can be partially extended by treatment with 1M GdnHCl, which expands the FN dimer by disrupting the electrostatic interactions that maintain its compact conformation, while keeping the individual modules of FN intact (Baneyx et al., 2002; Johnson et al., 1999; Karuri et al., 2009; Khan...
et al., 1990). Increasing concentrations of GdnHCl, above 2M, progressively unfold FN molecules, with significant unfolding of type III modules observed in 4M solutions of GdnHCl (Baneyx et al., 2001). We wished only to partially extend the FN dimers, so 1M GdnHCl was used. GdnHCl-treated FN was crosslinked to the surfaces of polyacrylamide gels and the effect on FN matrix assembly was studied.

This chapter explores the effect of substrate stiffness on substrate FN conformation, and how altering its structure can affect cell behavior and FN matrix assembly. We tested the hypothesis that substrate rigidity affects the ability of cells to induce FN conformational changes that support fibril assembly. To do this, we compared FN matrix assembly on polyacrylamide gels coated either with untreated FN (in the compact conformation) or with FN that had been partially extended by treatment with GdnHCl.

* Portions of the results presented in this chapter have been previously reported (Carraher, CL and Schwarzbauer, JE (2011). Effects of Substrate Rigidity on Fibronectin Matrix Assembly. Mol. Biol. Cell 22 (suppl), Abstract No. 359.), or have been submitted for publication (Carraher CL, Schwarzbauer JE. (2013) Regulation of Matrix Assembly through Rigidity-dependent Fibronectin Conformational Changes. J. Biol. Chem., in revision.).
Results

The FN crosslinked to rigid substrates binds more full-length FN

In vitro studies of FN fibril formation suggest that cells extend FN into fibrils by pulling on substrate-associated FN (Ohashi et al., 2002), and FN fibril formation involves conformational changes in FN molecules, so we examined whether the substrate-bound FN had the same conformation on gels of varying stiffnesses. The conformation of the FN bound to the substrates of different stiffness was examined by measuring the binding of full-length FN to the substrate FN. Soft and rigid polyacrylamide gels were crosslinked with 20 µg/ml and 9 µg/ml solutions of unlabelled FN, respectively, so that equivalent amounts of FN would be crosslinked to both surfaces. The gels were incubated with either a low concentration (15 µg/ml) or high concentration (50 µg/ml) solution of biotinylated full-length FN for 1 hour. Attached biotinylated FN was then detected via ELISA (Figure 5.1A). At both concentrations of biotinylated FN, approximately 2-fold more FN bound to rigid substrate than to soft.

To ensure this difference wasn’t due to different amounts of FN being crosslinked to the substrates, substrate FN was quantified using an ELISA to detect binding of the polyclonal R184 antibody against the III₁₋₆ domain of FN (Figure 5.1B). Equivalent amounts of FN were crosslinked to both the soft and rigid substrates.

This distinct binding could be due to a difference in the conformation of the substrate FN crosslinked to the surfaces of soft and rigid gels. The substrate FN on rigid gels might be in a conformation that exposes more FN-binding sites, allowing more biotinylated FN to attach.

The anti-FN MAB 1890 antibody shows variable binding to FN

The difference in substrate FN conformation was studied using Millipore’s MAB 1890 antibody, against the III₃ module of FN (Klein et al., 2003). When MAB 1890 was used to detect extracellular FN via immunofluorescence, it failed to stain fibrillar FN (Figure 5.2A). NIH 3T3 cells were plated on soft (1 kPa) polyacrylamide gels and on rigid glass (approximately 60 GPa, or 60,000,000 kPa) (Soga, 1985), and 12 hours later,
Figure 5.1 – Substrate FN on rigid substrate binds more full-length FN. A, Biotinylated full-length FN was incubated with soft (0.3 kPa) and rigid (91 kPa) substrates, and the amount of bound FN was measured by absorbance values at 405 nm. B, The amount of FN crosslinked to the surfaces of the substrates was determined by binding of the polyclonal R184 antibody against the III_{1-6} domain of FN. (mean +/- S.E.M., n=2)
Figure 5.2 – The MAB 1890 antibody does not stain fibrillar FN. A, NIH 3T3 cells were plated on soft (1 kPa) and rigid (60 GPa) glass substrates, and after 12 hours, the cells were fixed and extracellular FN was stained with both R457 and MAB 1890. B, HT1080 cells were plated on rigid (34 kPa) substrate, and after 12 hours, the cells were fixed with 3.7 % formaldehyde, permeabilized and stained with R457 and MAB 1890.
cells were fixed and stained with both MAB 1890 and the polyclonal antiserum R457 against the 70-kDa domain of FN. R457 staining of the unpermeabilized cells revealed short fibrils associated with cells on soft substrate and an extensive fibrillar network on glass. In contrast, no MAB 1890 staining is seen with cells on glass, and only patchy, cell-associated staining is visible on soft substrate. MAB 1890 failed to stain fibrillar FN and seemed to stain only cell-surface FN on soft substrate.

Even though MAB 1890 did not stain extracellular fibrillar FN, it was able to detect intracellular FN in permeabilized cells (Figure 5.2B). HT1080 cells were plated on glass coverslips, and after 12 hours, were fixed, permeabilized, and stained with MAB 1890 and R457. As before, MAB 1890 did not stain the fibrillar FN that was visible with R457 staining. MAB 1890 staining was observed in punctate, perinuclear bunches associated with the cell, resembling proteins in secretory vesicles. This staining was only observed in cells that were permeabilized, so the protein is presumed to be intracellular. As opposed to extracellular fibrillar FN, intracellular FN would likely be in a compact conformation.

In addition, when MAB 1890 was used to probe a western blot, it failed to detect human plasma FN, rat plasma FN, bovine plasma FN and the III1-6 fragment of FN (data not shown). It was, however, able to bind human plasma FN in an immunoprecipitation pull-down assay (Figure 5.3). MAB 1890 was incubated with either 10 µg/ml human plasma FN or conditioned media containing human FN and then incubated with protein G sepharose beads. While no FN was detected from the conditioned media, a clear band could be seen from the MAB 1890 incubated with human plasma FN. If MAB 1890 only recognizes FN in a compact conformation, then it wouldn’t bind denatured FN on a western, but it should be able to bind compact FN in solution.

**Substrate FN conformation varies with substrate stiffness**

Variability in the binding of full-length FN to substrate FN on soft and rigid gels suggests that its conformation changes according to substrate stiffness. This stiffness-dependent conformational change was studied by measuring the variable presence of the MAB 1890 epitope on FN. Soft and rigid polyacrylamide gels were crosslinked with FN,
**Figure 5.3 – The MAB 1890 antibody binds soluble human plasma FN.** 1 µg/ml MAB 1890 was incubated with either 10 µg/ml human plasma FN or with conditioned media. It was then incubated with Protein G Sepharose beads, eluted and run on a western blotted with R457. For the negative control, 10 µg/ml human plasma FN was incubated with Sepharose beads in the absence of MAB 1890.
and FN was adsorbed onto glass coverslips. The gels and coverslips were blocked with BSA and then incubated with serial dilutions of the MAB 1890, and antibody binding was detected via ELISA. More MAB 1890 antibody bound to the substrate FN on soft substrate than on rigid substrate (Figure 5.4A). Even less antibody bound to the FN adsorbed onto glass, when FN is known to assume an extended conformation (Baugh and Vogel, 2004).

To ensure this difference was not due to less FN being crosslinked to or adsorbed on the rigid substrates, equivalent amounts of biotinylated FN were crosslinked or adsorbed in parallel with the unlabelled FN and then detected via ELISA (Figure 5.4B). Almost 2-fold more FN was crosslinked to the rigid substrate, and over 6-fold more FN was adsorbed onto the glass coverslip. Less MAB 1890 antibody bound to FN on rigid substrates, even though there was more FN present.

MAB 1890 recognizes the III_{5} module of FN. We examined whether this stiffness-dependent binding could be replicated on substrates crosslinked or adsorbed with the III_{1-6} fragment of FN, instead of full-length FN. Even a greater disparity in MAB 1890 binding was observed with the III_{1-6} fragment (Figure 5.5A). III_{1-6} was crosslinked onto soft and rigid polyacrylamide gels and adsorbed onto glass coverslips, and MAB 1890-binding was measured. Significantly more MAB 1890 antibody bound to the substrate FN on soft substrate than on rigid substrate, and much less bound to the FN adsorbed onto glass. This happened even though the surface density of III_{1-6} was much higher on the stiffer substrates (Figure 5.5B), as measured with the polyclonal R184 antibody against the III_{1-6} domain of FN. Thus, fibronectin conformation seems to vary according to the stiffness of the substrate it is bound to, with less antibody binding on more rigid substrates, when FN is known to assume a more extended conformation (Baugh and Vogel, 2004).

We hypothesized that the MAB 1890 epitope is present on compact FN molecules and absent when FN is extended, which would indicate that FN assumes a compact conformation on softer substrates and a more extended conformation on rigid substrates. We examined whether extending FN on soft gels could make cells on soft substrate behave as if they were on rigid substrate. To do this, we pre-extended FN with buffered
Figure 5.4 – More MAB 1890 binds substrate FN on soft gels. MAB 1890 and biotinylated FN were detected at a wavelength of 405 nm. Soft (1 kPa) and rigid (20 kPa) gels were crosslinked and glass was adsorbed with a solution of 15 µg/ml, 10 µg/ml and 5 µg/ml unlabelled or biotinylated FN, respectively. A, The gels and glass with unlabelled FN were incubated with serial dilutions of MAB 1890 antibody, and antibody-binding was detected by ELISA. B, The amount of bound biotinylated FN was measured by ELISA (mean +/- S.E.M, n = 2).
Figure 5.5 – More MAB 1890 binds substrate-III₁₋₆ on soft gels. MAB 1890 and III₁₋₆ were detected at a wavelength of 405 nm. Soft (1 kPa) and rigid (34 kPa) gels were crosslinked with and glass was adsorbed with a solution of 15 µg/ml, 6 µg/ml and 1.1 µg/ml III₁₋₆, respectively. A, The gels and glass were incubated with serial dilutions of MAB 1890 antibody, and antibody binding was detected by ELISA (mean +/- S.E.M, n = 2). B, The amount of bound III₁₋₆ was measured with the polyclonal R184 antibody, against the FN III₁₋₆ domain by ELISA (mean +/- S.E.M, n = 3).
1M GdnHCl prior to crosslinking it to the polyacrylamide gel substrate. Conformational differences in substrate-bound FN with and without GdnHCl-treatment were confirmed by an ELISA using MAB 1890. Untreated or GdnHCl-treated FN was crosslinked to soft gels or absorbed onto glass coverslips, and MAB 1890 binding was measured and normalized to the amount of total substrate FN. Compared to untreated FN, MAB1890 binding to GdnHCl-treated FN was significantly decreased (Figure 5.6). Binding of a control, non-conformation-dependent monoclonal antibody, 5E6, to untreated and GdnHCl-treated FN showed no change. As before, a minimal amount of MAB1890 bound to FN adsorbed onto glass. The decrease in MAB 1890-binding to GdnHCl-treated FN supports the theory that this antibody recognizes compact FN, since it is well-documented that 1M GdnHCl partially unfolds FN (Baneyx et al., 2002; Karuri et al., 2009). Therefore, we infer that GdnHCl-treated substrate FN on soft gels is in a more extended conformation than untreated substrate FN.

**Guanidine-HCl treatment increases cell spreading and cell adhesion**

To examine whether changing the conformation of substrate FN could elicit changes in cell behavior, polyacrylamide gels were crosslinked with different amounts of GdnHCl-treated or untreated FN. Increasing the concentration of FN crosslinked to the substrate was shown to increase cell adhesion (Figure 3.5) and cell spreading (Figure 3.6), so we investigated whether extending substrate FN with GdnHCl would also affect these cell behaviors. Cell spreading was observed on soft polyacrylamide gels that were crosslinked with low concentration or high concentration solutions of untreated FN, or with a low concentration solution of GdnHCl-treated FN. The addition of GdnHCl increased cell spreading on the soft substrate, even when compared to gels with a higher concentration of untreated FN (Figure 5.7A).

GdnHCl-treatment also increased the adhesion of cells to soft substrate (Figure 5.7B). Soft polyacrylamide gels were crosslinked with either low, medium, or high concentration solutions of untreated FN, or with a low concentration solution of GdnHCl-treated FN, and cell adhesion was measured. GdnHCl-treatment almost doubled the number of adherent cells, approaching a similar degree of adhesion attained on substrate
Figure 5.6 – Guanidine HCl-treatment changes FN conformation. Untreated or GdnHCl-treated FN was crosslinked to a soft (0.9 kPa) substrate or adsorbed onto glass coverslips. An ELISA was performed using anti-FN monoclonal antibody MAB 1890 or a control monoclonal antibody 5E6. Absorbance values at 405 nm were normalized to the amount of FN crosslinked to the surfaces of the gels or coverslips. Graph shows the values averaged between two experiments +/- S.E.M. * = p < 0.05 from untreated sample.
Figure 5.7 – Guanidine HCl-treatment increases cell spreading and adhesion. A, soft (1 kPa) gels were crosslinked with 5 µg/ml untreated FN, 45 µg/ml untreated FN, or 5 µg/ml GdnHCl-treated FN and were imaged 6 hours later. B, HT1080 cells were plated in media with dexamethasone and without serum on soft (1 kPa) gels crosslinked with either 5, 15, or 45 µg/ml untreated FN or 5 µg/ml GdnHCl-treated FN. Cells were counted and averaged from four random fields imaged after 1 h (mean +/- S.E.M., n = 4).
crosslinked with a solution of FN 10 times higher. This suggests that extension of substrate FN with GdnHCl impacts the way cells interact with it, possibly making it more accessible to cells.

**Partially unfolding substrate FN increases matrix assembly**

The effects of FN conformation on FN matrix assembly were examined. Untreated FN and GdnHCl-treated FN were crosslinked to soft, intermediate, and rigid polyacrylamide gels, and cells on these substrates were then compared for the ability to assemble a FN matrix. The cells were plated on the substrates and exogenous rat FN was added after 2 hours. Six hours after plating, the cells were fixed and stained for exogenous FN. FN fibrils were noticeably increased on all substrates with GdnHCl-treated FN compared to untreated FN substrates (Figure 5.8). Little staining is visible on the untreated soft and intermediate substrates, but FN fibrils were detected on GdnHCl-treated soft and intermediate substrates. Providing cells with an extended form of FN appears to enhance their ability to assemble FN into fibrils, even on the soft substrate.

The amount of stable DOC-insoluble FN matrix was used to compare assembly with or without GdnHCl treatment. NIH 3T3 cells were plated as above and were lysed for analysis of DOC-insoluble matrix by immunoblotting. DOC-insoluble matrix was significantly increased on GdnHCl-FN substrates (Figure 5.9A). No DOC-insoluble FN was detected on soft and intermediate substrates. However, on soft and intermediate gels with GdnHCl-treated FN, DOC-insoluble FN was detected and DOC-insoluble FN was also visibly increased on the rigid substrate.

DOC-insoluble FN band intensities were quantified from exposures that yielded signals within the linear range. Quantities for each band were normalized to GAPDH and then to the untreated rigid band intensity before being averaged over multiple experiments. Quantification of band intensities showed that DOC-insoluble FN on the soft and intermediate substrates with GdnHCl-FN was similar to DOC-insoluble FN on the rigid substrate with untreated FN (0.8-fold and 1.3-fold, respectively) (Figure 5.9B). Matrix on the rigid substrate with GdnHCl-FN was also significantly higher than from
Figure 5.8 – Guanidine HCl-treatment of substrate FN increases FN matrix assembly. NIH 3T3 cells were plated on untreated or guanidine-treated soft (0.9 kPa), intermediate (3.6 kPa), and rigid (9.4 kPa) gels and allowed to spread for 2 hours before the addition of 10 µg/ml rat FN. Cells were fixed and stained for rat FN after 6 hours. Scale bar, 25 µm.
Figure 5.9 – GdnHCl treatment increases assembly of stable FN matrix on soft substrates. A, NIH 3T3 cells were plated on untreated or guanidine-treated soft (0.9 kPa), intermediate (3.6 kPa), and rigid (9.4 kPa) gels and allowed to spread for 2 hours before the addition of 10 μg/ml rat FN. Cells were lysed 6 hours after plating and DOC-insoluble FN was analyzed by immunoblotting with IC3 antibody. B, Band intensities from immunoblots of DOC-insoluble FN were quantified with and without GdnHCl treatment. The band intensities were normalized to the signal for the untreated rigid sample. The same untreated rigid samples were used in Chapter 4 to quantify Mn$^{2+}$ treatment. Values are the mean +/- S.E.M. for at least two experiments.
cells on the untreated rigid substrate (3.2-fold). These results show that FN matrix assembly is affected by the conformation of the FN that cells attach to, regardless of substrate stiffness.

**Partial Unfolding of Substrate FN Stimulates Initiation of Matrix Assembly**

During the early stages of FN matrix assembly, cells begin to arrange FN molecules into fibrils at matrix assembly sites, which form at sites of cell-substrate contact (McKeown-Longo and Mosher, 1985). These FN-FN interactions are mediated by FN’s 70-kDa amino terminal domain, and the presence of a cell’s assembly sites can be detected by their association with the 70-kDa fragment of FN (McKeown-Longo and Mosher, 1985; Sottile et al., 1991). Softer substrates might inhibit the initial steps of FN matrix assembly by reducing assembly sites.

Cells on rigid substrates were found to form more FN matrix assembly sites than cells on soft substrate (Figure 5.10). Cells were plated on soft and rigid polyacrylamide gels and on rigid glass coverslips, and FN matrix assembly sites were detected with fluorescently-labeled 70-kDa. Essentially no fluorescence was detected on the soft substrate, but images of cells on the rigid and glass substrate revealed bright, spiky arrays on the edges of cells. These results indicate that the initial steps of FN matrix assembly are down-regulated on soft substrate.

To determine whether partially unfolding substrate FN on soft substrate would encourage the initiation of FN matrix assembly, cells were plated on soft substrates crosslinked with either untreated FN or GdnHCl-treated FN, and biotinylated 70-kDa was added. The cells were fixed 30 minutes later, and biotinylated 70-kDa was detected with fluorescently-labeled streptavidin. Images of cell-bound biotinylated 70-kDa revealed punctate staining primarily near the peripheries of cells on untreated FN, whereas assembly sites that formed on GdnHCl-treated FN were larger and were found under the cell bodies as well as at cell edges (Figure 5.11A).

Assembly sites were quantified from the images of cells on soft substrates with GdnHCl-treated or untreated FN. The average intensity per cell area was measured for each cell, revealing that the overall intensity of matrix assembly sites formed by cells on
Figure 5.10 – Cells on rigid substrate form more FN matrix assembly sites. CHOα5-17 cells were pated on soft (0.9 kPa), rigid (91 kPa), and glass substrates along with 20 μg/ml rat plasma FN and 40 μg/ml AlexaFluor488-conjugated 70-kDa. Cells were fixed 2 hours later. Bright field images show the cell shape. Cells on rigid (91 kPa) gels were not visible in bright field images, so cell shape was visualized using auto-fluorescence.
Figure 5.11 – Enhanced formation of FN matrix assembly sites on a soft substrate with GdnHCl FN. NIH 3T3 cells were plated on untreated or GdnHCl-treated soft (0.9 kPa) substrates and allowed to spread for 2 hours before the addition of 20 µg/ml biotinylated-70-kDa and 10 µg/ml rat FN. Cells were fixed and probed with fluorescently-labeled streptavidin 30 minutes later. A, Representative images of assembly sites of cells on untreated or GdnHCl-treated FN. Scale bars, 10 µm. B, Fluorescent signal intensity of FN matrix assembly sites was measured for 17 cells in each condition, and the average intensity per pixel was calculated.

** p < 0.01
GdnHCl-treated FN substrates was almost 2-fold that of assembly sites on cells plated on untreated FN substrates (Figure 5.11B). These results demonstrate that the initial steps of FN matrix assembly on a soft substrate are influenced by the conformation of the substrate FN.
Discussion

We examined how the conformation of the FN crosslinked to the substrates is affected by the stiffness its environment, and how this conformation affects stiffness-dependent FN matrix assembly. Our results determined that partially unfolding the substrate FN increased FN matrix assembly on all substrates and increased the amounts of DOC-insoluble FN on all substrates. The amount of DOC-insoluble FN on soft and intermediate GdnHCl-treated substrates is comparable to that on rigid untreated substrate, indicating that GdnHCl-treatment of substrate FN has an impact on the step in FN matrix assembly that is inhibited on soft substrates.

Figure 5.12 explains how the conformation of the substrate FN can affect FN matrix assembly. Compact FN dimers are in a conformation that masks many FN-binding sites, thus preventing FN-FN associations and fibril formation. If the substrate FN is extended, previously hidden FN-binding sites are revealed. Other FN molecules bind to these exposed FN-binding sites, and fibrils are formed. In my experiments, substrate FN was extended either by treatment with GdnHCl or by crosslinking to rigid substrate. In both cases, the extended conformation of FN may have been maintained by multiple attachments of a single FN molecule to the surface of the gel. GdnHCl-treatment likely extended the FN, increasing the length of the molecule and the surface area available for contact with the gel-bound Sulfo-SANPAH crosslinker. Since rigid polyacrylamide gels have more acrylamide than soft gels, they might have more Sulfo-SANPAH attachments sites, which could increase the density of the crosslinker and thereby increase the probability that a single FN molecule is crosslinked to the gel in more than one place.

Just by changing the conformation of FN attached to the substrate, cells on softer substrates assembled a similar amount of FN as cells on rigid substrate. As demonstrated by the increase in FN assembly sites on GdnHCl-treated substrates, partial unfolding of substrate FN leads to an increase in the amount of exposed FN-binding sites, either directly, by crosslinking FN to the substrate in a conformation in which it displays previously buried binding sites, or indirectly, by disrupting the electrostatic interactions that hold FN in the compact form, consequently reducing the force needed to unfold FN.
Figure 5.12 – Model of effect of substrate FN conformation on FN matrix assembly. GdnHCl-treatment partially unfolds the substrate FN, exposing previously unavailable FN-binding sites. Exposure of FN binding sites encourages FN self-association and fibril formation. (adapted from Erickson 2000)
These two mechanisms are not mutually exclusive, as both result in the unmasking of FN-binding sites without the need for vigorous intracellular forces to unfold the FN dimers via strong integrin bonds.

In the initial stages of FN matrix assembly, cells begin to form fibrils with the surface-adsorbed FN (Ohashi et al., 2002), and these nascent fibrils are likely the precursors to stable DOC-insoluble fibrils. This initial step in FN matrix assembly involves extension of the FN molecules to reveal FN binding sites (Singh et al., 2010). If extending the conformation of substrate FN rescues FN matrix assembly on softer substrates by exposing FN-binding sites, then its effects should be detected in the earliest stages of assembly, when FN fibrils are just beginning to form. This was the case, as soft substrates with GdnHCl-treated substrate FN had significantly more matrix assembly sites than soft substrates with untreated FN. These results show that FN matrix assembly is affected by the conformation of the FN attached to the substrate, regardless of its stiffness, and that FN matrix assembly can be upregulated on the soft substrate by manipulating the conformation of the FN crosslinked to it. Thus, exposure of FN-binding sites is the key step that is affected in stiffness-dependent FN matrix assembly.

It was also shown that the conformation of FN varies according to the stiffness of the substrate it is crosslinked to, with rigid substrates seeming to increase the extension of FN. This conclusion is supported by the results that show variability in binding of both full-length FN and MAB 1890 antibody to substrate FN on gels of different stiffnesses. A greater amount of full-length FN bound to substrate FN on rigid gels than on soft gels, indicating that the substrate FN on rigid gels display more FN-binding sites than on softer gels. In addition, changes in MAB 1890 epitope exposure also corresponded with changes in substrate stiffness. As shown by IF and ELISA, MAB 1890-binding was greatly reduced in instances where FN is known to assume an extended conformation: in fibrillar form (Baneyx et al., 2001), when adsorbed onto glass (Baugh and Vogel, 2004), and when partially denatured by GdnHCl (Baneyx et al., 2002). Likewise, IF and immunoprecipitation demonstrated that MAB 1890 bound to compact FN in its intracellular and soluble forms (Hynes, 1990a). Therefore, the increase in MAB 1890-binding on soft gels implies that substrate FN on soft gels is in a more compact conformation that the substrate FN on rigid gels.
In these experiments, FN is likely more extended on rigid substrates due to single FN molecules being crosslinked to the gel in multiple places, but this stiffness-dependent conformational change mimics what happens in the presence of cells. Newly deposited FN fibers are extended much more rapidly when cells were seeded onto rigid matrix, when compared to FN fibers formed on softer matrix (Kubow et al., 2009), and FN tends to be more extended in fibrils formed on stiffer substrates (Antia et al., 2008). Stiffness-dependent conformational changes in FN could have important biological implications, as a rigid environment could increase unfolding of FN, exposing FN-binding sites and enhancing FN matrix assembly. Binding sites for cells and other molecules could also be affected, and cells could potentially detect differences in stiffness through their ability to interact with certain FN domains.
Chapter 6

Discussion

Summary of findings

Here we show, using polyacrylamide substrates of different stiffnesses, that the rigidity of the cellular environment regulates ECM assembly. Cells assemble a greater amount of stable FN matrix on a rigid substrate than on softer substrates. Increasing cell contractility was not sufficient to rescue FN matrix assembly on a soft substrate. However, stimulation of integrin activity to increase ligand-binding strength or partial extension of substrate FN significantly increased FN matrix assembly on softer substrates. These results indicate that assembly on a soft substrate is limited by the strength with which cells bind to FN and that extension of substrate-bound FN dimers bypasses the need for strong FN binding. Furthermore, partial opening of substrate FN promotes formation of matrix assembly sites, demonstrating that substrate stiffness exerts its effects during the initial steps of FN assembly.

These results establish a model whereby FN matrix assembly is restricted on soft substrate due to a lack of exposure of FN-binding sites (Figure 6.1). The substrate FN on soft gels assumes a conformation in which 1) the FN-binding sites remain cryptic, and 2) integrins do not bind with the strength needed to forcefully unfold the substrate FN and thus expose the FN-binding sites. In contrast, integrins are able to bind to FN on a rigid gel with sufficient strength to support FN unfolding. Additionally, substrate FN on rigid gels could be in a more extended conformation that more readily displays FN-binding sites. It is this unmasking of FN-binding sites that sustains FN fibril formation and matrix assembly. Cells on soft substrate can be compelled to assemble as much FN matrix as cells on rigid substrate by either strengthening the FN-integrin bonds with Mn$^{2+}$-treatment or unmasking FN-binding sites with GdnHCl-treatment. Stimulating cell contractility with either LPA or Calyculin A stimulates FN matrix assembly only on rigid
Figure 6.1 – Model of FN matrix assembly on soft and rigid substrates. By increasing integrin binding or by partially unfolding the substrate FN, cells on soft substrate are able to assemble as much FN matrix as cells on rigid substrate.
substrates. Thus, the difference in FN matrix assembly on soft and rigid substrates can be attributed to a deficiency in binding to and unfolding FN on soft substrates.

**Increased cell contractility does not rescue FN matrix assembly on soft substrate**

By measuring FN matrix assembly on soft, intermediate, and rigid polyacrylamide gels, it was determined that cells assemble less FN matrix on softer substrates. Since the cytoskeleton and actin-mediated cell contractility have been shown to play a role in FN matrix assembly (Zhang et al., 1994; Zhang et al., 1999; Zhong et al., 1998), we tested whether FN matrix assembly could be enhanced on soft substrates by stimulating contractility with LPA or Calyculin A. Increasing cell contractility was not able to substantially increase FN matrix assembly on the softer substrates, indicating that decreased cell contractility is not the primary reason for down-regulation of FN matrix assembly.

The studies on FN-integrin adhesions conducted by Friedland et al. provide a suitable explanation for this finding (Friedland et al., 2009). They measured the number and strengths of FN-integrin bonds formed by cells on polyacrylamide substrates ranging from approximately 1 kPa to 180 kPa that were crosslinked with FN. The strength of the bonds were determined using a spinning disc device, which measures the force required to break the adhesive bonds, thus detaching the cells (Boettiger, 2007). In addition, strong FN-integrin bonds were quantified by measuring the amount of integrins that could be covalently crosslinked to their FN ligands (Friedland et al., 2009). The number of α5β1 integrins that could be crosslinked was proportional to the number of strong bonds (Shi and Boettiger, 2003). It was found that the total number of adhesive FN-integrin bonds that formed was independent of substrate stiffness; however, the number of strong FN-integrin bonds increased with substrate stiffness (Friedland et al., 2009). Thus, cells on soft substrates form an equivalent amount of FN-integrin bonds as cells on rigid substrates, but the proportion of weak bonds was higher on softer substrates. They also found that downstream signaling was mediated by FN-integrin bond strength (Friedland et al., 2009). FAK phosphorylation was dependent on strong FN-integrin bonds, and phosphorylation increased in a stiffness-dependent manner. The inability of
LPA or Calyculin A to stimulate FN matrix assembly on soft substrate can be attributed to a lack of strong FN-integrin bonds and the resulting reduction in downstream signaling. According to this model, cells do engage substrate FN on soft gels, but these weak bonds are not sufficient to sustain downstream signals (Friedland et al., 2009), and our results indicate that they are also not able to convert FN fibrils into a mature insoluble matrix.

Increasing cell contractility may increase the force with which the cell pulls on the ECM, but without resistance from a rigid substrate, the bond is not reinforced and would remain weak (Choquet et al., 1997). By measuring the movement of and force applied to cell-associated FN-coated beads, Choquet et al. demonstrated that FN-integrin bonds strengthen proportionally in response to a resisting force (Choquet et al., 1997). The cells bound the FN-coated beads and transported them across the cell surface, but when the movement of the beads was restricted with an optical trap, the cells would increase the force applied to the FN-integrin linkage, pulling harder and thereby increasing the strength of the linkage. If the cell was able to overcome the resisting force, movement of the bead could not again be restricted by a resisting force less or equal to the one it had overcome, demonstrating that the bond had been strengthened. In the case of cells plated on FN-coated polyacrylamide gels, the resisting force is dependent on the stiffness of the substrate. Rigid substrates provide a higher resisting force than soft substrates, as they resist deformation (strain) to a greater degree in response to an applied force (stress). Following this reasoning, when cells on soft gels bind substrate FN, they would sense a relatively weak resisting force, if at all, and the FN-integrin linkage would not be strengthened. Stimulating cell contractility with LPA or Calyculin A would encourage the cell to pull on the substrate FN, but soft substrates readily deform upon applied force. This deformation of the soft substrate hinders the bond from experiencing a resisting force, and thus, prevents the bond from strengthening.

Stimulation of contractility has been shown to increase matrix assembly on rigid substrates (Zhang et al., 1994; Zhang et al., 1997; Zhong et al., 1998), and increasing cell contractility in our experiments led to a considerable increase in FN matrix on the rigid substrate. However, neither activation of Rho nor inhibition of MLC phosphatase was sufficient to promote assembly on a soft gel. This result suggests that, in the absence of
essential extracellular events, stimulating intracellular pathways to cell contractility is unable to rescue assembly.

**Integrin stimulation rescues FN matrix assembly on soft substrates**

Stimulating integrins with Mn$^{2+}$ induced assembly on soft and intermediate substrates to levels similar to matrix on the rigid untreated substrate or on a soft GdnHCl-FN substrate, indicating that integrin binding is impaired on soft substrate. Friedland et al. demonstrated that the total number of FN-α5β1 bonds is independent of substrate stiffness, but the number of strong FN-α5β1 bonds increases with substrate stiffness (Friedland et al., 2009). In addition, they determined that formation of strong bonds is dependent on the synergy site in FN (Friedland et al., 2009). While cells are able to adhere to FN through the binding of integrins to the RGD site in the III$^{10}$ module of FN (Hynes, 1990a), strong FN-integrin bond also require participation from the synergy site in the III$^{9}$ module of FN (Figure 1.1) (Friedland et al., 2009). Mn$^{2+}$ activation of integrins has been shown to compensate for weak integrin-FN binding in the absence of a synergy site (Danen et al., 1995) and to promote assembly of a recombinant FN that lacks the synergy site (Sechler et al., 1997). These data explain why cells are able to assemble FN matrix on a rigid substrate independent of Mn$^{2+}$ treatment and why they require integrin activation for assembly on soft substrate. Based on the findings of Friedland et al., cells on rigid substrates should have a sufficient number of strong FN-integrin bonds, which would allow them to apply enough force to unfold FN and expose FN-binding sites. However, cells on a soft substrate would bind FN weakly, and Mn$^{2+}$ treatment would strengthen these bonds and enable them to cause FN conformational changes.

**Partially unfolding substrate FN rescues FN matrix assembly**

Changing the conformation of substrate FN resulted in an increase in FN matrix assembly on softer substrates, equivalent to levels on rigid gels with untreated FN. Soluble FN is kept in a compact conformation through electrostatic interactions between the III$_{2,3}$ and III$_{12,14}$ domains and possibly through other intramolecular interactions (Johnson et al., 1999). GdnHCl disrupts these interactions, extending the FN dimer and
exposing cryptic FN-binding sites (Baneyx et al., 2002; Khan et al., 1990; Pace, 1986). In particular, FN-binding sites in the III_{1-2} domain are exposed by denaturation or stretch (Hocking et al., 1994; Karuri et al., 2009; Zhong et al., 1998). Conformational changes provide sites for association with other FN molecules via the N-terminal matrix assembly domain of FN and in this way initiate fibril formation.

Facilitation of a conformational change in FN by treatment with GdnHCl stimulated both the initiation of assembly through the formation of assembly sites and the assembly of a DOC-insoluble matrix on soft substrates. Conformational changes during assembly are normally induced by integrin binding (Singh et al., 2010). Therefore, our results indicate that substrate stiffness affects the ability of cells to induce extension of FN dimers, thereby limiting the exposure of FN-binding sites and affecting FN matrix assembly.

**GdnHCl-treatment bypasses the need for integrin stimulation**

The correspondence in effects of Mn^{2+} and GdnHCl-FN treatments suggests that assembly steps that rely on integrin-dependent conformational changes in FN occur inefficiently on the soft substrate. Given the relationship between integrin binding and FN conformational changes during assembly, these two treatments that stimulate assembly in a stiffness-dependent manner seem to be correcting the same deficiency. Cells on soft substrate bind to FN and are able to attach and spread, but they do not bind with maximal strength (Friedland et al., 2009). Mn^{2+} activation of integrins enhances FN-integrin binding (Gailit and Ruoslahti, 1988; Mould et al., 1995) and likely induces sufficient FN-integrin engagement to initiate FN unfolding.

FN fibrils form when cells forcefully unfold FN molecules, revealing cryptic FN-binding sites that allow intermolecular bonds to form between FN dimers (Singh et al., 2010). It is known that initiation of FN matrix assembly requires integrin binding to both the III_{9} and III_{10} modules of FN (Aota et al., 1994; Garcia et al., 2002; Sechler et al., 1997), which would form a strong FN-integrin bond (Friedland et al., 2009). The integrin cytoplasmic tails connect with the cytoskeleton, and FN-integrin binding contributes to the formation of focal adhesions that mediate this connection (Dubash et al., 2009). Fibril formation is promoted when actin filaments translocate FN-bound integrins from
these adhesion and across the cell surface, likely pulling on the integrin-bound FN (Ohashi et al., 2002; Pankov et al., 2000). And stimulating actin-based cell contractility is sufficient to induce conformational changes in FN that expose cryptic FN-binding sites (Zhong et al., 1998) and initiate fibril formation (Zhang et al., 1994; Zhong et al., 1998). Thus, a current model of FN matrix assembly suggests that cells mediate FN fibril formation because they bind FN with integrins and apply a force to the integrin-bound FN that partially unfolds it, thereby exposing previously hidden FN-binding sites (Singh et al., 2010). It has also been shown that the strength of the FN-integrin bond is important for FN matrix assembly (Garcia et al., 2002; Sechler et al., 1997; Singh et al., 2010), and Mn²⁺-treatment stimulates FN matrix assembly, presumably by strengthening the FN-integrin bond (Danen et al., 1995; Gailit and Ruoslahti, 1988; Mould et al., 1995). However, the key step in the process of FN fibril formation is the exposure of FN-binding sites, as FN molecules do not self-associate in the absence of available FN-binding sites (Hynes, 1990a). It is our hypothesis that crosslinking of GdnHCl-FN to the polyacrylamide gel surface directly affects FN-FN interactions by providing a substrate with FN in a conformation that displays previously buried FN-binding sites, a step that is otherwise cell-mediated. GdnHCl treatment would unmask FN-binding sites without the need of cells to unfold the FN dimers using strong integrin bonds. This would explain why FN matrix assembly and the formation of FN assembly sites are increased on soft substrate crosslinked with GdnHCl-treated FN. Thus it appears that the requirement for increased integrin binding strength can be bypassed by making FN-binding sites accessible.

Increases in FN matrix observed with chemical extension of FN are similar to those obtained by treating cells with Mn²⁺, suggesting that the effects of integrin stimulation are due to an increased ability of cells to extend substrate FN. The stimulatory effects of extending FN conformation without increasing integrin-FN binding support a current model of matrix assembly. In this model, fibril formation is initiated when FN-binding sites that are sequestered in the compact FN dimer become accessible by disruption of intramolecular interactions (Singh et al., 2010). Extension of FN with GdnHCl circumnavigates the need to forcefully unfold compact FN, and thus allows cells to form fibrils even with fewer tensioned FN-α5β1 bonds.
**Fibronectin could be a mechanosensing protein**

Several intracellular proteins involved in mechanotransduction have been reported to become mechanically extended in response to force, revealing cryptic interaction sites that mediate their activity (del Rio et al., 2009; Golji and Mofrad, 2010; Sawada et al., 2006). For example, the phosphorylation of the central domain of pCas130, a focal adhesion adaptor protein, varies in response to applied force (Sawada et al., 2006). Stretching the pCas130 molecule exposes a tyrosine residue that is the target for protein kinases such as FAK or Src, and this stretch-dependent phosphorylation supports downstream signaling (Sawada et al., 2006). In vivo, the pCas130 molecule is believed to be stretched when force is applied to a pCas130-containing focal adhesion. The N-terminal and C-terminal domain of pCas130 contain different binding sites for several focal adhesion proteins, including actin-binding proteins (Defilippi et al., 2006), so each end of the molecule is likely associated with different parts of the focal adhesion (Sawada et al., 2006). One end of the molecule might be anchored to the cytoskeleton, while the other end could be bound to an integrin-bound protein, so either external force applied to the integrin or internal force from the cytoskeleton could unfold the central pCas130 domain. Similar to these intracellular proteins, the correlation between substrate stiffness and FN conformational changes may allow cells to use FN itself to probe the stiffness of the environment. Cells sense the stiffness of their environment by pulling on their substrate and feeling the resistance (Discher et al., 2005). FN in a rigid environment might be more extensible than in a soft environment, providing a FN-dependent environmental signal to cells.

FN matrix assembly is upregulated on rigid substrates, demonstrating that the stiffness of the cellular microenvironment influences the way that it interacts with FN. Since FN molecules in fibrils are in extended conformations (Baneyx et al., 2001; Peters et al., 1998; Smith et al., 2007), stiffness-dependent FN matrix assembly could lead to stiffness-dependent FN conformations. FN in softer environments would be more likely to assume a compact conformation, since FN matrix assembly is downregulated on soft substrate, and FN in rigid environments would be more likely to assume an extended conformation, since fibril assembly is enhanced on rigid substrate. Because FN conformation could differ with substrate stiffness, cells might be able to detect
differences in stiffness through their varied ability to expose and interact with certain FN domains. For example, accessibility of the cell-binding domain of FN has been shown to vary according to its conformation (Altroff et al., 2004; Ugarova et al., 1995), and cryptic binding sites for growth factors and other ECM proteins have been identified (Mitsi et al., 2006; To and Midwood, 2011). Stiffness-dependent conformational changes could regulate the exposure of binding sites within a FN molecule, and in this way, FN conformation itself can act as a microenvironment mechanosensor. Thus our results provide an extracellular mechanism by which cells can sense and respond to the physical properties of their environment.

**Stiffness-dependent FN matrix assembly could play a role in disease progression**

Tissues become progressively more rigid during pathological progression. For example, stiff ECM has been shown to promote tumor progression by breast cancer cells (Levental et al., 2009; Paszek et al., 2005), and FN contributes to development of a tumorigenic phenotype by mammary epithelial cells (Williams et al., 2008). In addition, progression of liver fibrosis has been found to correlate with a progressive increase in liver stiffness (Yeh et al., 2002; Yin et al., 2007), and interestingly, an increase in liver stiffness has been shown to precede the pathological deposition of ECM proteins (Georges et al., 2007). Whether cells increase FN fibril formation in response to sensing a rigid pericellular environment is an important question, especially because FN assembly precedes and often seeds assembly of other ECM proteins, such as collagen I and collagen III (Sottile et al., 2007; Velling et al., 2002). Collagen has also been shown to modulate FN matrix assembly (Dzamba et al., 1993). Pathological increases in tissue stiffness are often a result of increased collagen deposition. Stiffness-dependent FN matrix assembly could create a pathological feedback loop: FN matrix assembly would promote collagen deposition, which then increases tissue stiffness, and in turn, upregulates FN matrix assembly. This could make FN a master regulator of stiffness-dependent ECM deposition. Our finding that the terminal event in stiffness regulation of matrix assembly is a FN conformational change implicates FN in a mechanosensing mechanism that may control disease progression. Future studies on stiffness-dependent
collagen assembly and how it is affected by FN matrix assembly would provide valuable insight into the mechanisms and possible treatments of diseases.
References


