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THE $\alpha 4 \beta 1$ INTEGRIN AND THE EDA DOMAIN OF FIBRONECTIN REGULATE A PROFIBROTIC PHENOTYPE IN DERMAL FIBROBLASTS*

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Abstract

Prompt deposition of fibronectin-rich extracellular matrix is a critical feature of normal development and the host-response to injury. Fibronectin isoforms that include the EDA and EDB domains are prominent in these fibronectin matrices. We now report using human dermal fibroblast cultures that the EDA domain of fibronectin or EDA-derived peptides modeled after the C-C' loop promote stress fiber formation and myosin-light chain phosphorylation. These changes are accompanied by an increase in fibronectin synthesis and fibrillogenesis. These effects are blocked by pretreating cells with either siRNA or blocking antibody to the $\alpha 4$ integrin. Our data indicate that the interaction between the $\alpha 4 \beta 1$ integrin and the EDA domain of fibronectin helps to drive tissue fibrosis by promoting a contractile phenotype and an increase in fibronectin synthesis and deposition.

Keywords

Fibronectin; Stress Fibers; Contractility; Rho Kinase; Fibrosis; $\alpha 4 \beta 1$ integrin

1. Introduction

Fibronectin is a ubiquitous, multifunctional glycoprotein that orchestrates a variety of physiological processes. It is a disulfide-linked dimer that consists of three types of repeating structural domains: Type I, II and III. The Type III domains all have a common molecular structure comprised of seven anti-parallel beta-strands, A-B-E and C'-C-F-G, that are arranged into two distinct beta-sheets (Petersen et al., 1983). Fibronectin exists in both a

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Authors' Contributions

Studies were designed by LVDW. Data collection: AVS, RK, JHP, KS. Data Analysis: AVS and RK. Data interpretation: LVDW, PJM-L. Drafting manuscript: AVS, LVDW, and PJM-L. Revising and editing manuscript: PJM-L.

soluble form which circulates in the blood plasma, as well as an insoluble polymerized form which is an essential component of tissue extracellular matrix (ECM). Matrix fibronectin not only provides physical support for surrounding cells, it provides critical biochemical and mechanical cues that dictate cell behavior (Bradshaw and Smith, 2013). During periods of active tissue remodeling, resident cells synthesize distinct isoforms of fibronectin which are deposited into the matrix. These isoforms arise by differential RNA splicing and are useful biomarkers in the identification of angiogenic blood vessels, inflammatory reactions and fibrotic tissue (Bissell and Radisky, 2001; Khan et al., 2012; Brem et al., 2000).

Fibronectin isoforms result from alternative splicing of pre-mRNA which results in the inclusion of one or both of two additional Type III domains, extra domain-A and -B (EDA and EDB) (Hynes, 1990). The splicing of the EDA and EDB domains is tightly regulated during embryogenesis with a high-degree of tissue-specific expression that decreases with the age of an organism (Bennet and Schultz, 1993). The impact of these fibronectin isoforms on tissue physiology is not well understood. Adult, healthy tissue is essentially devoid of these extra domains until periods of active tissue remodeling, with re-expression seen following tissue injury and during wound healing. EDA null mice exhibit abnormal wound healing (Muro et al., 2003); however, the specific mechanisms by which EDA⁺-fibronectin promotes wound repair are not well understood.

Early in vitro studies have linked a variety of cellular events and molecular outcomes to the expression of EDA⁺-fibronectin, including cell cycle progression, adhesion and migration (Inoue et al., 2001; Manabe et al., 1997; Manabe et al., 1999). EDA⁺-fibronectin is also thought to play a role in myofibroblast differentiation. Myofibroblasts are alpha-smooth muscle actin (α -SMA)-expressing cells that differentiate from normal fibroblasts in the presence of EDA⁺ fibronectin, mechanical tension and TGF- β (Serini et al., 1998; Hinz et al., 2001). Myofibroblasts have a key role in wound healing where they mediate contractile forces required for wound closure (Powell et al., 1999). More recent studies in EDA null mice have documented a role for the EDA isoform of fibronectin in the generation of pathologic tissue fibrosis and inflammation (Booth et al., 2012; Kohan et al., 2011; Brem et al., 2000; Muro et al., 2008). EDA⁺-fibronectin also promotes the stromal reaction, a fibrotic condition seen in many cancers, which contributes to the creation of a microenvironment conducive for tumor progression (Xiang et al., 2012; Ou et al., 2013; Kelsh and McKeown-Longo, 2013). In diseased states, myofibroblast contractility and enhanced deposition of extracellular matrix substantially alter tissue mechanics and enable inappropriate mechanical signals resulting in pro-fibrotic environments which can lead to excessive tissue scarring and organ dysfunction [reviewed in (Edsberg et al., 2012)]. These fibrotic lesions contain extensive ECM deposition with high levels of EDA⁺-fibronectin (Muro et al., 2008), thus potentially contributing to the generation and persistence of myofibroblasts within the tissue (Hinz, 2010).

Integrins are a family of transmembrane dimeric receptors that couple the ECM to the intracellular environment. The integrins act to transduce cues from the ECM to the inside of the cell and vice versa to facilitate remodeling of the ECM. Integrins $\alpha 9\beta 1$, $\alpha 4\beta 1$ and $\alpha 4\beta 7$ have been identified as receptors for the EDA domain (Liao et al., 2002; Shinde et al., 2008; Kohan et al., 2010). Yet, the specific role of these integrins in mediating the effects of EDA

in biological and pathological events remains poorly understood. In the present study we evaluated the contribution of the $\alpha 4\beta 1$ interaction with the EDA domain to a pro-fibrotic phenotype in dermal fibroblasts. We demonstrate that $\alpha 4\beta 1$ mediates EDA-induced stress fibers, myosin light chain phosphorylation, fibronectin synthesis and fibrillogenesis. Additionally, our data demonstrate that the $\alpha 4\beta 1$ binding site in EDA within the C-C' loop is sufficient for inducing this profibrotic response and identifies an $\alpha 4\beta 1$ -EDA⁺-fibronectin axis as a potential regulator of dermal fibrosis.

2. Results

2.1. The EDA Domain of Fibronectin Promotes Stress Fiber Assembly and Fibronectin Fibrillogenesis in Human Dermal Fibroblasts

To determine a potential role for the EDA domain of fibronectin in tissue fibrosis, dermal fibroblasts were seeded onto glass slides coated with plasma fibronectin (pFN) or co-coated with pFN and the EDA domain. Cells seeded on pFN in the presence of EDA showed a marked increase in actin stress fibers (Fig. 1A, panels a, b; g, and h). The increase in actin organization was accompanied by a marked increase in the association of phosphorylated myosin light chain (pMLC) with stress fibers consistent with the EDA promoting an increase in cellular contractile force (Fig. 1A, panels d and e). Another Type III domain of fibronectin, III-4, was used as a negative control. The III-4 domain did not promote either stress fiber formation or MLC phosphorylation (Fig. 1A, panels c, f, and i). Cells seeded directly onto the EDA domain in the absence of plasma fibronectin attached but did not spread (data not shown). Similar results were obtained when human fetal lung fibroblasts were used (data not shown). Western blot analysis of cell lysates indicated that cells plated on surfaces coated with a mixture of pFN and EDA exhibited a significant increase in the level of phosphorylation of MLC when compared with cells seeded on pFN alone (Fig. 1B, C). Previous studies have shown that changes in cell contractility parallel the formation of a fibronectin-rich matrix in the extracellular milieu (Chrzanowska-Wodnicka and Burridge, 1996; Zhang et al., 1997). To assess possible effects of EDA on fibronectin matrix formation, both pFN and cell synthesized fibronectin (cFN) were visualized by indirect immunofluorescence (Fig. 1D). As shown in panel a, there was minimal reorganization of substrate plasma fibronectin into fibrils after an overnight incubation. In contrast, cells seeded onto a substrate coated with both pFN and EDA (panel b) exhibited a more extensive reorganization of substrate plasma fibronectin into fibrils. The dark areas on the substrate represent the pFN coat which had been cleared from the substrate and reorganized into fibrils (Christopher et al., 1997). These fibrils could also be stained using an antibody directed at the EDB domain which specifically recognizes the cellular form of fibronectin (Fig. 1D, panels c and d), indicating that the newly assembled fibrils consist of plasma fibronectin which has been reorganized on the substrate as well as newly synthesized fibronectin. Taken together, these findings suggest that the EDA domain of fibronectin stimulates both actin stress fiber formation as well as fibronectin fibrillogenesis.

The EDA domain of fibronectin is a known ligand for the $\alpha 4\beta 1$ integrin receptor (Liao et al., 2002). Inhibition of $\alpha 4\beta 1$ function using a blocking antibody also prevented the EDA induced increase in fibronectin fibrillogenesis (Fig. 1E, panels a and b). Phase contrast

images showed that antibody treatments did not affect overall cell adhesion (Fig. 1E, panels c and d). These data indicated that the EDA domain mediated increases in stress fibers and fibronectin fibril formation depend on the $\alpha 4\beta 1$ integrin.

2.2 EDA Mediated Stress Fiber Assembly and Fibronectin Synthesis Depend on the $\alpha 4\beta 1$ Integrin

To further examine the contribution of this integrin receptor to EDA mediated assembly of stress fibers, the $\alpha 4$ subunit was knocked down in human dermal fibroblasts using siRNA. Expression levels of $\alpha 4$ before and after knockdown were monitored by flow cytometry. Dermal fibroblasts exhibited abundant surface expression of $\alpha 4$ (Fig. 2A, panel b), which was markedly downregulated following siRNA treatment (Fig. 2A, panel d). Cells treated with a control non-targeting siRNA did not exhibit any significant change in surface expression of $\alpha 4$ (Fig. 2A, panel c). Integrin $\alpha 4$ -knockdown cells and non-targeting siRNA control cells were cultured in wells coated with a mixture of pFN and EDA domain. As shown in Fig. 2B, increased staining for actin (panels a and b) and phosphorylated MLC (panels f and g) was seen when cells were seeded onto substrates coated with pFN and EDA as compared with cells seeded onto pFN alone. The EDA domain did not increase either actin (compare panels a and d) or p-MLC (compare panels f and i) staining under conditions of $\alpha 4$ knockdown, indicating that the EDA enhancement of stress fibers was dependent on the $\alpha 4$ integrin. The III-13 module was included in these studies as a negative control for the $\alpha 4\beta 1$. The III-13 module is known to stimulate stress fiber formation through interactions with syndecan-4 and therefore should not be sensitive to $\alpha 4$ knockdown (Saoncella et al., 1999; Woods et al., 2000). As shown in Fig. 2 (panels c and h), cells seeded onto a mixture of pFN and III-13 exhibited a slight increase in staining for both actin and p-MLC when compared with pFN alone, however the effect was not as dramatic as that seen with EDA. Interestingly, the staining for both actin and p-MLC was further increased under conditions of $\alpha 4$ knockdown when cells were seeded on pFN and III-13 (panels e and j). We also evaluated the ability of the EDA domain to effect stress fiber formation when it was presented to the cells in the context of the full length FN molecule. In this experiment, cells were seeded onto substrates coated with pFN or full-length recombinant cellular FN containing the EDA domain (EDA⁺cFN_{rec}). Cells seeded onto full-length recombinant EDA containing FN (EDA⁺cFN_{rec}) exhibited more intense stress fiber staining for both actin and pMLC (Fig. 2C, panels b and e). This enhanced staining for stress fibers was not seen under conditions of $\alpha 4$ knockdown (panels c and f). These data indicate that the binding of $\alpha 4\beta 1$ integrin to the EDA domain of fibronectin, whether in the form of an isolated domain or within the context of the intact molecule, resulted in an increased assembly of actin and pMLC into stress fibers.

To determine whether EDA was affecting expression levels of fibronectin, cells were cultured on plates coated with a mixture of pFN and EDA or pFN and III-13. Fibronectin gene expression was assessed by quantitative RT-PCR. In the presence of EDA, cells exhibited a dose-dependent increase in fibronectin mRNA levels when compared with cells seeded on pFN+III-13 (Fig. 3A). Under conditions of $\alpha 4$ knockdown (Fig. 3B), the EDA domain had no effect on fibronectin expression. These data indicate that the EDA domain promotes an increase in fibronectin gene expression which is dependent on the $\alpha 4$ integrin.

2.4 EDA-Derived Peptides and the EDA Domain Elicit Similar Biological Responses on Fibroblasts

To identify the binding domain for the $\alpha 4\beta 1$ integrin within the EDA domain, we utilized a panel of single and double point mutants of the EDA domain and synthetic peptides modeled after the EDA C-C' loop (Shinde et al., 2008). Because the EDA domain of fibronectin is also a ligand for the $\alpha 4\beta 7$ integrin and Toll-Like 4 Receptor (TLR4), we used blocking antibodies to $\alpha 4\beta 7$, TLR4 and $\alpha 4\beta 1$ to determine whether either $\alpha 4\beta 7$ or TLR4 contribute to the binding of dermal fibroblasts to the EDA domain. As shown in Fig. 4A, blocking antibody to $\alpha 4\beta 7$ (FIB504) or TLR4 had no effect on the binding of cells to EDA. In contrast, binding was completely inhibited using a blocking antibody to $\alpha 4\beta 1$ (P1H4). These data indicate that $\alpha 4\beta 1$ is the integrin mediating fibroblast binding to EDA. Human lung fibroblasts were then tested for their ability to bind to a panel of EDA GST fusion proteins containing various mutations in the C-C' loop. Fusion proteins containing a mutation in the D41 residue were significantly less effective in mediating cell adhesion, suggesting that sequences within the C-C' loop are required for $\alpha 4\beta 1$ binding (Fig. 4B). To confirm this observation, synthetic peptides representing the C-C' loop as well as a control peptide were used to inhibit the binding of cells to the EDA domain. Specific sequences are provided in Fig. 4C. Both cyclic and linear forms of the peptide representing the C-C' loop of the EDA domain completely inhibited EDA dependent cell adhesion while a control scrambled peptide had no effect (Fig. 4D). These data indicate that the primary binding site for $\alpha 4\beta 1$ within the EDA domain is found within the C-C' loop.

We then assessed whether or not the synthetic C-C'-derived peptide from the C-C' loop could promote actin stress fiber formation and FN reorganization into fibrils. Wells were coated with plasma fibronectin in the presence of either the C-C' peptide or a scrambled version of the C-C' peptide. The cells treated with the C-C' peptide showed increased staining for actin (Fig. 5A, panels a and b) and phosphorylated myosin light chain (Fig. 5A, d and e). The scrambled peptide had no effect (Fig. 5A, panels c and f). Similarly, the C-C' peptide stimulated an increase in fibronectin fibril formation (Fig. 5B, panels a and b). Fibronectin fibers stained positively for EDB-containing fibronectin indicating that cell-derived fibronectin was contributing to fibrillogenesis (Fig. 5B, panels d and e). The scrambled peptide had no effect (Fig. 5B, panels c and f). Taken together, the results indicate that the residues in the C-C' loop of the EDA segment bind to $\alpha 4\beta 1$ to stimulate stress fiber assembly, myosin-light chain phosphorylation, fibronectin synthesis and fibrillogenesis.

3. Discussion

This study documents a role for the $\alpha 4\beta 1$ integrin in the progression of a fibrotic phenotype in dermal fibroblasts. We show that the interaction between the $\alpha 4\beta 1$ integrin and the alternatively spliced EDA domain of fibronectin stimulates an increase in the assembly of actin and p-MLC into stress fibers as well as an increase in both fibronectin expression and fibrillogenesis. Mutational analysis identified the C-C' loop as the binding site for $\alpha 4\beta 1$ in the EDA domain. Peptides representing the C-C' loop in the EDA domain were able to promote stress fiber assembly and fibronectin fibrillogenesis indicating that the binding of

$\alpha 4\beta 1$ to the C-C' loop of the EDA domain is sufficient to induce a fibrotic phenotype. These observations are consistent with earlier studies which have linked the interaction between $\alpha 4\beta 1$ and the EDA isoform promoting increased cellular contractility, a process which has been linked to the assembly of both stress fibers (Amano et al., 1997; Leung et al., 1996) and fibronectin matrix (Zhang et al., 1997; Zhong et al., 1998; Yoneda et al., 2007).

Expression of the EDA isoform of fibronectin is tightly regulated appearing in specific spatio-temporal patterns during development and wound repair (French-Constant and Hynes, 1989; French-Constant et al., 1989; Muro et al., 2003; Singh et al., 2004). The EDA form of fibronectin is found in fibrotic tissue and in the stroma of solid tumors; however, its role in these pathologies is not well understood. Tissue fibrosis is typically preceded by injury and chronic inflammation and can be considered as a dysregulation of the normal tissue repair process [reviewed in (Lee and Kalluri, 2010)]. The initial stage of fibrosis is characterized by the activation of inflammatory processes including the infiltration of immune cells, epithelial-mesenchymal transition, cytokine production and angiogenesis. Later stages are characterized by excessive deposition of extracellular matrix and the accumulation of differentiated myofibroblasts, which can lead to increased tissue stiffening and changes in mechanical properties. In some cases, inflammatory and fibrotic loops are established which become self-perpetuating. When left uncontrolled, the accumulation of extracellular matrix and increased contractile forces can lead to excessive tissue contraction resulting in major organ system failure [reviewed in (Hinz et al., 2012; van de Water et al., 2013)].

Numerous studies have pointed to the EDA isoform of fibronectin as a contributing factor in the development of fibrosis. Genetic studies in EDA null mice have documented a role for the EDA⁺-fibronectin in several models of tissue fibrosis. EDA⁺-fibronectin is required for the increase in matrix deposition and accumulation of myofibroblasts seen in subepithelial airway fibrosis (Kohan et al., 2011; Hirshoren et al., 2013). In the absence of EDA, mice were protected from cardiac fibrosis associated with chronic allograft rejection (Booth et al., 2012) and infarct (Arslan et al., 2011). The $\alpha 4\beta 1$, $\alpha 4\beta 7$ and $\alpha 9\beta 1$ integrins have been identified as receptors for the EDA domain of fibronectin (Liao et al., 2002; Shinde et al., 2008; Kohan et al., 2010). The specific contribution of each receptor to the development of tissue fibrosis is not well understood, but recent data has linked the $\alpha 4\beta 7$ integrin to myofibroblast differentiation and the $\alpha 9\beta 1$ integrin has been linked to epithelial mesenchymal transition (EMT). In lung fibroblasts, EDA⁺-fibronectin induced an $\alpha 4\beta 7$ dependent increase in cell contractility, collagen and smooth muscle actin, three markers of myofibroblast differentiation (Kohan et al., 2010). The interaction of EDA fibronectin with the $\alpha 9\beta 1$ integrin receptor, which is customarily expressed on epithelial cells, has recently been shown to promote EMT in lung cancer cells and colorectal cancer by inducing the expression of mesenchymal markers including smooth muscle actin (Sun et al., 2014) and vimentin (Ou et al., 2014). Epithelial cells undergoing EMT are also thought to contribute to the pool of myofibroblasts which accumulate within fibrotic tissue (Guarino et al., 2009). In the current study, we show that the binding of EDA to $\alpha 4\beta 1$ on human dermal fibroblasts induced an increase in actin assembly, p-MLC, fibronectin synthesis and matrix deposition. However, we did not observe any induction of smooth muscle actin (unpublished observations) suggesting that the binding of the $\alpha 4\beta 1$ integrin to the EDA domain of

fibronectin is not sufficient to induce the complete differentiation of dermal fibroblasts into myofibroblasts. Our results do suggest that the interaction between the EDA domain of fibronectin and the $\alpha 4\beta 1$ integrin contributes to fibrosis by promoting tissue stiffening through the generation of contractile force and increased matrix deposition.

Chronic inflammation is a common aspect of tissue fibrosis. In addition to $\alpha 4\beta 1$, the EDA domain of fibronectin is also a ligand for Toll-Like Receptors (TLR), pattern recognition receptors which regulate the innate immune system. Earlier studies have shown that the EDA domain of fibronectin induces the expression of proinflammatory cytokines in immune cells through the activation of TLR4 (Okamura et al., 2001; Gondokaryono et al., 2007; McFadden et al., 2010). We have recently shown that the EDA domain activates TLR4-dependent signaling in human dermal fibroblasts and induces the expression of several proinflammatory cytokines including IL-8 and TNF α (Kelsh et al., 2014). In another study, EDA⁺-fibronectin was shown to induce the TLR4 dependent synthesis of collagen and smooth muscle actin in skin fibroblasts, implicating the TLR4-EDA axis in the regulation of a fibrotic response in fibroblasts (Bhattacharyya et al., 2014). The ability of EDA⁺-fibronectin to induce both inflammatory and fibrotic responses in fibroblasts suggests that fibroblasts, which are the primary source of EDA⁺-fibronectin, establish both TLR4 and $\alpha 4\beta 1$ dependent autocrine loops which fuel both inflammation and fibrosis. Taken together these studies point to EDA fibronectin as a critical link between the processes of tissue inflammation and fibrosis.

4. Experimental Procedures

4.1 Reagents

The anti-diphos MLC antibody was a kind gift from Dr. Peter Vincent (Albany Medical College). The anti-myosin antibody (MY-21) and the mouse IgG antibodies were from Sigma (St. Louis, MO). The anti-p190RhoGAP mouse antibody was from BD Transduction Laboratories (San Jose, CA). Anti-EDB (BC.1) antibody was from SantaCruz Biotech, Inc. (Santa Cruz, CA). The anti-human integrin $\alpha 4$ monoclonal antibody (MAB16983Z) and the $\alpha 4$ blocking antibody (HP2/1) were from Chemicon (Bellierica, MA). Blocking antibody to TLR4 was from R&D Systems (Minneapolis, MN). The fibronectin antibody specific for the 40 kDa gelatin binding fragment was described previously (Klein et al., 2003). The secondary antibodies anti-rabbit HRP, anti-mouse HRP were from Calbiochem (Danvers, MA). Alexa Fluor 594 Phalloidin, Alexa Fluor 594 goat anti-mouse IgG, Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 488 goat anti-rabbit IgG and Hoechst 33342 were from Molecular Probes (Grand Island, NY). The BCA Protein Assay Kit, Super Signal West Pico, West Femto and Restore Western Blot Stripping Buffer were from Pierce Biotechnology, Inc. (Rockford, IL). Y-27632, the selective inhibitor of the Rho-associated protein kinase p160ROCK, was purchased from Sigma. All synthetic peptides and oligo-nucleotides were synthesized by SigmaGenosys. All the peptides were analyzed by mass spectrometry and HPLC analyses and were >95% pure. iScriptTM cDNA synthesis kit and iQTM SYBR[®] Green Supermix was purchased from Bio-Rad (Hercules, CA). Purescript[®] RNA Purification Kit was from Qiagen (Valencia, CA). Complete protease inhibitor was obtained from Roche

Diagnostics (Indianapolis, IN). All other reagents were at least reagent grade and obtained from standard suppliers.

4.2 Cell culture

Human dermal fibroblasts and human fetal lung fibroblasts were maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), and 1% penicillin and streptomycin (Invitrogen). Cells were incubated in a humidified incubator (Thermo Electron Corporation Waltham, MA) at 37°C in 5% CO₂.

4.3 Production and Purification of Plasma Fibronectin and Recombinant Fibronectin Domains

Human plasma fibronectin was purified from a fibronectin- and fibrinogen-rich byproduct of Factor VIII production by ion-exchange chromatography on DEAE cellulose (Amersham Biosciences, Pittsburgh, PA) as previously described (Zheng and McKeown-Longo, 2002). Recombinant His-tagged III-13 and EDA were prepared as described previously (Kelsh et al., 2014). Recombinant His-tagged III-4 was prepared as previously described (Shinde et al., 2008).

To prepare full-length recombinant human FNs, two dihydrofolate reductase-deficient CHO (CHO DG44CHO) cell lines, transfected with pAIFNAC encoding recombinant full-length human ED+FN were cultured in the presence of methotrexate in α -minimal essential medium containing ribonucleosides and deoxyribonucleosides (GIBCO, BRL, Gaithersburg, MD) (Lasarte et al., 2007) and 2% FBS depleted FN by passage over gelatin-Sepharose column (Pharmacia Biotech, Uppsala, Sweden). To purify recombinant FN, aliquots of conditioned medium were applied to gelatin-Sepharose columns with resin volumes equal to one-half of the applied medium. After washing with Tris-buffered saline, columns were eluted with 8 M urea and peak fractions by A₂₈₀ were pooled and dialyzed versus PBS, aliquoted and stored at -70°C. Purity and splicing composition of eluted FNs was confirmed by SDS-PAGE with Coomassie staining and western blotting, respectively. Antibody IST-9 against the EDA domain was used to detect EDA-FN. Protein concentration was determined by BCA assay (Pierce).

Recombinant GST-fusion proteins were purified as described previously (2). In brief, bacteria were grown (Circle Grow medium, Bio 101 Systems, Morgan Irvine, CA) with ampicillin (100 μ g/ml), induced, harvested, lysed and the clarified supernatants were applied to glutathione-agarose [1 ml, 50% slurry pre-equilibrated with Dulbecco's phosphate buffered saline (DPBS)] (1.5 h, room temperature), washed, poured into a column and eluted (25 mM glutathione, 120 mM NaCl, and 100 mM Tris-HCl, pH 8.0). Eluted proteins were dialyzed (DPBS), quantitated and the purity determined (>95%) by SDS-PAGE.

4.4 Cell Adhesion Assays

Recombinant EDA (10 μ g/ml) or control domains were coated on polystyrene, non-tissue culture treated plates (Corning) (37°C, 1 h). Wells were washed, blocked (1% BSA in PBS, 37°C, 1 h). Cells were resuspended in serum-free DMEM and pre-stained (Hoechst 33342,

1:1000). For peptide inhibition assays, fibroblasts were preincubated (30 min) with either the cyclized or linear versions of EDA peptides (2 mM) prior to seeding. Experiments were conducted in either triplicate or quadruplicate and included BSA-coated wells as blank. Cells plated onto the wells were centrifuged (top side up; $10 \times g$, 5 min), incubated at 37°C for 1 h. Non-adherent cells were removed by centrifugation (top side down; $50 \times g$, 5 min). Adherent cells were then quantitated in the fluorescent plate reader (Molecular Devices, Sunnyvale, CA).

Fibroblasts were pre-incubated for 1 h in suspension with PBS, 10 $\mu\text{g/ml}$ anti- $\alpha 4$ (P1H4) from EMD Millipore (Billerica, MA), anti- $\beta 7$ (FIB504) from Biolegend (San Diego, CA) or control Mouse IgG, and seeded onto EDA-coated wells for an hour. Adherent cells were washed in PBS twice and fixed in PBS containing 4% formaldehyde for 30 min. Fixed cells were then stained with 0.05% toluidine blue for 2 h and washed three times in water. 10% acetic acid was then used to extract the toluidine blue and its absorbance was measured at 650 nm. The absorbance obtained at 405 nm was subtracted from each measurement to correct for light scattering.

4.5 Immunofluorescence Assay for Visualization of Stress Fibers and Fibronectin Matrix

Dermal fibroblasts were seeded onto chambered glass slides (Lab-Tek, Nalge Nunc International, Rochester, NY) were coated with a mixture of either pFN or PFN and EDA or III-4 domains and allowed to adhere (7 h). Cells were washed, fixed (4.0% paraformaldehyde, 15 min), permeabilized (0.5% Triton X-100, 10 min), washed (0.025% Tween 20/PBS), blocked [5% nonfat dry milk, 10% heat-inactivated goat serum in 0.025% Tween 20/PBS, 1 h] and stained with Alexa Fluor 594 phalloidin (1:100, Molecular Probes) to visualize F-actin. Phospho-myosin light chain (MLC) was visualized using antibodies directed against the diphosphorylated form of MLC. Antibodies were diluted in antibody dilution buffer (1% nonfat dry milk in 0.025% Tween 20/PBS). Slides were washed, stained with Hoechst dye 33342 and Alexa Fluor labeled secondary antibodies, mounted (ProLong[®] Gold, Invitrogen) and examined (Nikon Eclipse 80i, Nikon Instruments and Spot RT digital camera, Diagnostic Instruments, Sterling Heights, MI). For detection of fibronectin matrix, cells were allowed to adhere for 17 h and stained with either polyclonal antibodies to pFN (Christopher et al., 1997) or a monoclonal antibody (BC.1) directed against the EDB domain. In some experiments, cells were preincubated with either cyclized peptide or scrambled EDA peptides prior to seeding.

4.6 Knockdown of the $\alpha 4$ Integrin Subunits Using Small Interfering RNAs

Dermal fibroblasts were seeded at low density in complete medium, either not transfected or transfected with 25 nM ON-TARGET *plus* siRNA to $\alpha 4$ integrin or transfected with a non-silencing control siRNA (Dharmacon) using Oligofect-AMINE (Invitrogen). The cells were returned to a 5% CO₂ incubator, harvested after 72 h and either subjected to flow cytometry or immuno-fluorescence assays. Expression levels of $\alpha 4$ integrin was analyzed by Flow cytometry using a BD FACS Canto System and data analyzed using BD FACS Diva software. To each of the pre-blocked (1% BSA/PBS) flow tubes (BD Bio-sciences, Franklin Lakes, NJ), cells (100 μl) were added and incubated with either anti-human integrin $\alpha 4$ (Chemicon International) or mouse IgG (Sigma) antibody (10 $\mu\text{g/ml}$) (0.5 h) on ice. The

cells were washed twice with ice-cold PBS, and Alex Fluor 488 Goat anti-mouse (1:200) (Molecular Probes) was then added for 30 min on ice. The cells were washed and resuspended in 600 µl of ice-cold 1% formaldehyde/PBS and stored at 4°C until analysis.

4.7 Western Blotting

Cell lysates were electrophoresed into SDS-PAGE gels under reducing or non-reducing conditions and transferred to Immuno-Blot PVDF membranes (Bio-Rad). Membranes were blocked (1% BSA PBS/TBS Tween-20, 1 h), incubated overnight at 4°C with primary antibodies diluted in 1% BSA PBS, washed, then incubated for 1 h with secondary antibodies diluted in 1% nonfat milk PBS/TBS Tween -20). Bound antibodies were detected by enhanced chemiluminescence using Super Signal West Pico and West Femto reagents. Densitometric analysis was performed using a Fluor-S Multi-Imager and Quantity-One software (Bio-Rad). Membranes were then stripped and reprobed with an antibody against total protein to verify equal loading.

4.8 Quantitative RT-PCR

Cells were cultured on indicated ECM proteins and total cellular RNA was extracted after 17 h using an RNeasy extraction kit (Qiagen). RNA integrity was assessed by denaturing agarose gel electrophoresis and purity was measured via Nanodrop. 1.5 µg of isolated RNA was reverse transcribed to produce cDNA templates using an RT² First Strand Kit (Qiagen) according to the manufacturer's instructions. Fibronectin and -actin RT² qPCR Primer Assays (Qiagen) were used for cDNA amplification. qRT-PCR was carried out using RT² SYBR Green Mastermix (Qiagen) in a MyiQ Cyclor System (Bio-Rad Laboratories). The relative expression ratio of the target gene to the housekeeping gene was calculated using the 2^{-CT} method. The results are representative of values from 3 independent experiments.

4.9 Quantitation and Statistical Analysis

Statistical analysis was done using either paired t-test or Student's t-test for dependent samples. For other experiments, the significant differences from control were determined using a one-way ANOVA. The levels of significance were set at p<0.05.

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Abbreviations

α-SMCA	α-smooth muscle cell actin
cFN	cellular fibronectin
DPBS	Dulbecco's phosphate-buffered saline
ECM	extracellular matrix

EDA	fibronectin extra domain A
EDB	fibronectin extra domain B
FN-III	fibronectin type III repeat
FNs	fibronectins
MLC	myosin light chain
pFN	plasma fibronectin
ROCK	Rho-kinase
siRNA	small interfering RNA.

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Highlights

- The fibronectin EDA domain promotes a profibrotic phenotype in dermal fibroblasts.
- The EDA dependent profibrotic phenotype requires the $\alpha 4$ integrin receptor
- The fibronectin EDA domain binds the $\alpha 4\beta 1$ integrin within the C-C' loop
- The C-C' loop peptide is sufficient for the profibrotic response

Fig. 1. The EDA domain of fibronectin promotes stress fiber formation and MLC phosphorylation in dermal fibroblasts

A) Human dermal fibroblasts were seeded on glass coverslips co-coated with pFN (10 $\mu\text{g/ml}$) and either the EDA or the III-4 domain of fibronectin (10 $\mu\text{g/ml}$). After a 7 h incubation, cells were fixed, permeabilized and dual stained for F-actin and phospho-MLC. Phalloidin staining of actin was imaged at both 40x (a, b, c) and 100x (g, h, i). pMLC was visualized by indirect immunofluorescence (d, e, f). Scale bar = 50 μm . Data are representative of four independent experiments. B) Cells were seeded onto coated substrates for 7 h and the lysates analyzed by Western blotting for pMLC. Membranes were stripped and reprobed for total MLC to ensure equal loading. Representative blot from three independent experiments is shown. C) Quantitation of pMLC bands from 3 experiments. The intensity of each band was normalized against total MLC and compared against the pFN control which was set at 1. Data analyzed by Student's t-test. Error bars indicate standard error from 3 pooled experiments (* $p < 0.05$). D) Cells were seeded on glass coverslips coated with pFN (10 $\mu\text{g/ml}$) or a mixture of pFN (10 $\mu\text{g/ml}$) and EDA (40 $\mu\text{g/ml}$). Cells were incubated for 17 h, fixed, permeabilized and immunostained for total fibronectin (a, b) or cellular fibronectin using antibody to the alternatively spliced EDB domain (c, d). E) Dermal fibroblasts were seeded on glass coverslips coated with a mixture of pFN (10 $\mu\text{g/ml}$) and EDA (40 $\mu\text{g/ml}$). Cells were preincubated with either blocking antibody (HP2/1) to $\alpha 4\beta 1$ (b, d) or a control (IgG) antibody (a, c). Cells were incubated on substrates for 17 h and then fixed, permeabilized and stained for total fibronectin by indirect immunofluorescence. Scale bar = 50 μm . The data are representative of three independent experiments.

Fig. 2. EDA dependent stress fiber formation and FN matrix deposition are $\alpha 4$ -dependent
 A) Dermal fibroblasts were treated with either non-targeting control siRNA (c) or $\alpha 4$ siRNA (d) for 72 h, fixed, stained with a monoclonal antibody to the $\alpha 4$ integrin. $\alpha 4$ expression was analyzed by FLOW cytometry for $\alpha 4$ integrin subunit (b, c, d). Cells stained with control IgG (a) served as an isotype control. Data are representative of six independent experiments.
 B,C) $\alpha 4$ knockdown cells or control cells were seeded onto glass coverslips coated with pFN, a mixture of pFN and EDA, a mixture of pFN and III-13, or recombinant full-length EDA⁺FN and incubated for 7 h. Cells were fixed, permeabilized and stained for F-actin or p-MLC. Data are representative of 3 separate experiments.

Fig. 3. EDA upregulates FN mRNA levels

A) Dermal fibroblasts were seeded on surfaces coated with either pFN (10 µg/ml) or a mixture of pFN (10 µg/ml) and either the EDA domain (10 µg/ml or 40 µg/ml) or III-13 (40 µg/ml). B) Dermal fibroblasts in which the $\alpha 4$ integrin had been knocked down and their corresponding control cells were seeded onto substrates coated with either pFN (10 µg/ml) or a mixture of pFN (10 µg/ml) and EDA (40 µg/ml). Cells were incubated for 17 h, lysed and mRNA extracted. Quantitative RT-PCR was performed and FN mRNA levels were analyzed relative to control cells [cells on pFN + III-13 (A) or pFN (B)] and balanced with the housekeeping gene, β -actin, using $2^{-\Delta CT}$. Error bars indicate standard deviation of quadruplicate samples for one of three representative experiments. * $p < 0.01$ versus control cells on pFN by one-way Anova analysis with Newman-Keuls multiple comparison test.

Fig. 4. Binding of fibroblasts to EDA depends on $\alpha 4\beta 1$ binding to the C-C' loop

A) Human dermal fibroblasts were allowed to attach to wells coated with His-tagged EDA in the presence of a blocking antibody to $\alpha 4\beta 7$ (FIB504), TLR4 or $\alpha 4\beta 1$ (P1H4). IgG served as negative control. B) Recombinant GST-tagged EDA fusion proteins containing the designated mutations within the C-C' loop were coated (5 $\mu\text{g}/\text{ml}$ at 37°C for 1 h) onto 96-well microtiter plates. Human lung fibroblasts were stained with Hoechst 33342, washed and read in a fluorescence plate reader. C) GST-EDA or FN-III4 fusion proteins were coated (10 $\mu\text{g}/\text{ml}$, 37°C, 1 h) onto 96-well microtiter plates. Dermal fibroblasts were seeded onto coated wells in the presence or absence of the designated peptides. Cells were pretreated with the cyclized EDA-derived peptide or reduced and alkylated (linear) peptide (2 mM) for 0.5 h at 37°C. Data representative of three independent experiments; error bars represent standard errors.

Fig. 5. The EDA C-C' peptide promotes stress fiber assembly, MLC phosphorylation and FN fibrillogenesis

Dermal fibroblasts were pre-incubated (2 mM, 0.5 h) with either cyclized EDA peptide or scrambled EDA peptide and seeded onto plasma fibronectin coated substrates for 7 h. A) Cells were dual stained for actin with phalloidin and immunostained for pMLC. Exposure times for images were normalized to cell seeded on plasma fibronectin in the absence of peptides. Scale bar = 50 μm. Representative images from three independent experiments are shown. B) Cells were co-immunostained for total FN and cell-derived EDB containing FN. Similar results were obtained using human lung fibroblasts (data not shown). Exposure times for images were normalized to those for cells seeded on plasma fibronectin alone. Scale bar = 50 μm. Representative images from three independent experiments are shown.