IUBMB *Life*, 63(7): 538–546, July 2011

Critical Review

Fibronectin Splice Variants: Understanding Their Multiple Roles in Health and Disease Using Engineered Mouse Models

Eric S. White[1] and Andrés F. Muro[2]

[1]*Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, University of Michigan Medical School, Ann Arbor, MI*

[2]*Mouse Molecular Genetics Laboratory, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy*

Summary

The extracellular matrix (ECM) is a highly dynamic network of proteins, glycoproteins, and proteoglycans. Numerous diseases result from mutation in genes coding for ECM proteins, but only recently it has been reported that mutations in the *fibronectin (FN)* gene were associated with a human disorder. *FN* is one of the main components of the ECM. It generates protein diversity through alternative splicing of a single pre-mRNA, having at least 20 different isoforms in humans. The precise function of these protein isoforms has remained obscure in most cases. Only in the recent few years, it was possible to shed light on the multiple roles of the alternatively spliced FN isoforms. This substantial progress was achieved basically with the knowledge derived from engineered mouse models bearing subtle mutations in specific FN domains. These data, together with a recent report associating mutations in the *FN* gene to a form of glomerulopathy, clearly show that mutations in constitutive exons or misregulation of alternatively spliced domains of the *FN* gene may have nonlethal pathological consequences. In this review, we focus on the pathological consequences of mutations in the *FN* gene, by connecting the function of alternatively spliced isoforms of fibronectin to human diseases.

IUBMB *Life*, 63(7): 538–546, 2011

Keywords disease models; genetic models; alternative splicing; protein function; complex diseases.

Abbreviations EDA, Extra Domain A of FN; EDB, Extra Domain B of FN; RGD, Arg-Gly-Asp main cell binding domain of FN; RGE, Arg-Gly-Glu, mutated version of the FN cell binding domain; TGF, Transforming Growth Factor

Received 14 February 2011; accepted 7 April 2011

Address correspondence to: Andrés F. Muro, International Centre for Genetic Engineering and Biotechnology, Padriciano 99, I-34149 Trieste, Italy. Tel: +39-040-3757312. Fax: +39-040-226555.
E-mail: muro@icgeb.org

ISSN 1521-6543 print/ISSN 1521-6551 online
DOI: 10.1002/iub.493

THE EXTRACELLULAR MATRIX AND DISEASE

The extracellular matrix (ECM) is a highly dynamic three-dimensional network of proteins, glycoproteins, and proteoglycans. It provides structural support and anchorage for organs, tissues, cell layers, and individual cells. The ECM plays essential roles in fundamental cell processes such as cell growth, cell migration, polarity, embryogenesis, hemostasis, wound healing, and maintenance of tissue integrity, by sensing different environmental stimuli and providing specific inputs to the surrounding cells (*1, 2*). In addition, the ECM captures a wide range of cellular growth factors acting as a local reservoir for them. Changes in the physiological conditions and specific stimuli can trigger protease activities that cause the local release and activation of those factors, communicating instructive signals for development, tissue homeostasis, and basic cell functions, modifying its composition and ability to exert mechanical forces. ECM proteins are generally very complex, containing multiple conserved domains; frequently, inclusion or exclusion of these conserved domains is controlled by highly regulated alternative splicing of their pre-mRNAs. These large fibrous multidomain proteins (collagens, elastins, laminin, tenascin, and fibronectin (FN), among others) are recognized by specific cellular receptors, mainly belonging to the integrin family.

The critical importance of the ECM is demonstrated by the occurrence of numerous diseases resulting from mutations in genes coding for ECM proteins, including collagenopathies (osteogenesis imperfecta, Ehlers-Danlos syndrome VII, IV, etc.), skin defects (epidermolysis bullosa, cutis laxa, Ehlers-Danlos Syndrome I, II, III, etc.), fibrillinopathies (Marfan Syndrome, etc.), basement membrane defects, and osteoarthritis (for a more comprehensive list see ref. *3*). In the case of *FN*, only very recently it has been reported that mutations in specific FN domains are the cause of nephropathies with FN deposition (*4*) suggesting that some disease forms may be caused by subtle mutations in previously unexpected genes.

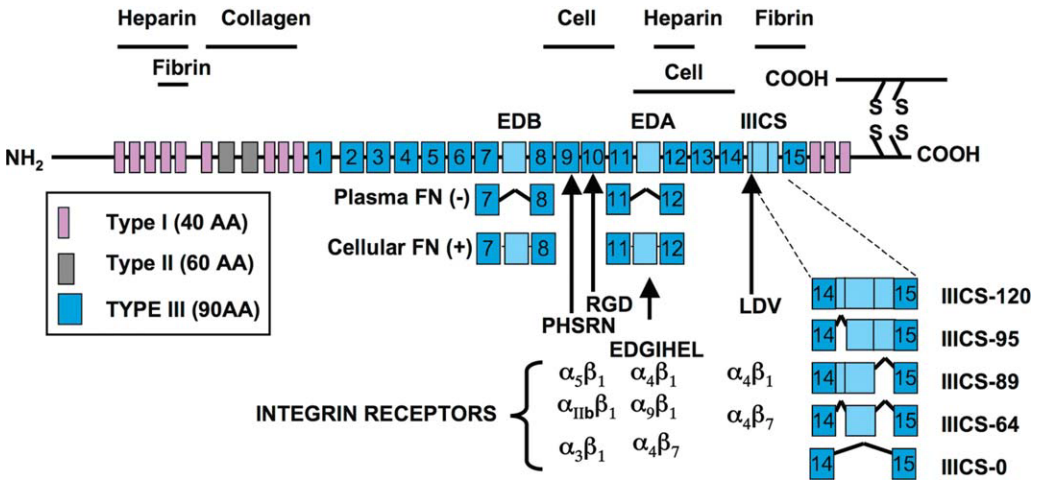

Figure 1. Fibronectin primary structure. The scheme shows a representation of a fibronectin dimer and its interactions. The different types of homologies (12 type I, two type II, and 15 type III) are represented. Numbering of type III homologies excludes EDA and EDB domains. Type I, II, and III domains are constituted of 40, 60, and 90 amino acids, respectively. Constitutive (RGD) and alternatively spliced (LDV), synergy (PHSRN), and EDA (EDGIHEL) cell-binding sites are indicated, together with their integrin receptor partners ($\alpha_4\beta_7$ integrin recognizes the EDA domain, but the precise aminoacids involved have not been yet determined). EDA and EDB splicing is similar in all species (either total inclusion or exclusion), whereas that of the IIICS region is species-specific (five variants in humans, three in rodents, and two in chickens). (Modified from ref. *14*, with permission from © John Wiley & Sons, Ltd.)

FN is one of the best-characterized examples of ECM proteins that has been studied using mouse models generated by gene targeting, bearing conditional, or subtle mutations in different domains of the gene (*5–12*). This type of approach remains the best one to understand the *in vivo* function of specific gene products. The present review focuses on the knowledge obtained from these engineered mouse models to understand the role of FN isoforms in health and pathological processes.

FIBRONECTIN STRUCTURE

FNs are a group of closely related glycoproteins made of two nearly identical ~250 kDa subunits. Monomers, which are linked together by disulfide bonds near the C-terminus, are made of repeating units of three different types of homologies: type I, II, and III (*13, 14*). Many of these independently folded domains are present in different ECM proteins supporting the hypothesis that these structural repeating units might have reassorted among different genes by exon shuffling during evolution (*15*). Type III modules are the most abundant modules in the FN molecule, and are also found in many different proteins across a wide range of species, whereas type I modules are found only in vertebrates. Although there is a single 75-kb gene coding for FN (*7*), up to 20 different protein variants are observed in humans. Protein diversity is obtained by alternative splicing of two type III exons, called Extra Domains A and B (also EIIIA and EIIIB), and of a segment connecting two other type III repeats, called type III connecting segment (IIICS) (Fig. 1). FN is found either as a soluble dimer in plasma, secreted by hepatocytes directly into circulation (plasma FN, or pFN), or deposited as insoluble fibrils in the ECM of tissues (cellular FN, or cFN). FN found in the ECM is partially derived from a variety of fibroblast-like surrounding cells, whereas a fraction is supplied by the plasma. The two FN isoforms differ in the presence of the EDA and EDB domains: (*a*) pFN lacks the alternatively spliced EDA and EDB sequences and (*b*) cFN contains variable proportions of these domains. FN has different functional domains that directly bind it to a variety of molecules such as fibrin, collagen, and heparin. These domains participate in the assembly of the ECM, FN fibrillogenesis and are recognized by cellular receptors, mainly belonging to the integrin family (Fig. 1). The majority of these domains are constitutively included in the mature FN molecule, such as the RGD cell-binding domain, which is recognized by the $\alpha5\beta1$ integrin. However, in some cases, their presence and affinity for their ligands can be regulated by alternative splicing, as discussed below.

ARE THE EDA AND EDB DOMAINS OF FN DISPENSABLE DURING EMBRYO DEVELOPMENT?

The *in vivo* importance of fibronectin was demonstrated by the drastic consequences observed in engineered FN mouse strains bearing mutations within different regions of the gene: (*a*) null mutation of the *FN* gene results in early embryonic lethality (*7*); (*b*) replacement of the EDB exon by the neomycin-resistance gene or by a longer exon including the EDB and flanking exons result in a functional-null genotype with early embryonic lethality (*8*); and (*c*) mutant mice bearing a homozygous mutation in the RGD cell-binding site (RGE) die at embryonic day 10, although the absence of a functional RGD

motif in FN did not compromise assembly of an FN matrix (*11*). All these mutants show vascular defects comparable with defects observed in integrin α5 null embryos (*16*).

The absence of reports describing mutations in the *fibronectin* gene in humans and the *in vivo* results mentioned above strongly supported the speculation that even minor modifications in the FN primary sequence might have lethal consequences. However, recent *in vivo* data derived from patients with glomerulopathy with associated FN deposits (*4*) and from engineered mice suggest that mutations in constitutive exons or misregulation of alternatively spliced domains may have pathological nonlethal consequences.

The EDA and EDB FN domains show a very high degree of homology among vertebrates (*14*), and are cassette-type exons which can be independently spliced-in or out from the pre-mRNA (*17*). They are very abundant in embryonic stages, supporting the hypothesis that they have an important role during embryogenesis and in vascular development, cell migration, and cell differentiation (*17, 18*). They show a very tight tissue-specific regulation and expression, their inclusion decreases with age of the animal, and adult tissues tend to be devoid of these extra domains (*17, 19, 20*). Both domains are not expressed in hepatocytes, but mice engineered to constitutively include the EDA exon have an 80% reduction in circulating pFN, possible caused by selective retention and degradation of EDA^+ FN by hepatocytes (*21*). Similarly, these domains are scarcely expressed in other adult tissues. They are upregulated in specific conditions such as tissue repair, tissue fibrosis, angiogenesis, and cell migration. However, homozygous mutant mice constitutively lacking the EDA or EDB domains (individually), or constitutively expressing the EDA domain do not show any degree of embryonic lethality, grow up without any obvious defect and reproduce normally (*6, 9, 12*). Therefore, one must conclude that single deletion (or constitutive inclusion) of the EDA or EDB domains are not as critical as previously expected or that, alternatively, other proteins may compensate for the lack of the specific FN isoforms in the mutant embryos. Simultaneous deletion of both EDA and EDB exons from the *FN* gene results in embryonic lethality with incomplete penetrance, displaying multiple embryonic cardiovascular defects (see below; ref. *5*).

THE FIBRONECTIN ISOFORMS PARTICIPATE IN LYMPHATIC VALVE FORMATION

The similarity of the RGD-to-RGE mutant and alpha5 integrin-null embryonic phenotype (*11*) to that of FN-null mice (*7*) confirmed the direct involvement of FN in vascular morphogenesis, suggesting that FN function during this process is mainly mediated by the $\alpha5\beta1$ integrin.

However, the role of FN isoforms in these processes seems less clear. The evident upregulation of the EDA and EDB exons during physiological embryonic angiogenesis and tumor angiogenesis has been documented in several publications (*22–24*), encouraging the use of these domains as vascular markers of solid tumors and metastases (*25, 26*). In spite of this, a detailed *in vivo* analysis of physiological and tumor angiogenesis using engineered mice lacking either the EDA or the EDB segments showed no involvement of either domain (individually) in angiogenesis (*6, 9, 12, 27, 28*).

In contrast, recent work on a mouse model bearing the simultaneous deletion of both EDA and EDB exons from the *FN* gene (*5*) started to shed light on the role of FN isoforms in vascular development. In this engineered mouse model, the phenotype showed embryonic lethality at E10.5 with incomplete penetrance, displaying multiple cardiovascular defects, together with a reduction in the number of α-SMA positive cells at E9.5. These results support a role of EDA- and EDB-containing fibronectin isoforms in cardiovascular development and suggest the presence of modifier genes affecting the severity of the phenotype (*5*). These domains may have some redundant function, because inclusion of either of them into the FN molecule is sufficient to attain normal blood vessel development. The mechanisms are still unclear but may be related to a global change in FN conformation necessary to attain enhanced FN properties, not achievable after the deletion of both the EDA and the EDB exons.

EDA^+ FN was shown to have a direct and crucial role in lymphatic valve morphogenesis (*29*) suggesting it may be involved in primary lymphedema. Deposition of EDA^+ FN in the valve-leaflet ECM and its interaction with α9 integrins are necessary for normal lymphatic valve formation. Deficiencies in this interaction, by the absence of either the ligand or the receptor, produce defective valve leaflets unable to avoid pathological retrograde flow of the lymphatic fluid (*29*). Consistent with this observation, $\alpha9\beta1$ integrin-deficient mice develop normally, are viable, and die shortly after birth primarily due to extensive bilateral chylothoraces occurring with defective lymphatic system development (*30*). These results strongly implicate α9 integrins (an EDA receptor), EDA^+ FN, and their interactions as important contributors to lymphatic development and sufficiency.

The absence of EDA^+ FN may also be associated with thoracic aortic aneurysm formation in patients with bicuspid aortic valve (BAV), but not with those having tricuspid aortic valve (*31*). Cultured medial cells from BAV did not increase EDA expression on TGF-$\beta1$ stimulus, supporting the hypothesis that a defect in the regulatory pathway governing FN alternative splicing *in vivo* may be associated with the presence of BAV. However, the molecular causes of these defects are still unknown and need further analysis.

THE ABSENCE OF THE EDA DOMAIN PREVENTS TISSUE FIBROSIS: FN-TGF-$\beta1$ INTERACTION

The involvement of EDA-containing FN (EDA cFN) in important pathological processes such as atherosclerosis (*12, 32*), lung fibrosis (*33, 34*), and liver fibrosis (*35*) has been well described. Mechanistically, EDA cFN appears to enhance differentiation of lipocytes into α-smooth muscle actin (SMA)-

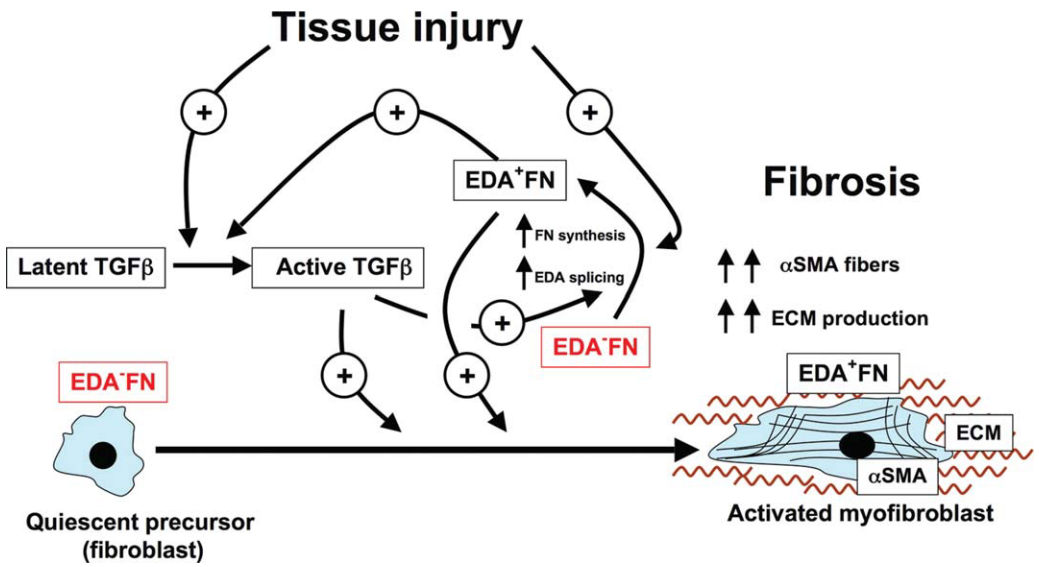

Figure 2. Role of EDA$^+$FN in lung fibrosis. Fibroblast differentiation into myofibroblasts occurs after tissue injury, which triggers activation of latent TGF-β into active TGF-β, FN synthesis, and EDA inclusion into FN. A loop is established, because the presence of EDA$^+$FN itself is necessary to activate latent TGF-β into active TGF-β, which in turn stimulates EDA inclusion into FN. Both the active forms of TGF-β and EDA$^+$FN are necessary to differentiate fibroblasts into myofibroblasts, which is prevented in the presence of only EDA-lacking FN in the ECM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

expressing myofibroblasts (*35*). Further work has shown that TGF-β1, together with EDA cFN, induces the differentiation of fibroblasts into α-SMA-expressing myofibroblasts (*36*). The physiologic relevance of this mechanism has been highlighted in experimental models of lung fibrosis (*34*) and allergen-induced airway fibrosis (*37*), in which EDA-deficient mice were unable to generate α-SMA-expressing myofibroblasts at sites of injury, thereby preventing the development of tissue fibrosis. Similarly, EDA cFN is involved in the development of atherosclerosis, because EDA-null mice develop significantly less atherosclerosis than wild-type (WT) mice (*12, 32*). Most recently, investigators have shown that loss of the EDA exon also improves survival and protects against cardiac dysfunction post-myocardial infarction in a murine model (*38*). However, effects on TGF-β1 expression and/or activity were not assessed in this manuscript. In total, these observations suggest the importance of EDA cFN as a permissive substrate for TGF-β1-induced myofibroblast differentiation, macrophage lipoprotein metabolism, tissue fibrosis, and postmyocardial infarction matrix remodeling. It is notable that previous studies have shown that picomolar amounts of active TGF-β1 are capable of driving the alternative splicing of EDA cFN such that the EDA-containing isoform is predominantly expressed (*39*). Thus, TGF-β1 likely drives myofibroblast activation and subsequent fibrosis via the production and subsequent signaling of EDA cFN. A general schematic of the role of EDA cFN in myofibroblast differentiation is presented in Fig. 2.

TGF-β exists in an ECM-bound complex containing TGF-β, latency-associated peptide (LAP), and latent TGF-β-binding protein-1 (LTBP-1). TGF-β1 activation, the rate-limiting step for its bioavailability, results from release of the mature TGF-β peptide from its LAP (*40*) due to either proteolytic or conformational modifications. For example, both thrombospondin-1 and the epithelial cell-expressed $\alpha v\beta 6$ integrin are known to bind LAP to induce conformational changes that release the mature peptide from complex (*40, 41*). Similarly, certain proteases like membrane-type 1-matrix metalloproteinase and certain furin-like enzymes proteolytically result in latent TGF-β activation (*42*). Intriguingly, our previous work demonstrates that although total TGF-β production is equivalent between EDA-deficient and WT mice, activation of TGF-β appears to be significantly impaired in EDA-deficient mice (*34*). The mechanism(s) behind EDA cFN-regulated TGF-β activation is currently unclear, but may reflect localization of latent TGF-β complexes to the ECM. Indeed, data suggest that LTBP-1 requires a fibronectin substrate for binding and localizing latent TGF-β complexes to the ECM in order for activation via $\alpha v\beta 6$ integrins (*43*). EDA-null and WT mice express equivalent amounts of total fibronectin in their ECM (*9*), suggesting that perhaps LTBP-1 binds to EDA cFN with greater affinity than to EDA-lacking FN, or that enhanced stiffness of EDA cFN compared to EDA-lacking cFN allows for enhanced TGF-β activation. A graphic representation of TGF-β activation and a possible role for EDA cFN is shown in Fig. 3.

As mentioned previously, TGF-β1 induces EDA cFN production and the two together drive fibroblast activation and (when unchecked) tissue fibrosis. However, the mechanism(s) by which this occurs has remained somewhat poorly described.

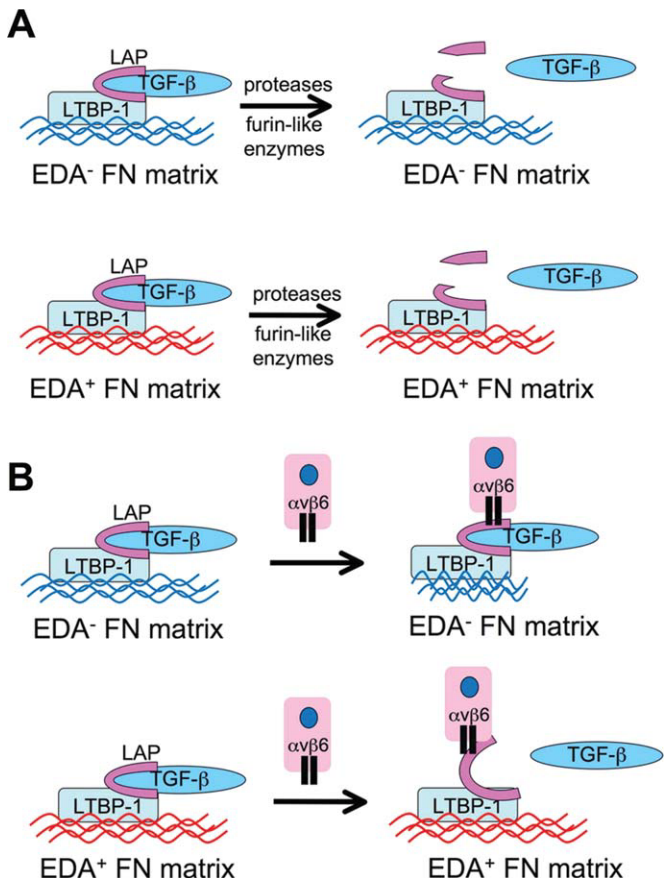

Figure 3. (A) Proposed mechanism of proteolytic TGF-β activation. TGF-β is maintained in a latent state by complexing with latency activating peptide (LAP) and latent TGF-β-binding protein-1 (LTBP-1) on a FN matrix (either without EDA [upper panel] or with EDA [lower panel]). In the presence of proteases, furin-like enzymes, and acids (among other stimuli), LAP is cleaved thereby releasing active TGF-β. (B) Proposed mechanism of integrin-mediated TGF-β activation. TGF-β again is maintained in a latent state in complex with LAP and LTBP-1 on a FN matrix. (upper panel) EDA-lacking FN folds into compact forms; thus, LAP-integrin $\alpha_v\beta_6$ ligation under shear force induces matrix alteration and failure to release active TGF-β. (lower panel) In contrast, the more rigid, cross-linked EDA$^+$ FN resists the shear forces of integrin $\alpha_v\beta_6$–LAP interactions, resulting in a conformational shift in LAP and release of active TGF-β. Other mechanisms may be operative as well. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

THE PRO-THROMBOTIC ROLE OF THE EDA$^+$FN ISOFORM

Among its wide range of functions, FN is also involved in platelet thrombi formation. It is a ligand of platelet surface receptors (*44*) and is covalently crosslinked to fibrin by coagulation factor XIII (for a more comprehensive review see ref. *45*).

However, little insight into the role of FN in thrombosis and hemostasis has been derived from genetic studies in humans, and the final *in vivo* demonstration of the role of FN and FN isoforms in thrombosis and hemostasis was possible only after the generation of mouse strains bearing specific mutations in the *FN* gene. In fact, the stabilization of platelet thrombi by crosslinking of plasma fibronectin to fibrin clots was definitely demonstrated *in vivo* using engineered mouse strains having different pFN concentration (*46, 47*).

The EDA$^+$ FN isoform is particularly interesting from the thrombus-formation point of view, because it is normally absent from plasma, but is present in platelets α granules and is highly increased in some disease states such as atherosclerosis, pulmonary and acute vascular injury, diabetes, thrombocytopenic purpura, and ischemic stroke (*12, 44, 48–51*).

Once again, analysis of mice constitutively producing EDA$^+$ FN in plasma provided important information about its function, revealing a prothrombotic activity of this isoform (*52*). Homozygous EDA$^{+/+}$ mice, notwithstanding the fact that they have only 20–30% of the pFN levels of WT mice (*9, 21*), showed accelerated thrombosis *in vivo* and *in vitro* at arterial shear rates when compared to WT mice (*52*). These results support the hypothesis that secretion of the EDA$^+$ FN prothrombotic isoform in plasma is negatively selected during evolution. In fact, a more extended (or activated) fibronectin form in plasma may have negative consequences such as increased risk of thrombosis, because of augmented exposition of binding sites and consequent increased binding by cellular receptors.

However, hemostatic parameters obtained from EDA$^{+/+}$ mice are normal, indicating that FN does not alter normal hemostasis, similar to what is observed in humans. Both mice and humans present a wide range of FN concentration in plasma (*53*) suggesting that platelet thrombus formation is not a simple function of plasma FN concentration. Whether the observations in mice are translatable to human patients has yet to be tested; however, these results strongly suggest that pathological increases in plasma levels of EDA cFN may be an important risk factor for thrombosis, especially in the disease states mentioned above. Such disease states predispose to vessel thrombosis, and therefore, the conditions of those patients may worsen because of increased risk of thrombosis caused by elevated plasma EDA$^+$ FN levels.

Evidence supporting FN as a risk factor for thrombosis has recently been published. Pecheniuk et al. (*54*) showed that a group of patients with idiopathic venous thromboembolism had elevated plasma FN levels but, unfortunately, the levels of EDA$^+$ FN were not determined.

RECEPTORS FOR EDA AND EDB cFN

Given that EDA cFN and EDB cFN are capable of directing various phenotypic behaviors different than EDA- or EDB-lacking pFN, it is not surprising that these isoforms might bind and signal via different integrin and nonintegrin receptors than pFN. As shown in Table 1, cells bind fibronectin through a variety of cell-surface receptors, including members of the

Table 1
Known cellular receptors for pFN, EDA cFN, and EDB cFN

Receptor	pFN	EDA cFN	EDB cFN
	$\alpha3\beta1$		
	$\alpha4\beta1$		
	$\alpha5\beta1$*		
	$\alpha8\beta1$	$\alpha4\beta1$	
Integrin	$\alpha v\beta1$	$\alpha4\beta7$	$\alpha?\beta1$
	$\alpha v\beta3$	$\alpha9\beta1$	
	$\alpha v\beta6$		
	$\alpha v\beta8$		
	$\alpha v\beta7$		
	$\alpha IIb\beta3$		
Non-integrin	Ku	TLR4	

*Denotes the main fibronectin receptor. α? Denotes the unkown alpha subunit partnering with β_1 integrin.

integrin superfamily. Although $\alpha5\beta1$ integrin is considered as the "classic" fibronectin receptor (*55*), it does not bind the EDA domain. Rather, the $\alpha4$ integrins ($\alpha4\beta1$ and $\alpha4\beta7$), $\alpha9\beta1$ integrin, and the toll-like receptor 4 (TLR4) appear to be receptors for the EDA domain (*14*). Although it is thought that $\beta1$ integrins are capable of binding EDB [based on data showing experimentally activated $\beta1$ integrins bind various fibronectin type III repeats (*56*)], no specific EDB receptor has yet been identified.

The $\alpha4$ and $\alpha9$ integrins are not specific for the EDA domain; thus, determining the functions of these receptors with respect to EDA cFN has been difficult. For instance, $\alpha4$ integrins also bind the variably spliced IIICS of fibronectin (*57*). However, data suggest that EDA–$\alpha4\beta1$ integrin interactions may mediate cell adhesion (*58*) and leukocyte recruitment (*59*). Classically, $\alpha4\beta7$ integrin is thought to be leukocyte restricted (*60*), where it is important for binding mucosal addressin cell adhesion molecule to effect lymphocyte homing (*61*). However, one recent study demonstrates the expression of $\alpha4\beta7$ integrin on lung fibroblasts, which appears to mediate the effects of EDA cFN in driving myofibroblast differentiation (*62*). EDA–$\alpha9\beta1$ integrin interactions appear to occur on various epithelial and endothelial cells, where they mediate cellular migration (*63*) and FN matrix assembly (*29*). Unpublished data also suggest that the interaction between $\alpha9$ integrin and the FN EDA domain is important for the development of venous valves in mice (T. Makinen, personal communication).

TLR4 is the most-recently identified receptor for the EDA domain (*64*). As the first description of the EDA domain activates TLR4, numerous functions have been ascribed to this interaction such as arterial remodeling in atherosclerosis (*65*), induction of cytotoxic T cell responses following antigen stimulation (*66*), stimulation of mast cells (*67*), and priming of leukotriene synthesis in neutrophils and monocytes (*68*). Taken together, these data suggest that EDA^+ FN is an important mediator of a number of inflammatory and other immunologic responses.

SIGNALING MECHANISMS INVOLVED IN EDA-MEDIATED FIBROBLAST-TO-MYOFIBROBLAST DIFFERENTIATION

Fibroblast-to-myofibroblast differentiation is a complex process by which resting or quiescent fibroblasts acquire features of smooth muscle cells, including upregulating the contractile proteins smooth muscle myosin and α-smooth muscle actin, as well as enhancing ECM protein synthesis (*69*). Induction of myofibroblast differentiation by TGF-β is a well-described event and requires the presence of EDA cFN (*62, 70*) as well as adhesive signaling. Previous studies show that in the presence of TGF-β, lung fibroblasts in suspension do not upregulate expression of α-SMA (*71*), suggesting that either mechanical tension or adhesion signaling through focal adhesions is also necessary for the myofibroblast phenotype. In fact, both are likely necessary. For example, activation of focal adhesion kinase (FAK) or suppression of the FAK inhibitor, focal adhesion kinase-related nonkinase, is required for myofibroblast differentiation (*71–73*) in part through the upstream effects of protein kinase C ε (PKC-ε) and downstream activities of the mitogen-activated protein kinase pathways. Other evidence implicates the phosphoinositol-3-kinase (PI3K) pathway in myofibroblast differentiation, because our lab has shown that inhibition of the PI3K antagonist phosphatase and tensin homolog on chromosome 10 also promotes myofibroblast differentiation *in vitro* and *in vivo* (*74*). Similarly, mechanotransductive effects on stiff substrates likely encountered in fibrosing tissues and on plastic dishes also enhance myofibroblast differentiation through FAK and other pathways (*75*). Taken together, these data suggest that myofibroblast differentiation following TGF-β stimulation relies heavily on ECM adhesive signaling.

CONCLUDING REMARKS

Targeted mutations in the *FN* gene have been very informative about the roles of various FN isoforms in normal and pathological situations, although many questions remain unanswered and need further investigation. Many of these mutations produce clear phenotypes, supporting the idea that these highly conserved domains and the mechanisms involved in their regulation are positively selected in evolution, conferring an adaptive advantage. However, in some cases the differences were absent or subtle, or relied on an exogenous stressor or perturbation in homeostasis to become evident. This indicates that certain gene mutations are clinically silent until an environmental trigger induces an alternative phenotype, and argues that genetically mutated animals should be evaluated under a variety of conditions before concluding that a specific mutation does not possess a significant phenotype. Moreover, it is generally accepted that the process of evolution will not retain certain genes or mutations within genes if no selective advantage is conferred.

However, the identification of such defects in the human population may be hampered by genetic heterogeneity and by the presence of modifier genes.

The generation and analysis of new mouse models, together with a novel approach based in sequencing the genome of patients suffering from thrombosis, lymphedema, fibrosis, and other FN-related diseases will certainly provide useful information regarding possible involvement of alternative splicing of FN in disease states.

ACKNOWLEDGEMENTS

AFM was supported by the Friuli-Venezia Giulia Regional grant. Dr. White's research on tissue fibrosis and extracellular matrix is supported by grants from the National Institutes of Health (R01 HL085083, RC2 HL101740) and the Drews Sarcoidosis Research Fund at the University of Michigan.

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