

Invited Review

New insights into form and function of fibronectin splice variants

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Abstract

The extracellular matrix (ECM) is a highly dynamic structure that not only provides a physical framework for cells within connective tissues, but also imparts instructive signals for development, tissue homeostasis and basic cell functions through its composition and ability to exert mechanical forces. The ECM of tissues is composed of, in addition to proteoglycans and hyaluronic acid, a number of proteins, most of which are generated after alternative splicing of their pre-mRNA. However, the precise function of these protein isoforms is still obscure in most cases. Fibronectin (FN), one of the main components of the ECM, is also one of the best-known examples of a family of proteins generated by alternative splicing, having at least 20 different isoforms in humans. Over the last few years, considerable progress on elucidating the functions of the alternatively spliced FN isoforms has been achieved with the essential development of key engineered mouse strains. Here we summarize the phenotypes of the mouse strains having targeted mutations in the *FN* gene, which may lead to novel insights linking function of alternatively spliced isoforms of fibronectin to human pathologies.

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Introduction

The complexity of higher eukaryotes is achieved with a finite number of genes, which generate protein diversity by a number of mechanisms. Among them, the most prominent one is alternative splicing of the pre-mRNA. It is estimated that over 60% of genes undergo alternative splicing [1]. In most cases, it occurs in a tissue-specific and developmentally regulated manner and is also regulated by different stimuli. However, in the majority of cases the *in vivo* functions of protein isoforms generated by alternative splicing remain not well defined.

Fibronectin (*FN*) is an excellent model in which to study gene function regulated by alternative splicing. FN is a multifunctional glycoprotein found in plasma and in the extracellular matrix (ECM) of tissues. It is expressed by multiple cell types and plays a key role in cell adhesive and migratory behaviour. Indeed, fundamental processes such as embryogenesis, haemostasis, wound healing and maintenance of tissue integrity are all processes that depend on interactions between cells and the ECM [2]. The critical importance of FN *in vivo* was conclusively demonstrated by the lethal effect of the murine *FN*-null mutation: mice lacking *FN* died from severe defects in embryonic development [3] (Table 1). In humans, the clinical significance of FN

is evidenced by recent data demonstrating that certain types of glomerulopathy result from mutations in the *FN* gene [4].

Although *FN* was one of the first genes reported to undergo alternative splicing [5–7] and is one of the best-studied models of alternative splicing [8–31], the *in vivo* relevance of alternatively spliced isoforms was poorly understood prior to the development of mouse models having targeted mutations of the *FN* gene. Conclusive evidence for the role of FN in some of the processes mentioned previously was obtained, taking advantage of the gene targeting methodology, which remains the best approach to understand the *in vivo* function of specific gene products. Here we discuss the recent advances in our understanding of the function of FN isoforms, largely derived from the use of mouse models having targeted mutations of the *FN* gene.

Fibronectin protein structure

FN is present only among vertebrates and its appearance in evolution correlates with the emergence of organisms with endothelial cell-lined vasculature [32,33]. The functional FN molecule consists of two similar or identical subunits of 220–250 kDa that

Table 1. Description of the main phenotypes observed in the different mouse models having targeted mutations in the *FN* gene

Animal model	Main phenotype	Reference
<i>FN</i> -null (<i>FN</i> promoter and first exon deletion)	Embryonic lethal E8.5/severe mesodermal defects during gastrulation Differences in penetrance of the phenotype associated to genetic backgrounds <i>Modifier gene</i> : identification of new genes that may play a role in heart development <i>Thrombosis</i> : defects in thrombosis of heterozygous mice were rescued by infusion of pFN	[3] [131] [132] [93]
<i>FN</i> -null EDB (<i>EDB</i> -cDNA fusion)	Embryonic lethal/mesodermal defects, similar to the <i>FN</i> -null strain. The presence on the <i>Neo</i> gene in the intron probably affected the normal processing of the <i>FN</i> gene	[121]
pFN KO (conditional 'floxed' <i>FN</i> gene)	<i>pFN depletion</i> : increased neuronal apoptosis and larger infarction areas following transient focal cerebral ischaemia/normal haemostasis and wound healing <i>pFN depletion and thrombosis</i> : role for pFN in thrombus stability/delayed occlusion of injured arteries <i>pFN depletion and angiogenesis</i> : antiangiogenic factors complex with adhesive plasma proteins to create active antiangiogenic substances. <i>cFN KO in cartilage</i> : normal skeletal development <i>pFN depletion and S. pyogenes virulence</i> : plasma fibronectin bound to the bacterial surface down regulates <i>S. pyogenes</i> virulence by limiting bacterial spread	[82] [92] [133] [134] [135]
<i>EDB</i> -null (deletion of <i>EDB</i> exon)	No obvious <i>in vivo</i> phenotype was observed using healing of bone fractures, behaviour, organogenesis and tumorigenesis models. Reduced growth and deposition of FN in the pericellular matrix in <i>EDB</i> -null MEFs <i>Tumorigenesis and angiogenesis</i> : No differences were observed <i>Thrombosis</i> : No differences were observed	[79] [88] [93]
<i>EDA</i> -null/ <i>EDA</i> knock-in (deletion of floxed <i>EDA</i> exon/optimization of <i>EDA</i> splicing sites)	Both strains had reduced lifespan/ <i>EDA</i> -null showed wound healing defects/ <i>EDA</i> ^{+/+} mice had decreased FN levels in plasma and tissues but showed normal wound healing <i>Splicing in vivo</i> : constitutive absence or inclusion had no effect in <i>EDB</i> or <i>IIICS</i> splicing <i>Splicing in vitro</i> : <i>EDA</i> inclusion had a minor effect in <i>IIICS</i> inclusion in MEFs <i>Behaviour</i> : <i>EDA</i> deletion affected motor coordination and probably balance/ <i>EDA</i> knock-in mice had reduced mobility in the open field test <i>Hepatocyte-specific deletion of the EDA exon</i> : plasma and tissue levels returned to normal, suggesting that an important fraction of the FN present in the ECM of tissues (up to 50–60% in some tissues) derives from plasma <i>Atherosclerosis</i> : both constitutive absence or inclusion of the <i>EDA</i> exon produced reduced atherosclerosis due to diminished take up of cholesterol by macrophages <i>Thrombosis</i> : despite the 75–80% reduction in pFN, <i>EDA</i> ^{+/+} mice had increased thrombosis and thromboembolism, and augmented <i>in vitro</i> thrombus formation in a perfusion chamber, suggesting a new pro-thrombotic role for <i>EDA</i> + FN <i>Lung fibrosis</i> : <i>EDA</i> -null mice were protected against bleomycin-induced lung fibrosis, with reduced <i>in vivo</i> and <i>in vitro</i> activation of myofibroblasts (determined by α -SMA levels) associated to a diminished activation of latent TGF β	[78] [11] [136] [112] [111] [120] [97] [86]
<i>EDA</i> -null (deletion of <i>EDA</i> exon)	<i>Atherosclerosis</i> : <i>EDA</i> -null mice showed reduced atherosclerosis (in an ApoE background) associated to a diminished uptake of cholesterol by macrophages <i>Tumorigenesis and angiogenesis</i> : no differences in tumour growth and levels of α -SMA in pericytes were observed <i>Thrombosis</i> : no differences were observed in occlusion time and formation of thrombus of injured arteries	[80] [88] [93]
<i>EDA</i> and <i>EDB</i> KO (deletion of <i>EDA</i> and <i>EDB</i> exon from the same allele)	Mutation was embryonic lethal with partial penetrance, associated to the genetic background. Multiple severe cardiovascular defects were observed, reminiscent of, but milder than, <i>FN</i> -null and α 5-integrin KO mice. Exclusion of both exons apparently did not affect protein synthesis, secretion or cell surface deposition of FN. Mice had reduced α -SMA-positive cells surrounding the aorta	[81]
<i>RGD</i> (mutation of <i>RGD</i> to <i>RGE</i>)	The mutation of the main cell binding site was embryonic lethal at E10. Embryos had shortened posterior trunk, absent tail bud-derived somites, and severe vascular defects resembling the phenotype of α 5-integrin-deficient mice. Assembly of matrix in mutant embryos or cells was apparently normal	[62]

are held together by two disulphide bonds near their carboxyl-termini, forming a dimer (Figure 1A). Each monomer is comprised of a combination of three different types of homologous repeating domains, termed Types I, II and III [2]. The 15 Type III modules constitute the largest part of the FN polypeptide and are clustered in the central part of the protein (Figure 1A). Individual FN Type III repeats have a

high degree of structural homology, despite displaying only 20–40% identity in amino acid sequence. They are composed of ~90 amino acids organized in seven anti-parallel β strands with exposed loops between these strands and without disulphide bonds [34] (Figure 1B, C). Domains may undergo structural modifications, exposing cryptic sites, induced by mechanical forces producing 'stretching' of the FN

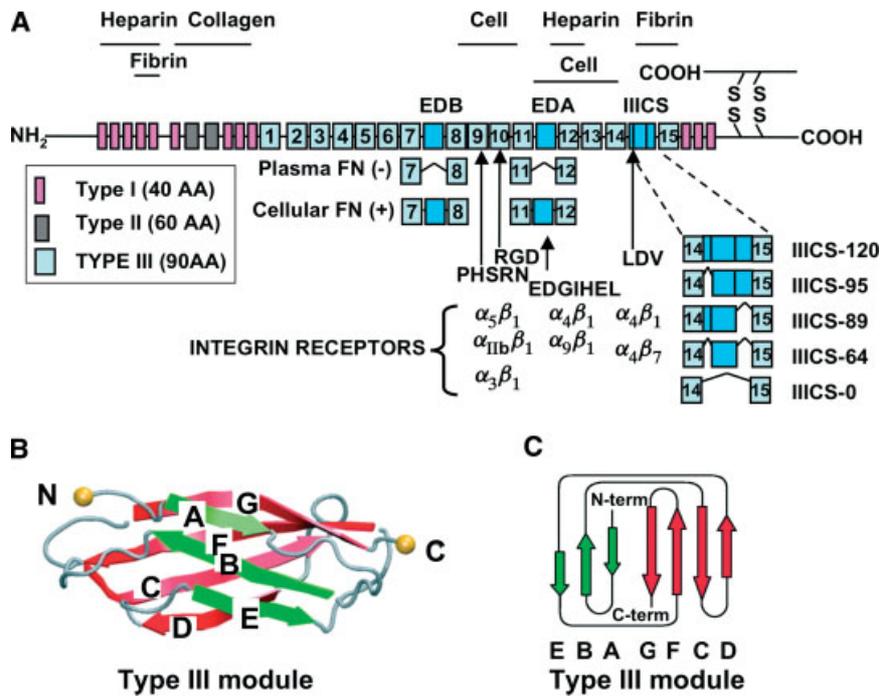


Figure 1. Fibronectin primary structure. The scheme shows a representation of a fibronectin dimer and its interactions (A). The different types of homologies (12 Type I, two Type II and 15 Type III) are represented. Numbering of Type III homologies excludes EDA and EDB domains. Type I, II and III domains 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. EDA and EDB splicing is similar in all species (either total inclusion or exclusion), while that of the IIICS region is species-specific (five variants in humans, three in rodents and two in chickens). Type III homologies are organized in seven anti-parallel β strands (spatial and planar representations are shown in B and C, respectively)

molecule, by proteolysis [35,36], or by incorporation of one (or both) of the alternatively spliced Type III domains (extra domains A and B, denominated EDA, EIIIA or EDI and EDB, EIIIB or EDII, respectively) [37].

Broadly, two variations of FN exist: plasma FN (pFN), a dimeric and soluble form secreted by hepatocytes directly into the circulation, lacking the alternatively spliced EDA and EDB sequences; and cellular FN (cFN), found in the ECM of tissues as a multimeric form assembled into fibrils which contain variable proportions of the EDA and EDB domains. FN fibrils are prominent in loose connective tissue, granulation tissue, embryonic basement membranes and on the surface of numerous cell types in culture [2,38].

FN gene structure and alternative splicing

FN is produced after transcription of a single gene, composed of 47 exons that spans over 90 kbp in the genome. Type III domains of FN are encoded by two exons with the exceptions of the EDA and EDB and the ninth Type III domain, each of which is encoded by a single exon. Multiple FN mRNAs, and consequently multiple protein isoforms, can arise due to alternative splicing within a single pre-mRNA (Figure 1A). The EDA and EDB exons can be included or excluded from FN mRNA [5,7,17,27], while the Type III connecting segment (IIICS) element (also termed the variable, or V, region) undergoes a more complicated

splicing pattern: it can be completely included (V120) or excluded (V0), or partially included, according to the species (Figure 1). Adding to the complexity, in zebrafish (a more distant species in evolution), two FN genes are present (termed *FN1a* and *FN1b*) [39,40]. *FN1a* undergoes no alternative splicing (and thus includes EDA, EDB and the V region constitutively), while the EDB and V regions are alternatively spliced in *FN1b* [39]. Interestingly, the complexity of V-region alternative splicing and the total number of FN isoforms seems to correlate with the evolutionary scale. In fact, only one V-region variant is found in the *FN1a* gene of zebrafish and two in the *FN1b* gene (for a total of five FN isoforms, from two independent genes) [39], whereas two V variants are present in frogs [41] and chickens (producing up to six FN isoforms) [25], three in rats and mice (with 12 FN isoforms) [7] and five in humans (generating up to 20 FN isoforms) [42].

Inclusion of the alternatively spliced regions is elevated during embryonic development [2] and decreases substantially after birth and with ageing [2,11,26,43]. However, the ‘embryonic’ splicing pattern is temporally re-established in adult life in certain circumstances, such as tissue repair, tissue fibrosis and angiogenesis. An excellent example is skin wound healing, where the inclusion of the EDA and EDB domains is increased in the cells at the base of the wound [16]. Similarly, both exons are up-regulated in regenerating liver, but with different kinetics [44–46]. The

re-appearance of EDA cFN has been also observed in lung fibrosis, prior to the appearance of collagen. These tissue repair situations are also characterized by the differentiation and activation of myofibroblasts. These processes require TGF β , which promotes EDA exon incorporation into the mature pre-mRNA [47,48] through an as-yet unknown mechanism.

Alternative splicing of EDA and EDB is modulated by members of the family of splicing regulators denoted SR proteins (for serine- and arginine-rich proteins) through distinctive mechanisms (Figure 2). In the case of EDA, regulatory sequences are located within the exon itself; in the case of EDB, distinct regulatory sequences are located within the exon and within the downstream intron.

The EDA exon is one of the first reported examples where regulatory sequences are located within the exon itself [49]. It contains a purine-rich region recognized by SR proteins, notably SF2/ASF, enhancing exon recognition and subsequent inclusion into the mature mRNA (Figure 2A) [9,10,12,22]. As additional steps of regulation and complexity, the activity of SR proteins is modulated in response to extracellular stimuli, by SR protein kinases [50,51], and by PKB/Akt [52]. The efficiency of exon recognition is affected by the promoter type and velocity of the RNA polymerase II that transcribes the gene [12,13,53,54].

The FN pre-mRNA is characterized by a particular secondary structure in the region of the EDA exon, which assures proper display of the exon splicing enhancer (ESE) region in a loop region of a stem-loop structure [23]. This particular secondary structure of the mRNA is stabilized by a nearby sequence

(ESS, exonic splicing silencer). On the contrary, the recognition of the EDB exon depends on the presence of TGCATG repeats in the downstream intron (ISE, intronic splicing enhancers) [18,19]. The recognition of these elements by SRp40 enhances EDB inclusion into the mRNA (Figure 2B) [55]. However, in regenerating liver, a second mechanism of EDB regulation has been identified which is functionally similar to EDA: the increased inclusion of EDB is mediated by SRp40 recognition of a specific purine-rich sequence present within the EDB exon [46].

Less attention has been paid to the mechanisms regulating alternative splicing of the IIICS region. One regulator of alternative splicing, termed mammalian homologue of suppressor-of-white-apricot (SWAP), influences IIICS splicing in a way that favours complete skipping of the IIICS region, generating the IIICS-0 form; in contrast, the SF2/ASF splicing factor stimulates inclusion of the complete IIICS region [56]. However, the sequences involved in the regulation of the IIICS region remain to be determined.

Interactions of FN with cellular receptors

FN interacts with cellular receptors known as integrins through specific sites of the protein. Integrins are cell-surface heterodimeric transmembrane receptors consisting of an α - and a β -subunit that link the ECM with the intracellular actin cytoskeleton and regulate specific signal transduction cascades. Although a large number of integrins can bind FN [57], the classic fibronectin receptor is the α 5 β 1 integrin. This integrin recognizes the well known RGD cell-binding site

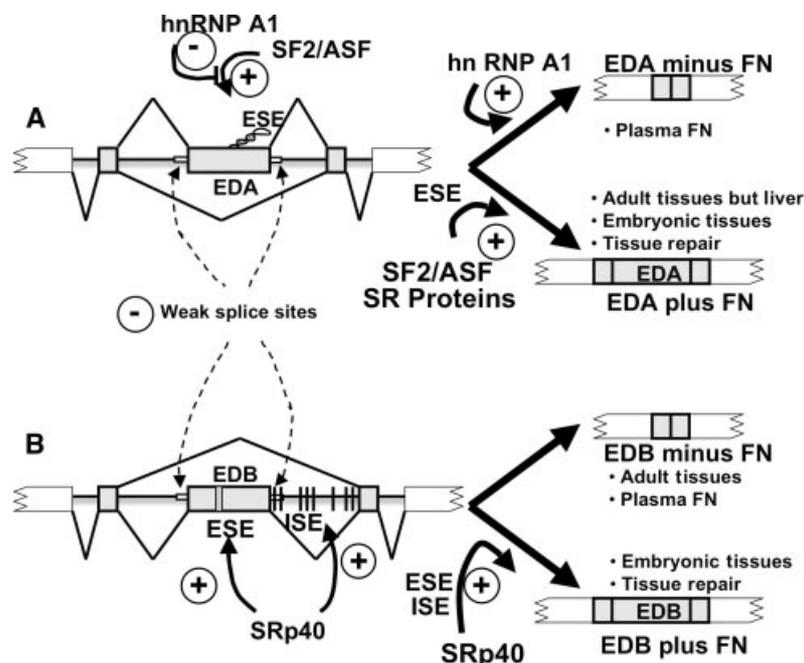


Figure 2. Mechanisms of EDA and EDB alternative splicing. (A) EDA exon splicing is positively regulated by the splicing factor SF2/ASF, which recognizes the exonic splicing enhancer (ESE) displayed in a loop region of stem-loop structure of the FN pre-mRNA. Skipping of the EDA exon is favoured by hnRNP A1. (B) The splicing factor SRp40 promotes EDB exon inclusion by binding to a purine-rich sequence (ESE) within the EDB exon itself, or by recognizing the intronic splicing enhancers (ISE) located in the adjacent downstream intron. The presence of weak splice sites favours skipping of the EDA and EDB exons

located in a mobile and exposed loop region [34] in the tenth Type III repeat, along with a synergy sequence (PHSRN) located in the adjacent ninth Type III module [58–60] (see Figure 1A). The RGD sequence is the prototypic integrin recognition sequence found in other ECM proteins, such as tenascin, fibrinogen, thrombospondin, vitronectin and von Willebrand factor (vWF) [61]. The functional importance of the RGD sequence has been confirmed recently through experiments demonstrating that knock-in mice with an *RGD* → *RGE* mutation in the *FN* gene die at embryonic day 10 (E10) (Table 1), resembling the phenotype of $\alpha 5$ -integrin-null animals [62]. However, FN from these animals can be assembled into fibrils *in vivo* and *in vitro*, suggesting that RGD is dispensable for this function.

In addition to its cell-binding properties, FN also binds other glycoproteins, including other ECM molecules, as well as components of the complement and coagulation systems [2,38]. Notably, FN binds quite strongly to fibrin and fibrinogen shortly after tissue injury and, along with activated platelets, makes up the bulk of the provisional haemostatic clot. The FN–fibrin meshwork undergoes covalent cross-linking to stabilize the clot and to allow for reparative cell migration. Moreover, within the ECM, FN can cross-link collagens, heparan sulphate proteoglycans and itself to provide a stable matrix platform on and in which cells reside.

Other cell-binding sites are present in alternatively spliced regions of FN, conferring the capacity to regulate cell-binding ability and affinity by alternative splicing. *In vitro* experiments have determined that the EDGIHEL sequence present in the EDA domain (Supplementary Figure 1, available at <http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2388.html>) is recognized by the $\alpha 4\beta 1$ and $\alpha 9\beta 1$ integrins [63–66]. A second cell-binding region is present in the IIICS segment, which contains the LDV and REDV sequences, recognized by the leukocyte $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins [67]. In contrast,

the cellular receptor(s) for the EDB domain remain largely unknown.

Both the EDA and the EDB exons show a very high degree of homology among vertebrates. Indeed, they have 53.3% and 71.4% of amino acid sequence identity, and 87.8% and 94.5% similarity for EDA and EDB, respectively, among 22 vertebrate species ranging from humans to frogs (see Supplementary Figures 1 and 2, available at: <http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2388.html>), in contrast to the lower conservation observed with the only other single-exon Type III domain, the ninth Type III repeat (41.1% identity and 75.5% similarity). Amino acid sequence identity decreases to 30% and 38.5% (and 63.3% and 79.1% similarity) for the EDA and EDB, respectively, if we consider the two *FN* genes of zebrafish. The evolutionary distance of the EDA and EDB among vertebrates is schematically shown in Figure 3.

The high level of conservation of the EDA and EDB sequences during evolution and of their tightly regulated patterns of splicing, in contrast with their low homology within a species (only 28% homology between human EDA and EDB amino acid sequences), strongly suggests a conserved but distinct role of these domains.

Functions of the EDA domain

Numerous functions have been ascribed to the EDA domain, including cell adhesion [68,69], wound healing [16,70], matrix assembly [71], dimer formation [72], protein secretion [73], cytokine-dependent matrix metalloproteinase expression [74], cell differentiation [45,75], tissue injury and inflammation [76,77], cell cycle progression and mitogenic signal transduction [69]. However, most of these studies were done using *in vitro* cell culture systems. Thus, the development of genetically modified animal models was a requisite to specifically address these issues *in vivo*. The

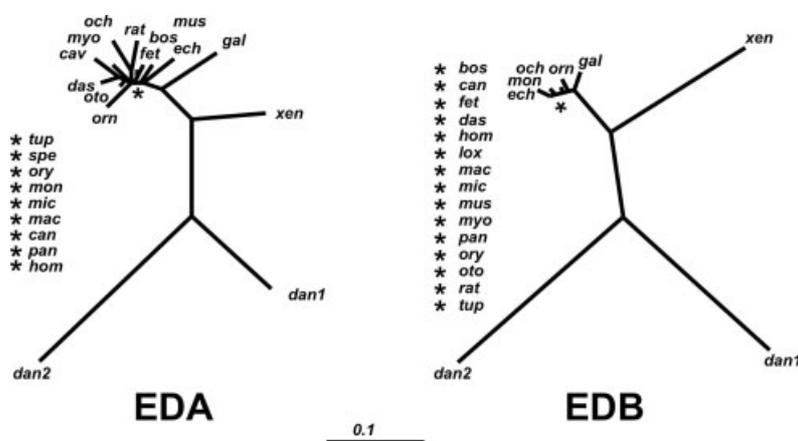


Figure 3. Unrooted phylogenetic diagram showing the relationships of the EDA and EDB domains among vertebrates. The tree was constructed by comparing the amino acid sequence of the EDA and EDB domains with the ClustalW2 software (<http://www.ch.embnet.org/software/ClustalW.html>) by the neighbour-joining method [137]. The dnd file generated was plotted using the TreeViewPPC package. The abbreviations are described in the legend to Supplementary Figure 1

importance of correct EDA splicing is strongly supported by the observation that mice either lacking or constitutively expressing the EDA exon displayed a substantially reduced lifespan [78] (Table 1).

Elevated levels of EDA cFN are found in plasma and affected tissues of patients with certain disorders, such as psoriasis, rheumatoid arthritis, diabetes and cancer, although the functional role of EDA cFN in these disease states is still obscure. Embryonic development is not possible without *FN* [3], but mice bearing single deletions of EDB or EDA are born normally [78–80]. In contrast, simultaneous deletion of both the EDA and EDB exons in the *FN* gene leads to embryonic lethality (albeit with incomplete penetrance) at E10.5 displaying severe cardiovascular defects (Table 1) (see below and [81]).

EDA cFN domain has an essential role in lung fibrosis

Fibronectin is a moderately abundant protein in blood (approximately 300–400 µg/ml and 580 µg/ml for humans and mice, respectively). Following tissue injury, pFN is extravasated from injured vessels and, together with platelets and fibrin, forms the haemostatic plug. This FN-rich clot serves as a provisional matrix to support cell migration during the wound closure process. However, mice lacking pFN undergo normal healing of cutaneous wounds [82], suggesting that cFN produced locally at the site of injury is sufficient to guide a normal healing process. Indeed, the importance of the EDA domain in cutaneous healing *in vivo* was shown in mice lacking the EDA domain (*EDA*^{-/-}), which demonstrated abnormal healing, ulceration and inflammation at the sites of wounding [78].

An important functional role for the EDA domain in the *in vitro* differentiation of fibroblasts into myofibroblasts has been suggested [45,75]. Myofibroblast differentiation and ultimate removal, when dysregulated in pathological situations, may lead to tissue fibrosis. One such threatening form of fibrosis in the lung is idiopathic pulmonary fibrosis (IPF). IPF is largely untreatable and patients ultimately die from unrelenting ECM deposition in the lung, resulting in progressive respiratory failure [83,84]. In IPF, EDA cFN is deposited before collagens in regions of active fibrosis [85], which correlates with increased expression of markers of fibroblast activation [α -smooth muscle actin (α -SMA)].

The link between EDA cFN and myofibroblast differentiation *in vivo* has been recently demonstrated using the well-described intratracheal bleomycin model of lung fibrosis. In *EDA*^{-/-} mice receiving intratracheal bleomycin, lung fibrosis was completely prevented, suggesting that EDA cFN is necessary for the development of pulmonary fibrosis [86]. Failure to develop lung fibrosis in *EDA*^{-/-} mice correlated with diminished activation of latent TGF β and decreased lung fibroblast responsiveness to active TGF β *in vitro*

(Figure 4, Table 1). Notably, *EDA*^{-/-} lung fibroblasts could undergo TGF β -induced myofibroblast differentiation, but only when plated on a matrix containing EDA cFN (Figure 4) [86]. These findings provide important new clues to the mechanisms of tissue fibrosis. The observation that EDA cFN is important for latent TGF β activation and myofibroblast differentiation supports the previously recognized crucial role of TGF β and the ECM in lung fibrosis. Further, it implicates EDA cFN in the pathogenesis of lung fibrosis. It is notable that, when the same EDA-engineered mice were used in an allergen-induced chronic asthma model, we again observed that EDA cFN is critical for myofibroblast differentiation and subsequent airway fibrosis [87].

The role of the EDA or EDB domains in α -SMA expression appears to be less important for non-fibroblast cells in other experimental models, such as vasculogenesis (Table 1). In fact, analysis of α -SMA in pericytes around blood vessels showed no differences between EDA-null, EDB-null and control mice, suggesting that neither EDA nor EDB are required for α -SMA expression in this model [88]. In addition, neovascularization in retinas, pancreatic tumours and transplanted melanomas were not affected by the absence of the EDA or EDB splice variants [88]. However, mice bearing a targeted deletion of both EDA and EDB exons display a reduced number of α -SMA-positive cells immediately surrounding the dorsal aorta, perhaps caused by a delay in the recruitment or differentiation of these cells [81] (see below). Based on the above studies, it is clear that the EDA domain is important for lung myofibroblast differentiation during experimental and possibly human lung fibrosis and that the absence of EDA cFN protects against lung fibrosis.

The role of FN and FN isoforms in thrombosis

Plasma FN participates in blood coagulation and thrombosis as a ligand of platelet surface receptors, and cross-links to fibrin. Platelets store a mixture of cFN (synthesized by megakaryocyte precursors) and pFN (endocytosed from plasma) in their α -granules [82,89], which is released and deposited on platelet surfaces only after activation by thrombin. pFN plays a role in thrombosis and haemostasis, an observation supported by numerous reports (see references in [90]). However, conclusive *in vivo* evidence of its role derives from three important observations. First, mice lacking both fibrinogen and vWF, key molecules involved in platelet aggregation and thrombus formation, still formed thrombi following ferric chloride arterial injury, thereby suggesting that FN could be a ligand supporting platelet aggregation [91]. Second, conditional pFN knock-out mice formed thrombi that continuously shed platelets (or groups of platelets) in the same model of arterial injury, reducing their

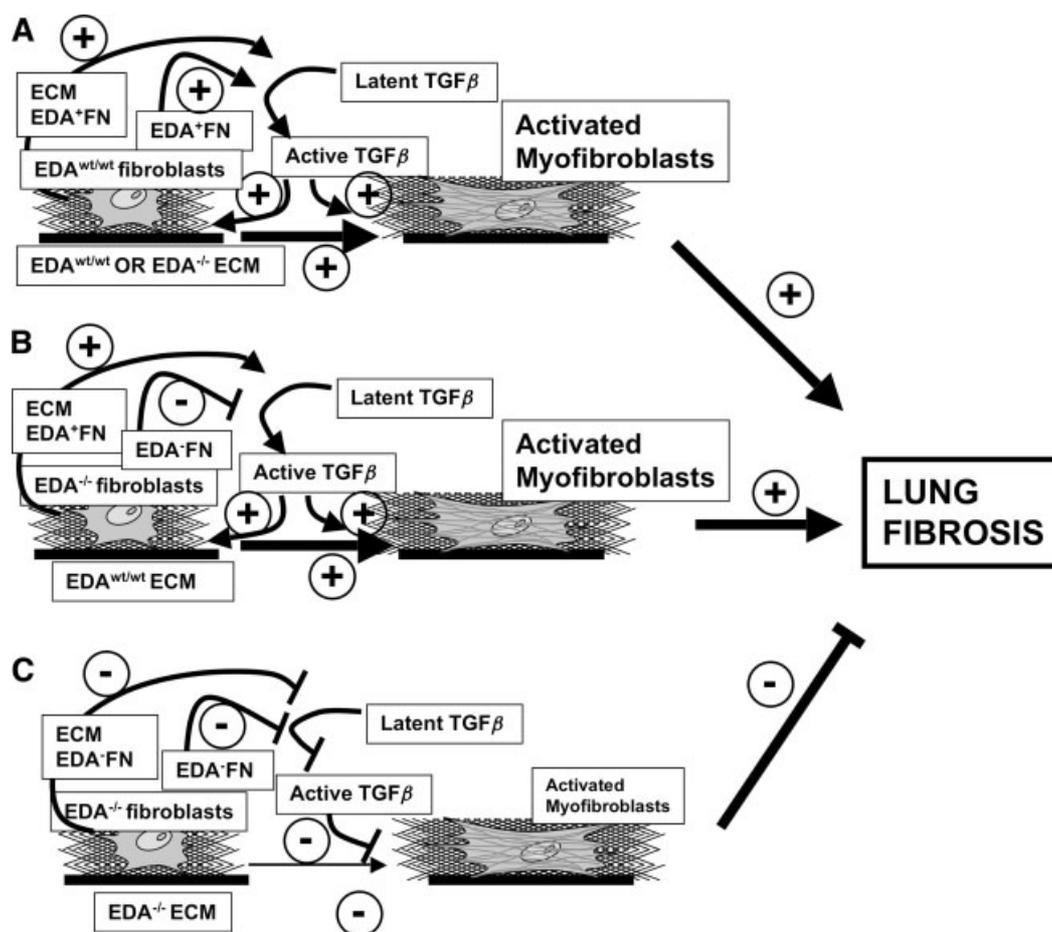


Figure 4. Role of EDA cFN in lung fibrosis. Fibroblast differentiation into myfibroblasts occurs only on the presence of EDA cFN, which could be either produced and secreted by the fibroblast [$EDA^{wt/wt}$ fibroblast (A)] or already present in the ECM, since $EDA^{-/-}$ fibroblasts differentiate into myfibroblasts only when plated on a EDA cFN (A, B). EDA cFN secreted by the cells or already present in the ECM activates latent $TGF\beta$ (A, B) and fibroblast differentiation proceeds, leading to lung fibrosis. In the absence of EDA cFN, produced either by the $EDA^{-/-}$ cells or by the ECM prepared from $EDA^{-/-}$ fibroblasts (C), the absence of latent $TGF\beta$ activation limits differentiation into myfibroblasts, preventing lung fibrosis

growth and delaying the occlusion time of the vessels [92] (Table 1). Finally, *FN*-null heterozygous mice (having reduced levels of pFN) also displayed a striking reduction in thrombus initiation and growth following ferric chloride arterial injury, a defect rescued after adding pFN back into the bloodstream [93] (Table 1). Together, these results indicate that pFN increases the stability of adherent platelet aggregates that form in response to experimental vascular injury. Since there is a considerable range in pFN concentration in humans and increases in its levels have been associated with coronary artery disease [94,95], these reports established an experimental link between pFN concentration and arterial disease in larger populations [96].

As might be expected, since pFN does not include the EDA or EDB domains, no differences in vascular thrombosis were observed in the $EDA^{-/-}$ and $EDB^{-/-}$ mouse strains [93]. However, somewhat surprisingly, the constitutive inclusion of EDA into the FN molecule in $EDA^{+/+}$ mice conferred pro-thrombotic activity, adding an extra level of complexity to fibronectin–platelet interactions [97]. These

animals, despite having only 20% of normal pFN levels, developed occlusive thrombi more quickly in ferric chloride-damaged arteries and were more susceptible to pulmonary emboli after infusion of collagen and epinephrine into the bloodstream (Table 1). *In vitro*, platelets from $EDA^{+/+}$ mice covered collagen-coated surfaces to a greater degree than wild-type platelets [97].

It is unclear why circulating EDA cFN may be pro-thrombotic, although different lines of evidence point towards a major conformational change in the FN molecule when the alternatively spliced domains are included, which could result in enhanced FN functions. Normally, pFN circulates in blood in a closed, non-active form with the RGD cell-binding loop unavailable for cell binding (Figure 5A). However, structural studies suggest that insertion of an extra domain may induce a conformational change that affects the exposure of the RGD site [98] or other epitopes [37], thereby enhancing the potential interaction between circulating FN with surface integrins on blood-borne effector cells [99–102] (Figure 5B). Since EDA cFN

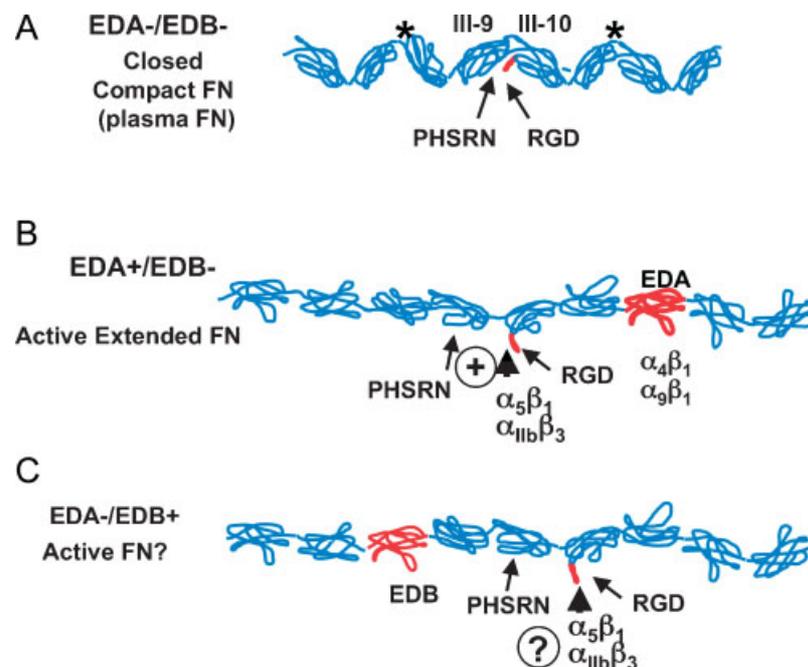


Figure 5. Schemes of the possible conformational changes of FN by the inclusion of the extra domains EDA and EDB. In the 'closed' or 'compact' conformation of pFN the RGD loop and nearby synergy sites are not available for interaction by integrins (A). The inclusion of the EDA domain in EDA cFN (B) triggers a conformational change enhancing the exposition of the RGD loop and the synergy site, and the binding of integrin receptors (indicated). The model postulates that inclusion of the EDB domain may also enhance the exposition of the RGD loop and the synergy site (C). Asterisks in pFN indicate the expected position of the EDB (left asterisk) and EDA (right asterisk) domains if inserted (A)

is more potent in promoting cell adhesion and spreading than FN lacking EDA [100], it is possible that circulating EDA cFN is able to serve as a nidus for platelet and fibrin(ogen) aggregation, resulting in enhanced thrombosis and vascular occlusion.

The inclusion of the EDA domain may trigger a global conformational change in the FN molecule, resulting in enhanced platelet integrin–FN interaction via $\alpha_{11b}\beta_3$ or other RGD-binding integrins. This hypothesis is indirectly supported by the observation that specific EDA-binding integrins (the $\alpha_9\beta_1$ and $\alpha_4\beta_1$ integrins) are not present on platelets. The main platelet integrin receptor involved in thrombus formation is $\alpha_{11b}\beta_3$, although $\alpha_V\beta_3$ and $\alpha_5\beta_1$ may also bind the RGD sequence. Global conformational changes (which may also occur due to forces applied during the platelet-activating process) result in enhanced RGD binding by integrins, and may indeed be responsible for pro-thrombotic properties of EDA cFN [35,36,103]. If true, then one would expect a similar finding in mice constitutively expressing EDB cFN (Figure 5C). This hypothesis has yet to be tested. However, the concept that EDA and EDB deficiency results in impaired RGD binding and FN function may account for the embryonic lethality of $EDA^{-/-}/EDB^{-/-}$ double-mutant mice [81].

These observations confer pro-thrombotic activity to EDA cFN and suggest that increased plasma EDA cFN levels might be a risk factor for thrombosis. Indeed, elevated plasma EDA cFN levels are present in patients with diabetes [104], vascular injury [105],

and rheumatoid vasculitis [106], all conditions which predispose to vessel thrombosis. Moreover, one recent study suggests that patients with acute stroke receiving tissue plasminogen activator (t-PA) for thrombolysis may be at increased risk for brain haemorrhage in the setting of elevated plasma cFN levels [107], indicating that circulating cFN may also predict worse outcomes for patients with vascular diseases.

Secretion of pFN from hepatocytes

pFN is synthesized by hepatocytes [108]. While the EDA and EDB exons are spliced out from the pre-mRNA in normal liver, a fraction of mRNA contains the IIICS region. Interestingly, soluble FN is secreted as a heterodimer in which one subunit must contain the IIICS segment and the other may not, as IIICS-0/IIICS⁺ and IIICS⁺/IIICS⁺ combinations [109]. Although IIICS-0 subunits are synthesized, IIICS-0 homodimers seem to be degraded along the secretory pathway [109]. Of particular note, IIICS-0 homodimers, much like circulating EDA cFN dimers, possess enhanced pro-thrombotic properties [110]. *In vivo* and *in vitro*, $EDA^{+/+}/EDA^{+/+}$ dimers produced by $EDA^{+/+}$ hepatocytes are not efficiently secreted into either plasma or culture medium and appear to be selectively degraded, perhaps due to an unfolded or misfolded conformation [111]. Consequently, plasma FN concentrations in $EDA^{+/+}$ mice are only 20–25% of that found in control animals [78,111].

It is tempting to suggest that negative selection for the pro-thrombotic forms of pFN has occurred through evolution. As such, hepatocytes may have developed at least two mechanisms to avoid the secretion of EDA⁺ or IIIICS-0/IIIICS-0 FN isoforms into the bloodstream. The first one is the total exclusion of the EDA exon from the FN mRNA by the splicing machinery [6], and the second one is the apparent intracellular degradation of these pro-thrombotic isoforms [109,111]. The pro-thrombotic properties of EDA⁺ and IIIICS-0 FNs may help explain the splicing patterns of pFN and cFN. It will be interesting to determine whether the EDB⁺ FN isoform also possesses pro-thrombotic properties.

Mice lacking regulated splicing of the EDA exon have defects in behaviour and motor coordination

Mice lacking regulated splicing of the EDA exon show no evident morphological alterations in the brain [78,112]. Surprisingly, though, each mutant strain displayed different CNS-related defects, suggesting a different role for each FN isoform (Table 1). EDA^{+/+} mice displayed a reduction in horizontal exploratory activity, but not vertical exploratory activity, in the open field test. Conversely, in the same test, EDA^{-/-} mice showed a decrease in vertical exploratory activity but not in horizontal exploratory activity [112]. Moreover, only the EDA^{-/-} mice showed significant impairment in the accelerating rotarod test, a test that requires enhanced motor coordination skills and gives an idea of impaired cerebellar function. Therefore, regulated splicing of the EDA exon appears to be essential for normal brain function and behaviour in mice, and perhaps in other species. Although the reasons are unclear, constitutive presence or absence of EDA cFN may affect fine-tuning during the migration of neurons, axons and dendrites in the central nervous system. This is consistent with the previously-reported role for FN in regeneration and migration of axons in the central nervous system [113,114]. On the contrary, no behavioural alterations were observed in EDB^{-/-} mice [79], perhaps suggesting a less critical role for this isoform in brain development and function.

Although further anatomical, behavioural and electrophysiological studies are necessary to better understand the neurological basis of the motor impairments described above, these behavioural alterations may represent a disadvantage for mice living under conditions where natural selection works. While purely speculative, this might account in part for the strikingly high degree of conservation of the EDA and EDB exons among species (see Supplementary Figures 1 and 2) and the conserved pattern of expression of these splice variants [2,20,99].

EDA cFN in atherosclerosis

Abundant evidence links FN and its isoforms to the development of atherosclerosis. Atherogenesis

involves a series of events where strong interaction with ECM is required, such as recruitment of blood monocytes to the arterial intima, maturation to tissue macrophages, lipid accumulation leading to foam cell formation, and smooth muscle cell migration from the arterial wall [115]. Notably, lipid accumulation does not occur and foam cells do not form without stable monocyte interactions with tissues, suggesting the importance of specific ECM-dependent signalling [116]. Considerable amounts of FN are present in the normal arterial wall, and it is strictly devoid of the EDA and EDB domains. However, in atherosclerotic lesions and in experimentally induced thickening of the aorta, there is a marked increase in total and EDA cFN adjacent to smooth muscle cells [117,118]. One possible role for EDA cFN in this context may be to activate toll-like receptor (TLR) 4 [77], thereby triggering nuclear translocation of nuclear factor (NF)- κ B, a molecule central to inflammation and atherogenesis [119]. However, the precise role of EDA cFN in the development and progression of atherosclerosis was unclear prior to elegant studies in EDA^{-/-} mice crossed with the atherosclerosis-prone ApoE^{-/-} mice [80] (Table 1).

In these studies, ApoE^{-/-}/EDA^{-/-} mice developed smaller atherosclerotic lesions in the aortic tree than ApoE^{-/-} control animals. In an *in vitro* foam cell assay, macrophages derived from ApoE^{-/-}/EDA^{-/-} mice accumulated less modified cholesterol than control macrophages, suggesting a role for EDA cFN in plasma lipid metabolism and foam cell formation. However, a recent report questions whether it is the regulation of FN splicing, and not the splice variant produced, that is more important in atherosclerosis [120]. In this series of experiments, aged EDA^{-/-} or EDA^{+/+} mice on a pure C57Bl/6 genetic background were fed an atherogenic diet (Table 1). As one might expect, EDA^{-/-} mice developed smaller and fewer atherosclerotic lesions associated with decreased macrophage cholesterol uptake. Surprisingly, however, EDA^{+/+} mice *also* demonstrated a significant reduction both in lesion size and frequency, as well as macrophage cholesterol uptake [120]. The similar findings between EDA^{-/-} and EDA^{+/+} mice support the possibility that a common mechanism related to the genetically introduced abrogation of EDA alternative splicing might affect some of the processes involved in plaque formation. Although the molecular mechanisms for these observations have not yet been defined, they highlight the critical role of alternative splicing regulation in disease pathogenesis.

Functional role of the EDB domain

Although more than 20 years have passed since the identification of the EDB exon [17], the biological function(s) of FN isoforms containing this domain remain unknown. One attempt to elucidate the role of the EDB exon in the early years of gene targeting

unfortunately produced complete null mutations of the *FN* gene (due to the presence of the neomycin cassette in the flanking intron), showing a phenotype similar to that previously observed after the targeted mutation of the *FN* gene [121]. Subsequently, a second strain of *EDB*^{-/-} mouse was generated [79]; these mice developed normally and were fertile. Interestingly, in this strain no significant phenotype was observed *in vivo*, even after analysing a number of different models where the *EDB* was previously thought to participate, such as behaviour, angiogenesis, thrombosis, organogenesis, tumorigenesis and healing of bone fractures [79,88,93]. However, a mild *in vitro* effect in matrix assembly and proliferation was observed in *EDB*^{-/-} embryonic fibroblasts (MEF) [79] (Table 1). These cells grew more slowly and produced fibrils that were shorter and thinner than those deposited by control MEF. The putative role for *EDB* in *FN* matrix assembly agrees with a previous report showing that *EDB* cFN is incorporated more efficiently into the ECM [71]. In an analogous manner, *EDA*^{+/+} MEF grow at a faster rate than those prepared from *EDA*^{-/-} or wild-type embryos (A. F. Muro, unpublished observations), supporting previous *in vitro* data that favour a role for the *EDA* domain in cell proliferation [69]. However, no *in vivo* correlate has been identified in either *EDB*^{-/-}, *EDA*^{-/-} or *EDA*^{+/+} animals [78,79], suggesting that other influences on cell proliferation may compensate for a minor modulatory role of *EDA* and *EDB* cFN.

Despite the absence of an obvious phenotype in the *EDB*^{-/-} mice, the extremely high degree of amino acid sequence conservation among species (~95% among 22 different vertebrates; see Supplementary Figure 2, available at <http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2388.html>) highly suggests an important function for this segment that may become evident under other pathophysiological conditions or in response to external stress. Indeed, a similar situation occurs in tenascin-C knock-out mice. In these animals, lack of tenascin-C results in no abnormalities in development, life span or fertility [122,123]. However, well-defined phenotypes were observed using specific models, such as corneal wounding, behavioural tests [122–125] and more acute examination of brain neurochemistry and neuromuscular junctions [126–128], to name but a few. Thus, further investigation into the role of *EDB* cFN in pathophysiological or stressed models is necessary to better identify potential roles for this splice isoform.

EDA and EDB combined (double) knock-out mice

EDA cFN and *EDB* cFN are highly expressed in the presence of tissue and vascular remodelling. They are found around embryonic vessels but are absent in normal arteries and veins of adult humans or mice [16,129]. The domains reappear during pathological

tissue regeneration and angiogenesis, such as in the processes of liver regeneration, wound healing and tumour development.

With the aim of determining *in vivo* *EDA* and *EDB* functions, a mouse strain with the simultaneous deletion of both domains was generated recently [81]. Mice harbouring the *EDA*^{-/-}/*EDB*^{-/-} double mutation were embryonic lethal, with about 80% penetrance, in a mixed c129 and C57Bl/6 genetic background (Table 1). No defect in production or cell-surface association of *FN* was observed, suggesting that lack of *EDA* and *EDB* together accounted for the defect in viability. Most of the affected embryos had a variety of cardiovascular defects, reminiscent of, but milder than, the phenotype observed in *FN*-null embryos [3]. Detailed analysis of *EDA* and *EDB* cFN expression showed their presence around the dorsal aorta in wild-type littermates at E9.5 and E10.5, associated with α -SMA expression, suggesting that *EDA* and *EDB* cFN may play a role in the development of aortic vessels during embryogenesis. *EDA*^{-/-}/*EDB*^{-/-} double mutants had a reduction in the number of α -SMA positive cells at E9.5 with an abnormal rounded morphology, but relatively equal numbers of α -SMA-expressing cells by E10.5, suggesting perhaps a defect in recruitment or in differentiation [81].

Instead of forming distinct vessel tubes, small vessels in *EDA*^{-/-}/*EDB*^{-/-} double mutant mice were not formed properly. The formation of endothelial cell sheets instead of tubes may be due to defective endothelial apical-basolateral polarity in the absence of *EDA* and *EDB* [81]. Polarized secretion of both *FN* and *EDA* cFN isoforms have been reported in endothelial cells and in airway epithelial cells, respectively [73,130]. Therefore, vascular defects of the double-mutant mice could be related to a defect in endothelial cell polarity, resulting in aberrant endothelial tube formation.

Since the absence of either the *EDA* or *EDB* exons did not produce any obvious defects in angiogenesis or in the levels of α -SMA-positive cells around blood vessels in embryos or in regions of neo-angiogenesis [78–80,88,120], it is reasonable to hypothesize that the roles of the *EDA* and *EDB* domains may be different, although they may have some redundant function. Furthermore, the presence of only one domain is sufficient to attain normal blood vessel development, which is absent after the concerted deletion of both domains.

While the phenotypic ramifications of knocking out both the *EDA* and *EDB* domains of *FN* are not fully understood, we are able to appreciate some of the implications of this model. First, it is clear that *FN*, and more specifically the *EDA* and *EDB* domains, are critical for heart and blood vessel development. Indeed, the presence of at least one of the extra domains is required for normal cardiac and vascular development.

Further, it appears that EDA and/or EDB cFN is necessary for the recruitment or differentiation of vessel-associated α -SMA cells. It is also possible that genetic background of the animal may influence the role of FN in development; *FN*-null mice created on pure C57Bl/6 mice had less severe defects in cardiac development, whereas the same mutation on a 129/Sv background resulted in severe defects in myocardial and endocardial morphogenesis [131,132]. Further detailed *in vivo* and *in vitro* analysis of this strain will provide insights in the molecular mechanisms underlying the roles of the EDA and EDB domains in the process described above.

Concluding remarks

The ECM is clearly a dynamic structure that provides instructive signals to cells during homeostasis as well as during disease states. FN, a major component of the ECM, has been extensively studied in an attempt to determine the functions of the >20 possible isoforms in humans. Through rigorous investigation, it is becoming clear that the alternatively spliced EDA and EDB domains possess distinct properties that allow for variation in function. Transgenic mice bearing targeted mutations in the FN gene display, in most cases, clear phenotypes; however, these phenotypes may be drastically different under conditions of stress or disease. The strikingly high conservation of both exons during vertebrate evolution and the conserved patterns of splicing in different organisms strongly support the idea of evolutionarily conserved functions. Moreover, it suggests that regulated splicing of the *FN* gene confers adaptive advantages in mice, and perhaps in humans.

More precise definition of the functions of the alternatively spliced FN isoforms will await further detailed *in vivo* and *in vitro* studies of the existing models and the generation of new engineered mouse strains having isoform-specific mutations in the *FN* gene. Those analyses will help us to better elucidate the molecular interactions and signalling pathways activated by alternatively-spliced isoforms of FN, the role of alternative splicing mechanisms in modulating cell and organism phenotype, and possibly the identity of currently unknown mechanisms associating FN functions and polymorphisms to pathological states in humans.

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Supplementary Information

Supplementary material may be found at the web address: <http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2388.html>

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