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## Fibronectins in Vascular Morphogenesis

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### Abstract

Fibronectin is an extracellular matrix protein found only in vertebrate organisms containing endothelium-lined vasculature and is required for cardiovascular development in fish and mice. Fibronectin and its splice variants containing EIIIA and EIIIB domains are highly upregulated around newly developing vasculature during embryogenesis and in pathological conditions including atherosclerosis, cardiac hypertrophy and tumorigenesis, however, their molecular roles in these processes are not entirely understood. We review genetic studies examining functions of fibronectin and its splice variants during embryonic cardiovascular development, and discuss potential roles of fibronectins in vascular disease and tumor angiogenesis.

### Keywords

alternative splicing; angiogenesis; cardiovascular development; endothelium; integrins; pericyte; vascular smooth muscle

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Blood vessel formation is an essential developmental process required for the survival of all vertebrate organisms [1]. Formation of vascular networks involves extensive interactions of endothelial cells with their environment. These interactions are positively and negatively modulated through the binding of endothelial cell integrins to extracellular matrix proteins, regulating vessel number, size, permeability, vessel sprouting and regression [2,3]. Timely activation and cessation of vessel formation is necessary to enable vascular networks to grow and change with the developing organism during embryogenesis as well as to respond to physiological and pathological instances when formation of new blood vessels is needed in the adult. One of the key steps in the formation of a functional vascular tree is the recruitment and association of endothelial and perivascular cells (also called pericytes and/or vascular smooth muscle cells) [4–6]. This review will highlight the emerging role of fibronectin in mediating the crosstalk between endothelial and perivascular cells and will discuss functions of fibronectins gleaned from genetic knockout studies.

### Functions of fibronectin in vascular morphogenesis

Fibronectin (FN) is an extracellular matrix protein, essential for blood vessel morphogenesis; it is incorporated between endothelial and perivascular cells. Genetic studies demonstrate the requirement for FN in cardiovascular development: absence of FN leads to embryonic lethal vascular defects [7–9]. These defects vary in severity depending on the genetic background indicating the existence of genetic modifier(s) acting in concert with FN during vascular morphogenesis [7]. In FN-null embryos derived from 129S4 strain of mice, endothelial cells are present within the embryo-proper but are not assembled into dorsal aortae, indicating the

requirement for FN in vasculogenesis, assembly of blood vessels directly from endothelial precursors. In embryos derived from the C57BL/6J genetic background, dorsal aortae form in the absence of FN; however, the endothelial linings of the dorsal aortae and the heart appear collapsed and separated from the apposing tissue, suggesting defects in vessel lumen formation [7]. This phenotype could also indicate that absence of FN causes defective interactions between endothelial and mesenchymal cells (in the case of dorsal aortae) and endocardial and ventricular myocardial cell layers (in case of the heart). These observations suggest that the role(s) of FN in vascular morphogenesis may be in modulating the formation of vascular lumens and/or modulating interactions between endothelial and perivascular cells. Importantly, formation of vascular networks is also defective in FN-null embryoid bodies (EBs) indicating that vascular defects in FN-null embryos reflect a direct role of FN in vascular morphogenesis and are not simply a consequence of secondary effects resulting from decreased haemodynamic force due to cardiac insufficiency in these embryos [10,11].

The severity of cardiac defects in FN-null embryos also depends on the genetic background. In FN-null embryos derived from 129S4 genetic background, heart formation is blocked before cardiac progenitors on either side of the embryonic midline fuse to form a tube, resulting in *cardia bifida*. On a C57BL/6J background, the heart tube forms in the absence of FN [7]. Our genetic mapping studies identified a one-megabase region on chromosome four, which contains modifier(s) regulating the severity of heart defects in FN-null embryos [12]. This modifier(s) may modulate the function of FN during blood vessel morphogenesis as well.

Experiments performed on the zebrafish *natter* mutant, a null mutation in the FN1 gene, also showed that FN1 is essential for the formation of a single heart tube from the bi-lateral cardiac primordia [13]. These experiments demonstrated that FN is essential for cell polarity within cardiac precursors. In the absence of FN, components of tight junction and adherens junction complexes are downregulated and mislocalized with respect to each other [13]. These defects in cellular polarity presumably lead to defective migration of bi-lateral cardiac progenitors at the midline toward each other to form a single cardiac vessel. It is likely that in FN-null mouse embryos from 129S4 genetic background, the absence of FN leads to altered cardiac cell polarity giving rise to *cardia bifida*. Interestingly, defects in heart development in *natter* mutants are also dependent on genetic background, leading to formation of a single heart tube in some *natter* fish [13]. This suggests that the modifier(s) interacting with FN in heart development may be evolutionarily conserved between species. Since early heart tube in either a zebrafish or a mouse embryo resembles a blood vessel (with endothelial cells forming a tube on the inside and a layer of muscle cells on the outside), determining the identity of genetic modifier(s) interacting with FN during heart development is most likely to provide further insights into the function of FN during vascular morphogenesis.

Analysis of mutations in FN and its main receptor, integrin  $\alpha5\beta1$ , in zebrafish and *Xenopus* strongly suggested that this receptor-ligand pair functions in establishing and/or maintaining cell polarity [14,15]. In *Xenopus*, FN and integrin  $\alpha5\beta1$  are required for polarized lamellipodial protrusions and for polarized orientation of mitotic spindles during axis elongation through convergent extension [15]. Integrin  $\alpha5$ -null mutants in zebrafish exhibit loss of polarized somite boundaries and randomized centrosome positioning [14]. Interestingly, recent studies showed that genetic ablation of mouse ZO-1, a component of tight junctions in mature epithelia, leads to severe defects in embryonic and vascular morphogenesis, resembling defects observed in FN-null embryos [16]. ZO-1 is one of the three ZO (zonula occludens) proteins, important for localization and organization of claudins in the process of tight junction formation during establishment/maintenance of apicobasal cell polarity [17,18]. ZO-1 null mouse mutants exhibit syncytial yolk sac vasculature, defects in vascular remodeling both in yolk sac and head vasculature, shortened anterior-posterior axis and absence of turning. It would be interesting to determine whether FN is required for localization of ZO-1 to endothelial cell-cell junctions

and whether FN functions in establishing and/or maintaining endothelial cell polarity during vascular morphogenesis.

Collapsed blood vessel lumens observed in FN-null embryos could potentially be due to defective endothelial cell polarity. Experiments using three-dimensional culture systems *in vitro* and in zebrafish showed that endothelial lumen formation involves polarized movements of vacuoles followed by their fusion and requires the function of genes involved in cell polarity such as Cdc42, Rac1, Par3, Pak2, Pak4 [19–21]. The functions of these proteins could be activated following the binding of FN to endothelial integrin  $\alpha 5\beta 1$  [22]. Taken together, these studies suggest that FN may function in vascular morphogenesis through mediating endothelial cell polarity.

## Fibronectin-binding Integrins

Some, and possibly most, of FN function(s) in vascular morphogenesis are mediated by integrin  $\alpha 5\beta 1$ . Integrin  $\alpha 5\beta 1$  is expressed by vascular endothelial cells and binds FN [23]. Genetic studies demonstrated that among all integrin alpha-encoding genes, ablation of integrin  $\alpha 5$  produces the most severe vascular defects; these defects are slightly milder but overall comparable with defects observed in FN-null embryos [3]. Similar to embryos lacking FN, integrin  $\alpha 5$ -null embryos display dilated and improperly patterned yolk sac vessels [24]. Vascular networks in the heads of integrin  $\alpha 5$ -null embryos are less intricate, exhibiting decreased vessel branching and sprouting suggesting that, similar to FN, integrin  $\alpha 5$  is required for angiogenesis [10]. *In vitro* experiments using integrin  $\alpha 5$ -null embryonic stem (ES) cells showed decreased vascular network formation in  $\alpha 5$ -null EBs compared with controls [10]. In addition, teratomas generated using  $\alpha 5$ -null ES cells were largely devoid of ES cell-derived blood vessels suggesting that integrin  $\alpha 5$  may also play a role in vasculogenesis [25]. Importantly, these experiments demonstrated that, similar to FN, integrin  $\alpha 5$  is required for vascular morphogenesis.

Integrin  $\alpha 5\beta 1$  binds FN by interacting with the RGD sequence within the Xth type III repeat of FN and this binding is greatly facilitated by the synergy site located within the IXth FN type III repeat [26]. The RGD sequence is required for the binding of FN to  $\alpha 5\beta 1$ , as well as for  $\alpha 5\beta 1$ -mediated FN matrix assembly and signaling [27]. Mutation of the aspartic acid to glutamic acid in this sequence, (RGD to RGE) abolishes these functions. Generation of mice in which the RGD sequence was replaced with the RGE sequence provided further insights into the function of FN-integrin interactions in vascular morphogenesis [28].

The gross appearance and the onset of embryonic lethality in RGE/RGE mutant mice is very similar to  $\alpha 5$ -null embryos suggesting that the binding of FN to integrin  $\alpha 5\beta 1$  through the RGD motif mediates most, if not all, of the functions of integrin  $\alpha 5\beta 1$  during embryogenesis and vascular morphogenesis [28]. Interestingly, similarly to the phenotypic variability in vascular morphogenesis observed in FN-null and integrin  $\alpha 5$ -null mutants, the severity of phenotype in RGE/RGE embryos is also dependent on the genetic background: it is milder in C57BL/6J strain of mice (R. Fassler, personal communication). This suggests that the modifier(s) affecting FN function during vascular morphogenesis is(are) genetically downstream of integrin  $\alpha 5\beta 1$ -FN interactions or act in a parallel pathway. A more detailed analysis of vascular development in these and  $\alpha 5$ -null embryos is required to determine more precisely the role of integrin  $\alpha 5\beta 1$ -FN interactions during vascular morphogenesis.

The vascular phenotype of FN-null mutants is more severe than the phenotypes of either  $\alpha 5$ -null or RGE/RGE mutants, suggesting that in addition to signaling through integrin  $\alpha 5\beta 1$ , FN has other functions required for vascular morphogenesis. Integrins  $\alpha 4\beta 1$ ,  $\alpha v\beta 3$ , and  $\alpha v\beta 5$  are expressed on endothelium and can bind FN, and while, individually,  $\alpha 4$  or  $\alpha v$ -containing

integrins are not required for early vascular morphogenesis (integrin  $\alpha 4$ -null and integrin  $\alpha v$ -null embryos show defects in head vessels after e10.5) [29,30], the deletion of FN negates interactions of all of the above integrins on endothelial cells with surrounding FN. The absence of these interactions could give rise to the increased severity of vascular defects in FN-null embryos compared with  $\alpha 5$ -null mutants. Alternatively, the function of FN in pathways other than integrin signaling could be important for vascular morphogenesis. These functions may include (but are not limited to) binding to and presenting growth factors such as VEGF [31, 32]. Matrix-bound and soluble VEGF plays distinct and important roles both in embryonic and tumor angiogenesis [33–35].

Interestingly, *in vitro* three-dimensional culture experiments demonstrated that FN fibrillogenesis is required for vascular network formation [36,37]. However, vascular networks do form in FN-null or integrin  $\alpha 5$ -null embryos, suggesting that neither FN nor FN fibrillogenesis are essential for this process [7,10]. Moreover, the presence of FN fibrils is not sufficient for vascular development, since FN fibrils are formed relatively normally in RGE/RGE mutant embryos but these embryos still develop severe cardiovascular defects [28].

Integrins containing the  $\alpha v$  subunit also bind FN and play overlapping but distinct roles during embryonic development compared with integrin  $\alpha 5\beta 1$  [29,38,39]. Mice lacking both integrin  $\alpha v$  and integrin  $\alpha 5$  subunits fail to proceed through gastrulation [39], suggesting that  $\alpha v$  and  $\alpha 5$ -containing integrins perform distinct functions *in vivo*. *In vitro*,  $\alpha v$ -containing integrins can compensate for the absence of  $\alpha 5$  in some assays (e.g., FN fibrillogenesis) [24]. However,  $\alpha v$ -containing integrins cannot perform all of the  $\alpha 5\beta 1$  functions *in vivo*. Specifically, the presence of  $\alpha v$ -containing integrins (these integrins bind and assemble FN fibrils) does not rescue the vascular defects in  $\alpha 5$ -null embryos [24]. Interestingly, endothelial cells in which all  $\beta 1$ -containing integrins have been deleted showed that these cells still bind to FN (while they are unable to bind collagen or laminin, substrates for  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrins) [40]. The binding of FN to  $\beta 1$ -null endothelial cells is mediated by  $\alpha v$ -containing integrins  $\alpha v\beta 3$  and  $\alpha v\beta 5$ ; however, the binding of  $\alpha v$ -containing integrins to FN is not sufficient for vascular morphogenesis and does not rescue embryonic lethality in these embryos [40]. Taken together these studies indicate that engagement of  $\alpha v$ -containing integrins by FN is not sufficient to elicit intracellular signaling pathways orchestrating proper vascular morphogenesis. Phenotype of Tie2-Cre;  $\beta 1^{\text{flox/flox}}$  mutants, in which all  $\beta 1$ -containing integrins are deleted in endothelial cells closely resembles the phenotype of integrin  $\alpha 5$ -null mutants [40] and suggest that binding of FN to endothelial-expressed integrin  $\alpha 5\beta 1$  mediates the function(s) of FN in vascular morphogenesis.

## Functions of fibronectin splice variants in vascular morphogenesis

FN is a large modular glycoprotein composed of repeating subunits (Fig. 1) [27]. Biochemical studies in the past three decades have provided us with a general map of functions of some of the individual domains or their combinations [41]. Investigation of the function(s) of FN splice variants containing EIIIA and/or EIIIB alternatively spliced exons has received the most attention and has been recently extensively reviewed [42]. Here we will provide insights into the function of these splice variants from the most recent genetic studies.

Analysis of embryonic phenotypes of EIIIA and EIIIB-double-null mutants led to a number of key findings, which will help to decode the function of EIIIA- and EIIIB-containing FNs in vascular morphogenesis. The sequences of EIIIA and EIIIB are only 29% identical to each other, while individual EIIIA and EIIIB sequences are nearly 100% conserved among vertebrates, suggesting that inclusion or exclusion of EIIIA and EIIIB domains gives rise to FN proteins with distinct functions. However, mice lacking either EIIIA or EIIIB exons are

viable and fertile, suggesting that embryonic vascular development proceeds fairly normally in the absence of one of the alternatively spliced exons.

Embryos lacking both EIIIA and EIIIB exons die between e9.5 and e10.5 of gestation from severe cardiovascular defects [43]. We observed variability in the severity and the type of vascular defects in the double-null embryos probably due to the fact that the double-null mutation was generated in mouse ES cells derived from a mixed 129P2/C57BL/6J genetic background, and segregation of genetic factors in the EIIIA/EIIIB double-null embryos has contributed to this phenotypic variability [43]. In the most severely affected EIIIA/EIIIB double-null embryos vasculature appeared syncytial in the yolk sac, cephalic and trunk vascular beds, similar to FN-null mutants. Since FN protein is expressed in EIIIA/EIIIB double-nulls to a similar extent as in controls, vascular defects in EIIIA/EIIIB double-null mutants are specifically due to the absence of both EIIIA and EIIIB domains of FN. Staining of embryos and yolk sacs with an antibody recognizing *Pecam1* (a marker of vascular endothelial cells) showed that instead of an organized network of small and large vessels, mutant embryos contained sheets of endothelial cells with intervascular spaces that were few in number and diminished in size [43]. A similar phenotype has been observed in numerous other mutants including strains with genetic deletions in *VEGF-A*, *Ang1*, *TGF $\beta$ 1*, or *bFGF* [44–47]. Since binding of integrin  $\alpha 5\beta 1$  to FN mediates signaling of a number of growth factors including *PDGF-BB*, *EGF*, and *bFGF* [48], it is possible that FN and its splice variants modulate growth factor signaling during vascular morphogenesis. Reagents to detect *in vivo* activation of some of these growth factor pathways (e.g. *TGF $\beta$ 1*) are now available (e.g. antibodies recognizing phosphorylated *Smads*) and will allow testing of this hypothesis in the future.

Formation of vascular sheets instead of networks in EIIIA/EIIIB double-null embryos could also be due to the possibility that the absence of both EIIIA and EIIIB altered extracellular matrix mechanical properties promoting cell-cell adhesion at the expense of cell-matrix interactions and led to the formation of vascular sheets instead of vessels with distinct diameters [49]. Cell binding and migration cause stretching of extracellular matrix proteins, including FN [50,51]. The degree of rigidity or elasticity of extracellular matrix alters cellular responses in addition to, and also independent of, chemical composition of the matrix. For example, at identical substrate densities, plating endothelial cells on softer surfaces promotes cell-cell interactions and network formation, whereas plating endothelial cells on rigid surfaces promotes cell-matrix interactions, endothelial cell dispersal and inhibition of vascular network formation. Therefore, it would be interesting to determine whether deletion of both EIIIA and EIIIB leads to altered mechanical properties of vascular extracellular matrix, promoting formation of vascular sheets instead of distinct vessels. Alternatively, inclusion of EIIIA and EIIIB exons into FN proteins may have altered relative orientations between FN domains, leading to suboptimal presentation of the *RGD* motif (or other domains) to cells, thereby causing diminished integrin activation.

EIIIA and EIIIB domains may engage specific receptors on endothelial and/or perivascular cells promoting the functions of these domains in vascular development. The EIIIA domain of FN has been reported to bind integrins  $\alpha 4\beta 1$  and  $\alpha 9\beta 1$  as well as *Toll-4* receptor *in vitro* [52–54]; However, *in vivo*, the biological importance of these interactions is not yet clear [55]. It is possible that vascular defects in the heads of EIIIA/EIIIB double-null embryos are due in part to defective interactions of EIIIA with  $\alpha 4\beta 1$ , since this integrin is required for cephalic vascular morphogenesis - it mediates interactions of neural crest-derived pericytes with the endothelium of cephalic vessels [30]; Since  $\alpha 9\beta 1$  is important for the development of lymphatics [55] it remains to be investigated whether embryos in which EIIIA/EIIIB double-null mutation is embryonic lethal have altered specification of lymphatic endothelium. The binding partners of EIIIB are not known. Identification of specific morphogenetic abnormalities in EIIIA/EIIIB double-null embryos will lead to understanding of specific



biological properties affected by the absence of EIIIA and/or EIIBB and aid in identification of cell types whose biological function is influenced by EIIIA and/or EIIBB-containing FNs. These studies may lead to identification of new receptors for EIIIA and EIIBB (and/or confirmation of the proposed EIIIA receptors) as well as to a better understanding of the molecular roles of EIIIA- and EIIBB-containing FNs in vascular morphogenesis.

Most of the EIIIA/EIIBB double-null embryos develop hemorrhages, suggesting that the absence of both EIIIA and EIIBB leads to rupture and/or leakiness of vessels. However, not all vessels appear abnormal. For example, while perineural vascular plexuses in the head and trunk appear syncytial, intersomitic vessels seem to form normally. In addition, cephalic vessels in mildly affected double-null mutants appear to end abruptly (giving rise to blunt-ended vessels) and some head vessels appear to be acutely constricted. Such defects were not found in other embryonic locations in these mutants [43]. Currently, it is not clear why the absence of both EIIIA and EIIBB affects only a subset of vessels. A more detailed investigation into expression patterns of EIIIA and EIIBB FN mRNA and protein in e8.5 and e9.5 embryos will aid in the identification of the type and location of defective vessels in EIIIA/EIIBB double-null mutants, and will likely point to potential mechanistic functions for these splice variants.

## Interactions of mural and endothelial cells

Immunohistochemical analyses using limited amounts of antibodies directed to either EIIIA or EIIBB, or recognizing all forms of FNs, demonstrated that FN proteins are enriched around dorsal aortae and branchial arch arteries within the anterior trunk of embryos (dorsal to the heart) at e10.5 [43]. Dorsal aortae are the first embryonic vessels to be invested with alpha smooth muscle actin ( $\alpha$ SMA)-expressing VSMCs [56]. In addition, prior experiments suggested that EIIIA-containing FNs may be important in facilitating differentiation of VSMC phenotype [57]. Our experiments showed a three-fold decrease in the number of  $\alpha$ SMA-expressing VSMCs around dorsal aortae in the mutants [43]. These results suggested that EIIIA and/or EIIBB splice variants of FN are important in a process of formation of aortic VSMC layer. Their role may be in any of the following biological processes: 1) migration of VSMC progenitors to the dorsal aorta; 2) differentiation of perivascular cells to adopt aortic VSMC characteristics; 3) proliferation and/or survival of VSMCs or their precursors. FN and its receptors are known to play a role in all of these types of processes *in vitro* and *in vivo*. Interestingly, the absence of all FN forms leads to defective association of aortic endothelial cells with the adjacent mesenchyme [7]. This observation further strengthens the notion that FN and its splice variants are important for endothelial-perivascular cell interactions during morphogenesis of the vascular tree.

In the past few years, there has been a considerable advance in our understanding of mechanisms important for differentiation of pericytes/VSMCs and a number of genetic studies clearly showed the role for Notch and TGF $\beta$  signaling in this process [4,58–60]. Genetic and fate-mapping studies in mice and chicks identified a number of embryonic sites that contain precursors to VSMCs [61]. However, the mechanism of recruitment of VSMC progenitors from their places of origin to blood vessels is not well understood. Recent studies showed that PDGF-B (platelet-derived growth factor B) is important for the recruitment of VSMCs to blood vessels in normal embryos [62] and in a subcutaneous tumor model using T421 fibrosarcoma cells [63]. Nevertheless, whether PDGF-B functions in directed migration of precursor cells toward endothelial tubes remains unknown [4,63].

Recent studies showed that VSMCs of a segment of aorta located dorsal to the heart are derived from the hypaxial myotome of somites expressing transcription factor Pax3 (but not from Pax3-expressing neural crest cells) [64]. These studies showed that somite-derived cells expressing EGFP knocked into the Pax3 locus migrate to dorsal aortae to form VSMCs expressing  $\alpha$ SMA

[64]. However, the expression of Pax3 ceases upon the transit of these cells toward the dorsal aortae [64]. Future experiments such as genetic fate-mapping studies using mice expressing Cre recombinase driven by Pax3 promoter (Pax3-Cre) will be necessary to delineate the role (s) of EIIIA and/or EIIIB in the process of recruitment of VSMC progenitors to dorsal aortae. In addition, since EIIIA and EIIIB-containing FNs are also expressed around branchial arch arteries, it would be interesting to determine whether the absence of these splice variants in the double-null mutants leads to defective recruitment of VSMCs to these vessels as well. Unlike VSMCs of dorsal aortae, VSMCs of branchial arches are derived from a subpopulation of neural crest cells termed cardiac neural crest [65]. These cells originate from the dorsal aspect of the neural tube between the otic vesicle and the posterior boundary of the third somite. Following their migration, these neural crest cells are recruited to the branchial arch arteries and form a VSMC layer. Future fate-mapping studies using Wnt1-Cre and/or P0-Cre strains in conjunction with Rosa26-lacZ reporter strain [65] will be necessary to determine the role of EIIIA and/or EIIIB in the formation of VSMC layer of branchial arch arteries. Such fate-mapping studies will also provide insights into the role of FN during various stage(s) of arterial VSMC development.

*In situ* hybridizations in chicken embryos detecting mRNA of FN and its splice variants showed that endothelial cells of extraembryonic blood vessel secreted FN during embryogenesis [66], however, it remains unclear whether any embryonic vessels express FN mRNA. If endothelial cells of dorsal aortae also secrete FNs, than one could envision that expression of FNs by the aortic endothelial cells may facilitate recruitment of the nearby mesenchymal cells to the aortae or may act upon mesenchymal cells adjacent to dorsal aortae to induce their differentiation. This model implies that perivascular cells and/or their precursors express receptors for FN and/or EIIIA and EIIIB splice variants. However, if instead perivascular cells or their precursors produced FN proteins, one might hypothesize that these proteins are involved in bi-directional communication between perivascular and endothelial cells. Past genetic data showed that binding of growth factors produced by perivascular cells to their receptors expressed on endothelial cells is essential for vascular morphogenesis [67]. The most well-known examples of these interactions are VEGFs and angiopoietins produced by the perivascular cells and their receptors, VEGFRs and Tie2, expressed on endothelial cells, respectively [67]. If mRNAs for FN and its splice variants are produced by the mesenchymal cells adjacent to dorsal aortae, it would be expected that the receptors for FN and its splice variants are expressed on endothelial cells. Indeed, during vascular remodeling following balloon catheterization, FN and its splice variants are expressed by the proliferating VSMCs and receptors known or believed to bind FN's RGD or EIIIA sequences ( $\alpha 5\beta 1$  or  $\alpha 4\beta 1$  and  $\alpha 9\beta 1$ ) are expressed by endothelial cells [68–70]. Taken together, detailed examination of spatial and temporal expression of FN mRNAs and proteins during embryonic vascular morphogenesis will aid in understanding the functions of these splice variants as well as in determining the identity and functional significance of cellular receptors for EIIIA and EIIIB.

A more detailed analysis of VSMC recruitment to the aortae in mice carrying single-exon deletions of either EIIIA or EIIIB may give further insight into the individual roles of these highly conserved domains in vascular morphogenesis. These analyses are important even though EIIIA-null and EIIIB-null mutants are viable and fertile. We noticed that VSMC recruitment to dorsal aortae is defective even in phenotypically normal EIIIA/EIIIB double-null embryos (these embryos presumably would have survived gestation to produce viable and fertile mice). In addition, *in situ* hybridization studies (reviewed below) showed that upregulation of EIIIA-containing and EIIIB-containing forms of FN follow a different time course after vascular injury suggesting that EIIIA-FNs and EIIIB-FNs have non-overlapping functions.

Association of endothelial and perivascular cells is crucial during embryogenesis as well as after birth, for physiological and pathological processes that require new blood vessel formation or remodeling [4,67,71–73]. For example, association of these cell types is required during the physiological process of retinal vascularization in a newborn and disruption of this process leads to endothelial cell death and vessel regression [74]. Similarly, during tumor angiogenesis, association of endothelial and perivascular cells is required for tumor vessel stability; disruption of this process leads to regression of a select subset of tumor vessels [5, 75].

## Fibronectin and its Splice Variants in Pathology

### Cardiovascular Disease

A number of cardiovascular diseases stem from inappropriate recruitment and proliferation of VSMCs during vascular remodeling [76]. For example, migration and proliferation of VSMCs are key factors in the pathology of atherosclerosis [77]. Accumulation of these cells and secretion of extracellular matrix proteins (including FN and its splice variants [78]) results in the formation of the fibrous cap, which if untreated leads to narrowing and eventual occlusion of the affected arterial blood vessels [77]. Breaking of the fibrous cap causes thrombosis and accounts for 50% of the stroke cases in the United States (the third most common cause of death in this country) [79]. EIIIA<sup>+</sup> FNs are expressed within atherosclerotic lesions [78], however their functions are unknown and their effects on the progression of atherosclerotic lesions are controversial since both the absence of EIIIA [80,81] or its constitutive presence have been shown to have protective effects [80]. The functions of EIIIB within atherosclerotic lesions have not been examined.

Importantly, a large percentage (up to 40%) of current surgical procedures to treat atherosclerosis including balloon angioplasty, bypass surgery, and endarterectomy fail within the first year after the treatment due to re-occlusion (i.e. restenosis) of the treated artery, grafted vein, or implanted stent [79,82]. This happens as a result of VSMC migration into the site of injury and their excessive proliferation following treatment [77]. Interestingly, while there is little or no expression of EIIIA and EIIIB splice variants in normal adult vessels (mouse or human), mouse models of balloon angioplasty showed upregulation of these splice variants as soon as four days after injury [83].

mRNAs of FN and its splice variants were upregulated as soon as one day following coarctation of the ascending aorta. This injury creates cardiac pressure overload leading to cardiac hypertrophy. Following this injury, FN mRNAs were expressed in smooth muscle cells of coronary arteries but not veins, suggesting that expression of FN and its splice variants plays a role during arterial but not venous vessel remodeling following injury [84]. Aortic injury resulting from balloon catheterization (this injury approximates endothelial injury following atherosclerotic plaque rupture) results in 3–4 fold upregulation of EIIIA and EIIIB splice variants compared with uninjured vessels [85]. Interestingly, the inclusion of the EIIIA exon into FN mRNA is detectable by the fourth day post-injury, before the formation of neointima, while the expression of EIIIB is not detectable at that time. By the seventh day, neointima is detected in injured vessels and so is the expression of EIIIB. The expression of both EIIIA and EIIIB seems to occur in the vascular smooth muscle cells, and the delay in the appearance of EIIIB compared with EIIIA suggests that these splice variants have non-overlapping roles in vessel healing and/or pathology of atherosclerosis [85].

Similarly, upregulation of EIIIA-containing FNs precedes inclusion of EIIIB into FN mRNA following transplantation of cardiac allografts or isografts [86]. Interestingly, in both allografts and isografts, these splice variants were upregulated in the epicardium [86], the site containing progenitor cells with the potential to give rise to coronary vascular smooth muscle cells, cardiac



fibroblasts and ventricular myocardial cells [87,88] and in zebrafish, these cells have the capacity to differentiate into coronary vascular endothelial cells during cardiac regeneration [89]. These progenitors are mobilized following injury, and thus EIIIA and/or EIIIB splice variants may play a role in this process by affecting migration, proliferation, differentiation and/or survival of these progenitors. Taken together, the time-delay in the onset of expression of EIIIA and EIIIB-containing FNs implies that these splice variants play non-overlapping functions *in vivo*. The availability of viable EIIIA/EIIIB double-null mutants will help determine the role of these splice variants in vascular morphogenesis following injury and could point to specific individual roles of EIIIA and EIIIB in this process.

## Tumorigenesis

Striking upregulation of EIIIA and EIIIB-containing FNs has been observed around tumor vasculature in both human and mouse tumors [90–103]. These splice variants are sometimes called “oncofetal fibronectin isoforms” to signify their prominent expression in embryos and in tumors [104] but not around normal adult vasculature. Correlation of expression of EIIIB and EIIIA with the presence of tumor angiogenesis led to the development of anti-EIIIB and EIIIA antibodies as tumor-targeting and tumor-imaging reagents [105–108].

Individual deletion of EIIIA or EIIIB exon from the *FN* gene did not have a significant effect on tumor progression in the transgenic Rip1-Tag2 model of pancreatic islet carcinogenesis [103]. Interestingly, pericytes expressing  $\alpha$ SMA were abundant around tumor vasculature in either EIIIA-null or EIIIB-null mice, and RNase protection assay revealed that absence of either EIIIA or EIIIB did not quantitatively affect the levels of  $\alpha$ SMA in these tumors [103]. These experiments suggest that the presence of either EIIIA or EIIIB-containing FNs is sufficient for the formation of a layer of pericytes around tumor vasculature. Since some EIIIA/EIIIB double-null mice are viable and fertile, it would be interesting to determine whether the absence of both EIIIA and EIIIB forms of FN impairs tumor angiogenesis.

In the course of tumor progression, a thick sleeve of extracellular matrix, which includes FN and its splice variants, surrounds tumor vessels [103,109]. Interestingly, a thick layer of pericytes also surrounds those tumor vessels expressing high levels of FN. Tumor vessels not expressing FNs have a single pericyte layer (or no pericytes at all) [103]. The functional distinction between these types of tumor vessels is not clear. Our immunohistochemistry experiments in embryos showed that FN and its splice variants are preferentially assembled around dorsal aortae and not anterior cardinal veins suggesting that tumor vessels surrounded by a coat of pericytes and FN may have characteristics of arterial vessels. Alternatively, upregulation of EIIIA and/or EIIIB FNs may function in recruitment and/or differentiation of pericytes by tumor blood vessels. Understanding the process of recruitment of pericytes

to, and their association with, blood vessels is essential for the design of effective drugs to block tumor angiogenesis and tumor progression. Since the absence of both EIIIA and EIIIB led to defective recruitment of vascular smooth muscle cells to embryonic dorsal aortae, we hypothesize that EIIIA and/or EIIIB are required for the recruitment of pericytes to tumor blood vessels as well.

Elimination of tumor vessels has been a major goal of anti-angiogenic therapy [110]. Drugs and antibodies that interfere with VEGFR or PDGFR receptor signaling (these agents affect proliferation and survival of tumor blood vessel endothelial cells and association of endothelial cells with pericytes, respectively), led only to a transient decrease in tumor growth [110]. Interestingly, blockade of VEGFR signaling leads to a transient “normalization” of tumor blood vessels: enrichment in the number of tumor vessels surrounded by pericytes, tighter endothelial-pericyte associations, and decrease in the number of vessels without the pericyte

coat [109]. This normalization allows for more efficient delivery of chemotherapeutic drugs to kill tumor cells. This concept has been successfully tested by clinical trials [111].

Drugs interfering with PDGFR signaling (these drugs were hypothesized to inhibit interactions of tumor blood vessels with pericytes) also give rise to “normalized” tumor blood vessels. While vessels in untreated tumors are surrounded by a coat of vascular smooth muscle cells loosely associated with the blood vessel wall, tumors receiving drugs blocking PDGFR signaling contain blood vessels that are tightly associated with the surrounding pericytes [110]. Since EIIIA- and EIIBB-containing FNs are expressed around vessels surrounded by a thick coat of pericytes, and since anti-angiogenic therapy leads to enrichment of tumor blood vessels surrounded by pericytes, it would be interesting to determine whether blood vessels expressing EIIIA and/or EIIBB FNs are especially refractory to anti-angiogenic treatments described above. If found to be the case, further understanding of the role of EIIIA and EIIBB FNs in the process of tumor angiogenesis may lead to the design of drugs targeting all types of tumor vasculature.

## Conclusions and Perspectives

FN was discovered over thirty years ago as a protein abundantly secreted by tumor cells [27]. Intense investigation of the function of FN *in vitro* led to the discovery of integrins and other proteins involved cell motility and in the bi-directional communication between cells and their environment. However, understanding the molecular function of FN in the context of a developing vertebrate organism has proven to be challenging. Recent genetic studies in fish, frogs and mice have suggested that FN functions in maintaining or establishing cell polarity and highlighted the role of FN and its splice variants in development of vascular smooth muscle cells. Future studies involving an extensive temporal and spatial analysis of FN mRNA and protein expression along with genetic fate-mapping studies and *in vivo* assessment of growth factor signaling pathways in FN-null mutants will lead to a more detailed understanding on the function of FN in embryonic and cardiovascular development.

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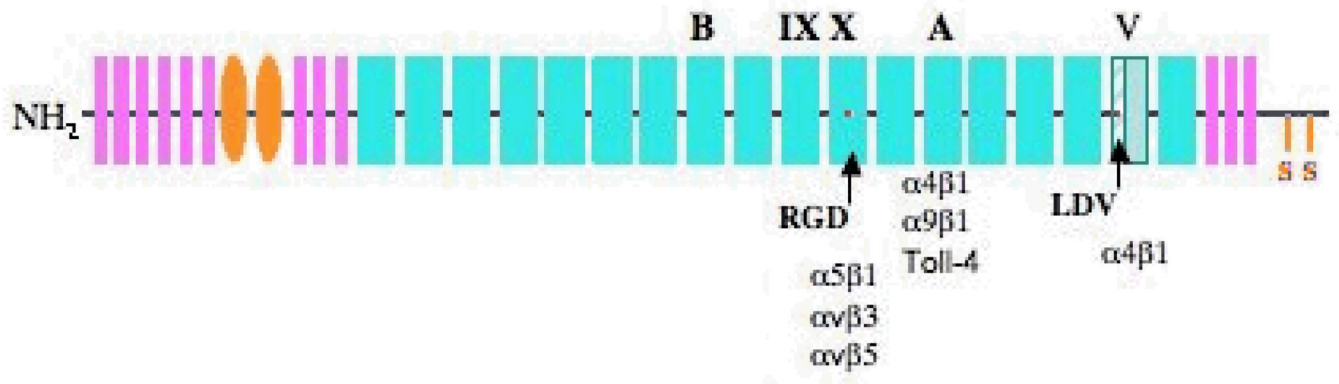
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**Figure 1. Schematic structure of FN**

FN is composed of structural repeats of three types. Type I repeats are designated in pink, type II repeats are in orange and type III repeats are in cyan (the 9<sup>th</sup> and 10<sup>th</sup> repeats are marked). Alternatively spliced EIIIA, EIIIB and V regions are marked. The stippled part of the V region can be included (V120 isoform) or excluded (V95 isoform) in the mouse. Red dots indicate integrin-binding sites in the 10<sup>th</sup> type III repeat and in the V region. Integrins known to bind these sites and EIIIA are listed.