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Collaboration of Fibronectin Matrix with other Extracellular Signals in Morphogenesis and Differentiation

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Abstract

Tissue formation and cell differentiation depend on a properly assembled extracellular matrix (ECM). Fibronectin is a key constituent of the pericellular ECM, forming essential connections between cell surface integrin receptors and structural components of the ECM. Recent studies using vertebrate models, conditional gene knockouts, tissue explants, and cell culture systems have identified developmental processes that depend on fibronectin and its receptor $\alpha 5\beta 1$ integrin. We describe requirements for fibronectin matrix in the cardiovascular system, somite and precartilag development, and epithelial-mesenchymal transition. Information about molecular mechanisms shows the importance of fibronectin and integrins during tissue morphogenesis and cell differentiation, as well as their cooperation with growth factors to mediate changes in cell behaviors.

Introduction

The extracellular matrix (ECM) is a critical regulator of cell behaviors [1,2]. Cell- ECM binding provides chemical signals, structural support, and local cues organized to promote integration of environmental information by intracellular pathways. Cell interactions with the ECM are dynamic, usually involving new ECM protein polymerization and matrix remodeling. Cell-mediated assembly of ECM proteins into multimeric structures directs cell and tissue organization and controls cell differentiation, making the assembly process a key player in tissue morphogenesis. Assembly of the ubiquitous ECM protein fibronectin (FN) into fibrils requires coordination of FN expression levels with integrin receptor activity and connections to cytoskeletal components [3,4]. FN fibrils then form the foundation upon which collagens and many other ECM proteins are deposited. This review summarizes recent data on the critical role of FN matrix during tissue development with emphasis on how the ECM cooperates with other molecules to promote morphogenetic processes.

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Fibronectin matrix in cardiovascular development

FN dimers are assembled into fibrils primarily by the integrin receptor $\alpha 5\beta 1$ and both ligand and receptor play central roles in cardiovascular development. FN-null mice die as embryos with defects in formation of the heart and early embryonic and extraembryonic blood vessels [5,6]. A closer examination of heart development in the absence of FN showed defects in the cardiac outflow tract and right ventricle that were linked to decreased proliferation of cardiac precursor cells [7]. Similar cardiac defects were observed in conditional mouse knockouts of FN or $\alpha 5$ targeted to the pharyngeal region [8] as well as in fibroblast growth factor 8 (Fgf8) hypomorphs [9]. Transcriptional targets of Fgf8 signaling were down-regulated in FN-null or $\alpha 5$ -null embryos implicating FN matrix in the potentiation of Fgf8 signaling at least for this stage of heart development [7].

FN and $\alpha 5$ have also been linked to the migration of cardiac precursor cells. In zebrafish between 18 and 24 hpf, myocardial precursors migrate to the midline and then fuse to form a single heart tube [10]. Knockdown of the transcription factor Snail1b causes a reduction in $\alpha 5$ expression and discontinuous FN matrix in the region of cell migration [11]. Both FN matrix assembly and cardiac fusion were rescued by injection of $\alpha 5$ mRNA indicating that precursor cell migration depends on Snail1b-mediated regulation of $\alpha 5$ expression and subsequent FN matrix assembly. Perhaps Snail1b is acting downstream of sphingosine-1-phosphate (S1P). Mutation of the S1P transporter Spns2, which is required for S1P secretion, combined with knockdown of FN caused a severe, two-heart phenotype in zebrafish [12]. Cardiac progenitor cell differentiation appeared normal but cell movements needed for cardiac fusion did not occur.

Analysis of epicardial cell migration in vitro has uncovered a novel mechanism that is executed by two proteins, Bves and NDRG4, previously implicated in vesicular trafficking [13–15]. Knockdown of the Bves/NDRG4 complex increased FN accumulation in cytoplasmic vesicles and decreased deposition of vesicular FN onto the substrate [13]**. These changes in FN subcellular localization accompanied loss of directional persistence in cell migration suggesting an interesting model in which autocrine FN recycling supports de novo matrix assembly to direct epicardial cell migration.

Conditional knockout of $\alpha 5$ in the anterior mesoderm, the source of cardiac tissue and the anterior vasculature, identified a surprising connection between mesoderm and neural crest development. Differentiation of cardiac neural crest into vascular smooth muscle cells (VSMCs) was affected by the absence of this integrin in mesodermal cells which caused morphogenetic defects in aortic arch arteries [16]**. These results suggest a model in which the ECM assembled by $\alpha 5\beta 1$ on mesodermal cells influences neural crest-derived cells to develop into VSMCs. FN-binding integrins are also needed in the neural-crest derived VSMCs. Double knockout of $\alpha 5$ and αv integrins in VSMCs, both of which bind to the RGD domain of FN, caused embryonic lethality with cardiovascular defects similar to the anterior mesoderm $\alpha 5$ knockout mice [17]; single integrin knockout showed no defects in cardiac development. Both VSMCs and the vessel wall ECM were abnormal in $\alpha 5/\alpha v$ -null mice, which corresponded with severe defects in FN matrix assembly in cultured double-null VSMCs [17]*. Matrix incorporation of a number of other ECM proteins was deficient.

Of particular note is latent transforming growth factor β (TGF β) binding protein (Ltbp1); its deficiency disrupted Smad signaling downstream of TGF β , a likely contributor to the observed cardiovascular defects [17]. Together these analyses of cardiac neural crest differentiation demonstrate a central role for integrins and their FN matrix in controlling cell differentiation by providing pericellular signals from the ECM itself as well as by presenting exogenous factors like TGF β . Other FN-dependent signals may participate in development of a VSMC-phenotype [18,19]. Interestingly, activation of Notch was limited to those neural crest cells that express FN and $\alpha 5\beta 1$ and Notch activation was needed for VSMC differentiation by neural crest. These findings suggest the novel idea that FN induces an autocrine signaling response by cells [19]*. Future interpretations of FN's effects should consider potentially distinct roles of paracrine and autocrine mechanisms of FN action.

Synergy between FN-integrin and homophilic cadherin interactions

Matrix assembly and integrin activity determine somite border formation and recent genetic studies in zebrafish identified Rap1 GTPase as an inside-out activator of $\alpha 5$ integrin in this process. Whereas loss of $\alpha 5$ has an effect on anterior somite formation with a modest reduction in FN matrix [20], knockdown of both Rap1 and $\alpha 5$ caused loss of all somite borders and complete disruption of FN matrix [21]. The regulation of integrin activation and FN matrix assembly during somitogenesis occurs by a unique mechanism. Homophilic cell-cell interactions between cadherin 2 molecules in the presomitic mesoderm stabilize the association of $\alpha 5$ integrins on adjacent cells to maintain them in an inactive state [22]**. The unusual integrin-integrin association was suggested when cells lacking $\alpha 5$ were introduced into a mutant host zebrafish that lacks proper segmental patterning and these cells rescued $\alpha 5$ activation and FN matrix formation. Cadherin 2-null zebrafish have ectopic FN matrix assembly and $\alpha 5$ clustering, supporting its role as an integrin regulator [22]. Eph/ephrin signaling can activate integrins at somite boundaries [23] suggesting that a pathway from ephrin through Rap1 activates $\alpha 5$ and promotes FN matrix assembly during morphogenesis of somite boundaries.

Cadherins have also been linked to FN matrix assembly in chondrocyte differentiation and salivary gland cleft formation. The early events in chondrogenic differentiation such as cell condensation and changes in gene expression are recapitulated in a micromass culture system using mesenchymal stem cells [24]. We showed that cell aggregation depends on FN matrix assembly [25]. As mesenchymal stem cells condense, FN and N-cadherin levels are up-regulated and matrix assembly increases. Once cells have condensed into an aggregate, N-cadherin is down-regulated but FN matrix assembly continues. During this period, cells proliferate and initiate the differentiation program, which requires induction of the Sox9 transcription factor, which is essential for cartilage-specific gene expression. Either the blockade of FN polymerization [25] or the knockdown of N-cadherin [26] in micromass cultures prevents cell condensation. Incomplete condensation prevents up-regulation of Sox9 expression and cell differentiation into chondrocytes. These data and the role of cadherins in somite morphogenesis suggest that cadherin interactions bring or keep cells juxtaposed during the initial stages of morphogenesis but then FN matrix assembly takes over to maintain cell connections while allowing cell rearrangements needed for subsequent steps in tissue development.

Differential regulation of cadherins and FN matrix assembly may be a common mechanism for cell aggregation as it has been connected to differences in cohesion of glioblastoma cells that vary in invasiveness [27]. Cell clustering, as shown on a collagen matrix substrate, depends not only on FN and $\alpha 5\beta 1$ but also on development of a pro- contractile phenotype. LPA treatment stimulates contractility and clustering concomitant with FN matrix assembly [28]. In fact, the clustering appears to depend on a balance between pro-contractile signals that promote matrix formation and pro-migratory signals such as PDGF and the matrix metalloprotease MMP-2 that induce matrix turnover [29]. This balance may be a common mechanism for cell clustering in development and disease.

Regulated contractility is also proposed to explain cleft formation during branching morphogenesis in salivary gland explants. A localized increase in FN fibril formation decreased expression of E-cadherin allowing cleft formation sites to form on the surface of the explant [30]. Myosin phosphatase appears to be a central regulator of this process. By binding to either myosin light chain or a deacetylase (HDAC6), it controls the balance between cell contractility and microtubule acetylation [31]**. Binding to HDAC6 caused hyperacetylation of microtubules, which increased adhesion maturation and induced FN matrix formation over the entire surface of the explant resulting in decreased cleft formation. On the other hand, dephosphorylation of myosin light chain by the phosphatase reduced contractility sufficiently to allow localized changes that promote cleft formation [31].

Another type of FN-cell interaction involving exosomes has recently been implicated in intercellular communication. Exosomes are endosome-derived extracellular vesicles involved in intercellular signaling and delivery of cargo such as signaling proteins, lipids and miRNAs, to neighboring cells [32]. Tumor-derived exosomes can promote tumor progression and cell invasion [33]. FN has now been shown to play an important role as a mediator of myeloma-derived exosome targeting. Interestingly, FN binds to heparan sulfate on the surface of exosomes and acts as a bridge to heparan sulfate proteoglycans on the target cells [34]*. Blocking antibodies against FN prevented exosome delivery suggesting a potential new approach to modulate tumor progression and other functional effects downstream of exosome targeting.

Cooperation between FN matrix and growth factors

TGF β has essential roles in both normal and pathogenic cell differentiation and tissue morphogenesis. It is a well-known inducer of expression of genes involved in cell adhesion and matrix assembly. In myofibroblast differentiation, for example, expression of ECM proteins such as FN and type I collagen and of cell contractility molecules (e.g., α -smooth muscle actin) are up-regulated [35,36] and FN matrix assembly is enhanced [37]. However, in some cells, co-regulation of ECM molecules by TGF β can modulate the extent of FN assembly. Hyaluronan (HA) and FN were both increased in the matrix of TGF β -induced human lung myofibroblasts. However, FN levels could be further enhanced by degradation of HA or inhibition of HA synthase (HAS) [38] suggesting that HA in the matrix has an inhibitory effect on FN assembly. Changes in ECM protein levels are not necessarily a direct effect of TGF β stimulation. For example, the transcription factor SOX4 is induced by TGF β and then up-regulates mesenchymal genes including FN [39]. Therefore, it is important to

control the timing of experiments in order to distinguish signals directly downstream of TGF β from those that are several steps removed from TGF β action.

During development, FN matrix potentiates morphogenetic signals such as Fgf8 and S1P, as mentioned above. Epithelial-mesenchymal transition (EMT), another important morphogenetic process, is induced by TGF β with dramatic changes in gene expression and cell phenotype [40]. FN matrix signaling has recently been shown to cooperate with TGF β to stimulate EMT [41]**. While definitive changes in EMT can take many hours or days in some cases, significant up-regulation of key EMT proteins, including FN, Snail, and N-cadherin, was detected within 6 hrs of TGF β treatment in mammary epithelial cells attached to FN but not in cells on laminin [41]. Smad2 phosphorylation was also enhanced by FN. Notably, interactions with FN allowed cells to respond to lower concentrations of TGF β indicating that FN and TGF β signals cooperate to help cells reach a threshold needed for induction of EMT. While these results implicate the canonical Smad pathway in the cooperative effects, a non- canonical pathway with PEAK1 as a node connecting TGF β receptor signals with integrin signals through Src and ERK has also been reported [42]. Another EMT inducer, the matrix metalloprotease MMP-3, also relies on FN-cell interactions since EMT was activated by MMP-3 treatment of mammary epithelial cells only when the cells were growing on a FN substrate; cells on laminin did not undergo EMT [43]. These results with mammary epithelial cells and EMT show that cell-FN interactions cooperate with, and prolong the cellular responses to, growth factors and other extracellular inducers.

Concluding remarks

While FN matrix stimulates cells directly through $\alpha 5\beta 1$ integrin to the actin cytoskeleton and intracellular pathways, it also collaborates with other extracellular signals to regulate morphogenesis and differentiation. The effects of soluble extracellular signaling factors are potentiated by outside-in signals involving FN matrix to promote cell growth, motility, and differentiation (Figure 1A-1). Intracellularly, $\alpha 5\beta 1$ is affected by other pathways to induce assembly of a FN matrix. In vivo evidence shows that inside-out signaling can be downstream of transcriptional regulation, other transmembrane receptor signals, and cell contractility (Figure 1A-2). Integrin-cadherin collaboration appears to have a dual role in morphogenesis (Figure 1B), maintaining integrins in an inactive state during somitogenesis and bringing cells together to induce matrix assembly in precartilagel condensation. Outside-in and inside-out signals have been described before but these latest results show mechanistic significance in discrete developmental events. Furthermore, not only does FN enhance the effects of other signals, it also appears to collaborate with itself to promote cell migration and differentiation through autocrine signaling.

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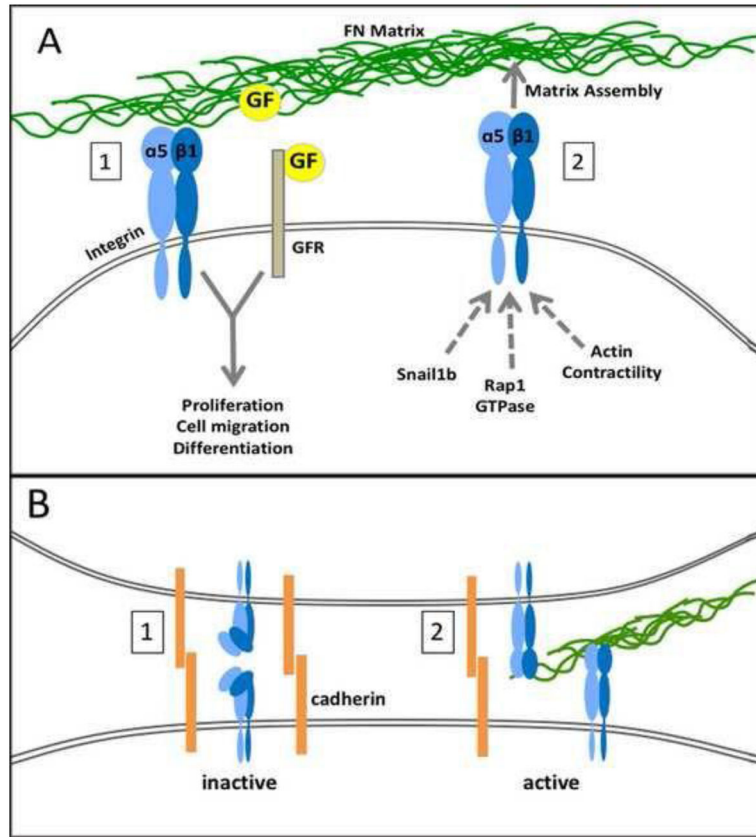


Figure 1.

Figure 1A. Bidirectional signaling by a FN matrix.

(1) Outside-in signaling is initiated by $\alpha 5 \beta 1$ (blue) binding to FN matrix (green). FN matrix provides binding sites for soluble growth factors (GF) which facilitates GF binding and activation of its receptor (GFR). Intracellular integration of FN and GF signals stimulates specific cell responses. Pathways mentioned in the text that collaborate with FN matrix include $TGF\beta$, Fgf8, S1P, and Notch.

(2) Inside-out signaling promotes FN matrix assembly by activating $\alpha 5 \beta 1$ integrins. Mechanisms include stimulation of integrin activity by cytoplasmic pathways that involve Rap1 GTPase or actin contractility and by $\alpha 5$ expression induced by Snail1b transcription factor.

Figure 1B. Collaboration between integrins and cadherins to regulate FN matrix assembly.

(1) Homophilic interactions between cadherins (orange) on neighboring presomitic mesodermal cells maintain integrins (blue) in an inactive folded conformation.

(2) Downregulation of cadherins allows integrin-mediated FN matrix assembly between neighboring cells, as observed in precartilaginous condensation.