Fibronectin fibrillogenesis regulates three-dimensional neovessel formation

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During vasculogenesis and angiogenesis, endothelial cell responses to growth factors are modulated by the compositional and mechanical properties of a surrounding three-dimensional (3D) extracellular matrix (ECM) that is dominated by either cross-linked fibrin or type I collagen. While 3D-embedded endothelial cells establish adhesive interactions with surrounding ligands to optimally respond to soluble or matrix-bound agonists, the manner in which a randomly ordered ECM with diverse physico-mechanical properties is remodeled to support blood vessel formation has remained undefined. Herein, we demonstrate that endothelial cells initiate neovascularization by unfolding soluble fibronectin (Fn) and depositing a pericellular network of fibrils that serve to support cytoskeletal organization, actomyosin-dependent tension, and the viscoelastic properties of the embedded cells in a 3D-specific fashion. These results advance a new model wherein Fn polymerization serves as a structural scaffolding that displays adhesive ligands on a mechanically ideal substratum for promoting neovessel development.

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Under quiescent conditions, the endothelium of the patent vasculature exists as a two-dimensional (2D) monolayer that rests atop a self-organized basement membrane that serves to separate the cells from the underlying interstitial matrix [Jain 2003; Carmeliet 2005]. During angiogenesis and vasculogenesis, this structural arrangement is disrupted as endothelial cells embed themselves within a three-dimensional (3D) extracellular matrix (ECM) dominated by either cross-linked networks of the clotting protein, fibrin, or the triple-helical matrix molecule, type I collagen [Jain 2003; Carmeliet 2005]. Within this extrinsic matrix, endothelial cells are exposed to angiogenic growth factors that initiate the migratory, proliferative, and tubulogenic responses required for the development of the neovasculature [Jain 2003; Carmeliet 2005]. Growth factors acting alone, however, are unable to trigger angiogenesis [Chen et al. 1997; Huang et al. 1998; Geiger et al. 2001; Discher et al. 2005; Ingber 2006; Vogel and Sheetz 2006].

Using traditional, 2D culture systems, the responsiveness of adherent cell populations to soluble growth factors is modulated by the composition, density, and topology of adhesive ligands as well as the mechanical properties of the supporting matrix itself [McBeath et al. 2004; Discher et al. 2005; Engler et al. 2006; Vogel and Sheetz 2006]. However, an increasing body of evidence indicates that standard 2D culture systems do not recapitulate accurately cell behavior displayed in the 3D in vivo setting [Walpita and Hay 2002; Hotary et al. 2003; Chun et al. 2006; Nelson and Bissell 2006; Yamada and Cukierman 2007]. Indeed, when cells are cultured within the confines of 3D extracellular matrices in vitro, cell–matrix adhesive interactions are established that more closely approximate those observed in vivo and, in coincident fashion, the cell cytoskeleton reorganizes into patterns that mimic those exhibited by intact tissues [Walpita and Hay 2002; Hotary et al. 2003; Beningo et al. 2004; Debnath and Brugge 2005; Meshel et al. 2005; Chun et al. 2006; Yamada and Cukierman 2007]. Despite
the described contrasts between 2D and 3D cell behavior, the mechanisms by which proangiogenic endothelial cells remodel the surrounding 3D ECM to generate an adhesive platform that allows them to interpret the biochemical and mechanical inputs critical to neovessel formation remain undefined. Here, we demonstrate that the endothelial cell-dependent unfolding and pericellular polymerization of the soluble glycoprotein, fibronectin (Fn), plays a required—and 3D-specific—role in triggering neovascularization. By constructing a pericellular scaffold of polymerized Fn fibrils, endothelial cells are able to assemble a functional cytoskeletal–actomyosin complex and modulate their intracellular viscoelastic properties to engage the mechanotransduction-sensitive programs that drive 3D neovessel formation.

Results

Endothelial cell tubulogenesis in 3D

When embedded in a 3D gel of cross-linked fibrin and stimulated with a cocktail of proangiogenic factors in serum-containing media, human endothelial cells assume a spheroid configuration during the first 8–12 h of culture (Fig. 1A). Consistent with reports that endothelial cell rounding is incompatible with proliferative activity (Folkman and Moscona 1978; Chen et al. 1997; Ingber 2006), no increase in cell number is detected until after a 48-h culture period, whereupon the embedded cells display a stretched phenotype (Fig. 1A,B). Endothelial cell number subsequently increases after the 2-d lag period, and a tubulogenic program is engaged that leads to the formation of an anastomosing network of patent neovessels by day 6 (Fig. 1B,C). Neovessel formation in vitro recapitulates the in vivo program that is dependent on both α5β1, a Fn-specific integrin, as well as the Fn RGD domain contained within its primary cell-adhesion module (Supplemental Fig. 1; Kim et al. 2000; Francis et al. 2002, Hynes 2002, Takahashi et al. 2007). Furthermore, as observed in vivo (Clark et al. 1982; Risau and Lemmon 1988; Neri and Bicknell 2005), endothelial cell morphogenesis occurs in tandem with the assembly of a network of Fn fibrils that not only enmesh the stretched endothelial cells observed at 48 h, but also ensheath the tubules formed at the end of the 6-d culture period (Fig. 1D).

Figure 1. Endothelial cell tubulogenesis and Fn matrix assembly. (A) Endothelial cells (1.5 × 10⁵) were embedded in a 3D fibrin gel in the presence of 20% human serum and a cocktail of VEGF, HGF, TGFα, TGFβ1, and heparin. Phase contrast micrographs are shown following 0, 2, 4, and 6 d in culture. (B) Endothelial cell growth in the presence or absence of the provasculogenic cocktail was assessed by protease digestion of the 3D fibrin matrix followed by counting of cells. (C) Following 6 d in culture, patent endothelial cell tubules are formed as assessed in H&E-stained cross-sections or TEMs. (D) Confocal laser micrographs of pericellular assembly of FITC-labeled Fn matrix following 8 h, 2 d, and 6 d of culture. Bar, 30 µm.
The 3D-specific regulation of endothelial cell function by Fn fibrillogenesis

Under 2D culture conditions, cell shape changes critical to signal transduction, migration, and proliferation are dictated by the composition, organization, and rigidity of the surrounding ECM (Chen et al. 1997; Discher et al. 2005; Bershadsky et al. 2006; Vogel and Sheetz 2006; Cavalcanti-Adam et al. 2007). As changes in the morphology of 3D-embedded endothelial cells correlate with Fn matrix assembly, we first sought to characterize the functional role that the fibrillogenesis process plays in the tubulogenesis program. To block Fn matrix assembly without affecting the initial binding of soluble Fn binding to \( \alpha_5\beta_1 \), endothelial cells were incubated with (1) monoclonal antibodies directed against Fn domains embedded within, or near, the Fn III\(_{1,2} \) modules that are critical for regulating Fn-Fn interactions (i.e., monoclonal antibody L8 or 9D2), (2) a 70-kDa N-terminal Fn fragment that interferes with the polymerization of intact Fn dimers by competing for matrix assembly sites on the endothelial cell surface, or (3) a 49mer peptide (termed the functional upstream domain, or FUD) derived from the Streptococcus pyogenes adhesion F1 protein, which binds directly to the N-terminal matrix assembly domain of Fn (Chernousov et al. 1991; Tomasini-Johansson et al. 2001, 2006; Mao and Schwarzbauer 2005). As exemplified by the addition of FUD (but not the control peptide Del29, wherein FUD residue 29 is deleted), the ability of fibrin-embedded endothelial cells to assemble a Fn matrix is blocked completely in the presence of inhibitors of Fn fibrillogenesis (Fig. 2A). In the absence of Fn fibrillogenesis—and despite the presence of a surrounding 3D fibrin matrix, serum, and exogenously provided proangiogenic growth factors—the endothelial cells are unable to undergo the expected shape change and retain a spherical morphology (Fig. 2A). Coincident with the block in Fn matrix deposition, the 3D migratory and proliferative responses of the embedded endothelial cells are blunted significantly and tubulogenesis is effectively terminated (Fig. 2B,C; Supplemental Fig. 2). Under these conditions, where Fn/\( \alpha_5\beta_1 \) interactions are left intact, no increase in apoptosis (as assessed by TUNEL staining) is detected in the absence of Fn fibrillogenesis (data not shown). Furthermore, the ability of Fn matrix inhibitors to block neovessel formation is not restricted to the specific use of a fibrin gel suspension system. Similar, if not identical, results are obtained when neovessel formation is initiated with spheroids of endothelial cells embedded in 3D fibrin gels (Korff and Augustin 1998) or, alternatively, when type I collagen, the major ECM macromolecule found in mammalian tissues (Hotary et al. 2003; Chun et al. 2004, 2006), is used as the supporting matrix (Fig. 2D,E). Although Fn matrix assembly can regulate type I collagen deposition (Velling et al. 2002), neovessel formation is unaffected in the presence of the collagen synthesis inhibitor, cis-hydroxyproline (data not shown).

While the findings presented thus far support a required role for Fn fibrillogenesis in regulating endothelial cell behavior within the confines of a 3D ECM, endothelial cells are likewise able to assemble Fn matrices when cultured atop physiologic substrata (Fig. 2; Christopher et al. 1997; Bourdouloues et al. 1998). Consistent, however, with the fact that cell behavior can be affected differentially under 2D versus 3D culture conditions (Walpita and Hay 2002; Hotary et al. 2003; Debnath and Brugge 2005; Chun et al. 2006; Larsen et al. 2006; Yamada and Cukierman 2007), the inhibition of Fn matrix assembly during endothelial cell culture atop fibrin gels did not affect cell shape, migration, or proliferation (Fig. 2F–H).

Given the demonstrated requirement for Fn matrix assembly in 3D neovessel formation in vitro, a functional role for fibrillogenesis in tissue sites undergoing active angiogenesis in vivo was assessed. To this end, 3D composite gels of fibrin and type I collagen were placed atop the chorioallantoic membrane of live chicks and angiogenesis initiated by the application of a cocktail of growth factors in the presence of FUD or the Del29 peptide control. Under control conditions, angiogenic vessels infiltrated the ECM construct in tandem with the deposition of a dense network of Fn fibrils (Fig. 3A). In the presence of FUD, however, Fn matrix assembly is almost completely inhibited and neovessel formation is ablated (Fig. 3A,B). As monoclonal antibody L8 only recognizes unfolded Fn epitopes that are exposed during Fn fibrillogenesis (Chernousov et al. 1991; Zhong et al. 1998), Fn matrix assembly in the context of human tumor angiogenesis was also assessed. Immunostaining of a series of renal cell carcinomas (stages GI–IV; \( n = 18 \)) and invasive ductal breast carcinomas (\( n = 8 \)) demonstrates that vascular wall L8 immunoreactivity is dramatically increased in tissues undergoing active vasculo/angiogenesis (Fig. 4). In both renal cell carcinoma and invasive ductal breast carcinoma specimens, all blood vessels and vascular channels are strongly L8-reactive with additional stromal staining seen in some cases of breast cancer (Fig. 4). In normal tissues, immunoreactivity for the L8 Fn epitope is observed infrequently as small streaks in <10% of the vessels (Fig. 4).

Fibrillogenesis and endothelial cell cytoskeletal organization

Changes in cell geometry impact on the signaling cascades that control cell migration, proliferation, and morphogenesis (Chen et al. 1997; Tan et al. 2003; McBeeath et al. 2004; Ingber 2006). In vivo, integrins and growth factors collaborate in the activation of mitogen-activated protein kinase [MAPK] pathways, which regulate the angiogenic response (Eliceiri et al. 1998; Geiger et al. 2001; Hoang et al. 2004; Ingber 2006). To determine the degree to which endothelial cell responses to growth factor and integrin–ligand signals are linked to Fn matrix assembly, the phosphorylation of the MAPKs ERK1, ERK2, JNK, and p38 were monitored in the absence or presence of fibrillogenesis inhibitors during the 48-h period that precedes proliferative responses. In control cultures, sustained MAPK activation is maintained throughout the 48-h incubation period in a fashion that recapitulates the...
in vivo setting (Fig. 5A; Eliceiri et al. 1998; Corson et al. 2003). However, independent of the marked changes in endothelial cell morphology and cytoskeletal organization associated with the inhibition of Fn fibrillogenesis, phosphorylation patterns of ERK1/2, JNK, and p38 are largely unaffected (Fig. 5A).
Despite the comparable initiation of signal transduction cascades in endothelial cells competent or incompetent for Fn matrix assembly, cell responses to integrin and growth factor-mediated signals are also dictated by the organization of actin cytoskeletal architecture (Chen et al. 1997; Huang et al. 1998; Bershadsky et al. 2006; Ingber 2006). In tandem with the ability of growth factor-stimulated endothelial cells to adopt an elongated phenotype in 3D culture, a reticulated pattern of well-organized stress fibers is resolved by F-actin phalloidin staining when cells are cultured in the presence of the Del29 control peptide (Fig. 5B). In 3D culture, stress fibers terminate at specialized β1 integrin- and vinculin-rich sites of cell–matrix interactions, termed 3D adhesions (Geiger et al. 2001; Larsen et al. 2006). As such, endothelial cells transfused with a GFP-tagged vinculin expression vector or alternatively immunostained with an activated β1 integrin-specific monoclonal antibody target both vinculin as well as activated β1 integrins into stitch-like structures at the endothelial cell periphery (Fig. 5C). In the absence of Fn fibrillogenesis, however, stress fiber formation is suppressed completely and actin staining is confined to the cortical envelope in a punctate network (Fig. 5B). Furthermore, specific interactions between either activated β1 integrins or vinculin and F-actin networks can no longer be discerned (Fig. 5C). Endothelial cells alternatively cultured atop fibrin matrices in a 2D configuration assemble a well-organized stress fiber-focal adhesion network whose organization is unaffected by inhibitors of Fn fibrillogenesis (Fig. 5D).

**Fn matrix assembly induces myosin-dependent tractional forces**

Adhesive interactions between cells and their surrounding matrix allow for the generation of tractional forces...
that regulate cell fate and function (McBeath et al. 2004; Discher et al. 2005; Engler et al. 2006; Larsen et al. 2006; Vogel and Sheetz 2006; Yamada and Cukierman 2007).

Given a 3D-specific requirement for Fn matrix deposition in the assembly of organized cell–matrix adhesive sites, the ability of embedded endothelial cells to generate tractional forces on the fibrin matrix was determined in the presence or absence of Fn fibrillogenesis inhibitors. In stressed ECM gels wherein cells are permitted to exert isometric tension, the degree of force exerted by cells on the surrounding fibrillar network can be assessed by monitoring gel contraction after the matrix is released from the surrounding culture dish (Corbett and Schwarzbauer 1999; Even-Ram et al. 2007). As shown in Figure 6, growth factor-stimulated endothelial cells cultured in control gels for 48 h were able to actively contract the released fibrin gel. In contrast, each of the Fn fibrillogenesis inhibitors markedly attenuated the ability of the embedded endothelial cells to generate tractional forces under 3D [Fig. 6A], but not 2D [data not shown], culture conditions.

Tractional forces exerted at cell–matrix adhesion sites require the activation of an actinomyosin motor complex whose assembly is tightly linked to actin cytoskeleton organization, nonmuscle myosin II isoform expression, and the rigidity of the surrounding substratum (Meshel et al. 2005; Engler et al. 2006; Even-Ram et al. 2007; Yoneda et al. 2007). Given that...
Fn matrix rigidity or adhesivity can affect the expression of gene products critical to the generation of tractional forces (Ben-Ze’ev et al. 1980; Deroanne et al. 2001; Engler et al. 2006), we monitored \[\text{F-actin}, \text{F-actinin}, \text{myosin light chain-2 (MLC2)}, \text{as well as nonmuscle myosin IIA and IIB isoforms (NMMIIA and NMMIIB, respectively)}\] protein levels in 3D-embedded endothelial cells (Fig. 6B). Significantly, whereas each cytoskeletal component is expressed in growth factor-stimulated endothelial cells actively assembling a Fn matrix, endothelial cells cultured in the presence of FUD or L8 express markedly reduced levels of β-actin, α-actinin, and MLC2 (Fig. 6B; Engler et al. 2006; Clark et al. 2007; Even-Ram et al. 2007).

**Figure 5.** The 3D Fn matrix is required for endothelial cell cytoskeletal organization and adhesion. (A) Levels of phosphorylated ERK1/2, JNK, and p38 were determined by immunoblot analysis in lysates of endothelial cells embedded in fibrin gels in the presence of either control IgG or mAb L8 for 0 h, 2 h, 1 d, or 2 d. Total ERK1/2 serves as the loading control. (B) Endothelial cells were cultured in 3D fibrin gels in the presence of the FUD or control peptides for 2 d. F-actin cytoskeletal organization was monitored following staining with Alexa 488-conjugated phalloidin and confocal laser microscopy. Bar, 20 µm. (C) Endothelial cells in 3D fibrin matrices in the presence or absence of FUD were either stained with an antibody against activated β1 integrin [green, left panels] or transfected with a GFP-tagged vinculin expression vector [pRK-vinculin-EGFP, green, right panels]. Following counterstaining with Alexa 594-labeled phalloidin [red], fluorescence was monitored by confocal laser microscopy. Bar, 10 µm. (D) Endothelial cells were cultured atop a 2D fibrin gel for 2 d in the presence of either Del29 or FUD peptides. In tandem with staining with Alexa 594-labeled phalloidin to monitor cytoskeletal organization, active β1 integrin and vinculin distribution were monitored by staining with an antibody against active β1 integrin [left panels] or assessing GFP-vinculin localization [right panels], respectively, by confocal microscopy. Bar, 20 µm.

**Sensing extracellular stiffness through Fn fibrillogenesis**

Endothelial cells cultured atop highly malleable surfaces retain a spheroid configuration, fail to organize stress fibers, and are unable to exert tractional forces (Folkman and Moscona 1978; Chen et al. 1997; Georges and Janmey 2005; Engler et al. 2006)—a phenotype identical to that observed in 3D-embedded, Fn matrix assembly-incompetent endothelial cells. As the cell’s internal stiffness—a viscoelastic property governed by cytoskeletal assembly, actin cross-linking, and the production of actomyosin-dependent stress—changes as a function of the perceived stiffness of the surrounding substratum (Solon et al. 2007), intracellular nanorheology was used to
monitor the micromechanical properties of 3D-embedded endothelial cells. One-hundred-nanometer-diameter fluorescent polystyrene beads were ballistically injected into the endothelial cell cytoplasm before the cells were cultured within the 3D fibrin matrix to circumvent the endocytic pathway and subsequent directed motion of the beads (Tseng et al. 2002). After 72 h, beads dispersed uniformly in the cytoplasm (e.g., Fig. 6C), and their (random) displacements within the cytoplasm (Fig. 6D) were analyzed with the appropriate software (see details in Materials and Methods). As shown in Figure 6E, relative to control endothelial cells, the mean square displacement (MSD) of the beads is significantly increased in cells treated with the FUD peptide, indicating a significant relative cytoplasmic softening compared with that of cells where Fn matrix assembly is intact. Elastic moduli (G, expressed in dyne per square centimeter), which quantify the local resistance of the cytoplasm against small random forces acting on the surface of the beads, were derived from MSD curves to quantify cellu-
lar mechanical properties. The elastic modulus of the cytoplasm of FUD-treated cells is significantly lower than that of control cells \(P < 0.001\), indicating a pronounced defect in internal stiffness and the cell’s ability to sense a sufficiently rigid substratum (Fig. 6F).

In the absence of Fn fibrillogenesis, an impaired ability of embedded endothelial cells to generate myosin-dependent forces and increase cytoplasmic stiffness would be predicted to affect both Fn unfolding (Wu et al. 1995; Zhong et al. 1998; Baneyx et al. 2002, Yoneda et al. 2007) as well as the ability of the cells to properly register the mechanical properties of the surrounding substratum (Discher et al. 2005, Engler et al. 2006). As such, the rheological and functional characteristics of endothelial cells were assessed in the presence of the specific myosin ATPase inhibitor, blebbistatin (Engler et al. 2006; Clark et al. 2007). Blebbistatin-treated endothelial cells phenocopy Fn matrix assembly-incompetent cells and fail to increase cytoplasmic stiffness, undergo cell shape change, assemble a pericellular Fn matrix, or reorganize cytoskeletal architecture (Fig. 6E–G). Consequently, endothelial cell tubulogenesis is blocked completely (Fig. 6H). Hence, myosin ATPase activity and Fn matrix assembly play required roles in regulating the endothelial cell’s ability to match internal stiffness with that of the surrounding substratum so as to propagate the mechanotransduction-initiated signals critical to neovessel formation.

Discussion

By modulating the responsiveness of endothelial cells to soluble agonists, the biomechanical properties of the ECM play critical roles in regulating vasculogenesis and angiogenesis (Chen et al. 1997; Huang et al. 1998; Georges and Janmey 2005). In 3D culture, endothelial cell responses to proangiogenic agonists are similarly disengaged when pericellular Fn matrix assembly is blocked and the cells are unable to use the multiple ligands presented by dense matrices of fibrin or type I collagen as structural platforms in either the in vitro or in vivo settings. Recent studies demonstrate that cells “sense” the mechanical properties of the surrounding matrix via a myosin II-dependent pathway (Discher et al. 2005; Solon et al. 2007). Consequently, the adherent cells alter their intracellular stiffness to match that of the supporting ECM in order to initiate the appropriate acytomyosin-dependent responses (Discher et al. 2005; Engler et al. 2006; Solon et al. 2007). Given that (1) the 3D behavior of Fn fibrillogenesis-incompetent endothelial cells phenocopies that of cells cultured atop suboptimal substrata and (2) internal cellular stiffness is modulated as a function of the perceived stiffness of the extracellular substratum (Engler et al. 2006; Solon et al. 2007), we reasoned that 3D analyses of microrheological responsiveness might uncover a required role for Fn matrix assembly in allowing endothelial cells to discern the biomechanical characteristics of the pericellular environment. Embedded in a 3D matrix, cells are inaccessible to conventional physical probes, including atomic force microscopy, glass microneedles, membrane-bound magnetic beads, or micropipette suction (Tseng et al. 2002). Using intracellular nanorheology, however, the random movements of submicron particles microin-
jected into the cytoplasm can be monitored as a means to determine local viscoelasticity [Tseng et al. 2002, Pan-orchan et al. 2007]. Based on such analyses, we demonstrate that 3D-embedded endothelial cells sense and respond to a sufficiently rigid scaffolding only when a network of Fn polymers is interposed between the cell and surrounding matrix of fibrin and/or type I collagen.

The ability of Fn matrix assembly to regulate endothelial cell mechanotransduction signaling is likely ascribed to either of two, not necessarily mutually exclusive, processes. First, as Fn fibrils are cross-linked into insoluble mats by both covalent and noncovalent interactions (Mao and Schwarzbaeur 2005), the assembled matrix may increase local mechanical rigidity to a degree necessary to support the tractions forces and intracellular stiffening generated during cell spreading [Davidson et al. 2004]. Indeed, in preliminary studies designed to directly monitor changes in the rigidity of the pericellular matrix, we found that endothelial cells modulate the local mechanical rigidity of the ECM by a process dependent on Fn fibrillogenesis [R.G. Rowe, J.B. George, D. Wirtz, and S.J. Weiss, unpubl.]. Second, Fn matrix assembly allows for an increase in the local concentration and order of potential αβ integrin-binding sites that would serve as a structural platform for cell spreading (Geiger et al. 2001; Mao and Schwarzbaeur 2005). Unfolded Fn molecules could further alter cell function by displaying matricryptic heparin-binding sites (Gui et al. 2006). As neither mechanically soft matrices displaying high adhesivity nor rigid matrices decorated with suboptimal levels of proadhesive binding sites will induce changes in cytoskeletal architecture and intracellular stiffness [Discher et al. 2005; Solon et al. 2007], endothelial cells apparently use Fn matrix assembly to purposesfully deposit an elastic layer of proadhesive binding sites whose concentration and topology support neovessel formation.

In vivo, Fn is expressed transiently around the developing vasculature during both vasculogenesis and angiogenesis [Clark et al. 1982, Risau and Lemmon 1988; Neri and Bicknell 2005]. As the vascular structures mature, Fn expression wanes, and the maturing blood vessels are invested with a type IV collagen/laminin-rich basement membrane [Risau and Lemmon 1988; Jain 2003]. Fn is re-expressed, however, in the neovascular surrounding wound sites or tumors [Neri and Bicknell 2005]. Significantly, large deposits of unfolded Fn could be detected in association with neovessels infiltrating cancerous tissues. While Fn fibrils are also found, as expected, in association with fibroblasts, it is important to stress that we were unable to identify a similarly critical role for Fn matrix assembly in fibroblast function—under either 2D or 3D culture conditions [Supplemental Fig. 3]. These findings reinforce earlier reports where inhibitors of Fn fibrillogenesis did not affect fibroblast behavior in 3D collagen gels [Halliday and Tomasek 1995]. Similar analyses of Fn matrix assembly in wild-type and Fn knockout embryos indicate that distinct differences exist between embryonic fibroblast and endothelial cell behavior in vivo and in vitro [George et al. 1993; Deroanne et al. 1996; Hocking et al. 2000; Sottile et al. 2000; Deroanne et al. 2001; Francis et al. 2002]. Cell type-specific responses to Fn fibrillogenesis are likewise consistent with more recent findings that mesenchymal cell populations display singular mechanical requirements for optimizing their responses to matrix-derived signals [McBeath et al. 2004; Georges and Janmey 2005; Engler et al. 2006]. Interestingly, a unique role for Fn in the specific regulation of endothelial cell behavior is supported by the fact that unlike most ECM molecules, Fn expression is limited to vertebrates, and its appearance in evolution coincides with the appearance of the vasculature [Hynes and Zhao 2000].

With increasing frequency, the morphology, adhesive interactions, and behavior of cells are recognized as being uniquely affected by the dimensionality of their surrounding matrix environment [Hotary et al. 2003, Debnath and Brugge 2005; Chun et al. 2006; Nelson and Bissell 2006, Yamada and Cukierman 2007]. Under 3D conditions, we posit that the random intertwining of the surrounding fibrin or collagen fibrils precludes the high-density, planar display of adhesive ligands on a rigid template that can support integrin clustering and/or cellular traction. These data are consistent with a model wherein the endothelial cell has elected to assemble its own pericellular matrix of polymerized Fn to meet the spatial and structural constraints placed on transmitting extracellular signals to intracellular compartments and effectors. By regulating cell shape, stiffness sensing, and mechanotransduction, Fn matrix assembly is shown to serve as a multifaceted and 3D-specific regulator of neovessel formation that may prove useful as a target for therapeutic intervention in pathologic states.

Materials and Methods

Endothelial cell isolation and 2D/3D culture

Endothelial cells were isolated from human umbilical cord veins by collagenase digestion and cultured in Medium 199 (Gibco) containing 20% human serum, 50 µg/mL endothelial cell growth supplement (BD Biosciences), 100 U/mL penicillin, and 100 µg/mL streptomycin ([Hiraoka et al. 1998]. For 2D/3D culture, endothelial cell monolayers (no later than third passage) were suspended by mild trypsinization and dispersed within or plated atop fibrin (5 mg/mL) or collagen (2.2 mg/mL) gels (prepared as described, Hiraoka et al. 1998, Hotary et al. 2003), and stimulated with a cocktail of growth factors including 100 ng/mL human vascular endothelial growth factor (VEGF) (Genentech), 50 ng/mL human hepatocyte growth factor (HGF) (Genentech), 10 ng/mL human TGFα (Biosource), 0.5 ng/mL TGFβ1 (R&D Systems), and 100 µg/mL heparin (Sigma). In selected experiments, endothelial cell spheroids were prepared and suspended in 3D fibrin gels.

To monitor Fn matrix assembly or to assess the impact of inhibiting Fn matrix assembly on endothelial cell function, human serum was depleted of Fn by gelatin-sepharose affinity chromatography [Amersham] and supplemented with either 20 µg/mL human plasma Fn (Sigma) or fluorescein isothiocyanate (FITC)-labeled Fn. Where indicated, endothelial cells were incubated with mouse IgG (Pierce), monoclonal antibody L8, monoclonal antibody 9D2 (final concentration of 100 µg/mL), the 70-kDa Fn fragment (75 µg/mL, Sigma), the FUD peptide...
3D fibronectin matrix assembly

(250 nM), blebbistatin (50 µM, Calbiochem), or cytochalasin D (10 µM, Sigma) during 3D culture. Cell number in 3D cultures was determined by hemocytometry after dissolving gels with 2 mg/mL bacterial collagenase (Worthington) while the number of patent tubules was determined in randomly selected cross-sections.

To monitor endothelial cell contractility, 2D or 3D cultures cast in 24-well plates at 2 d incubation [prior to commencement of 3D proliferation] were detached from the tissue culture plate and cells were allowed to contract the matrices for 10 h, at which time gel diameter was measured. To assess migration in 2D, endothelial cells were plated atop a fibrin substrate in the presence of a glass coverslip. After attachment, the coverslip was removed and migration into the cell-free area monitored over 2 d. To monitor 3D migration, endothelial cells were suspended in 3D fibrin gels in 96-well tissue culture plates. After formation of the fibrin gel, the gels were removed and embedded within fibrin gels in 24-well plates. Migration from the inner gel into the outer gel was monitored over 6 d.

Antibody purification and fluorescent Fn labeling

9D2 monoclonal antibody was purified from the 9D2 hybridoma cell line (Sottile et al. 1998) by Staph protein A/G affinity chromatography following the manufacturer’s protocol (Pierce). The L8 monoclonal was purified as described previously (Chernousov et al. 1991). Human plasma Fn (Sigma) was labeled with FITC as described [McKeown-Longo and Mosher 1985].

Western blot and immunofluorescence

For Western blotting, the following antibodies were used: ERK1/2 (Santa Cruz Biotechnology), phospho-p42/44 MAPK (Thr180/Tyr182), β-actin, α-actinin (Cell Signaling Technologies), NMMIIA, and NMMIIB (Covance). Phosphorylated JNK was detected following pull-down with c-Jun fusion protein beads (Cell Signaling Technologies) and probed with phospho-SAPK/JNK1/2 (Thr183/Tyr185) polyclonal antibody (Cell Signaling Technologies).

For active β1 integrin immunofluorescence, 3D cultures were fixed in 4% formaldehyde, permeabilized with 0.25% Triton X-100, and incubated overnight with an anti-active β1 monoclonal antibody [Chemicon] followed by an Alexa 488-conjugated secondary antibody (Molecular Probes). For visualization of the F-actin cytoskeleton, cultures were stained with Alexa 594- or Alexa 488-conjugated phalloidin (Molecular Probes) following permeabilization. To examine the 3D Fn matrix, fibrin gels supplemented with 20 µg/mL FITC-labeled Fn were fixed with 4% formaldehyde. All 3D gels were analyzed by confocal microscopy.

Histology and transmission electron microscopy (TEM)

3D cultures were fixed in 4% formaldehyde, processed for parafin-embedded sectioning and standard haematoxylin and eosin (H&E) staining. TEM was carried out as described previously (Hirooka et al. 1998; Hotary et al. 2003).

Plasmid constructs and transfection

The pRK/EGFP-vinculin vector was introduced into endothelial cells by lipofectamine-mediated transfection [Invitrogen]. Cells were used for 3D culture 24 h following transfection.

Chorioallantoic membrane angiogenesis assay

3D matrices of type I collagen or a type I collagen/fibrin composite matrix were cast in transwell tissue culture inserts (24-well size) perforated with a 25-gauge needle. A 30-µl Matrigel (BD Biosciences) reservoir was placed atop the matrix containing 200 ng of VEGF, 100 ng of HGF, and either the del29 or FUD peptides. The entire apparatus was placed atop the dropped CAM of 10- to 11-d-old fertile chicken eggs. Following incubation in a humidified incubator for 3 d at 37°C, the matrices were harvested and processed for sectioning and H&E staining.

3D intracellular microtiroughology

The micromechanical properties of the cytoplasm of endothelial cells embedded in the 3D were measured using the method of ballistic intracellular nanorheology (Panorchan et al. 2006, 2007). To our knowledge, this is the only method capable of probing the intracellular micromechanics of single cells embedded in a 3D matrix. Briefly, 100-nm-diameter polystyrene fluorescent beads were ballistically injected in the cytoplasm of cells using a biologic particle delivery system (Bio-Rad) [Lee et al. 2006]. The cells were then incorporated in the 3D fibrin matrix. After 72 h of incubation, the beads (between five and 10 per cell) had dispersed uniformly in the cytoplasm and their centroids were tracked for 20 sec with high spatial (~5 nm) and temporal (~33 msec) resolutions using a Cascade 1k CCD camera [Roper Scientific] mounted on a Nikon Eclipse TE2000-E epifluorescence microscope and controlled by the software MetaView [Universal Imaging]. The MSDs of individual beads were computed from the time-dependent (x,y) coordinates of the beads’ centroid displacements. The MSDs were verified to have a slope <1; i.e., no directed motion of the beads was ever observed. A simple mathematical manipulation detailed in Tseng et al. (2002) was used to transform MSDs into elastic modulus [reported here] and viscous modulus of the cytoplasm. The number of individual cells and total number of beads tracked for each type of cell are indicated in the legend for Figure 6. Mean values, standard error of measurement (SEM), and statistical analysis of bead displacements were calculated and plotted using Graphpad Prism (Graphpad Software). Two-tailed unpaired t-tests were conducted to determine significance, which was indicated using the standard Michelin Guide scale.

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