

A Balance of Substrate Mechanics and Matrix Chemistry Regulates Endothelial Cell Network Assembly

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Abstract—Driven by specific extracellular matrix cues, endothelial cells can spontaneously assemble into networks. Cell network assembly is, in part, dictated by both substrate stiffness and extracellular matrix chemistry; however, the balance between substrate mechanics and matrix chemistry in promoting cell network assembly is not well understood. Because both mechanics and chemistry can alter cell–substrate and cell–cell adhesion, we hypothesized that cell network assembly can be promoted on substrates that minimize cell–substrate adhesivity while promoting cell–cell connections. To investigate these hypotheses, bovine aortic endothelial cells (BAEC) were seeded on variably compliant polyacrylamide (PA) substrates derivatized with type I collagen and observed over time. Our results indicate that cell network assembly can be induced on substrates that are sufficiently compliant (Young’s modulus, $E = 200$ Pa) and present significant amounts of substrate-bound ligand, and on substrates that are stiffer ($E = 10,000$ Pa) but which present less adhesive ligand. In both of these cases, cell–substrate adhesivity is decreased, which may enhance cell–cell adhesivity. Moreover, our data indicate that fibronectin polymerization stabilizes cell–cell contacts and is necessary for network formation to occur regardless of substrate compliance or the density of substrate-bound ligand. These data demonstrate the balance between substrate mechanics and chemistry in directing cell network assembly.

Keywords—Polyacrylamide gel, Substrate compliance/stiffness, Fibronectin polymerization, Angiogenesis.

INTRODUCTION

Angiogenesis is the process of new blood vessel formation. Regulated by factors including shear stress, growth factors, and cytokines, endothelial cells (ECs) migrate from pre-existing vessels, proliferate, and differentiate to form new blood vessels.⁶ The extracellular matrix (ECM), which includes collagen and fibronectin (Fn),^{15,26} provides ECs with chemical and mechanical cues that drive migration and invasion,² events integral

to new vessel formation. While these events occur *in vivo*, ECs can be induced to assemble into capillary-like networks that mimic angiogenesis *in vitro*¹⁸ under the appropriate conditions. Because vascularization is critical to most successful tissue engineering, understanding and controlling these conditions may be critical to the *in vitro* development of transplantable organs.

Cell network assembly has been shown to be influenced by ECM protein type,^{32,33} ECM concentration^{13,31} and substrate stiffness^{3,27} through their effects on cell shape,¹² motility, and differentiation.¹⁶ Tissue formation may arise from the optimization of mechanical and chemical input from both cell–cell cohesivity and cell–substrate adhesivity.^{9,25} Recent evidence suggests that cellular organization may be directed in part by substrate mechanics; in contrast to compliant substrates, rigid gel substrates promote cytoskeletal and focal adhesion organization and cell spreading.⁵ On compliant substrates, cells tend to aggregate rather than migrate away from each other. Substrate mechanics may have a direct effect on EC sorting and subsequent *in vitro* organization. Recent data from our lab suggests that ECs on compliant substrates prefer cell–cell connections and cluster while those on stiff substrates prefer cell–substrate connections and migrate away from each other.²³ ECs exhibit an increase in projected area and the appearance of actin stress fibers with increasing substrate stiffness,³⁵ and others have suggested that EC capillary morphogenesis may be modulated by a balance between substrate stiffness and traction force generation.²⁷ Taken together, these data indicate that substrate compliance can directly affect cell organization.

Endothelial cell network assembly is further influenced by ECM concentration. EC attachment increases with increasing collagen concentration and promotes the formation of capillary networks at intermediate adhesivity.¹³ It has been suggested that Fn may control EC assembly by supporting tension-dependent cell

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shape changes.¹¹ While it is well accepted that both matrix chemistry and mechanics affect endothelial network formation, the balance between substrate mechanics and matrix chemistry in promoting endothelial cell network assembly is not well understood.

In this study, the effects of substrate mechanics and matrix chemistry on EC adhesion were investigated to understand the integration of mechanical and chemical signals that mediate cell network assembly. Bovine aortic endothelial cells (BAEC) were seeded on variably compliant polyacrylamide (PA) substrates derivatized with type I collagen. Our results indicate that cell network assembly occurs on compliant substrates and not on stiff substrates at a high concentration of matrix collagen, that cell network assembly is induced on stiff substrates by lowering the concentration of collagen, and that overall cell network assembly is dependent on the ability of BAECs to polymerize Fn. These results indicate that ECs may integrate mechanical and chemical cues to achieve a balance in cell–cell vs. cell–substrate adhesion; when cell–substrate adhesivity is low, cell–cell connectivity is increased, and conversely, when cells are firmly adherent to a substrate, they are less likely to assemble.

MATERIALS AND METHODS

Coverslip Activation

Coverslips were prepared as previously described.^{24,34} Square glass coverslips (No. 2, 22 × 22 mm, VWR, West Chester, PA) were passed through the flame of a Bunsen burner, coated with 0.1 N NaOH (Sigma-Aldrich, St. Louis, MO), and allowed to dry. The coverslips were coated with 3-aminopropyl-trimethoxysilane (Sigma-Aldrich, St. Louis, MO), washed in 18.2 MΩ cm purified deionized water, and incubated with a coating of a 0.5% solution of glutaraldehyde (70% aqueous stock solution, Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline ((PBS), Invitrogen, Carlsbad, CA) at room temperature for 30 min. The coverslips were washed with 18.2 MΩ cm purified deionized water and allowed to dry overnight.

Poly(acrylamide) Gel Synthesis

Gels of various Young's moduli (200, 1000, 2500, 5000, and 10,000 Pascals) were synthesized by varying the ratio of acrylamide to bis-acrylamide in the gel solution mixture.³⁵ Gel mixtures were prepared with 3–7.5% acrylamide (40% w/v solution), 0.04–0.35% N,N'-methylene-bis-acrylamide (2% w/v solution), 0.05% N,N,N,N-tetramethylethylenediamine (Bio-Rad, Hercules, CA), and 30 mM 4-(2-hydroxy-

ethyl)-1-piperazineethanesulfonic acid ((HEPES), pH 6.0, Sigma-Aldrich, St. Louis, MO). The solutions were adjusted to pH 6.0 with 2N HCl (Sigma-Aldrich, St. Louis, MO) and degassed for 30 min. Additional solution components were 20 μmol/mL N-6-((acryloyl)amido)hexanoic acid ((N-6), synthesized in our lab according to the method of Pless *et al.*²²) dissolved in ethanol (Sigma-Aldrich, St. Louis, MO), and 2% 0.5 μm diameter fluorescent beads (Molecular Probes, Carlsbad, CA).

Polymerization was initiated by the addition of a 0.1% ammonium persulfate (Bio-Rad, Hercules, CA) solution in water to the acrylamide mixture. A total of 20 μL of the mixture was pipetted onto an activated coverslip and a circular coverslip (No. 2, 18 mm diameter, VWR, West Chester PA) was used to flatten the drop. Polymerization was allowed to occur for 30 min at room temperature. The circular coverslip was removed, and the gel was incubated with 1, 5, 10, 50, or 100 μg/mL of type I rat-tail collagen (Becton Dickinson, Franklin Lakes, NJ) in HEPES (pH 8.0, Sigma-Aldrich, St. Louis, MO) for two hours at 4 °C. The un-reacted N-6 linker was capped with 0.1% ethanolamine (Sigma-Aldrich, St. Louis, MO) in HEPES (pH 8.0, Sigma-Aldrich, St. Louis, MO). Gels were washed with sterile PBS and stored in six-well plates.

Cell Culture

BAECs were maintained at 37 °C and 5% CO₂ in Medium 199 (Invitrogen, Carlsbad, CA) supplemented with 10% FetalClone III (HyClone, Logan, UT), and 1% each of penicillin–streptomycin, MEM amino acids (Invitrogen, Carlsbad, CA), and MEM vitamins (Mediatech, Manassas, VA). BAECs were used from passages 8–12.

Fibronectin Inhibitor and Control

Fibronectin polymerization was inhibited by adding 500 nM pUR4B (a kind gift from Dr. Jane Sottile) to the BAEC suspension just prior to gel plating. A total of 500 nM III-11C was added to the BAEC suspension to serve as a control to pUR4B^{20,28} (also a kind gift from Dr. Sottile).

BAEC Network Assembly, Area, and Perimeter Studies

BAECs were plated on gels of various Young's moduli (200–10,000 Pa) at a density of 100,000 or 200,000 cells per well of a six-well plate for cell-assembly studies and 50,000 cells per well for area and perimeter studies. 10× magnification images of cells were captured with an Olympus IMT-2 inverted phase contrast microscope with a QImaging Retiga 1300

camera or a Zeiss Axio Observer.D1m inverted phase contrast microscope with an AxioCam camera for the duration of the experiment. Media were replenished every other day.

For cell-assembly studies, aspect ratios of BAECs with network morphology were measured with Image J (version 1.37, available from the National Institutes of Health, Bethesda, MD, at <http://rsb.info.nih.gov/ij/>). The aspect ratio was defined as the ratio of straight line-segment lengths drawn onto images of BAECs corresponding to the long and short axis of the cells. Aspect ratio measurements were constrained to BAECs that were in contact with at least two additional cells. Data were measured and pooled into two groups ($n = 150$ for each condition) representing the presence or absence of cell network assembly across multiple experiments and time points. For area and perimeter studies, Image J was used to outline and quantify cells. Area and perimeter measurements were constrained to BAECs that were not in contact with any other cells in pre-network, sub-confluent cultures. Data were measured ($n = 50$ for each gel compliance) across multiple experiments at 24 h after plating.

Statistical Analysis of Assembly, Area, and Perimeter Measurements

The natural log of aspect ratios, cell area, and cell perimeter measurements were taken to ensure normality of the data. Data were compared with analysis of variance and Tukey's Honestly Significantly Different test or Student's t test (Fig. 4d only) in JMP software (v.7, SAS, Cary, NC). All data were reported as mean \pm standard error of the mean (SEM).

Immuno- and Fluorescent Staining for Fibronectin Localization

BAECs on PA gels were fixed in 3.7% formaldehyde (Mallinckrodt Baker, Phillipsburg, NJ) at 4 °C overnight and washed with PBS as described elsewhere.¹⁷ Samples were incubated with 1% Triton in PBS and 0.02% Tween (Mallinckrodt Baker, Phillipsburg, NJ)/1% bovine serum albumin ((BSA), Sigma-Aldrich, St. Louis, MO) in PBS for 1 h. Gels were incubated 1:50 with a mouse monoclonal fibronectin primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS/3% BSA in a humidified chamber at 4 °C overnight. A 1:200 fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS/3% BSA was applied to the samples for 1 h at room temperature. BAEC filamentous actin was stained with Alexa Fluor 546 phalloidin (Molecular Probes, Carlsbad, CA) 1:25 in PBS and nuclei were

stained with 4',6-diamidino-2-phenylindole ((DAPI), Sigma-Aldrich, St. Louis, MO) 1:10 in 18.2 M Ω cm purified deionized water. Fluorescent localization was visualized with a Zeiss Axio Observer.Z1m with a Hamamatsu ORCA-ER camera. Images were pseudo-colored with Axiovision software v. 4.6.

RESULTS

Compliant Substrates Promote BAEC Network Assembly

Previous data suggest that compliant substrates promote cell network assembly of a variety of cell types,^{9,32} including endothelial cells.^{3,27} However, in endothelial cell network formation, the relative contributions of matrix mechanics and chemistry are unclear. PA gels permit the independent manipulation of the substrate stiffness and matrix chemistry to study the effects of mechanics and chemistry on cell network assembly.^{5,9} Here, PA gels were synthesized with Young's Moduli of 200, 1000, 2500, 5000, and 10,000 Pa, derivatized with 100 μ g/mL of type I collagen, and seeded with BAECs. On 200 and 1000 Pa gels, BAECs organized into two-dimensional (2D) network structures characterized by cords and looping-cell morphologies (Figs. 1a and 1b, respectively), often seen in tube formation assays. Cords and loops of BAECs presented as early as 24 h after plating and endured for the duration of the experiment. On stiffer gels (2500, 5000, and 10,000 Pa), BAECs failed to assemble into cords or loops and appeared uniformly distributed throughout the course of observation (Figs. 1c–1e, respectively).

Time-lapse microscopy of cell network assembly on compliant gels indicated that early cords or line segments of processional BAECs branched between nodes of cells and matured into closed-loop ring patterns of cells over time. Cells were observed to sprout from cords to create additional connections (Fig. 2).

Cell Network Assembly is Induced on Stiff Substrates by Decreasing Collagen I Concentration

Because ECs tended to form networks on compliant gels where cells are also less spread and adherent, we hypothesized that a decrease in cell–substrate adhesivity enhances cell network assembly. To test this hypothesis, PA gels of varying stiffness were synthesized and conjugated with decreased collagen (1 μ g/mL) to decrease cell–substrate adhesivity relative to substrates conjugated with 100 μ g/mL of collagen. Notably, lowering the concentration of collagen I shifted cell network assembly to gels of 2500, 5000, and 10,000 Pa (Figs. 3b–3d, respectively), where it was not

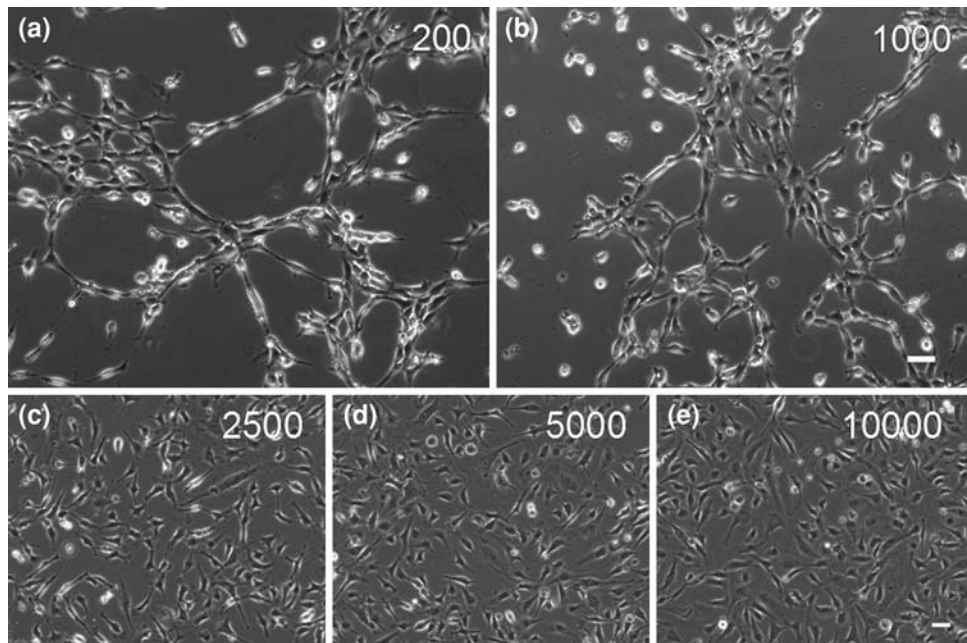


FIGURE 1. Compliant substrates promote BAEC network assembly. (a and b) Phase contrast images of BAECs on PA gels derivatized with $100 \mu\text{g}/\text{mL}$ of collagen I assemble into networks on 200 and 1000 Pa substrates, respectively. This phenotype was characterized by cords of cells and ring-like morphologies. (c–e) This organization was not present when substrate stiffness was increased to 2500, 5000, and 10,000 Pa, respectively. Bar = $50 \mu\text{m}$.

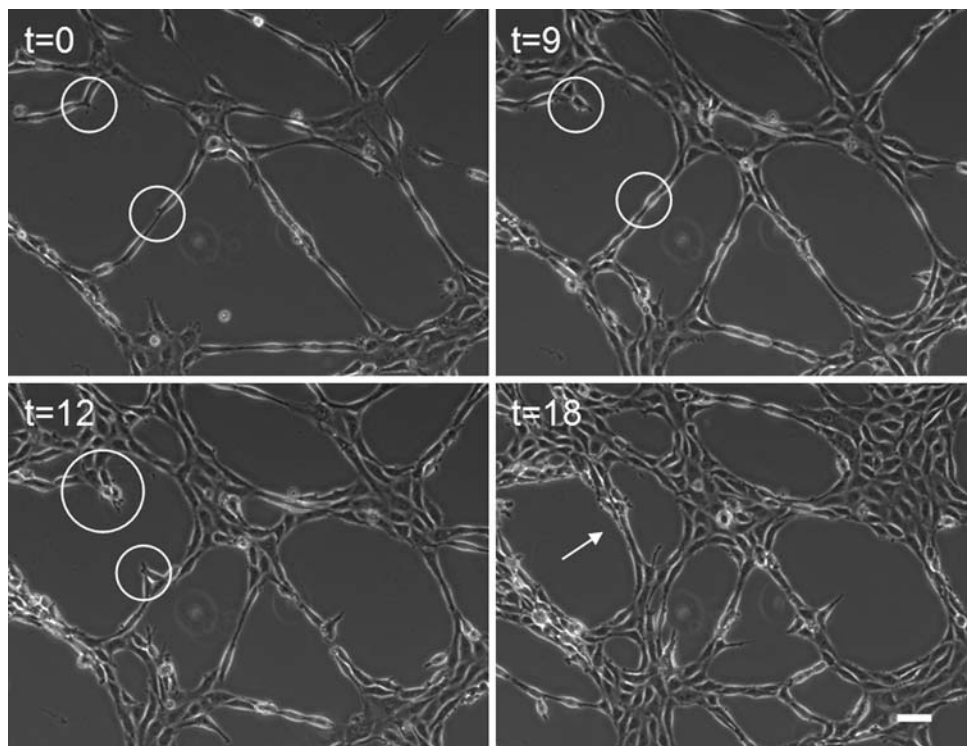


FIGURE 2. Network development over time. Time-lapse images of BAEC network development over an 18 h period. $T = 0$ corresponded to 96 h after PA gel seeding. Networks developed as cords of cells that joined together to form 2D ring-like morphologies over time. Circled regions highlight BAECs that formed an additional connection (arrow) by sprouting from existing cords. Time in hours. Bar = $50 \mu\text{m}$.

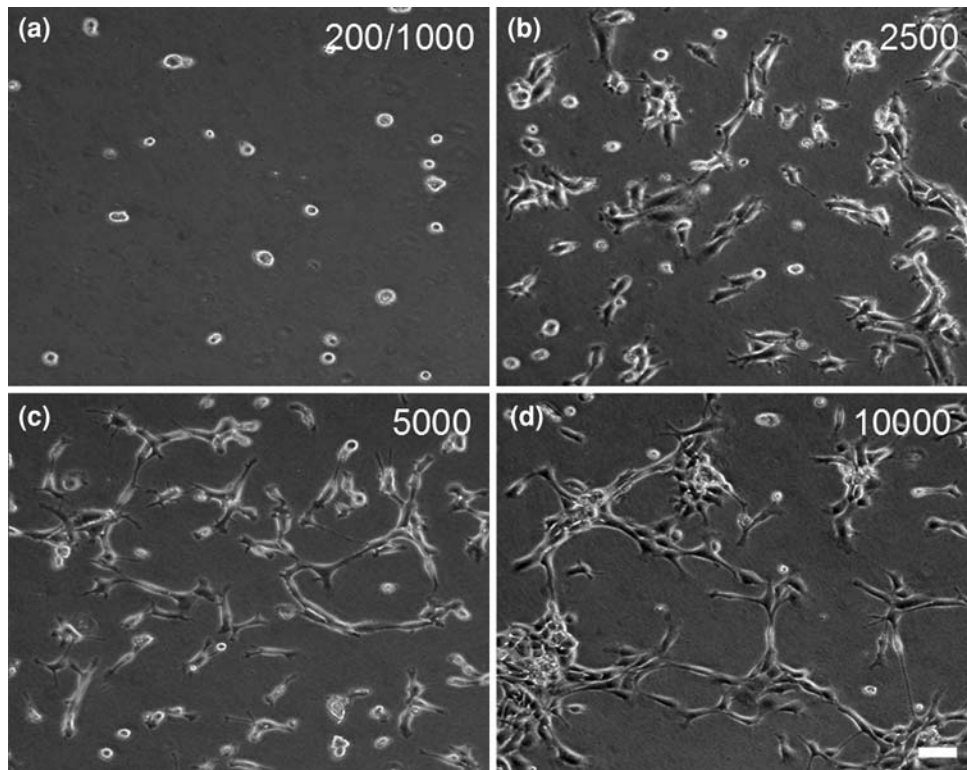


FIGURE 3. Cell network assembly is induced on stiff substrates by decreasing collagen I concentration. (a) BAECs on PA gels derivatized with $1 \mu\text{g/mL}$ of collagen I showed limited spreading and an inability to organize into networks but remained adherent to the gel. (b–d) Cell network assembly was shifted to 2500, 5000, and 10,000 Pa gels, respectively, in comparison to cells on gels derivatized with $100 \mu\text{g/mL}$ collagen. Bar = $50 \mu\text{m}$.

seen previously on gels with increased collagen (see Figs. 1c–1e). Cord development occurred on 2500 and 5000 Pa gels as well (Figs. 3b and 3c, respectively) but qualitatively did not develop into looping morphologies as complete as those on 10,000 Pa gels (Fig. 3d). On 200 and 1000 Pa gels, BAECs were adherent to the gel but retained a rounded morphology (Fig. 3a), and thus were unable to form loops. It should be noted that while decreasing the substrate adhesivity on a stiff gel increased network assembly, cell network assembly on stiffer gels derivatized with $1 \mu\text{g/mL}$ of collagen I were less extensive than assemblies formed at $100 \mu\text{g/mL}$ on compliant gels.

Ratio of Area to Perimeter Correlates with BAEC Network Assembly

To explore the mechanism of cell network assembly, we measured changes in cell morphology as a function of gel modulus and ligand density. Area and perimeter measurements were limited to BAECs that were not in contact with any other cells in pre-network, sub-confluent cultures. Cell area (Fig. 4a, white bars) and perimeter (Fig. 4b, white bars) increased with increasing substrate stiffness. When the collagen concentration was decreased to $1 \mu\text{g/mL}$, the cell area

(Fig. 4a, black bars) and perimeter (Fig. 4b, black bars) decreased. Cell adhesion and spreading were insufficient to measure on 200 and 1000 Pa gels derivatized with only $1 \mu\text{g/mL}$ of collagen. Notably, area and perimeter alone were not necessarily predictors of cell network assembly as cell areas of statistical similarity were observed in cases of both the presence and absence of cell network assembly (e.g. Fig. 4b, compare the statistically similar 2500 Pa gel white bar, where networks did not form, to the 5000 Pa gel black bar, where networks did form). However, it was determined that the ratio of BAEC area to perimeter did correlate with cell network assembly. Sub-confluent BAECs with a significantly lower ratio of area to perimeter developed into network structures (Fig. 4c, below the dashed line), whereas those with a higher ratio typically did not form network structures (Fig. 4c, above the dashed line). Therefore, the ratio of area to perimeter appeared to be a predictor of future endothelial cell network assembly, where rounder cells were less likely to form spontaneous networks.

Assembled BAECs have an Increased Aspect Ratio

To quantify cell network assembly, observed in Figs. 1 and 2, the aspect ratio of BAECs in cell

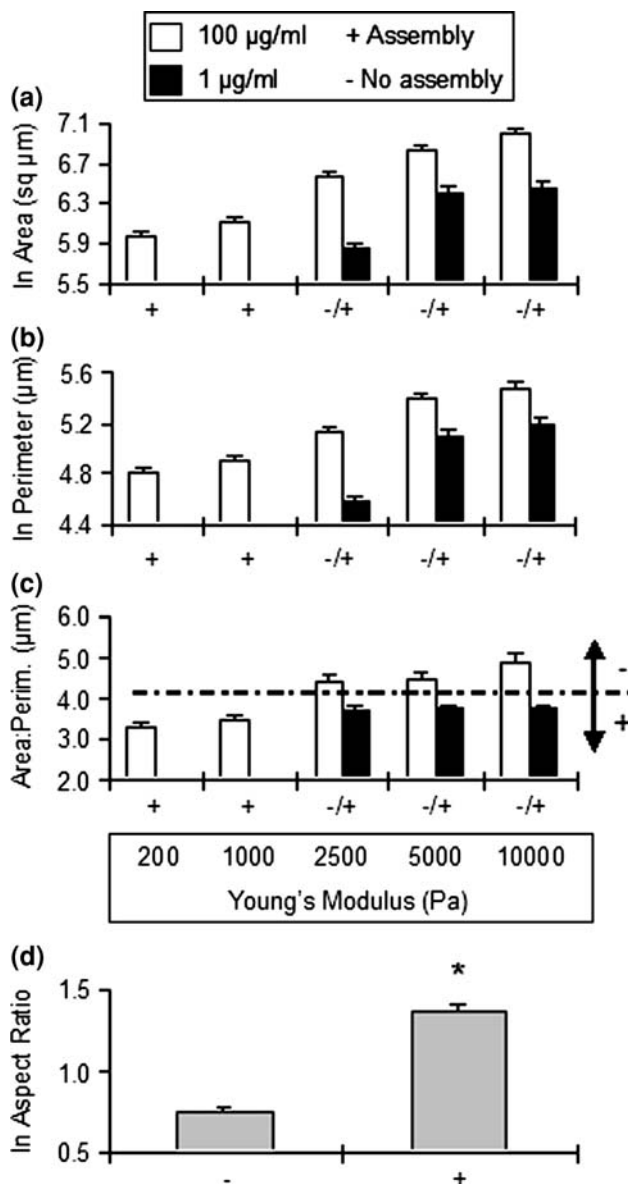


FIGURE 4. Ratio of area to perimeter predicts BAEC network assembly. (a and b) Cell area and perimeter increase on PA gels derivatized with 100 $\mu\text{g}/\text{mL}$ of type I collagen with an increase in substrate stiffness (white bars). Cell area and perimeter was reduced by lowering the collagen I concentration to 1 $\mu\text{g}/\text{mL}$ but still increased with substrate stiffness (black bars; insufficient spreading to measure on 200 and 1000 Pa gels). (c) Measurements of ratios of BAEC area to perimeter showed statistical significance between the occurrence (bars below dashed line) and absence (bars above dashed line) of cell network assembly. Note that the occurrence and absence of cell network assembly show statistical similarity within each group. (d) Aspect ratios showed statistical significance between the occurrence (+) and absence (-) of cell network assembly. “***” Indicates $p < 0.001$, +/- indicates the presence or absence of cell network assembly, respectively. Mean \pm SEM.

assemblies was measured (Fig. 4d). The aspect ratio was defined as the ratio of the long axis to the short axis of BAECs making at least two cell-cell contacts.

The aspect ratio of BAECs assembled in cords or loops (Fig. 4d, starred (+) bar) was significantly different than the aspect ratios of BAECs not arranged in networks (Fig. 4d, (-) bar). Cell network assembly was not quantified by more traditional methods including skeletonizing the micrographs²⁷ or measuring lengths of cords,¹ because in our samples cell network assembly was most extensive approximately 4 days after plating when cells on stiffer substrates were 100% confluent, and these measurements could not be done.

Fn Fibers Colocalize with BAEC Network Assembly

Recent data suggest that Fn is required for EC assembly in 3D.³⁶ While it is known that cell spreading and Fn polymerization are linked,¹⁰ and spreading increases with matrix stiffness,³⁵ it is unclear how matrix stiffness affects Fn polymerization during cell network assembly. To assay for the presence of Fn in networks formed on 2D compliant gels, BAECs on PA gels of varying compliance were stained with a FITC-conjugated anti-fibronectin antibody. Fn fibrils colocalized with BAECs organized into networks (Fig. 5a) whereas Fn was uniformly distributed in samples where networks did not form (Fig. 5b). Interestingly, Fn also colocalized with networks formed on gels conjugated with decreased collagen, however the Fn appeared more web-like (Fig. 5c) in comparison to the Fn fibrils formed by cells in networks on compliant gels.

BAECs do not Require Exogenous Fn to Assemble into Network-like Structures

Because BAEC network assembly was associated with Fn (see Fig. 5a), and others' data suggest that cell network assembly proceeds independently of exogenous layers of ECM,³² we sought to determine whether serum-derived Fn was *necessary* for 2D cell network assembly on compliant substrates. BAECs were seeded on variably compliant PA gels derivatized with collagen, either in the presence of media with fibronectin-free serum (experimental condition) or in the presence of media with complete serum (control).

Fn-free media appeared to have no effect on cell network assembly at a high collagen I concentration (100 $\mu\text{g}/\text{mL}$, as depicted in Fig. 1), however cell network assembly on stiff 10,000 Pa gels derivatized with low amounts of collagen was shifted to gels derivatized with 5 and 10 $\mu\text{g}/\text{mL}$ of collagen I where it was previously seen at 1 $\mu\text{g}/\text{mL}$. Despite the lack of exogenous Fn in the media, Fn staining still colocalized with networks of cells, indicating cells were secreting and polymerizing their own Fn (Fig. 6).

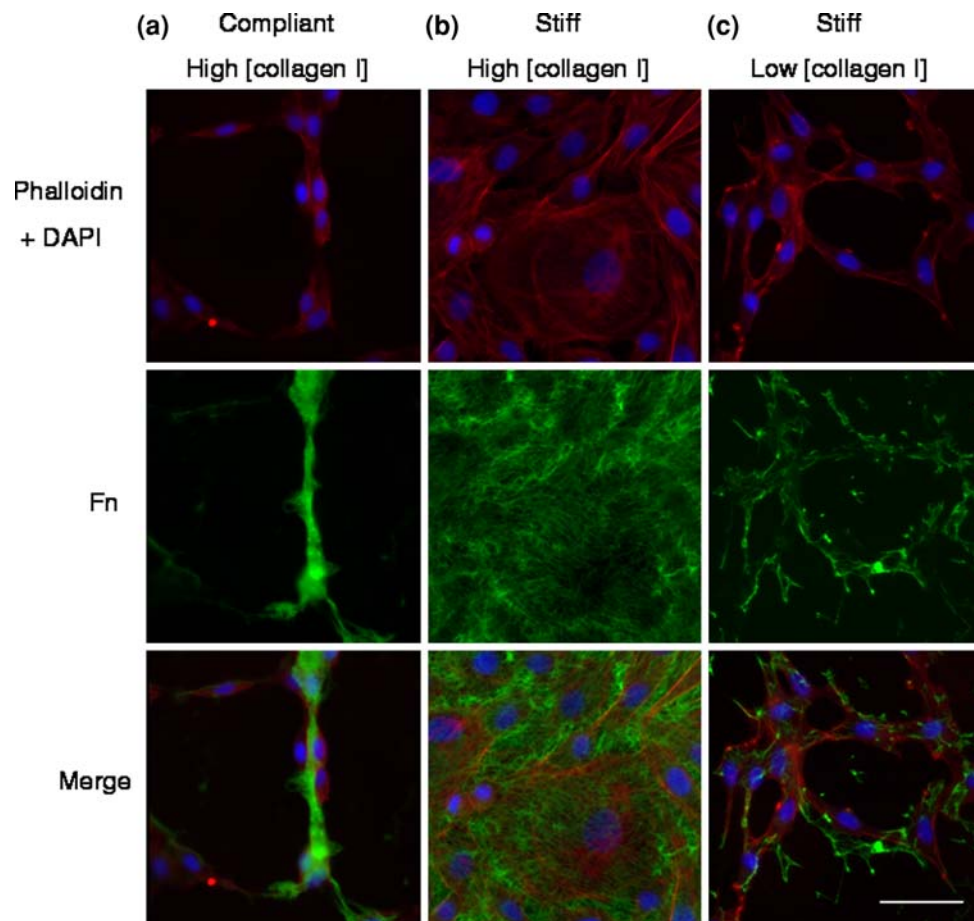


FIGURE 5. Fn colocalizes with BAEC network assembly. PA gels of 200, 1000, 2500, 5000, and 10,000 Pa were derivatized with 100 $\mu\text{g}/\text{mL}$ of type I collagen and plated with BAECs. Samples were fixed and stained with a FITC-conjugated anti-fibronectin antibody. (a) BAECs assembled into cords and loops that were colocalized with Fn. (b) Fn was uniformly distributed in samples where networks did not form. (c) Fn signal appeared web-like on 10,000 Pa gels when the collagen I concentration was lowered to 5 or 10 $\mu\text{g}/\text{mL}$. Bar = 50 μm .

BAEC Network Assembly on Compliant Substrates Requires Fn Polymerization

Our data indicate that BAEC network assembly is associated with Fn deposition (Fig. 5) and yet is independent of exogenous Fn from serum (Fig. 6). To determine whether Fn polymerization is requisite for cell network assembly, 500 nM pUR4B, a fibronectin polymerization inhibitor, or a control peptide, III-11C,^{20,28} was added to cultures on variably compliant PA gels.

In the presence of the Fn polymerization inhibitor, no stable networks formed on any type of gel, whereas in the presence of the control peptide, networks formed under the same conditions where they formed previously. On 200 Pa gels, where BAEC networks typically form, BAEC network assembly was very transient in the presence of pUR4B (Fig. 7a). Cells were adherent and appeared to form small cords, but BAECs in this configuration were not seamlessly well connected and were generally rounded in shape, unlike those observed

on 200 Pa gels without the inhibitor. Time-lapse images revealed that BAECs on 200 Pa gels treated with pUR4B appeared more motile than control cells in networks (data not shown) and failed to develop stable assemblies of cords or loops; instead, transient cell–cell connections formed that disassembled over time (Fig. 7a).

Immunostaining of cells treated with Fn inhibitor revealed a punctuate distribution of Fn surrounding the cells (Fig. 7b). This was in stark contrast to the fibrils formed between cells when the inhibitor was not added (see Fig. 5).

Interestingly, individual, subconfluent cells on stiffer, 10,000 Pa gels conjugated with 100 $\mu\text{g}/\text{mL}$ of collagen, that normally would not form networks, when treated with Fn polymerization inhibitor, displayed a morphology that was typically indicative of future cell network assembly (Fig. 8). Cells were more elongated and spindle-shaped. However, despite this pre-network-like morphology, these cells did not form

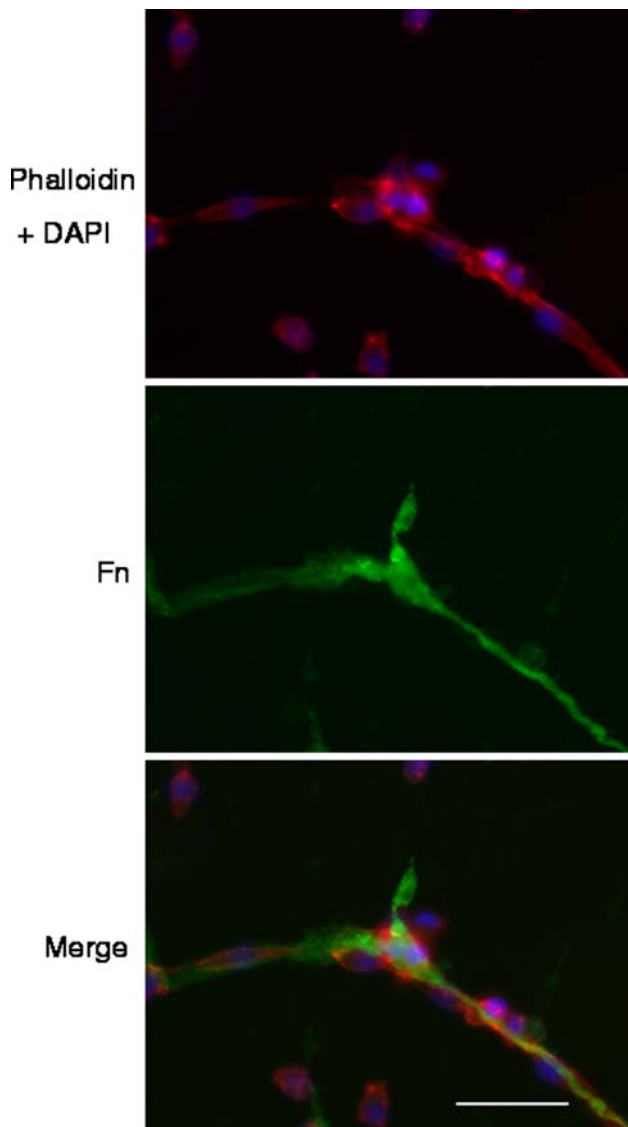


FIGURE 6. BAECs do not require exogenous Fn to assemble into network-like structures. BAECs were seeded on PA gels and derivatized with collagen I in media with fibronectin-free serum (experimental condition) or in media with complete serum (control). Samples were seeded with BAECs and stained with a FITC-conjugated anti-Fn antibody. In the presence of media containing Fn-free serum, cell network assembly occurred on 200 and 1000 Pa gels and was associated with Fn staining. Bar = 50 μm .

networks. Time-lapse microscopy of cells on these substrates in the presence of Fn polymerization inhibitor indicated that cells formed connections and elongated, but upon elongation, the connection between cell tethers either broke or one of the cells released from the substrate and rounded up (Fig. 8). In the presence of Fn polymerization inhibitor, these cells displayed impaired cell–cell and cell–substrate adhesion, perhaps attributed to their cell shape change and inability to form stable cell–cell connections.

DISCUSSION

To investigate the balance of substrate mechanics and matrix chemistry in mediating EC assembly, variably compliant PA gels were synthesized and derivatized with two (high and low) concentrations of type I collagen. Cell network assembly occurred on 200 and 1000 Pa gels, and not on 2500, 5000, or 10,000 Pa gels when the collagen concentration was 100 $\mu\text{g}/\text{mL}$. Cell network assembly was shifted to stiffer gels (2500, 5000, and 10,000 Pa) when the concentration of collagen was lowered to 1 $\mu\text{g}/\text{mL}$. We hypothesize that EC assembly results from a balance between cell–cell and cell–matrix interactions as modulated by substrate stiffness and ECM matrix chemistry, and that cell network assembly results from an optimization of mechanical input.⁹

The formation of cell assemblies due to substrate stiffness may be due to an alteration of associated focal adhesion and cytoskeletal proteins that mediate cell–substrate interactions through a tactile-sensing feedback mechanism linked to adhesion and net contractile forces.⁴ It is well established that focal adhesion size and number and stress fibers are reduced on compliant substrates³⁵ and substrates with less ligand available to bind.⁵ Cell network assembly is disrupted when traction forces are inhibited,³³ and it has been shown that a decrease in substrate rigidity, through a decrease in collagen content, induces EC cord-like morphologies with associated decreased concentrations of actin, talin, and vinculin.³ Additionally, type I collagen mediates capillary cell network assembly via $\beta 1$ integrin-activated Src and Rho activation that disrupts VE-cadherin interactions at the cell–cell junctions and induces actin stress fibers.¹⁹ Changes in these cell–cell-, cell–substrate-, and cytoskeletal-associated proteins may affect cell-generated tensile forces based on ECM density; high ECM density promotes cell spreading while lower concentrations permit EC shape changes that promote capillary tube formation.¹² Changing the density of ECM attachment sites causes cell shape changes that can affect differentiation of capillary cells^{11,14} resulting in enhanced tube formation.⁸ These results suggest that on a high collagen concentration-derivatized compliant substrate, and on a low collagen concentration-derivatized stiff substrate (conditions that promoted EC assembly), inadequate mechanical input, as mediated by substrate stiffness and matrix ligand concentration, drove BAECs to prefer cell–cell contacts that increased mechanical input and fostered cell network assembly.

Fn polymerization colocalizes with BAEC networks. It was shown previously that tube formation was associated with Fn^{7,21} and that BAECs form networks of cords independent of exogenous layers of

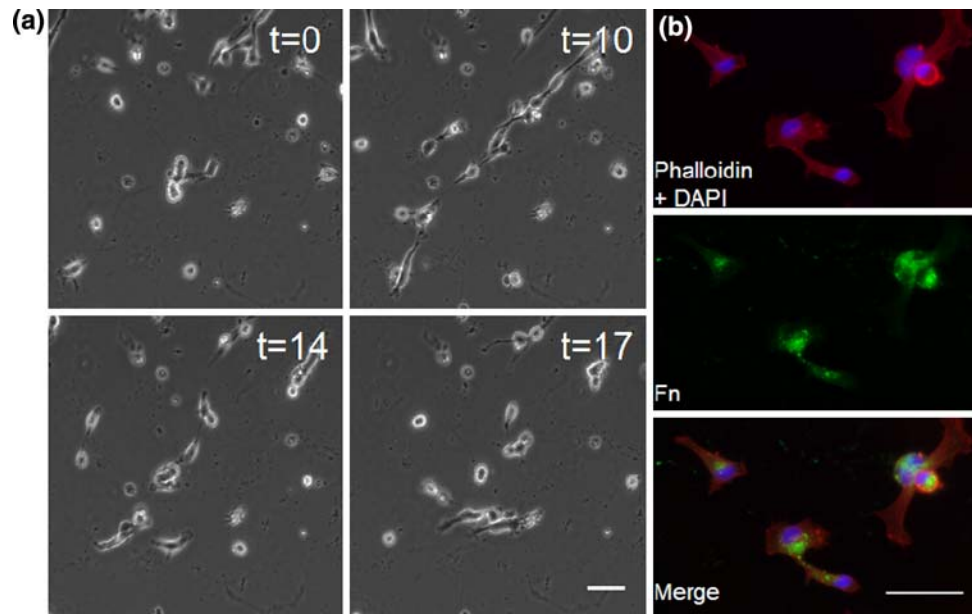


FIGURE 7. BAEC network assembly on compliant substrates requires Fn polymerization. (a) BAECs were seeded on PA gels with media containing 500 nM pUR4B fibronectin polymerization inhibitor or 500 nM Ill-11C control peptide. On 200 Pa gels in the presence of pUR4B, BAEC network assembly was disrupted with regions of transient assembly. Time-lapse images over a 17 h period revealed that BAECs treated with pUR4B appeared more motile than control cells in networks (data not shown) but failed to develop complete network structures; instead, cell clusters made transient cell–cell connections that disassembled over time. $T = 0$ corresponded to 48 h after plating. Time in hours. (b) On 1000 and 10,000 (1 $\mu\text{g}/\text{mL}$ of collagen I) Pa gels, cell network assembly was ablated in the presence of pUR4B. Fn surrounding cells treated with pUR4B appeared punctate. Bar = 50 μm .

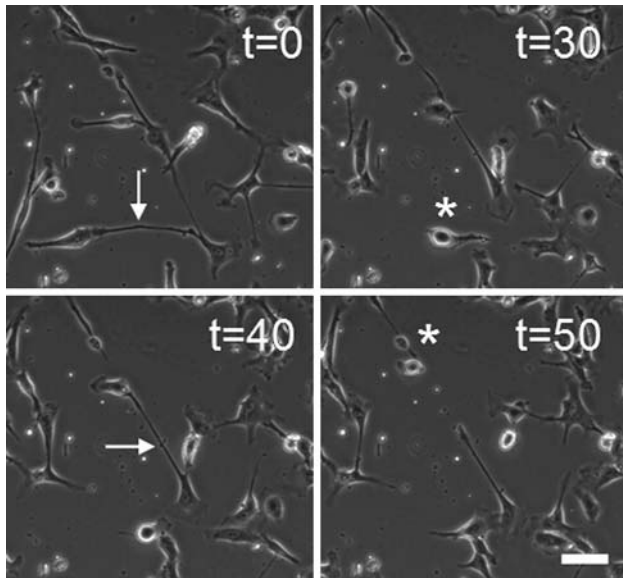


FIGURE 8. Inhibiting Fn polymerization disrupts the balance between cell–cell and cell–substrate adhesivity. BAECs were seeded on 10,000 Pa gels conjugated with 100 $\mu\text{g}/\text{mL}$ of collagen I in the presence of Fn polymerization inhibitor and recorded using time-lapse microscopy. When Fn polymerization is inhibited, cells appear more elongated and spindle-shaped with long cell–cell connections (arrows) which are typical of cells prior to network formation. However networks do not form; cell–cell connections are transient and tend to break and/or result in cells rounding and releasing from the substrate (asterisk). Time in minutes. Bar = 50 μm .

ECM.³² Cell network assembly on PA gels was associated with Fn deposition and independent of exogenous soluble Fn. On stiff 10,000 Pa gels, a low collagen concentration resulted in cell network assembly with or without Fn-containing serum used in the media. This result suggests that a decreased adhesivity (due to a decreased collagen concentration), and not soluble ECM, induced cell network assembly.

The inhibitor pUR4B is a 49-mer peptide that binds to, and inhibits, Fn matrix polymerization²⁸ by interfering with the interaction of Fn and molecules at cell-surface assembly sites.³⁰ It has been suggested that Fn polymerization is integral to maintaining ECM Fn,²⁹ and it has been shown to regulate neovessel formation by supporting cytoskeletal organization and the development of actomyosin-dependent tension.³⁶ Our data further show that BAEC 2D cell network assembly requires Fn polymerization. Figure 7a indicates that BAECs plated on 200 Pa gels, where networks normally form, when treated with pUR4B form transient assemblies characterized by rounded cells. Time-lapse images revealed that these BAECs appeared more motile, and made transient cell–cell connections compared with control cells in networks. These results suggest that BAECs require Fn polymerization to stabilize cell–cell interactions that promote cell network assembly. While pericellular Fn polymerization plays a role in 3D neovascularization³⁶

that may occur during angiogenesis, both the combination of a compliant substrate that drives BAEC proximity, and Fn polymerization which stabilizes cell–cell contacts, facilitate EC 2D assembly.

Our data also indicate that Fn polymerization plays a key role in the balance between cell–cell and cell–substrate adhesion that drives tissue assembly. When ECs are unable to polymerize Fn, their ability to spread is impaired,¹⁰ therefore the cells are generally smaller, appear more spindle-shaped and appear to be less adherent to the substrate. When cell–substrate adhesion is decreased, cells typically shift the balance to cell–cell adhesion,²⁵ and increased cell network assembly and aggregation. So we might expect that by inhibiting Fn polymerization, cell network assembly might increase due to a decrease in cell spreading and cell–substrate adhesion. However, Fn polymerization also appears to reinforce cell–cell connections (Fig. 7). Therefore, when Fn fibril formation is inhibited, cell–cell adhesions are unstable and transient. Clearly, there exists a balance where Fn polymerization supports and strengthens the formation of stable cell–cell contacts without enhancing cell spreading to the extent that cell–cell contact is no longer preferred over cell–substrate adhesion.

Together, these data indicate that substrate mechanics or decreased cell–substrate adhesivity through changes in matrix density can drive cells into a network-like assembly, and Fn polymerization is required to form stable cell–cell contacts. These results should help further guide the design of biomaterials intended to foster angiogenesis.

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