Extracellular Matrix Fibronectin Stimulates the Self-Assembly of Microtissues on Native Collagen Gels

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Fibronectin is an adhesive glycoprotein that is polymerized into extracellular matrices via a tightly regulated, cell-dependent process. Here, we demonstrate that fibronectin matrix polymerization induces the self-assembly of multicellular structures *in vitro*, termed tissue bodies. Fibronectin-null mouse embryonic fibroblasts adherent to compliant gels of polymerized type I collagen failed to spread or proliferate. In contrast, addition of fibronectin to collagen-adherent fibronectin-null mouse embryonic fibroblasts resulted in a dose-dependent increase in cell number, and induced the formation of three-dimensional (3D) multicellular structures that remained adherent and well-spread on the native collagen substrate. An extensive fibrillar fibronectin matrix formed throughout the microtissue. Blocking fibronectin matrix polymerization inhibited both cell proliferation and microtissue formation, demonstrating the importance of fibronectin fibrillogenesis in triggering cellular self-organization. Cell proliferation, tissue body formation, and tissue body shape were dependent on both fibronectin and collagen concentrations, suggesting that the relative proportion of collagen and fibronectin fibrils polymerized into the extracellular matrix influences the extent of cell proliferation and the final shape of microtissues. These data demonstrate a novel role for cell-mediated fibronectin fibrillogenesis in the formation and vertical assembly of microtissues, and provide a novel approach for engineering complex tissue architecture.

Introduction

MANY OF THE RECENT advances in regenerative medicine have been made as details of the mechanisms involved in tissue development have emerged.¹ Cell function is controlled through complex and dynamic interactions between cells and their surrounding extracellular matrix (ECM). This 3D network is composed of collagens, glycoproteins, and proteoglycans, and provides an adhesive substrate for the organization of cells into tissues.² Fibronectin is a principal component of the ECM and plays a key role in early embryonic development.³ In the ECM, fibronectin is organized as an extensive network of elongated, branching fibrils. Soluble fibronectins are assembled (polymerized) into insoluble ECM fibrils via a tightly regulated, cell-dependent process that can be rapidly up- or downregulated.⁴ Most adherent cells assemble a fibronectin matrix via a similar cellular mechanism involving the actin cytoskeleton and integrin receptors.⁴ In turn, ligation of ECM fibronectin fibrils by cell surface receptors stimulates actin-dependent processes, including cell spreading,⁵ growth,⁶ and migration,^{7,8} promotes cell cohesion,^{9,10} and regulates morphogenetic movements in *Xenopus* embryos.¹¹ Additionally, fibronectin matrix polymerization promotes the co-deposition of collagen fibrils into

the ECM,¹² stimulates collagen fibril contraction,¹³ and increases the tensile strength of cell-embedded collagen gels.¹⁴ These studies suggest that fibronectin matrix polymerization affects embryonic development and tissue morphogenesis through bidirectional control of ECM and cytoskeletal organization and function.

Cellular self-assembly describes the aggregation of individual cells into spheroids or mixed populations of cells into multi-layered tissue-like structures.¹⁵ Self-assembly is essential during development and morphogenesis, and is emerging as an attractive approach to engineering complex 3D tissues. During self-assembly, cell-cell and cell-ECM adhesions generate mechanical forces and chemical signals that control cell motility, cell proliferation, and tissue structure.^{9,16-18} The balance of adhesive and anti-adhesive forces is thought to be a critical determinant in tissue morphogenesis, and may play a role in cellular self-assembly.^{19,20} Using this premise, we developed a model of self-assembled microtissue by combining compliant, low-adhesive type I collagen substrates²¹ with the cell-mediated formation of highly adhesive fibronectin matrix fibrils.²² Addition of fibronectin to collagen-adherent fibronectin-null mouse embryonic fibroblast (FN-null MEFs) triggered cell proliferation and induced the formation of multicellular, dome-shaped

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microtissues, referred to as "tissue bodies." Altering fibronectin and collagen concentrations influenced the extent of cell proliferation as well as the ultimate shape of the tissue bodies. These studies demonstrate the importance of ECM composition and organization in tissue self-assembly and provide a useful model for studying cell motility, growth, and morphogenesis in three dimensions.

Materials and Methods

Reagents

Human plasma fibronectin was isolated from Cohn's fraction I and II.²³ Fibronectin fragments (60, 120, and 160/180 kDa) were generated proteolytically, as described.²⁴ Laminin was from BD Biosciences. Recombinant vitronectin was produced in bacteria and purified on heparin-Sepharose (GE Healthcare).²⁵ Type I collagen was extracted from rat tail tendons using acetic acid and precipitated with NaCl.²⁶ Recombinant His-tagged functional upstream domain (FUD, also referred to as pUR-4) and the control peptide, Del29 (provided by Dr. Deane Mosher, University of Wisconsin, Madison, WI), were expressed in bacteria and purified on nickel-Sepharose (GE Healthcare).²⁷ Antibodies and their sources are as follows: anti-fibronectin clone 9D2²⁸ (a gift from Dr. Deane Mosher, University of Wisconsin, Madison, WI); anti-fibronectin clone L8, (a gift from Dr. Michael Chernousov, Weis Center for Research, Geisinger Clinic, PA); anti-bromodeoxyuridine (BrdU) mAb, Alexa⁵⁹⁴-conjugated goat anti-mouse IgG, and Alexa Fluor⁴⁸⁸-conjugated goat anti-rabbit IgG (Invitrogen); and nonimmune mouse IgG and polyclonal anti-fibronectin (Sigma).

Cell culture

FN-null MEFs⁶ (provided by Dr. Jane Sottile, University of Rochester, Rochester, NY) were cultured on collagen I-coated dishes under fibronectin- and serum-free conditions using a 1:1 mixture of Cellgro[®] (Mediatech) and Aim V (Invitrogen).

Collagen gels

Native type I collagen gels were prepared by mixing 2×concentrated Dulbecco's modified Eagle's medium (DMEM; Life Technologies), type I collagen, and 1×DMEM on ice such that the final mixture contained 0.25–2.5 mg/mL collagen and 1×DMEM. The pH of the collagen solution was adjusted to 7.2–7.4 with 0.1 N NaOH. Aliquots (0.21 mL/cm²) of the collagen mixture were added to wells of 48-well tissue culture plates or 35 mm tissue culture dishes and allowed to polymerize overnight at 37°C and 8% CO₂. Collagen gels were then equilibrated for 1 h at 37°C in 8% CO₂ with a 1:1 mixture of Aim V/Cellgro. Polymerized collagen gels had an average thickness of 2 mm. To form thin films of monomeric collagen, tissue culture wells were coated overnight at 4°C with 1 mg/mL collagen in 0.02 N acetic acid, and then washed three times with ice-cold phosphate-buffered saline (PBS).

Cell growth assays

Monodispersed FN-null MEFs cells were seeded on polymerized collagen gels (5.26×10⁴ cells/cm²) in 48-well tissue culture plates. Control collagen gels received an equal vol-

ume of media without cells. Cells were allowed to adhere to the collagen substrates for 4 h at 37°C and 8% CO₂. Various concentrations of fibronectin (6.25–200 nM) or an equal volume of the vehicle control, PBS, were then added to wells. In some experiments, vitronectin (50 nM), laminin (50 nM), or fibronectin fragments (400 nM) were added to wells. In other experiments, 9D2 mAb, L8 mAb, or control IgG (208 nM), and FUD or Del29 peptides (25–125 nM) were added at the time of fibronectin addition. Cells were incubated for up to 6 days at 37°C and 8% CO₂, and cell number was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; USB).²⁹ Briefly, MTT was added to a final concentration of 5.3 mM per well and cells were incubated for 4 h. The medium was removed and collagen gels were digested with collagenase (from *Clostridium histolyticum*, type-I; Sigma) at a final concentration of 206 units/mL. Upon digestion of the collagen gels, acidified isopropanol (0.04 N HCl) was added to dissolve the formazan crystals. Absorbance values were obtained using a spectrophotometer, and the absorbance at the reference wavelength (700 nm) was subtracted from the absorbance at the test wavelength (570 nm). Relative cell number was determined by subtracting the average absorbance obtained from "no-cell" collagen gels from absorbances obtained with cell-seeded collagen gels.

Microscopy

Bright-field images were obtained using an Olympus BX60 microscope and photographed using a Spot digital camera (Diagnostic Instruments). To obtain single-channel fluorescent images, FN-null MEFs were seeded on polymerized collagen gels (5.26×10⁴ cells/cm²) in 35 mm tissue culture dishes. Four hours after seeding, fibronectin (25 nM) was added to wells and cells were incubated for 6 days. Cells were fixed and permeabilized with 4°C acetone/methanol (1:1) for 8 min at –20°C. Fibronectin was observed using an anti-FN polyclonal antibody followed by an Alexa⁴⁸⁸-labeled goat anti-rabbit secondary antibody. After staining, cells were examined with an Olympus BX61W confocal microscope equipped with an Olympus PlanF1 immersion objective (40×, 0.8 numerical aperture).³⁰ Fluorescence images were acquired by illuminating the sample with a 20-mW argon laser and imaging with a Nipkow disk confocal head and intensified charge-coupled device camera (XR Mega 10; Stanford Photonics). Images were recorded to a DVD recorder and processed offline using ImageJ (NIH).

Multi-channel imaging within microtissues was performed using multiphoton microscopy. To observe actively proliferating cells, BrdU (100 μM; BD Biosciences) was added to wells on day 1, 18 h before fixation. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.05% Triton X-100. Fibronectin was observed using an anti-FN polyclonal antibody followed by Alexa⁴⁸⁸-labeled goat anti-rabbit secondary antibodies. Cells were co-stained with an anti-BrdU monoclonal antibody and Alexa⁵⁹⁴-labeled goat anti-mouse secondary antibodies. Cell nuclei were labeled with 4',6'-diamidino-2-phenylindole (DAPI) (60 nM). Images were collected using an Olympus Fluoview 1000 AOM-MPM microscope equipped with a 25×, 1.05 NA water immersion lens (Olympus). BrdU, labeled with Alexa⁵⁹⁴, and DAPI were simultaneously excited at 780 nm using a femtosecond Mai Tai HP Deep See Ti:Sa laser (Spectra-Physics). The emitted

FIG. 1. Effect of fibronectin on cell proliferation and microtissue formation. FN-null MEFs were seeded on polymerized collagen gels (1 mg/mL) and allowed to adhere for 4 h. **(A)** Cells were treated with increasing concentrations of fibronectin (FN; 0–200 nM). Cell number was determined on day 6. Data are presented as fold increase in cell number over 0 nM FN $\pm$ standard error of the mean (SEM) of three experiments performed in triplicate. *Significantly different from 0 nM FN, ANOVA, $p < 0.05$. **(B)** Cells were treated with 100 nM fibronectin or an equal volume of the vehicle control, PBS. Cell number was determined at various times. Data are presented as fold increase in cell number over 0 h $\pm$ SEM of three experiments performed in triplicate. *Significantly different from PBS, Student's t -test, $p < 0.05$. **(C)** Representative phase-contrast images of cells treated with either 100 nM PBS or an equal volume of PBS for various times. Images were acquired at 0, 24, 72, and 144 h. Scale bar, 50 μ m. ANOVA, analysis of variance; FN-null MEFs, fibronectin-null mouse embryonic fibroblast, PBS, phosphate-buffered saline.

fluorescence was separated using a dichroic mirror (505 nm long-pass), a 609 nm bandpass filter (#FF01-609/54-25; Semrock) for BrdU, a 460 nm bandpass filter (#FF01-460-80; Semrock) for DAPI, and detected using two bi-alkaline photomultiplier tubes. Fibronectin labeled with Alexa⁴⁸⁸ was excited at 900 nm and filtered with a 519 nm bandpass filter (#BA495-546; Olympus) using the same apparatus.

Height measurements

Tissue body heights were measured using a confocal microscope and CCD camera (Dage-MTI CD72). The fine adjustment knob on the microscope was calibrated to 1 μm /graduation and the calibration was verified using microspheres (8 μm mean diameter $\pm$ 0.11 μm standard deviation; Bangs Laboratories). Tissue body heights were measured by recording the number of graduations needed to move from the plane of focus at the cell–collagen gel interface to the peak of the tissue body. For each experiment, 35 mm tissue culture dishes were divided into three equal regions, and heights from 10 isolated tissue bodies within each region were measured.

Statistical analysis

Growth assays and tissue body height measurements are expressed as mean $\pm$ standard error of the mean (SEM) and represent one of at least three separate experiments performed in triplicate. Statistical comparisons were performed using either one-way analysis of variance followed by Tukey's post test or Student's *t*-test for unpaired samples, as appropriate. Results were considered statistically significant when $p < 0.05$.

Results

Fibronectin increases cell proliferation and induces tissue body formation

Cell proliferation is an integrated response to physical and chemical signals arising from cell adhesion to ECM proteins. Both the macromolecular composition and 3D structure of complex ECMs influence cell proliferative responses. Previous studies have shown that fibronectin enhances the growth rate of cells seeded onto monomeric collagen.⁶ To determine the effect of fibronectin on the proliferation of cells adherent to polymeric collagen, FN-null MEFs were seeded on 2-mm-thick gels of native collagen and cultured in the absence or presence of fibronectin. FN-null MEFs do not express fibronectin and have been adapted to grow under serum-free conditions.⁶ Thus, the use of FN-null MEFs in the current study allows us to characterize cell behavior in the complete absence of fibronectin and to distinguish the effects of soluble fibronectin from ECM fibronectin. Addition of fibronectin to FN-null MEFs adherent to native collagen gels resulted in an increase in cell number with increasing concentrations of fibronectin (Fig. 1A). Beginning with a fibro-

nectin concentration of 25 nM, there was a statistically significant increase in cell number compared to control samples (Fig. 1A).

To quantify fibronectin-induced cell growth as a function of time, collagen-adherent cells were treated with either 100 nM fibronectin or the vehicle control, PBS. Cell number was determined on each of 7 days. FN-null MEFs adherent to polymerized collagen and cultured in the absence of fibronectin did not proliferate over the 144 h period (Fig. 1B; +PBS). In contrast, differences in cell number between vehicle-control and fibronectin-treated cells were significant beginning 72 h after fibronectin addition (Fig. 1B; +FN). At 144 h after fibronectin addition, there was an $\sim$ 2-fold increase in cell number compared to PBS controls (Fig. 1B). Large networks of well-spread cells formed on the surface of collagen gels within 24 h of fibronectin addition (Fig. 1C). By 72 h, fibronectin had induced the formation of 3D, multicellular structures (Fig. 1C). Taken together, these data indicate that fibronectin stimulates cell spreading, proliferation, and microtissue formation of cells adherent to polymerized type I collagen.

Microtissue morphology is dependent on fibronectin concentration

Effects of fibronectin on microtissue morphology were assessed initially by phase-contrast microscopy. Addition of fibronectin to FN-null MEFs adherent to collagen gels resulted either in the formation of compact 3D structures, termed tissue bodies (Fig. 2A, 12.5–50 nM fibronectin), or flattened sheet-like structures with broad surface areas (Fig. 2A, 100 and 200 nM fibronectin), depending on fibronectin treatment concentration. Addition of 6.25 nM fibronectin to collagen-adherent FN-null MEFs had no effect on cell morphology compared to the vehicle control, PBS (compare Fig. 2A with Fig. 1C; +PBS). To quantify the effect of fibronectin concentration on tissue body height, cells were fixed on day 6 and heights were measured using confocal microscopy. Tissue body height exhibited a biphasic response to fibronectin with peak height occurring at 25 nM fibronectin (Fig. 2B). As the concentration of fibronectin was increased from 25 to 200 nM, a dose-dependent decrease in tissue body height occurred (Fig. 2B). The average height of the flattened sheet-like structures formed in response to 200 nM fibronectin was $\sim$ 3 times that observed in the absence of fibronectin (0 nM FN; Fig. 2B), indicating that these sheet-like structures are composed of multiple layers of cells. Cell height in the presence of 6.25 nM fibronectin was not significantly different from that observed in the absence of fibronectin (Fig. 2B). The contrasting effects of fibronectin concentration on cell proliferation (dose dependence, Fig. 1A) and tissue body height (biphasic, Fig. 2B) indicate that the extent of vertical height is not determined solely by the extent of cell proliferation.

FIG. 2. Effect of fibronectin concentration on height of tissue bodies. FN-null MEFs were allowed to adhere to polymerized collagen gels for 4 h. Cells were then treated with various concentrations of fibronectin (FN; 0–200 nM). **(A)** Representative phase-contrast images of cells on day 6. Scale bar, 50 μm . **(B)** Heights of tissue bodies were determined on day 6. Data are presented as average height $\pm$ SEM and represent one of two experiments performed in triplicate. *Significantly different from 0 nM FN, ANOVA, $p < 0.05$. **(C)** Collagen-adherent cells were treated with 25 nM fibronectin and tissue body heights were determined at various times. Data are presented as average height $\pm$ SEM and represent one of two experiments performed in triplicate. *Significantly different from 0 h, ANOVA, $p < 0.05$.

FIG. 3. Cell proliferation and tissue body formation in response to extracellular matrix proteins. FN-null MEFs adherent to polymerized collagen gels (1 mg/mL) were treated with 50 nM sp. fibronectin (FN), vitronectin (VN), or sp. laminin (LN), or an equal volume of PBS. **(A)** Cell number was determined on day 6. Data are presented as mean fold increase in cell number over PBS control $\pm$ SEM of three experiments performed in triplicate. *Significantly different from +PBS, ANOVA, $p < 0.05$. **(B)** Representative phase-contrast images of cells after 6 days. Scale bar, 50 μ m.

To measure the vertical expansion of fibronectin-induced tissue bodies with respect to time, FN-null MEFs were seeded on polymerized collagen gels and treated with 25 nM fibronectin to induce tissue body formation. Tissue body height was measured over the course of 6 days. A monotonic increase in tissue body height was observed as time increased (Fig. 2C). A significant increase in tissue body height was observed within 48 h of fibronectin addition (Fig. 2C). Tissue bodies assembled in response to a single treatment of 25 nM fibronectin reached a peak height of $\sim 50 \mu$ m within 5 days (Fig. 2C).

Intact fibronectin is required for cell proliferation and tissue body formation

To determine whether stimulation of cell proliferation and tissue body formation is a unique response to fibronectin, the effects of other ECM proteins on proliferation and tissue body formation were assessed. Incubation of collagen-adherent FN-null MEFs with equal molar concentrations of either vitronectin or laminin had no effect on cell proliferation (Fig. 3A). In addition, neither vitronectin nor laminin triggered the formation of tissue bodies (Fig. 3B). These data suggest that cell proliferation and tissue body formation on

polymerized collagen I is not a general property of ECM molecules.

Ligation of integrin receptors by fibronectin fragments can stimulate actin cytoskeleton organization and activate intracellular signaling events that control cell proliferation.³¹ To determine whether fibronectin fragments stimulate the proliferation of cells adherent to polymeric collagen, FN-null MEFs were seeded on polymerized collagen gels in the absence or presence of intact fibronectin or equal molar concentrations of the 60-kDa (collagen-binding), 120-kDa (integrin-binding), or 160/180-kDa (collagen- and integrin-binding) fibronectin fragments. Incubation of cells with fibronectin fragments had no effect on cell proliferation (Fig. 4A), and did not promote cell spreading or tissue body formation (Fig. 4B). These data indicate that the interaction of cells with integrin- and/or collagen-binding fragments of fibronectin alone is not sufficient to stimulate cell proliferation or tissue body formation.

Cells in tissue bodies assemble an extensive 3D fibronectin matrix

The ability of cells to spread and assemble a fibrillar fibronectin matrix is influenced by the rigidity of the under-

FIG. 4. Effect of fibronectin fragments on cell proliferation and tissue body formation. FN-null cells adherent to polymerized collagen gels (1 mg/mL) were treated with either 200 nM fibronectin or equal molar concentrations of various FN fragments. **(A)** Cell number was determined on day 6. Data are presented as mean increase in cell number over PBS control $\pm$ SEM of three experiments performed in triplicate. *Significantly different from +PBS, ANOVA, $p < 0.05$. **(B)** Representative phase-contrast images of cells after 6 days in culture. Scale bar, 50 μ m.

FIG. 5. Cells in tissue bodies polymerize an extensive three-dimensional fibronectin matrix. FN-null MEFs adherent to polymerized collagen gels (1 mg/mL) were treated with 25 nM fibronectin. After a 6 day incubation, gels were fixed and permeabilized. The fibronectin matrix was observed along the z-axis at 10 μ m step sizes using a confocal microscope. Arrows denote elongated fibronectin fibrils. Arrowheads indicate short, stitch-like fibrils. Images represent one of three experiments performed. Scale bar, 50 μ m.

lying substrate.^{32,33} To determine whether FN-null MEFs adherent to polymerized collagen gels can assemble soluble fibronectin into matrix fibrils, the formation of fibronectin fibrils within tissue bodies was assessed using confocal immunofluorescence microscopy. As shown in Figure 5, fibronectin staining was observed throughout the tissue body. Elongated fibronectin matrix fibrils were localized to the periphery of the tissue bodies and were observed primarily at the interface of the tissue body and the collagen gel (Fig. 5; 20 and 30 μm ; arrows). Fibronectin staining was also visible within the central region of the tissue body where it formed shorter stitch-like fibrils that appeared to co-localize with regions of cell-cell contact (Fig. 5; 30 μm ; arrowheads).

A comparison of the time courses for fibronectin-induced cell growth (Fig. 1C) and tissue body height (Fig. 2C) reveals that tissue bodies form before the onset of significant changes in cell number, suggesting that cells assembled into tissue bodies may be actively proliferating. BrdU incorporation and multiphoton immunofluorescence microscopy were used to determine the location of actively proliferating cells relative to tissue bodies and fibronectin fibrils. Three-dimensional reconstruction of fibronectin-induced microtissues demonstrates that cells that had assembled into tissue bodies were actively proliferating (Fig. 6A, B). Fibronectin staining was clearly visible throughout the tissue body with fibronectin fibrils extending out onto the collagen substrate (Fig. 6A) and co-localizing with proliferating cells (Fig. 6B, arrows).

Cell proliferation and tissue body formation depend on fibronectin matrix formation

To determine whether assembly of ECM fibronectin fibrils is necessary for cell proliferation and tissue body formation in response to fibronectin, studies were conducted using two different approaches to inhibit fibronectin matrix assembly. The 9D2 and L8 mAbs inhibit fibronectin matrix polymerization without affecting the initial association of fibronectin with the cell surface,²⁸ or fibronectin-integrin interactions.⁷ The 49-residue peptide (FUD) from Adhesin F1 of *Streptococcus pyogenes* inhibits fibronectin matrix assembly by blocking the binding of the amino-terminus of fibronectin to cell surfaces.²⁷ To determine whether inhibitors of fibronectin matrix assembly block fibronectin-mediated cell proliferation and tissue body formation, FN-null MEFs adherent to polymerized collagen gels were treated with fibronectin in the presence or absence of 9D2 mAbs, L8 mAbs, or FUD peptides. Addition of 9D2 or L8 mAbs inhibited fibronectin-dependent cell proliferation (Fig. 7A) and tissue body formation (not shown). In contrast, nonimmune IgG had no effect on the fibronectin-mediated responses (Fig. 7A, and not shown). Similarly, addition of FUD peptides to fibronectin-treated, collagen-adherent cells inhibited cell proliferation (Fig. 7B) and tissue body formation (Fig. 7C) in a dose-dependent manner. The control peptide, Del29, did not alter the cellular responses to fibronectin (Fig. 7B, C), demonstrating the specificity of the FUD peptide. Addition of FUD or Del29 peptides to collagen-adherent cells in the absence of fibronectin had no effect on basal cell number or cell morphology as compared to PBS-treated controls (Fig. 7B, C). Addition of 125 nM FUD peptides to fibronectin-treated cells completely inhibited tissue body formation (Fig. 7C), whereas addition of 25 nM FUD altered the final morphology

FIG. 6. Cell proliferation in tissue bodies. FN-null MEFs adherent to polymerized collagen gels were treated with 25 nM fibronectin. After a 24 h incubation, BrdU was added and cells were incubated for an additional 18 h. Fibronectin (green) was observed using an anti-FN pAb followed by Alexa⁴⁸⁸-conjugated anti-rabbit IgG. Proliferating (BrdU-positive) cells (red) were observed using an anti-BrdU mAb followed by Alexa⁵⁹⁴-conjugated anti-mouse IgG. Cell nuclei were stained with DAPI (blue). **(A)** Fibronectin, BrdU, and DAPI were observed along the z-axis at 1 μm step sizes using two-photon microscopy. Collected images were then projected onto the z-plane using ImageJ. Scale bar, 50 μm . **(B)** Merged image showing fibronectin, DAPI, and BrdU staining along one slice of the tissue body shown in A. Arrows point to fibronectin-BrdU colocalization. Scale bar, 50 μm . BrdU, bromodeoxyuridine.

FIG. 7. Cell proliferation and tissue body formation depend on FN matrix polymerization. FN-null MEFs were seeded on polymerized type I collagen. After 4h, cells were treated with either PBS or fibronectin (FN; 25 nM). In **(A)**, 9D2, L8, or nonimmune mouse IgG (208 nM) was added to fibronectin-treated wells. Data presented as fold increase in cell number over PBS $\pm$ SEM of three experiments performed in triplicate. *Significantly different from PBS, ANOVA, $p < 0.001$. **(B)** FUD or Del29 (25 and 125 nM) peptides were added to fibronectin- and PBS-treated wells. Data are presented as fold increase in cell number over PBS $\pm$ SEM and represent one of three experiments performed in triplicate. *Significantly different from PBS, ANOVA, $p < 0.05$. **(C)** Representative phase-contrast images of cells treated with either PBS or FN in the absence or presence of FUD or Del29 peptides. Images were collected on day 6 of culture and represent one of three experiments performed. Scale bar, 50 μ m. FUD, functional upstream domain.

FIG. 8. Fibrillar collagen is required for fibronectin-induced microtissue formation. FN-null MEFs cells were seeded on either thick gels of polymerized collagen or thin coats of monomeric collagen, and incubated with fibronectin (25 nM) for 4 days. Representative phase-contrast images are shown. Scale bar, 50 μ m.

of tissue bodies and led to the formation of smaller, more compact structures (Fig. 7C). These data indicate that fibronectin matrix polymerization is essential for promoting proliferation and self-assembly of cells adherent to polymerized collagen.

Fibronectin-induced cell proliferation and tissue body formation are influenced by collagen organization and concentration

To determine whether the macromolecular organization of the collagen substrate is important for tissue body formation in response to fibronectin, FN-null MEFs were seeded onto either polymerized or monomeric collagen in the presence of fibronectin. Addition of fibronectin to cells adherent to polymerized collagen induced the formation of tissue bodies (Fig. 8). In contrast, addition of fibronectin to cells adherent to monomeric collagen resulted in monolayer growth of cells (Fig. 8). As reported previously,⁶ fibronectin enhanced the growth rate of FN-null MEFs adherent to monomeric collagen (data not shown). Together, these data indicate that fibronectin-induced cell proliferation is independent of substrate collagen structure, whereas formation of 3D tissue bodies in response to fibronectin requires polymeric collagen.

Increasing the concentration of collagen in polymerized collagen gels increases the linear modulus, failure stress, and fibril density, without affecting fibril diameter.³⁴ To determine how substrate properties of polymerized collagen affect

fibronectin-induced cell proliferation and tissue body formation, FN-null MEFs were seeded on polymerized collagen gels of increasing collagen concentration (0.25–2.5 mg/mL), in the absence or presence of 25 nM fibronectin. In the absence of fibronectin, cell proliferation was unaffected by increasing collagen concentration (Fig. 9A). In contrast, increasing the collagen concentration of the polymerized gel decreased fibronectin-induced cell proliferation in a dose-dependent manner (Fig. 9A). Similarly, the morphology (Fig. 9B) and height (Fig. 9C) of fibronectin-induced tissue bodies were dependent on collagen concentration. Tissue bodies formed on 0.25 mg/mL collagen gels were characterized by broad extensions surrounding a central 3D structure (Fig. 9B). In contrast, tissue bodies formed on 2.5 mg/mL collagen gels formed compact structures with few cell extensions onto the collagen substrate (Fig. 9C). Compared to tissue bodies formed on 1 mg/mL collagen, tissue body height was less on gels composed of 0.25 and 0.5 mg/mL collagen (Fig. 9C), consistent with the more flattened morphology observed in Figure 9B. Although the final shape of tissue bodies differed as collagen concentrations increased from 1.0 to 2.5 mg/mL, the vertical heights were not different (Fig. 9C). A comparison of Figure 9A and C at 2.5 mg/mL collagen indicates that fibronectin induced the formation of 3D structures in the absence of cell proliferation.

Thus far, our data indicate that increasing concentrations of substrate collagen inhibit the growth-promoting effects of fibronectin fibrils. To determine whether increasing the concentration of exogenous fibronectin could reverse the inhibitory effects of high concentrations of substrate collagen, FN-null MEFs were seeded on 2.5 mg/mL collagen gels and treated with up to 800 nM fibronectin. Addition of increasing concentrations of fibronectin to cells on 2.5 mg/mL collagen gels resulted in a dose-dependent increase in cell proliferation over basal levels (Fig. 10A) and similarly, reversed the morphological effects of high collagen concentrations, leading to tissue bodies with progressively flatter morphologies (Fig. 10B).

Discussion

In this study, we have used FN-null MEFs adherent to native collagen gels to investigate the functional role of fibronectin fibrillogenesis on cell proliferation, cellular self-assembly, and microtissue morphology. Our data demonstrate that fibronectin specifically stimulates proliferation and self-assembly of cells adherent to native collagen fibrils into tissue-like structures by a mechanism that requires fibronectin matrix assembly. These responses were specific to intact fibronectin, as neither fibronectin fragments nor other ECM proteins triggered microtissue formation. Fibronectin-induced tissue body formation occurred in the absence of cell

FIG. 9. Cell proliferation and tissue body formation depend on collagen concentration. FN-null MEFs, adherent to polymeric collagen gels of increasing collagen concentrations (0.25–2.5 mg/mL), were treated with fibronectin (FN; 25 nM) or an equal volume of PBS. (A) Cell number was determined on day 6. Absorbance values were normalized to values obtained for +PBS on 0.25 mg/mL collagen. Data are presented as average increase in cell number $\pm$ SEM of three experiments performed in triplicate. *Significantly different from respective +PBS control, $p < 0.05$. (B) Representative phase-contrast images of cells after 6 day in culture. Scale bar, 50 μ m. (C) Heights of tissue bodies were determined using confocal microscopy. Data are presented as average height $\pm$ SEM and represent one of three experiments performed in triplicate. *Significantly different from 1 mg/mL collagen, ANOVA, $p < 0.05$.

FIG. 10. Increasing fibronectin concentrations reverse effects of collagen on cell proliferation and microtissue shape. FN-null MEFs, adherent to 2.5 mg/mL polymerized collagen gels, were treated with increasing concentrations of fibronectin (25–800 nM), or an equal volume of PBS (0). **(A)** Cell number was determined on day 6. Absorbance values were normalized to values obtained for 0 nM fibronectin. Data are presented as an average increase in cell number $\pm$ SEM of three experiments performed in triplicate. *Significantly different from 0 nM fibronectin, $p < 0.05$. **(B)** Representative phase-contrast images of cells on day 6. Scale bar, 50 μ m.

proliferation, indicating that proliferation and cellular self-assembly are independent responses to fibronectin matrix assembly.

Unlike tissue spheroids, in which balls of compacted cells form floating aggregates,¹⁵ cells at the collagen gel interface

of fibronectin-induced tissue bodies were adherent and well spread on the collagen substrate. Altering either fibronectin or collagen concentration influenced the extent of cell proliferation as well as the ultimate shape of the microtissue. Increasing fibronectin concentrations induced a monotonic

increase in cell proliferation, but had a biphasic effect on microtissue height, where higher fibronectin concentrations produced flatter, sheet-like structures with broad bases. In contrast, increasing collagen concentrations resulted in a dose-dependent inhibition of fibronectin-induced cell proliferation, but increased tissue body height, producing monolith-like structures with small bases. Taken together, these data indicate that the relative proportion of collagen and fibronectin fibrils polymerized into the ECM influences the extent of cell proliferation and the final shape of microtissues.

Cellular self-assembly in response to matrix fibronectin occurred only on polymeric collagen gels and not on monomeric collagen substrates, implying that the macromolecular organization of collagen fibrils plays a permissive role in fibronectin-induced microtissue formation. Indeed, results from our study provide support for the concept that cellular self-assembly may be controlled through local differences in adhesive and anti-adhesive forces that occur between cells and their substratum. A similar phenomenon was reported recently for bovine aortic endothelial cells wherein decreased cell–substrate adhesivity corresponded to increased cell–cell adhesion and endothelial network formation.¹⁸ Others have demonstrated that decreasing substrate rigidity permits the concurrent development of cell–matrix and cell–cell adhesions, while increasing substrate rigidity favors cell–matrix adhesions.^{19,35} As such, the biphasic effect of fibronectin on tissue body height observed in the current study may be a consequence of enhanced rigidity or adhesivity in response to increased fibronectin fibril formation that, in turn, increased cell–matrix adhesions and decreased cell–cell adhesions, leading to flatter structures.

In contrast to the biphasic effect of fibronectin concentration on tissue body morphology (Fig. 2), increasing the collagen concentration of the polymerized substrate reduced fibronectin-mediated cell spreading and increased tissue body height, leading to more compact structures (Fig. 9). Increasing the collagen concentration of polymerized type I collagen gels increases both the stiffness of the gel and the density of the collagen fibrils, but does not affect collagen fibril diameter.³⁴ Others have shown that increasing substrate stiffness while maintaining ligand density promotes cell spreading and reduces cellular self-assembly.¹⁹ On the other hand, peak cell spreading on compliant collagen gels occurs at intermediate collagen densities³⁶ and is reduced at high collagen fibril densities.³⁷ Hence, in the current study, the reduction in tissue body area that occurred in response to increasing collagen concentrations was likely due to effects of increasing collagen fibril density, which in our model, predominated over the expected cellular response to increasing substrate stiffness. Importantly, these data indicate that cellular self-organization is an integrated response to chemical and mechanical signals arising from both fibronectin and collagen fibrils in the ECM. As such, it may be possible to direct the formation and shape of engineered tissues by spatially and/or temporally manipulating the composition and organization of the supporting ECM.

An extensive fibronectin matrix was polymerized by cells throughout the microtissues and was not limited to areas in direct contact with the collagen substrate or to well-spread cells. At least two distinct morphologies of fibronectin matrices were associated with tissue bodies. Elongated, fibrillar

fibronectin appeared localized to outer regions of the tissue body and co-localized with proliferating cells. Fibrillar fibronectin staining was also observed in tracks leading away from tissue bodies, suggesting active matrix polymerization by cells during migration to the central structure. In addition, a pericellular form of the fibronectin matrix localized to central regions of the tissue bodies and was associated with nonproliferating cells. We hypothesize that regional variations in the organizational patterns of fibronectin fibrils, due in part to local variations in tension or rigidity, may give rise to distinct fibronectin matrices and, in turn, distinct cell behaviors.

Smooth muscle cells and fibroblasts seeded on native collagen fibrils fail to spread and display a reduced proliferative capacity associated with increased expression of growth inhibitory signaling molecules.^{38,39} Similarly, in the present study, FN-null MEFs seeded on polymeric collagen gels in the absence of fibronectin failed to spread and did not proliferate. In contrast, initiation of cell-mediated fibronectin matrix polymerization overcame the inhibitory effect of polymerized collagen and promoted cell proliferation. The mechanisms by which fibronectin matrix polymerization initiates cell proliferation on fibrillar collagen are not known. Growth-promoting intracellular signaling events initiated by cell adhesion to fibronectin fibrils⁴⁰ may simply override growth-inhibitory signals induced by fibrillar collagen. Alternatively, binding of fibronectin to collagen⁴¹ may physically insulate growth-inhibitory epitopes of fibrillar collagen from cells or, conversely, form a pro-migratory complex that permits cell migration over the collagen substrate⁸ to initiate cell aggregation.

Several cell types, including endothelial cells, fibroblasts, and myocytes, sense and respond to substrate rigidity. For example, fibroblasts adherent to flexible collagen-coated polyacrylamide gels show reduced cell spreading and higher rates of motility than cells on more rigid collagen-coated substrates, which spread and form stable focal contacts.³³ Further, substrate rigidity and intracellular cytoskeletal tension generation strongly influence fibronectin fibril formation.^{32,42} In turn, the formation of a fibronectin matrix enhances actin cytoskeletal tension^{13,43} and increases the mechanical tensile properties of cell-embedded collagen gels.¹⁴ In the current study, the relative proportion of collagen and fibronectin fibrils polymerized into the ECM influenced the formation and shape of microtissues, with increasing fibronectin levels leading to progressively flatter structures. These studies indicate that the local balance of ECM-derived forces, influenced in part by the extent of fibronectin fibril formation, contributes to the microenvironment of the cell to locally influence cellular behaviors essential for tissue morphogenesis.

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Disclosure Statement

No competing financial interests exist.

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