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Collagen-Polymer Guidance of Vessel Network Formation and Stabilization by Endothelial Colony Forming Cells In Vitro

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Abstract

The dynamic process of vessel formation and remodeling is essential to embryonic development, post-natal tissue homeostasis and function, wound healing, and next-generation therapeutic vascularization and tissue engineering strategies. Here, uncommon collagen polymer building blocks, specified by their intermolecular cross-link composition, were used to tune the fibril microstructure and physical properties of collagen matrices for purposes of guiding three-dimensional (3D) lumenized vessel network formation by endothelial colony forming cells (ECFC) in vitro. A new and comprehensive 3D vessel morphometric analysis approach was also used to quantify vessel network morphology and architecture parameters. Results show that independent variation of collagen concentration (fibril density) and oligomer:monomer ratio (interfibril branching), two independent determinants of matrix stiffness, yield vessel networks with different architectures and persistence. Increases in fibril density decreased the overall number of vessel networks formed, while an increase in interfibril branching led to formation of highly branched vessel networks with increased vessel volume density. In general, increasing matrix stiffness, whether by modulation of fibril density or interfibril branching, contributed to increased lumen expansion and vessel segment elongation. Finally, oligomer, but not monomer, matrices induced maturation and stabilization (>14 days) of ECFC vessel networks, marked by changes in the temporal and spatial deposition of collagen type IV. Collectively, this work highlights that ECFC vessel morphogenesis is not dependent upon matrix stiffness alone, but rather the interplay of collagen fibril microstructure and matrix physical properties. Furthermore, it identifies oligomers and their associated intermolecular cross-links as new and important design parameters for vascular-inductive matrices for use in cell culture, regenerative medicine, and engineered tissue applications.

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Keywords

endothelial colony forming cells; microstructure; oligomers; type I collagen; vasculogenesis

Introduction

Compromised vascular perfusion is a major factor associated with progression of many disease states (e.g., peripheral vascular disease and ischemic heart disease), failure of tissue/organ transplants, and complications related to wound repair. In addition, a major challenge associated with development of clinical-scale engineered tissue replacements is the lack of a functional microvasculature that integrates with the host circulation. Vasculogenesis, the process by which blood vessels form *de novo* from individual endothelial cell precursors, represents a new and promising strategy for addressing these unmet needs. It has long been accepted that vasculogenesis occurs during early embryogenesis yielding the first blood vessels and establishing a primary vascular plexus.^[1] However, a growing body of preclinical and clinical data now shows that endothelial precursors contribute to vessel growth in adults via a process consistent with post-natal vasculogenesis.^[2–4] In fact, a specific population of endothelial cell precursors, known as human endothelial colony forming cells (ECFC), can be isolated from cord or peripheral blood by adherence to extracellular matrix (ECM) proteins. ECFC display a hierarchy of clonal proliferative potential and have the capacity to undergo vessel morphogenesis when cultured in three-dimensional (3D) collagen or fibrin matrices *in vitro*.^[5–7] Furthermore, upon implantation in immunodeficient mice, ECFC spontaneously form human blood vessels that anastomose and become an active part of the systemic circulation.^[5, 8] At present, significant advancements have been made in our understanding of the molecular and cellular events underlying vascular morphogenesis and stabilization.^[9–13] However, little is known regarding how biophysical cues inherent to the surrounding collagen-based ECM modulate this process and the complex cell signal interplay. This gap in knowledge precludes the design and development of vascular-inductive biomaterials that efficiently and predictably direct the localization, morphology, and architecture of lumenized vessel networks as well as their functional integration with the host vasculature.

In-vitro 3D models involving seeding endothelial cells (EC) or their precursors within polymerizable type I collagen and fibrin matrices have proven to be invaluable tools for deciphering signaling cascades controlling vessel morphogenesis.^[14, 15] These systems have facilitated elucidation of the program of cellular events, since lumen formation and vessel elongation occur specifically in 3D and not on 2D surfaces.^[16] Early model systems incorporated phorbol esters as medium supplements or other anti-apoptotic approaches to promote EC viability and vessel morphogenesis.^[14, 17, 18] These strategies were necessary since EC, in general, undergo rapid apoptosis when embedded in traditional monomer collagen formulations in absence of these additives.^[19, 20] Within these traditional model systems, EC exhibit vacuole formation, coalescence of vacuoles to form multicellular lumens, and sprouting and branching events that yield multicellular networks of interconnected tubes.^[11] Vessel formation reportedly reaches steady state within about 48 hours with no evidence of late-stage vessel maturation or vascular basement membrane (BM) deposition.^[21, 22] More recently, it has been documented that co-culture of EC with pericytes or other accessory cells (e.g., fibroblasts, mesenchymal stem cells) promotes not only EC survival within collagen and fibrin matrices,^[23–26] but also late-stage maturation events including vascular basement membrane deposition.^[24, 27] Within these co-culture systems, lumenized vessels form and are stable for several weeks.^[28] Based on this work, it is now generally accepted that such heterotypic EC-accessory cell association is required for vessel network maturation and stabilization.^[22, 24, 29]

One limiting factor in the study of vessel formation has been the lack of 3D matrix models in which specific structural and physical features of the surrounding matrix molecules can be quantified and systematically studied. We and others have established that collagen assembly is dependent upon a number of polymerization parameters including buffer composition, pH, and ionic strength, presence of copolymers (e.g., other collagen types) or accessory molecules (e.g., glycosaminoglycans), as well as collagen molecule integrity (e.g., presence or absence of telopeptides).^[30–38] As such systematic variation of specific polymerization parameters can be used to predictably control relevant physical properties (e.g., fibril density, fibril diameter, interstitial fluid viscosity, and matrix stiffness) of polymerizable collagen-fibril matrices.^[33, 34, 36, 37] In fact, an increase in collagen concentration has been routinely used to document how matrix stiffness, which occurs as a result of increased fibril density, effectively modulates early vacuolization in vitro as well as the number and morphology of vessel networks formed by ECFC and differentiated endothelial cells in vivo.^[4, 7, 21, 39–41] Another feature known to modulate collagen fibril microstructure and matrix biophysical properties but which has not been incorporated into 3D in-vitro models to date, is the number and type of collagen intermolecular cross-links. In fact, the inability to modulate these features when using traditional collagen formulations (e.g., monomer and atelocollagen) to design 3D cell microenvironments has been identified as a major shortcoming.^[42, 43] While the collagen molecule primary sequence is identical across tissues, posttranslational modifications and formation of enzyme-mediated (lysyl oxidase) intermolecular cross-links contribute to diversification of collagen type I molecules, collagen fibril assembly, and therefore matrix form and function.^[44, 45] It is postulated that, during collagen assembly, lysyl oxidase binds to and catalyzes cross-link formation between prefibrillar aggregates of staggered collagen molecules (monomers) to create covalently cross-linked oligomers.^[46] In turn, these different oligomer precursors (dimers or trimers) direct the progressive molecular packing and assembly that eventually gives rise to tissue-specific fibril architecture and matrix function.

Recently, we have shown that different acid solubilization and purification protocols can be applied to porcine skin to preferentially yield purified type I collagen monomer and oligomer formulations^[7, 34]. Both monomers and oligomers possessed intact telopeptide regions and contained reactive aldehydes generated from acid-labile, intermediate cross-links (e.g., Schiff base). Pig skin monomers, like conventional monomer preparations, represented individual, triple-helical collagen molecules. In contrast, oligomers comprised small aggregates of collagen molecules (e.g., trimers) which retain their covalent intermolecular cross-link (e.g., histidinyl-hydroxy-lysinonorleucine). From our work, it is apparent that intermolecular cross-links and oligomers play an important role in in-vitro collagen assembly and matrix physical properties.^[7, 34] We found that an increase in oligomer:monomer ratio (average polymer molecular weight), while maintaining a constant collagen concentration resulted in an increase in matrix stiffness; however, no correlative effect on fibril density or diameter was observed.^[7] Instead, early evidence suggested that the matrix stiffening observed with oligomers was associated with an increase in interfibril branching. In this way, variation in oligomer:monomer ratio and total collagen concentration provides independent control over interfibril branching and fibril density, respectively, two fibril-level design parameters which determine matrix stiffness.

We now extend this work by demonstrating that intermolecular cross-links and associated collagen oligomers constitute a new and important determinant of the biophysical properties and vascular-instructive capacity of polymerizable collagen matrices. In the present study we show how modulation of collagen concentration (fibril density) and collagen oligomer:monomer ratio (interfibril branching) affect in-vitro formation, persistence, and stabilization (basement membrane deposition) of vessel networks by ECFC. A new 3D morphometric analysis method was used to quantify and compare differences in 3D vessel

network architecture. Importantly, this work demonstrates for the first time the capacity to incorporate biophysical cues within polymerizable collagen matrices to predictably guide specific vessel architectures and vascular network stabilization with basement membrane deposition in absence of accessory cells, phorbol esters, or anti-apoptotic agents.

Materials and Methods

Preparation of Oligomer and Monomer Collagens

All type I collagens were derived from the dermis of market weight pigs. Oligomer collagen was prepared as described previously.^[34] Monomer collagen was prepared by extracting pig skin with 0.5 M acetic acid followed by salt precipitation, which selectively eliminates oligomers from solution.^[47] Purified oligomer and monomer collagens were lyophilized from 0.1 M acetic acid solutions. Lyophilized collagens were dissolved in 0.01 N hydrochloric acid (HCl). Collagen concentration was determined using a Sirius Red (Direct Red 80) assay as previously described.^[36] Collagen formulations were standardized based upon purity as well as polymerization potential.^[34] Here, polymerization potential is defined as the relationship between shear storage modulus (G') of polymerized matrices and collagen content of the polymerization reaction.

Preparation of 3D Collagen Matrices

All collagens were polymerized under identical reaction conditions to produce 3D matrices as described previously.^[7] Collagen solutions were diluted with 0.01 N HCl and neutralized with 10X phosphate buffered saline (PBS; 1X PBS had 0.17 M total ionic strength and pH 7.4) and 0.1 N sodium hydroxide to achieve neutral pH (7.4). Neutralized collagen solutions were kept on ice prior to the induction of polymerization by warming to 37°C. Due to the increased viscosity of collagen solutions, positive displacement pipettes (Microman, Gilson, Inc., Middleton, WI) were used to accurately pipette all collagen solutions.

Analysis of Collagen Fibril Microstructure

Collagen matrices were polymerized at desired collagen concentrations or matrix G' (stiffness) in Lab-Tek chambered coverglass slides (Nunc, Thermo Fisher Scientific, Rochester, NY), fixed with 3% paraformaldehyde in phosphate buffered saline (BSA), and blocked with 1% bovine serum albumin. Matrices then were immunostained with monoclonal anti-collagen type I (C2456, Sigma-Aldrich, St. Louis, MO) followed by AlexaFluor546 donkey anti-mouse secondary antibody (A10036, Life Technologies, Grand Island, NY). Confocal microscopy was performed on an Olympus Fluoview FV1000 confocal system adapted to an Olympus IX81 inverted microscope with a 60X UPlanSApo water immersion objective (Olympus, Tokyo, Japan). Images of the fibril microstructure were collected in combined reflection (fibril-based back-scattered light) and fluorescence (immunolabeled type I collagen) modes. Image stacks were collected from at least three random locations within each of three independent matrices per matrix formulation. Fibril volume fraction (fibril density), total fibril length, average fibril length, number of interfibril branches, and fibril diameter was determined by applying Imaris Filament Tracer (Bitplane, St. Paul, MN) to merged reflection/fluorescence images.

Oscillatory Shear Testing

Viscoelastic properties of polymerized collagen matrices were measured in oscillatory shear and unconfined compression formats on a stress-controlled AR2000 rheometer (TA Instruments, New Castle, DE) using a stainless steel 40-mm diameter parallel plate geometry as previously described.^[34] Each sample was polymerized (30 minutes) on the rheometer to standardize sample geometry and adherence to plates. A shear strain sweep

from 0.01% to 5% strain at 1 Hz (chosen from predetermined linear viscoelastic response regions) was used to measure shear storage modulus (G') and shear loss modulus (G''). Reported values are at 1% strain. Shear testing was performed on three independent matrices per matrix formulation ($n=3$).

Preparation of 3D Vascularized Tissue Constructs

Human umbilical cord blood ECFC were obtained from EndGenitor Technologies (Indianapolis, Indiana) and cultured as described previously.^[5] ECFC (5×10^5 cells/ml) were suspended in neutralized oligomer or monomer solutions matched at collagen concentration or matrix stiffness. Collagen concentration was also fixed, and oligomer:monomer ratio was varied to create matrices that were 0:100, 50:50, and 100:0. Collagen-ECFC suspensions were pipetted into 96-well tissue culture plates at 58 μ l/well, allowed to polymerize for 30 minutes at 37°C, and cultured in complete endothelial cell growth medium (EGM-2, Lonza, Walkersville, MD) at 5% CO₂ at 37°C. The short polymerization times for all collagen formulations (roughly 15 minutes or less) and the cell seeding density supported formation of tissue constructs in which individual cells were homogeneously distributed within the collagen fibril matrices. After 3, 7, or 14 days, tissue constructs were fixed with 3% paraformaldehyde in PBS and stained for confocal microscopy. All ECFC were used between passage 6 and 9.

Immunostaining of Vascularized Tissue Constructs for Basement Membrane Deposition

Deposition of basement membrane components, collagen type IV and laminin, was evaluated at days 3, 7, and 14 using immunofluorescence. Tissue constructs were fixed with 3% paraformaldehyde, blocked with 1% BSA, and stained with rabbit anti-human collagen type IV (ab6581, Abcam, Cambridge, MA) or rabbit anti-human laminin (L9393, Sigma-Aldrich) overnight at 4°C. Constructs then were rinsed with PBS and incubated with goat anti-rabbit-Qdot 605 (Q11402MP, Life Technologies, Carlsbad, CA) overnight at 4°C. After rinsing, constructs were counterstained with FITC-conjugated Ulex Europaeus Agglutinin 1 (UEA-1) lectin (L9006, Sigma-Aldrich, St. Louis, MO) and imaged using confocal microscopy.

Morphometric Analysis of ECFC Vessel Networks

For morphometric analysis of ECFC vessel networks, tissue constructs were fixed in 3% paraformaldehyde and stained with FITC-conjugated UEA-1 lectin. Tissue constructs were imaged using confocal microscopy in combined fluorescence and reflection modes for visualization of ECFC and collagen fibrils, respectively. Image stacks ($211.956 \mu\text{m} \times 211.956 \mu\text{m} \times 50 \mu\text{m}$) were collected from at least 5 locations within each of 3 independent tissue constructs per matrix formulation. Image files were imported into Imaris for 3D reconstruction and analyzed using Filament Tracer software to generate filamentous microstructures that approximated the vessel network volume as shown in Figure 1. Filament Tracer provided measurements for total vessel volume, total vessel length, average vessel segment length, average vessel segment volume, and average vessel diameter. Vessel volume percent was calculated by dividing the total vessel volume by the total image volume, and the average number of vessels per network, an indicator of network branching, was calculated by dividing the total number of lumenized vessels by the total number of networks counted.

Statistical Analysis

Measured values for vessel microstructure are reported as mean $\pm$ standard error (SE). Differences between groups were analyzed using Kruskal-Wallis one-way analysis of variance, and pairwise comparisons were made using Wilcoxon-Mann-Whitney test. The

Kolmogorov-Smirnov test was used for pairwise comparisons of lumen diameter distributions. For collagen microstructural-mechanical analysis, measured values are reported as mean $\pm$ standard deviation (SD). Multiple groups were compared using an analysis of variance (ANOVA) and a least squares mean comparison using the Tukey-Kramer method. SAS software (SAS Institute, Cary, NC) was used for all statistical comparisons. Differences were considered statistically significant when $p < 0.05$.

Results

Collagen Concentration Driven Increases in Matrix Stiffness Decrease the Number of Vessel Networks Formed but Enhance Lumen Expansion and Vessel Elongation

We and others have shown that collagen concentration driven changes in fibril density and therefore matrix stiffness modulate the density and morphology of localized vessels formed by transplanted human endothelial progenitors in vivo.^[39, 41] More recently, we documented that matrices prepared with oligomers induced a greater extent of ECFC vacuolization in vitro compared to monomers and that the number and size of vacuoles could be modulated with collagen concentration.^[7] Only oligomers contain covalent intermolecular cross-links between collagen molecules (e.g., trimers), while conventional monomers represent single collagen molecules. Here, we extended this work by focusing on late-stage events associated with matrix-induced vessel formation in vitro. Specifically, ECFC were cultured 7 days in monomer or oligomer matrices, each prepared at 0.5, 1.5, and 3.0 mg/ml, to determine how increases in collagen concentration (fibril density) affect the number, morphology, and architecture of vessel networks. A new morphometric analysis method was performed on 3D reconstructed vessel network images for quantification of relevant parameters including number of vessel networks, vessel volume percent, total vessel length, average vessel segment volume, average vessel segment length, average vessel diameter, and vessels per network (branching).

At all concentrations tested, formulation-dependent differences in the extent of multicellular lumen formation and vessel elongation were apparent. In general, ECFC showed lesser vacuolization and vessel network formation within monomer matrices (Figure 2). In fact, the vessel volume percent obtained for vascularized tissue constructs imaged at a relatively low magnification (image volume about $4 \times 10^{-2} \text{ mm}^3$) was roughly 6.3 times greater for oligomer (13.3%) compared to monomer (2.11%) prepared at the same stiffness (200 Pa). Only a few vessel networks within monomer matrices persisted at 7 days since the majority that formed regressed within the first 48 hours. In contrast, oligomer matrices supported early vacuolization as well as formation of extensive multicellular vessel networks (Figure 2) that persisted well beyond 14 days, confirming our previous findings.^[7] More detailed morphometric analyses (described below) were performed on day 7 vessel networks identified within monomer and oligomer constructs by obtaining image volumes at higher magnification representing about $2 \times 10^{-3} \text{ mm}^3$. It should be noted that since vessel networks within monomer were sparse and not uniformly distributed, total vessel length and vessel volume percent values obtained at high magnification overestimated those observed over a larger tissue construct volume (at low magnification). In contrast, high magnification values obtained for oligomer underestimated those measured over a larger tissue construct volume since vessel networks were extensive and more uniformly distributed. No significant contraction was observed at the construct level for any of the experimental conditions studied. As such cell-induced matrix compaction or reduction in construct volume did not contribute to observed formulation-dependent differences in ECFC response.

Although the number of ECFC vessel networks that formed within oligomer declined from roughly 4.72 to 1.43 per image volume as concentration increased from 0.5 to 3.0 mg/ml, the vessel networks became more extensive with a marked increase in branching (vessels per

network; Figure 3A) and a significant increase ($p < 0.05$) in average vessel segment length (Figure 3E). In addition, a statistically significant ($p < 0.05$) rightward shift in vessel diameter distribution was observed with increasing oligomer concentration (Figure 3B–C). In fact, average vessel diameters increased significantly ($p < 0.05$) as a function of oligomer concentration and ranged from $13.10 \pm 5.17 \mu\text{m}$ for 0.5 mg/ml to $17.05 \pm 5.64 \mu\text{m}$ for 3.0 mg/ml (Table 1). These observed increases in average vessel segment length and average vessel diameter, contributed to significant ($p < 0.05$) increases in average vessel segment volume as a function of oligomer concentration (Figure 3F). The increase in vessel segment volume was somewhat countered by a moderate but statistically insignificant decline in total vessel length (Figure 3D), thereby yielding only modest increases in the overall vessel volume percent (Figure 3G).

As stated previously, only a limited number of vessel networks persisted within monomer at 7 days. For monomer, morphological differences observed as a function of concentration were less apparent. The number of vessel networks decreased from roughly 3.2 to 1.78 per image volume, and vessel diameter distributions showed no statistical difference as a function of concentration (Figure 4A). As shown in Table 1, average vessel diameters in monomer covered a limited range from $12.10 \mu\text{m}$ to $13.77 \mu\text{m}$ over the concentrations tested. Average vessel diameter values for 3.0 mg/ml monomer were statistically larger ($p < 0.05$) than those for 1.5 mg/ml monomer, but not 0.5 mg/ml monomer. Interestingly, average vessel diameters in monomer were statistically smaller ($p < 0.05$) than those formed in oligomer at all concentrations except at 0.5 mg/ml (Table 1). A modest decrease was observed in total vessel length with increasing monomer concentration, but the difference was not statistically significant (Figure 4C). There was also no significant difference in the average vessel length (data not shown); however, average vessel segment volume increased significantly ($p < 0.05$), most likely due to differences observed in lumen diameter (Figure 4D). Furthermore, unlike oligomer matrices, vessel volume percent within monomer matrices showed a general, but statistically insignificant, decline with increasing collagen concentration (Figure 4E).

Increases in Interfibril Branching and Matrix Stiffness, as Modulated by Oligomer:Monomer Ratio, Enhances ECFC Vessel Network Formation

Recently, we suggested that oligomers, and their associated covalent intermolecular cross-links, work to stiffen polymerized collagen matrices via increasing the extent of interfibril branching independent of fibril density.^[7] Unfortunately, at that time, there were no reliable or direct methods for quantifying and comparing the number of interfibril branches as part of detailed fibril microstructure analyses. In the present study, filament tracer software was applied to high-resolution, 3D reconstructed images of fibril microstructures for purposes of quantifying relevant parameters, including fibril volume fraction, number of interfibril branches, and average fibril diameter. Here, oligomer:monomer ratio was varied while total collagen concentration was held constant (1.5 mg/ml). As shown in Figure 5, matrices produced with high oligomer to monomer ratios (100:0) represent highly interconnected networks with relatively short interfibril lengths and high matrix stiffness. On the other hand, matrices produced with low oligomer to monomer ratios (0:100) represent entanglements of lengthy fibrils with low matrix stiffness. Image-based quantification of relevant fibril microstructure parameters confirmed that the number of interfibril branches and matrix stiffness increased significantly with oligomer content ($p < 0.05$, Figure 5). No statistical differences were observed in fibril density or diameter as a function of oligomer:monomer ratio (Figure 5), confirming our previous supposition.^[7] It is important to note that both fibril density and interfibril branching are independent determinants of matrix stiffness. As such, variation of oligomer:monomer ratio independent of collagen concentration allowed uncoupling of fibril density and matrix stiffness.

When ECFC were seeded within matrices in which the oligomer:monomer ratio and therefore interfibril branching was varied, roughly equivalent numbers of vessel networks formed but interesting differences in vessel network architectures were observed (Figure 6A). Those structures that formed in 0:100 matrices appeared as short single vessels or branched networks of short vessels. Vessel networks formed in the 50:50 matrices represented long, extended vessel structures with few branches. In fact, 50:50 matrices exhibited networks with the greatest average vessel segment lengths ($p<0.05$; Figure 6D), albeit these networks were not as highly branched as those observed in 100:0 matrices. Vessel diameters were statistically similar for 100:0 and 50:50 matrices; however, those formed in 0:100 matrices showed a statistically significant leftward shift in population distribution and statistically smaller average lumen diameters ($p<0.05$; Figure 6B). 100:0 matrices induced the greatest total vessel length and vessel volume percent ($p<0.05$, Figure 6C,E). Vessel volume percent values observed for 0:100 and 50:50 matrices were statistically similar and roughly 2.5 fold less than 100:0 matrices.

Comparison of ECFC Vessel Network Architecture and Basement Membrane Deposition within Oligomer and Monomer Matrices at Matched Stiffness

A critical step in vessel network stabilization and maturation is deposition of a supportive basement membrane.^[12, 28] To better understand the differences in the vascular-inductive properties of monomer and oligomer matrices, late-stage vessel formation events including lumenized vessel architecture and vascular basement membrane deposition were compared for monomer and oligomer matrices at matched stiffness (200 Pa).

When analyzed based on matched matrix stiffness (200 Pa), oligomer showed more robust vessel formation in terms of number of vessel networks formed as well as number of vessels per network as illustrated in Figure 7A. Only sparse vessels (roughly 67% compared to oligomer) persisted after 7 days of culture within monomer, the majority of which consisted of short, unbranched vessels. Morphometric analysis of vessel networks showed that oligomer induced a vessel diameter distribution (Figure 7B) and an average vessel diameter that was significantly greater ($p<0.05$) than those in monomer. Average vessel diameters were $12.82\pm4.18\ \mu\text{m}$ and $14.79\pm4.81\ \mu\text{m}$ for monomer and oligomer matrices, respectively. In addition, oligomer induced a 3-fold higher total vessel length (Figure 7C), 1.6-fold higher average segment volume (Figure 7D), and a 5-fold higher vessel volume percent (Figure 7E) compared to monomer, all which were statistically significant ($p<0.05$).

We then compared the temporal and spatial basement membrane deposition, specifically collagen type IV and laminin, associated with ECFC vessel formation within monomer and oligomer matrices. ECFC were cultured in either oligomer or monomer matrices (200 Pa) for 3-, 7-, and 14 days, and constructs were immunostained with primary antibodies to basement membrane proteins, collagen type IV and laminin. A detergent-free immunostaining approach was used to only detect basement membrane components deposited extracellularly.^[24] Consistent with previous reports,^[17, 24] ECFC cultured in monomer matrices showed little to no basement membrane deposition. The low level immunofluorescence signals observed for both collagen type IV and laminin in monomer appeared confined within or highly associated with cells (Figure 8A, Figure 9). Furthermore, the low signal intensity and spatial distribution for both proteins remained relatively constant throughout the 14-day time course. On the other hand, ECFC in oligomer exhibited a progressive increase in collagen type IV deposition as well as time-dependent differences in the spatial distribution of collagen type IV (Figure 8A–B). At day 3, regional collagen type IV staining was observed highly associated with the cell membranes of ECFC, especially those that were vacuolated. As time continued and multi-cellular lumenized vessel networks formed, the overall level of deposition increased but a close association between collagen type IV and the vessel basal membrane were maintained. Collagen type IV

was more spread along the length of the vessel by Day 14, and as the vessel microstructure grew in size and complexity, a substantial increase in collagen type IV deposition was noted. Interestingly, collagen type IV not only covered the abluminal surface of the vessel structure but also was noted at measurable distances away from the network. We postulate that these areas marked sites where vessels formed and deposited basement membrane prior to regressing. Unlike collagen type IV, laminin deposition was limited in oligomer cultures and showed little to no change in spatiotemporal distribution over the time course studied (Figure 9). Laminin displayed a punctuate immunofluorescence pattern that was highly associated with the cell at all time points.

Discussion

This work provides new perspective into vascular-inductive matrix design by bringing to center stage the contribution of naturally-occurring collagen intermolecular cross-links and ECM biophysical signaling modalities to ECFC vessel formation, persistence, and maturation. Relatively uncommon oligomers allowed independent modulation of fibril density and interfibril branching, two microstructure features known to define the collagen matrix stiffness.^[7] A new and comprehensive 3D vessel morphometric analysis approach facilitated quantification and comparison of relevant vessel network morphology and architecture parameters. Major findings from the study include: 1) collagen intermolecular cross-links and associated oligomers, constitute a valid molecular polymerization parameter for tuning specific fibril- and matrix-level design properties which in turn can be used to guide ECFC vessel formation; 2) the extent, morphology, and architecture of vessel network formation by ECFC within collagen matrices can be predictably modulated through independent variation of collagen concentration (fibril density) and oligomer:monomer ratio (interfibril branching); 3) oligomer matrices induce more extensive and persistent vessel networks compared to corresponding monomer matrices prepared at matched collagen concentration or matrix stiffness; and 4) oligomer, but not monomer, matrices support stabilization and maturation of ECFC vessel networks via collagen type IV deposition and assembly.

A number of in-vitro angiogenesis and vasculogenesis models have been used to document the promorphogenetic effects of natural polymer matrices (e.g., collagen and fibrin) on vessel formation. Traditional angiogenesis models involve monitoring vessel sprouting and elongation from 1) EC seeded on top of 3D matrices or 2) EC spheroids or EC-coated microcarrier beads suspended within matrices.^[15, 26, 40, 51–54] In contrast, vasculogenesis models rely on measurement of vacuole and lumen formation following homogeneous distribution of individual EC or their precursors within 3D matrices. Three-dimensional collagen matrices prepared from conventional collagen monomer formulations are routinely employed in both angiogenesis and vasculogenesis models to represent the interstitial ECM.^[7, 17, 21, 40] Recent studies targeting late-stage vessel maturation and stabilization events have been conducted using in-vitro systems in which EC and accessory cells are co-cultured within collagen matrices.^[23, 24] since monomeric collagen alone induces rapid apoptosis and does not support vessel persistence.^[17, 21, 55, 56] EC-accessory cell interactions stimulate BM deposition and yield vessels that persist several weeks.^[28] Historically, any quantification of vessel morphogenesis within these in-vitro vasculogenesis and angiogenesis models has been limited to 2D morphometric analysis with measurement of vacuole density, vacuole area, vessel (lumen) diameter and/or total vessel length.^[21, 25]

In the present study, ECFC were suspended within matrices prepared from collagen monomers and oligomers purified from porcine dermis.^[7, 34] While both formulations retained intact telopeptide regions, only oligomers contained stable, mature covalent cross-links (histidinyl-hydroxy-lysineonorleucine) between collagen molecules (e.g., trimers).^[7]

While it is well documented that lysyl oxidase and intermolecular cross-links are critical to development and function of the vasculature and cardiovascular system,^[57] their influence on neovascularization has not been elucidated. Since these collagen polymers show different patterns of molecular packing and fibril formation, they could be applied effectively to define how fibril density and interfibril branching work independently to guide ECFC vessel morphogenesis.^[7] A comprehensive analysis of 3D vessel network morphology and architecture, including vessel volume percent, number of vessels per network, along with time-dependent changes in basement membrane deposition provided additional perspective into the long-term functional properties of the formed vessels. Consistent with the majority of studies performed to date, tissue constructs were maintained in a standard culture environment representing 5% carbon dioxide in air. As such, ECFC were exposed to an oxygen concentration (21%) which was higher than that (2–4%) found in tissues where angiogenesis typically occurs in vivo (e.g., tumors and wounds).^[58–60] Reduced oxygen levels (hypoxia) have been reported to promote angiogenic proliferation and sprouting by EC.^[61] In addition, recent results obtained from standard 2D in-vitro cultures suggest that ECFC may respond differently than mature EC under hypoxic conditions.^[62, 63] As such, further experimentation is needed to better define the effects of hypoxia on ECFC vessel formation and stabilization.

Collagen concentration and its associated changes in fibril density are routinely modulated to evaluate the effect of matrix stiffness on vessel morphogenesis.^[7, 21, 40, 53] Others have also sought to stiffen matrices to modulate vessel morphogenesis by increasing the amount of cross-links within collagen (e.g., non-enzymatic glycation), fibrin (e.g., Factor XIII treatment), and collagen/fibrin composites (e.g., glyoxal treatment).^[23, 51, 52] While the present work focuses on in-vitro model systems involving natural polymers, the physical properties of synthetic polymer matrices have also been shown to modulate vasculogenesis and angiogenesis.^[48–50] Here, ECFC vessel formation was evaluated within both monomer and oligomer matrices over a concentration range of 0.5 to 3.0 mg/ml. It was immediately apparent from this study that oligomer induced more robust vessel formation compared to monomer. Total vessel length decreased and vessel diameter increased as the concentration of each collagen was increased; however, vessel diameter increases were modest in monomer compared to oligomer. Average vessel diameters ranged from $12.10 \pm 4.11 \mu\text{m}$ to $13.77 \pm 4.71 \mu\text{m}$ and $13.10 \pm 5.17 \mu\text{m}$ to $17.05 \pm 5.64 \mu\text{m}$ for monomer and oligomer matrices, respectively. These vessel diameter values fall well within the range reported for capillary beds found in various tissues in the body (5–40 μm).^[64] Similar relationships between commercial monomer concentration, vessel length, and vessel diameter have been established previously using in-vitro angiogenesis and vasculogenesis assays.^[15, 21, 53] In fact, based on previous observations, it was postulated that lumen expansion and vessel elongation were inversely related.^[21] Expanded analysis of vessel network morphology and architecture revealed an interesting finding in the present study. It was apparent that the inverse relationship between concentration and total vessel length could be attributed to a decrease in the total number of networks formed within each collagen. Average vessel segment length, when analyzed for all networks or on a per network basis, increased with concentration, indicating that increased fibril density does not directly hinder vessel elongation. In fact, the lumen expansion and vessel elongation observed with oligomer concentration yielded an overall increase in average vessel segment volume and vessel volume percent, despite the decrease in the overall number of vessel networks. In contrast, vessel volume percent decreased monotonically with increasing monomer concentration, which could be attributed to the smaller number of vessel networks formed as well as the modest vessel diameter increases observed with increases in monomer concentration.

Interfibril branching is another fibril microstructure feature that contributes to matrix stiffness. As oligomer:monomer ratio was increased from 0:100 to 100:0, interfibril

branching increased from 3319.92 ± 712.98 branch points to 8133.83 ± 1672.08 branch points, matrix stiffness increased from 59.60 ± 13.07 Pa to 264.20 ± 37.15 Pa, and fibril diameter and density remained relatively constant, confirming our previous suppositions.^[7] When seeded within matrices with varied levels of interfibril branching but similar fibril densities, ECFC sensed and responded to differences in matrix physical properties as evidenced by different patterns of vessel formation. In this case, as matrix stiffness increased with interfibril branching, the number of vessel networks formed did not decrease but rather remained relatively constant over the range tested. Vessel networks in 0:100 oligomer:monomer matrices were not well developed and exhibited the smallest average vessel diameter, shortest total vessel length, and smallest vessel volume percent. An interesting finding was that ECFC in 50:50 oligomer:monomer developed a distinct morphology and formed vessel networks that were relatively long and unbranched. As oligomer:monomer ratio was increased to 100:0, vessel networks became more extensive and displayed the greatest total vessel length, vessel volume percent, and level of vessel branching. The observed increase in vessel network branching was reminiscent of in-vivo capillary networks and would likely contribute to increased perfusion, more uniform molecular diffusion, and greater anastomotic potential with the host vasculature.^[39]

Previously, it has been suggested that vessel morphogenesis is modulated by the cell-matrix force balance that develops as a result of cell-generated traction forces which are resisted by matrix stiffness.^[21] Therefore, we compared ECFC vessel morphogenesis within monomer and oligomer matrices prepared at matched stiffness (200 Pa). Consistent with previous reports,^[10, 65, 66] ECFC underwent rapid apoptosis and formed limited vessel networks within pig skin monomer in absence of phorbol esters and accessory cells. In contrast, extensive and highly branched vessel networks were formed in oligomer as evidenced by significant increases in vessel diameter, average vessel segment volume, total vessel length, and vessel volume percent. Monomer matrices require a substantially higher fibril density to generate the same matrix stiffness as oligomer matrices,^[34] which can constrain cell spreading and cell-cell associations necessary for vessel formation. In this way, modulation of interfibril branching independent of fibril density allowed matrix stiffness to be tuned to support necessary ECFC traction forces required for vacuole formation while preserving a spatial distribution of fibrils that fosters lumen expansion, vessel elongation, and formation of multi-cellular vessel networks. Since monomers form entanglements of lengthy fibrils, it may be that ECFC cannot generate sufficient traction forces to proceed through the vessel morphogenesis process. This notion is further supported by our published work that showed that vacuole density and total vacuole area for oligomer matrices was always significantly higher than those for monomer matrices, despite whether matrices were prepared at matched concentration or stiffness.^[7] Collectively, these results show that vessel morphogenesis is not driven by matrix stiffness alone but rather the interplay between fibril microstructure (density) and matrix stiffness. In fact, the ratio of matrix stiffness to fibril density may serve as a better predictor of vessel formation rather than each of the parameters alone.

Another critical, late-stage event associated with vessel morphogenesis, is vessel maturation and stabilization via basement membrane deposition.^[12, 67] While it is known that EC have the capacity to synthesize basement membrane components, Kusuma and co-workers recently reported that ECFC cultured on 2D collagen-coated surfaces could deposit BM proteins into an organized matrix whereas mature EC only expressed BM proteins intracellularly.^[62] Here, immunostaining was used to detect the spatial distribution of two BM proteins, collagen type IV and laminin, during ECFC vessel formation within monomer and oligomer over a 14-day period. Consistent with previous reports, monomer induced little to no collagen type IV or laminin deposition by ECFC in absence of accessory cells.^[24] However, we show, for the first time, that cell-instructive properties inherent to oligomer matrices are sufficient to induce collagen type IV deposition and assembly by ECFC. The

progressive assembly of basement membrane along the abluminal surface of the vessel network, likely contributed to improved ECFC survival and vessel network stability.^[28] It was somewhat surprising that little to no laminin was found associated with oligomer-induced ECFC vessel networks. However, these findings are consistent with recent studies by Jakobsson and co-workers that show laminin is not required for vascular development by embryonic stem cells but rather it appears to be involved in regulation of lumen diameter.^[68] Additional studies are needed to elucidate the mechanisms underlying oligomer induction of BM deposition by ECFC.

Based on our findings as well as the work of others, it is apparent that matrix-integrin-cytoskeletal signaling provides an important convergence point for the biophysical and biochemical signaling pathways that drive vascular development and stabilization.^[7, 15, 21, 69, 70] While the majority of previous studies have focused on molecular and cytoskeletal events downstream of integrin-binding, we now show how collagen building blocks specified by their intermolecular cross-link composition and molecular packing alter the cell-matrix force balance through modulation of collagen fibril microstructure and matrix physical properties. Mason and co-workers recently showed that non-enzymatic glycation of collagen with ribose increased matrix stiffness and increased lumen diameter and sprout length by BAEC in an angiogenesis assay.^[51] However, in that study, matrix stiffness was not altered by changes in collagen fibril architecture but rather an increase in the stiffness of individual fibrils. Our in-vitro findings that matrices devoid of mature, intermolecular cross-links induce ECFC vessel networks that exhibit decreased vessel diameters and rapid regression are consistent with animal studies where lysyl oxidase and associated enzymatic cross-link formation is reduced or knocked out.^[71–75] Furthermore, the concentration-dependent decrease in vessel network density and increase in vessel diameter observed with our in-vitro model parallels our previous in-vivo findings in which ECFC-collagen constructs were implanted subcutaneously within immunocompromised mice.^[39] Another interesting finding based on our results and the work of others is that an increase in matrix stiffness, despite the mechanism and the assay format, contributes to both lumen expansion and vessel elongation.^[7, 51] Membrane anchored collagenase membrane type I-matrix metalloproteinase (MT1-MMP) has been identified as a major molecular player associated with both of these processes.^[16, 22, 76] In addition, multi-molecular signaling complexes involving $\alpha_2\beta_1$ -integrin and MT1-MMP have recently been described.^[16] These complexes effectively coordinate integrin recognition, localized MT1-MMP collagen proteolysis, and small Rho GTPase (e.g., Cdc42, Rac, and Rho) signal transduction events necessary for vacuole and lumen formation in vitro.^[49, 77] The spatial and temporal activation of these and other complexes (e.g., integrin-mediated cell-matrix adhesions) may explain the differences in vessel network architecture observed with independent interfibril branching and fibril density modulation. Further investigations to elucidate mechanisms by which the cell-matrix force balance is linked to protease and small Rho GTPase activation are needed.

In summary, this work highlights that ECFC vessel morphogenesis is not dependent upon matrix stiffness alone, but rather the interplay of collagen fibril microstructure and matrix physical properties. Furthermore, it identifies oligomers and their associated intermolecular cross-links as critical determinants of ECM vascular-inductive properties.

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Figure 1. Morphometric analysis of ECFC vessel networks within collagen matrices
ECFC vessel networks were stained with FITC conjugated UEA-1 lectin and imaged using confocal microscopy (26 slices at 2 μm thickness/slice). Images were reconstructed in 3D using Imaris (A), covered with an isosurface (B), masked to create an additional channel (C), and then analyzed using filament tracer. Panel D shows the fit of the filaments in the cone geometry that approximates the morphology of vessel network volume. Scale bar = 20 μm .

Figure 2. ECFC show enhanced in-vitro vessel formation within monomer versus oligomer matrices

ECFC at 5×10^5 cells/ml were seeded within collagen monomer (A) and oligomer (B) matrices matched at G' of 200 Pa and cultured for 7 days. Constructs were stained with FITC-conjugated UEA-1 lectin and imaged using confocal microscopy. Panels A and B represent 3D rendered images of z-stacks (21 slices at $5 \mu\text{m}$ thickness/slice) showing lectin-based detection of 3D ECFC vessel networks. Scale bar = $100 \mu\text{m}$.

Figure 3. ECFC vessel network morphology is dependent on oligomer concentration

Schematic representations of 3D ECFC vessel network elongation and branching are shown for oligomer matrices at 0.5-, 1.5-, and 3.0 mg/ml (A). The distributions of vessel diameters (B) were measured for ECFC vessel networks following 7 days of culture within oligomer matrices at 0.5 mg/ml (black), 1.5 mg/ml (gray), and 3.0 mg/ml (white). Cumulative distributions of vessel diameters (C) show statistically significant shifts toward increased vessel diameters with increasing oligomer concentration (n = 123; 0.5 mg/ml vs 1.5 mg/ml, $p < 0.0001$; 1.5 mg/ml vs 3.0 mg/ml, $p = 0.0446$; 0.5 mg/ml vs 3.0 mg/ml, $p < 0.0001$). Total vessel length (D), average vessel segment length (E), average vessel segment volume (F), and vessel volume percent (G) were measured for all ECFC vessel networks at 0.5 mg/ml (black), 1.5 mg/ml (gray), and 3.0 mg/ml (white). As collagen concentration increased, average segment length and average segment volume increased significantly. Total vessel length decreased progressively over the concentration range. Vessel volume percent showed an increase from 0.5 mg/ml to 1.5 mg/ml, with no further increase at 3 mg/ml (Data represents mean $\pm$ SEM; n = 14; asterisk indicates statistically different pairwise comparisons, $p < 0.05$).

Figure 4. Monomer concentration has limited effect on ECFC vessel network morphology

The distributions of vessel diameters (A) were measured for ECFC vessel networks following 7 days of culture within monomer matrices at 0.5 mg/ml (black), 1.5 mg/ml (gray), and 3.0 mg/ml (white). Cumulative distributions of vessel diameters (B) show no statistical difference between any of the concentrations (n = 55; 0.5 mg/ml vs 1.5 mg/ml, $p=0.1718$; 1.5 mg/ml vs 3.0 mg/ml, $p=0.0518$; 0.5 mg/ml vs 3.0 mg/ml, $p=0.4374$). Total vessel length (C), average vessel segment volume (D), and vessel volume percent (E) were measured for all ECFC vessel networks at 0.5 mg/ml (black), 1.5 mg/ml (gray), and 3.0 mg/ml (white). As collagen concentration increased, total vessel length and vessel volume percent decreased with no significance. Average segment volume increased significantly with concentration (Data represents mean $\pm$ SEM; n = 14; asterisk indicates statistically different pairwise comparisons, $p<0.05$).

Figure 5. Increase in oligomer to monomer ratio yields increase in matrix stiffness through increased interfibril branching independent of fibril density

Reconstructed images of fibril microstructure for collagen formulations representing oligomer:monomer ratios of 0:100 (A), 50:50 (B), and 100:0 (C) polymerized at matched concentration (1.5 mg/ml). Matrix stiffness (G'), fibril volume fraction, number of interfibril branch points, and fibril diameter were quantified and compared. As oligomer content increased, the number of interfibril branch points and matrix stiffness increased significantly ($p < 0.05$). No statistical differences were noted for fibril density or diameter values (Data represents mean $\pm$ SD; $n = 3$; letters indicate statistically different groups for each parameter based upon Tukey-Kramer range testing; $p < 0.05$). Scale bar = 10 μm .

Figure 6. ECFC vessel network morphology changes with the addition of oligomers

Schematic representations of 3D ECFC vessel network elongation and branching are shown for 1.5 mg/ml matrices of 0:100, 50:50, and 100:0 oligomer:monomer ratios (A). Vessel diameter distributions (B), total vessel length (C), average vessel segment length (D), and vessel volume percent (E) were measured for ECFC vessel networks following 7 days of culture within 0:100 (white), 50:50 (gray), and 100:0 (black) oligomer:monomer collagen matrices prepared at 1.5 mg/ml. Total vessel length for 50:50 oligomer/monomer is significantly lower than oligomer and monomer. 50:50 oligomer/monomer also shows significantly higher average segment length and volume than the other matrices. Vessel volume percent is highest in oligomer matrices, but there is no difference observed between monomer and 50:50 oligomer/monomer matrices (Data represents mean $\pm$ SEM; n = 14; asterisk indicates statistically different groups, $p < 0.05$).

Figure 7. Comparison of ECFC vessel network lumen diameter and morphology for monomer and oligomer matrices prepared at matched stiffness (G')

Schematic representations of 3D ECFC vessel network elongation and branching are shown for oligomer and monomer matrices matched at 200 Pa (A). Vessel diameter distribution (B), total vessel length (C), average vessel segment volume (D), and vessel volume percent (E) were measured for ECFC vessel networks following 7 days of culture within monomer (black) and oligomer (white) matrices matched at 200 Pa. Total vessel length, average segment volume, and vessel volume fraction were significantly greater for oligomer matrices (Data represents mean $\pm$ SEM; n = 13 for C, D, E; asterisks indicates statistically different groups, $p < 0.05$).

Figure 8. Comparison of temporal and spatial patterns of collagen IV deposition by ECFC vessel networks within oligomer and monomer matrices

ECFC at 5×10^5 cells/ml were seeded in monomer and oligomer matrices matched at G of 200 Pa and cultured for 3, 7, and 14 days. Constructs were stained with FITC-conjugated UEA-1 lectin and anti-collagen type IV antibody. Samples were imaged using confocal microscopy (21 slices at 5 μ m thickness/slice) to show 3D ECFC vessel networks (green) and basement membrane deposition, collagen type IV (red). (A) ECFC deposition of collagen type IV increased dramatically with time within oligomer matrices. Collagen type IV is secreted from the cells, and Day 3, 7 oligomer cultures show close association of collagen type IV with the basal membrane of the cell/vessel. At Day 14, collagen type IV is associated with the basal membrane of the vessels, as well as in areas distant from the vessel network that could be potential vessel regression sites. ECFC in monomer showed little collagen type IV deposition at all time points. Panel B shows single slice and cross-section views (XY-, XZ-, and YZ planes) of ECFC vessel networks within oligomer. Specimens were stained with anti-collagen type IV antibody. Arrows in the XY plane indicate the lumen areas shown in XZ- and YZ planes. Scale bar = 50 μ m.

Figure 9. Comparison of temporal and spatial patterns of laminin deposition by ECFC vessel networks within oligomer and monomer matrices

ECFC at 5×10^5 cells/ml were seeded in monomer and matrices matched at G of 200 Pa and cultured for 3, 7, and 14 days. Constructs were stained with FITC-conjugated UEA-1 lectin and anti-laminin antibody. Samples were imaged using confocal microscopy (21 slices at 5 μ m thickness/slice) to show 3D ECFC vessel networks (green) and basement membrane deposition, laminin (red). Laminin deposition is largely cell associated and is deposited to a lesser quantity than collagen type IV. Punctate staining patterns were observed with both monomer and oligomer matrices and remained consistent over time. Scale bar = 50 μ m.

Comparison of average vessel diameter ($\pm$ SD) and associated p-values for ECFC vessel networks formed within monomer and oligomer matrices at specified concentrations (Day 7) (Data represents mean $\pm$ SD; n = 55; letters indicate statistically different concentration groups based upon Tukey-Kramer range testing within each collagen formulation; $p < 0.05$).

Table 1

Concentration (mg/ml)	Vessel Diameter (μ m)		P-Value
	Monomer	Oligomer	
0.5 mg/ml	12.4 $\pm$ 4.13 ^{A,B}	13.10 $\pm$ 5.17 ^Y	0.3580
1.5 mg/ml	12.10 $\pm$ 4.11 ^B	16.86 $\pm$ 5.81 ^Z	<0.0001
3.0 mg/ml	13.77 $\pm$ 4.71 ^A	17.05 $\pm$ 5.64 ^Z	<0.0001