Introduction The development of vasculature remains a major challenge in the generation of viable engineered tissue constructs. One aspect of this challenge is to form microvessels of varying diameters and promote their maturation to generate stable and functional microvasculature. Our approach utilizes hydrogel-based microfluidics lined with endothelial cells (ECs) to form arteriole/venule-like vessels, in conjunction with forming capillaries by harnessing the self-assembly behavior of ECs during co-culture with multipotent stromal cells (MSCs). Constructs with both capillaries and arteriole/venule-like vessels, however, require a suitable biomaterial that will
support this dual vascularization strategy. Hence, the motivation for the current study was to select a biocompatible material with the appropriate biochemical and mechanical properties for generating perfusable tissues using EC-lined microchannels and vascular self-assembly. We investigated fibrin and type I collagen for this application, given their established capacity to induce capillary self-assembly and provide mechanical strength, respectively. Collagen, fibrin, and collagen-fibrin co-gels were assessed for a number of functional characteristics relevant to the successful implementation of such a dual vascularization strategy.

Materials and Methods

Human bone marrow stromal cells and human umbilical vein endothelial cells were used as the MSC and EC populations in this study, while all hydrogel components were of bovine origin. Experiments exploring the roles MSCs play in EC self-assembly through network formation and sprouting were performed using 2.5 mg/mL fibrin in static 3D culture. Network formation was induced by
embedding ECs within hydrogels as single cell suspensions \( (3.0 \times 10^6 \text{ cells/mL}) \), while sprouting was induced by embedding EC-seeded microcarrier beads within hydrogels. Sprouting assays were performed with and without the protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA), as previous works have found it essential for robust sprouting within collagen-only hydrogels. For perfusion studies involving EC-lined microchannels, hydrogels were formed in polydimethylsiloxane chambers around a nylon line, followed by removal of the line to create a channel, which was then perfused using syringe or peristaltic pumps after EC seeding. Hydrogel microstructure was analyzed by scanning electron microscopy (SEM) after formalin fixation and critical point drying. Cell-mediated contraction of hydrogels was assessed under non-restrained conditions, while compression testing was performed using 20% strain. Depending on the number of conditions for individual experiments, Student's t-tests, or one-way or two-way ANOVA statistical tests were used to assess significance.

Results
MSCs were found to be essential for inducing capillary self-assembly through EC sprouting, and were required for the continued stabilization of endothelial networks, as without MSCs these networks would disassemble (Fig. 1) and ECs would become apoptotic. Fibrin and collagen-fibrin co-gels could facilitate EC sprouting in the absence of PMA, whereas collagen alone could not. Additionally, both fibrin and co-gels (2.5 mg/mL fibrin + 0.5-3.0 mg/mL collagen) were relatively resistant to cell-mediated contraction compared to collagen-only hydrogels when compared 30 hr after cell encapsulation (p ≤ 0.001) (Fig. 2a). Fibrin alone, however, was found to have insufficient mechanical strength to produce smooth perfusable channels; hence, collagen-fibrin co-gels were found to be optimal for this application. Furthermore, testing of co-gel mechanical properties revealed that increasing collagen content results in a higher compressive modulus, with moduli increasing from 0.25 to 0.53 kPa for co-gels containing 2.5 mg/mL fibrin and 0.5 or 4.0 mg/mL collagen, respectively (p ≤ 0.001) (Fig. 2b). Further physical characterization using SEM revealed that collagen hydrogels were composed of ~70-280 nm fibrils, while fibrin had a dense
amalgamated microstructure, and co-gels appeared to have collagen fibrils on their surface with diameters in the range of ~178-290 nm (Fig. 3a, b). Additionally, with increasing collagen content, EC network formation decreased within co-gels, in terms of nodes and total length (Fig. 2c, d); however, this decrease could be ameliorated by using a relatively high EC density (>7.5 \times 10^6 cells/mL). Finally, during perfusion of EC-lined channels in fibrin-collagen co-gels, ECs were found to sprout into the surrounding hydrogel forming a simple ex vivo microvaculature.

Discussion and Conclusion

This work revealed collagen-fibrin as a promising candidate for forming vascularized tissue constructs, in terms of its ability to facilitate EC sprouting behavior and its capacity to form perfusable microchannels. The fact that collagen-fibrin facilitates sprouting in the absence of PMA, unlike collagen alone, is highly beneficial given the potential deleterious side effects of using a PKC activator clinically. Also, given that fibrin polymerization is relatively rapid, co-gels may prove
to be a useful material for layer-by-layer formation of vascularized constructs using emerging bioprinting technologies, especially considering mutagenic ultraviolet light is not required for its polymerization (unlike other bioink materials). Hence, we are currently working towards adapting this approach to a custom 3D printing platform, in order to produce thick vascularized tissue constructs with high viability.
Figure 1. Effect of bone marrow stromal cell (BMSC) co-culture on endothelial cell 3D network formation over a period of 21 days in 2.5 mg/mL fibrin. Blue = nuclei.

Figure 2. Effects of hydrogel composition on cell-mediated contraction (a), compressive modulus (b), and endothelial cell 3D network formation (c, d).
Figure 3. SEM of collagen (1.0 mg/mL), fibrin (2.5 mg/mL) and collagen-fibrin co-gels (1.0 mg/mL and 2.5 mg/mL) processed by critical point drying (a). Collagen-like fibrils within co-gels are indicated by arrowheads. Fibril diameters of collagen and collagen-fibrin co-gels are shown in (b).

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