Guided sprouting from endothelial spheroids in fibrin gels aligned by magnetic fields and cell-induced gel compaction

Kristen T. Morina, Robert T. Tranquilloa,b,*

aDepartment of Biomedical Engineering, University of Minnesota, Minneapolis, MN, USA
bDepartment of Chemical Engineering & Materials Science, University of Minnesota, Minneapolis, MN, USA

ARTICLE INFO

Article history:
Received 24 February 2011
Accepted 5 May 2011
Available online 1 June 2011

Keywords:
Angiogenesis
Endothelial cell
Fibrin
Co-culture

ABSTRACT

An aligned engineered microvascular network is critical to the culture of thick or highly metabolic tissue in vitro due to the need for inlet and outlet sides for perfusion of the network. Contact guidance may be a way to achieve aligned networks, but the relationship between the alignment of endothelial sprouts and the alignment of extracellular matrix fibers has yet to be fully elucidated. The data presented here show that sprouts from human blood outgrowth endothelial cell spheroids align with fibrin fibers, and that the extent to which the sprouts align depends upon the strength of the fibril alignment. This was true for both magnetically-aligned fibrin and fibrin aligned via cell-induced gel compaction, although magnetically-aligned fibrin was more effective over the same culture period. The data also demonstrate that longer sprouts are grown when the fibrils, and thus the sprouts, are more strongly aligned. The formation of aligned endothelial sprouts using these methods can be an essential step in the generation of aligned microvascular networks.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

A major challenge that remains in tissue engineering is the provision of oxygen and nutrients to cells deep within a thick construct cultured in vitro prior to implantation. Tissues receiving oxygen through diffusion only are limited to a thickness of several hundred microns, which is insufficient for most tissue of human size. The thickness limitations may be even larger in highly metabolic tissue such as myocardium, skeletal muscle, and liver. For example, the intercapillary distance in human myocardium is only about 20 μm [1]. One solution to this problem of generating thick tissues in vitro is to create a microvascular network within the tissue. Such a network, if it could be perfused in vitro, would provide oxygen to the cells of a thick tissue both during culture. Perfusion could then be sustained transiently after implantation with percutaneous perfusion until inoculation occurs.

Many methods have been developed to create microvascular networks within engineered tissue, from the fabrication and endothelial seeding of tubes within the tissue [2] to the entrapment of single endothelial cells (ECs) within biopolymeric gels [3–6]. However, the entrapment of EC spheroids in biopolymeric gels (e.g. collagen or fibrin) is an attractive potential method of forming a microvascular network within engineered tissue because it mimics in vivo sprouting angiogenesis.

Korff and Augustin [7] first demonstrated that the outer layer of EC spheroids behaves similarly to the endothelium of a vessel with the center of the spheroid as the lumen of the vessel. The surface ECs become quiescent and express CD31 at cell–cell contacts, and I-CAM and V-CAM expression was inducible by TNF-α activation. They also showed improved survival of ECs when cultured in suspension as spheroids rather than as single cells. In vitro network formation by human umbilical vein EC (HUVEC) spheroids entrapped in collagen I gels has been shown [8], and the ability of nearby sprouts to anastomose within fibrin gel has been demonstrated [9]. In addition, vessel formation was shown in vivo using HUVEC spheroids in a Matrigel/fibrin matrix [10]. This spheroid technique required only approximately 10% of the cells required by groups using dispersed cells in similar in vivo studies to achieve a microvascular network connected to the host circulation [3,11–13], suggesting that fewer cells may be needed to form tubule networks in vitro if the ECs are entrapped as spheroids rather than single cells (relative numbers of cells used for in vitro dispersed and spheroid experiments were not described [8]). The co-entrapment of support cells such as fibroblasts or smooth muscle cells has been shown to aid vessel growth from EC spheroids [10].

* Corresponding author. Department of Biomedical Engineering, University of Minnesota, Minneapolis, MN, USA.
E-mail address: tranquillo@umn.edu (R.T. Tranquillo).

0142-9612/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2011.05.018
Autologously isolated ECs such as human blood outgrowth endothelial cells (hBOECs) are advantageous for the vascularization of engineered tissues to avoid immunorejection. hBOECs are a subset of blood mononuclear cells isolated by culturing in EC-favoring conditions for several weeks. The cells have cobblestone morphology, express vWF, CD34, VE-cadherin, flk-1, and PECAM-1, and notably do not express the monocyte markers CD45 or CD14 [14]. hBOECs also have a high proliferative potential (10^6 cells in 60 days) [14], making them ideal for tissue engineering applications.

With any method of microvascular network formation, the alignment of the formed microvessels is critical because it designates inlet and outlet sides of the tissue for in vitro perfusion. Alignment also mimics native structure in tissues such as myocardium, in which strong extracellular matrix fiber alignment correlates with strong cellular and vascular alignment in the same direction [15]. Studies of cells cultured in 3D environments in vitro suggest that cells are able to sense the alignment of nearby extracellular matrix fibers and align with the fibers, a phenomenon known as contact guidance [16]. For example, when magnetic fields were used to align the fibrils of collagen I gel to different extents, the alignment of entrapped fibroblasts coincided with that of the collagen fibrils [17]. Similarly, fibroblasts [18] and cardiomyocytes [19] aligned parallel to matrix fibers in fibrin-based tissue-engineered constructs, where the fibril alignment was created through mechanically-constrained cell-induced gel compaction. However, few studies have investigated the contact guidance response of ECs or EC sprouts. ECs elongated in the direction of fiber alignment when plated on aligned collagen I fibers [20] or aligned electroporetin polymer fibers [21]. Other studies have shown that stretching induces the alignment of both fibrin or collagen fibers and EC tubule structures within the gel, although cause and effect were not determined between the fiber and cell alignment [22,23]. Endothelial sprouts were also observed to be aligned with local elastin fibers in the rat mesentery, although again cause and effect were not established [24]. Very limited data is available on the alignment of sprouts from EC spheroids, but Korff et al. showed that EC spheroids entrapped in collagen I gels can exert traction forces and align the collagen fibrils between nearby spheroids [9]. The sprouts from these spheroids grew towards each other, but cause and effect between sprout growth and collagen fibril alignment and the level of tension in the collagen fibril network was not determined. Interestingly, this local rearrangement of fibrils was not seen when EC spheroids were entrapped in fibrin gel [5].

In the studies presented below, hBOEC spheroids and neonatal human dermal fibroblasts (nhDFs) were co-entrapped in fibrin gel aligned via magnetic force or cell-induced compaction. The alignment of the sprouts from the hBOEC spheroids was measured to determine the relationship between the alignment of fibrin fibrils and hBOEC sprouts. Sprout length, sprout cellularity, and nhDF alignment were also measured in order to investigate the mechanism of aligned sprouting.

2. Materials and methods

2.1. Cell culture

hBOECs were provided by Dr. Robert Hebbel at the University of Minnesota. They were cultured in “hBOEC medium” (ECM-2 (Lonza) with an additional 8% FBS and 1% penicillin-streptomycin) in type I rat tail collagen-coated flasks. Passages 8–12 were used for these experiments. nhDFs were purchased from Lonza and maintained in 50/50 DMEM/F-12 with 10% FBS, and 1% antibiotic-antimycotic. Passages 8–12 were used for these experiments.

2.2. Endothelial Cell Spheroid formation

hBOEC spheroids were made via the method of Laib et al. [25] by suspending 500 cells stained with CellTracker Green (Invitrogen) in a 25 μl drop of “spheroid medium” and placing the drops on a petri dish. Spheroid medium contained 80% hBOEC medium and 20% Ca-P coated tissue culture medium (1.25% w/v meniscus cellulose in EBM-2 (Lonza)). The dishes were inverted, and cultured overnight. The spheroids were harvested by covering the bottom of the dish with HBSS, and pipetting the volume into a conical tube. The spheroids had settled to the bottom of the tube within 15 min, from where they were removed and entrapped in fibrin gel.

2.3. Fibrin constructs with magnetic alignment

Due to the diamagnetic anisotropy of the fibrinogen molecule, fibrin gels can be aligned if they are polymerized in the presence of a strong magnetic field [26,27]. Teflon rings were attached to rectangular glass coverslips using sterile vacuum grease to create wells 1.5 cm in diameter. Fibrin gels (500 μl each) were formed within these wells by combining hBOEC spheroids, nhDFs, bovine fibrinogen (Sigma) in 20 mM HEPES-buffered saline, bovine thrombin (Sigma) in saline, and EGM-2 (Lonza) without FBS. Final gel concentrations were 3.3 mg/ml fibrin, 0.08 μM thrombin, 68% (v/v) EGM-2 without FBS, 80 spheroids/ml, and 250,000 nhDFs/ml. Gels were allowed to polymerize in the presence of a 9.4 T magnetic field, a 7 T magnetic field, or no magnetic field (isotropic control) for 15 min 1 ml of hBOEC medium was added to each well and was replaced every 2 days. Fibroblasts were included in these constructs because limited sprout growth was observed in constructs without co-entrapped nhDFs. hBOECs and nhDFs have local microscopic field magnetic field exposure on fibroblast and endothelial cell behavior [28–32], additional constructs were made with or without spheroids to test the effect of magnetic field exposure on sprout growth and proliferation, and on angiogenic factor production by nhDFs. Isotopic gels were formed as above and were exposed to a magnetic field and field-off periods of 15 or 20 minutes, or harvested from constructs containing only nhDFs using an RNaseA extraction kit (Quagen) after 1 and 3 days of culture, and quantitative RT-PCR was performed to investigate VEGF-A, hBF2, and Ang-1 production. The forward (f) and reverse (r) primers used were designed to amplify both the endogenous and transfected hBF2 and Ang-1 and are as follows: VEGF-A 5′TGGATCCATCGAGTTTCCGT TGTGT3′, CD14 5′TTGTGACGACGAGGACGAC 3′ and CD14 r 5′GGGAGGACGACGAGGACGAC 3′.

Because others have found effects of magnetic field exposure on extracellular matrix fibrils [21], other studies have found that stretching induces the alignment of both fibrin or collagen fibers and EC tubule structures within the gel, although cause and effect were not determined between the fiber and cell alignment [22,23]. Endothelial sprouts were also observed to be aligned with local elastin fibers in the rat mesentery, although again cause and effect were not established [24]. Very limited data is available on the alignment of sprouts from EC spheroids, but Korff et al. showed that EC spheroids entrapped in collagen I gels can exert traction forces and align the collagen fibrils between nearby spheroids [9]. The sprouts from these spheroids grew towards each other, but cause and effect between sprout growth and collagen fibril alignment and the level of tension in the collagen fibril network was not determined. Interestingly, this local rearrangement of fibrils was not seen when EC spheroids were entrapped in fibrin gel [5].

In the studies presented below, hBOEC spheroids and neonatal human dermal fibroblasts (nhDFs) were co-entrapped in fibrin gel aligned via magnetic force or cell-induced compaction. The alignment of the sprouts from the hBOEC spheroids was measured to determine the relationship between the alignment of fibrin fibrils and hBOEC sprouts. Sprout length, sprout cellularity, and nhDF alignment were also measured in order to investigate the mechanism of aligned sprouting.

2.4. Fibrin constructs with cell-induced alignment

Fibrin constructs were made in slab geometries by mixing hBOEC spheroids, nhDFs, bovine fibrinogen (Sigma) in 20 mM HEPES-buffered saline, bovine thrombin (Sigma) in saline, and EGM-2 (Lonza) without FBS. Final gel concentrations were 3.3 mg/ml fibrin, 1.25 μM thrombin, 68% (v/v) EGM-2 without FBS, 36 spheroids/ml, and 250,000 nhDFs/ml. Three geometries were used to create slabs with aspect ratios of 1, 2, and 3. The aspect ratio was defined as the ratio of the length of the construct to its width. Rectangular score marks were made on the bottom of a 150 mm petri dish in sizes 1 cm × 2 cm, 1 cm × 3 cm, and 1 cm × 4 cm. A 0.5 cm wide porous polyethylene space was fixed inside the score mark at each end using sterile vacuum grease. Gel-forming solution was mixed for 20 s and pipetted into the rectangular space between the spacers, giving initial construct sizes of 1 cm × 1 cm (250 μl gel), 1 cm × 2 cm (500 μl gel), 1 cm × 3 cm (750 μl gel), corresponding to aspect ratios 1, 2 and 3 respectively. Constructs were allowed to polymerize for 5 min at room temperature, after which 35 ml of hBOEC medium was added. hBOEC medium was replaced on the day after casting and every 2 days subsequently.

The following day, slides were lifted from the dish via the spacers and the spacers were reattached to a new dish, with the distance between the spacers remaining constant. This action detached the construct from the dish, allowing the cells to compact and align the fibrin fibrils between the two spacers.

2.5. Image acquisition and analysis

Constructs were cultured for 7 days, after which spheroids from each condition were imaged using confocal microscopy with a 10× objective. Z-stacks were used when required to ensure that all sprout lengths were included. The length and angle of each sprout was measured in the XY-projections of the spheroids using ImageJ (NIH). Sprouts were predominately linear structures. Therefore, a straight line was drawn over each sprout representing the average length and angle, and the length and angle of this line was recorded (Fig. S1). The X and Y components of each length were calculated and summed for all sprouts within a given construct. The sums were then used to calculate the average sprout anisotropy index, defined as the ratio of total X length to total Y length (where X is the direction of fibrin fiber alignment). With this definition, a high anisotropy index indicates strong sprout alignment, and an anisotropy index of 1 is perfectly isotropic.

For magnetically-aligned constructs, spheroids were also imaged on day 0. The X and Y diameters were measured and their ratio taken to determine whether any elongation of the spheroid in the direction of alignment had occurred. For nuclear labeling and nhDF alignment measurements, constructs were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X, and stained with...
rhodamine phalloidin and Hoescht 33342. Sprouts were then imaged with confocal microscopy using a 10× objective and 3× optical zoom. Nuclei fully within the boundaries of CellTracker Green staining were considered within the sprout and were manually counted.

F-actin expressing cells that did not contain the CellTracker Green were considered nhDFs. The nhDFs displayed elongated morphology, with nuclei also elongated in the same direction as the F-actin fibers. The angle of nuclear elongation was measured with ImageJ for nhDFs with nuclei at varying distances from the sprout. In the case where the sprout grew at an angle 15°–90° from the overall fibril alignment direction, the difference between the angle of each nucleus and that of the overall fibril alignment direction were computed. If the difference between the nucleus and sprout angles was smaller than the difference between the nucleus and overall fibril angles, the nhDF was considered to be more aligned with the sprout than the overall fibril alignment direction. For cases where the sprout grew at an angle less than 15° from the overall fibril alignment direction, the anisotropy index was calculated as above using a standard length for every cell.

2.6. Alignment mapping

The fibril alignment of each construct was measured via polarized light imaging [34]. Briefly, each sample was placed between two linear polarizers, and images were taken as one of the polarizers rotated. A custom Matlab script was used to calculate the average retardation and alignment direction at each pixel. The retardation normalized to tissue thickness is the tissue birefringence, a measure of fibril alignment strength. Although the quantitative relationship between fibril alignment distribution and birefringence is unknown, it is monotonic [35]. For magnetically-aligned samples, the average birefringence of the largest square fitting within the well was reported. For constructs with cell-induced alignment, the average birefringence (across the construct width) in the middle of the slab, where birefringence is the highest, was reported, and only spheroids in areas where the average birefringence (across the construct width) was within 10% of the middle value were analyzed. The Matlab script also generated alignment maps, in which line segments representing the local strength and direction of alignment overlay a grayscale image indicating the retardation of the construct at each pixel. Alignment mapping was performed on days 0 and 7 for magnetically-aligned constructs and only on day 7 for cell-aligned constructs.

2.7. Histology

Constructs were fixed in 4% paraformaldehyde and frozen in OCT embedding medium. 9 μm sections were taken both parallel and perpendicular to the direction of alignment using a cryostat. Sections were stained with hematoxylin and eosin.

2.8. Statistics

Data were analyzed in Minitab. Comparisons were made using ANOVA with Tukey post-hoc tests, and correlations using linear regression. Both comparisons and correlations were considered significant at p < 0.05. At least 30 sprouts were analyzed in each condition, with 4 magnetically-aligned constructs and 3 × 4 cell-aligned constructs per condition. PCR data were analyzed by the comparative C(T) method followed by ANOVA [36].

3. Results

3.1. Increasing fibril alignment via increased magnetic field strength during fibrin gel formation increased EC sprout alignment

Alignment mapping of magnetically-aligned and isotropic control constructs formed within wells made from Teflon rings verified that fibrin fibril alignment increased with magnetic field strength (Fig. 1A–C,G). The alignment was uniform across each construct. The spheroids were round immediately after gel formation regardless of the application of a magnetic field (data not shown). Decreases in fibril alignment occurred between days 0 and 7 for the 9.4 T and 7 T conditions; however, all measurements of the alignment strength of 9.4 T constructs were higher than those of 7 T constructs regardless of day, and likewise between 7 T and isotropic control constructs (data not shown). In all conditions, the constructs were thinner after 7 days of culture than immediately after gelation (data not shown), and thickness varied little across the construct. Representative images of spheroids in each condition after 7 days of culture are shown in Fig. 1D–F. Sprout anisotropy index positively correlated with increasing birefringence ($R^2 = 0.84$; Fig. 1H), indicating that the sprouts aligned with the fibrin fibrils.

Alignment mapping of constructs exposed to a 9.4 T magnetic field after gelation confirmed that the application of a magnetic field after gelation did not induce fibril alignment (data not shown). PCR analysis indicated that the 15 min magnetic field exposure itself did not have an effect on the production of VEGF-A, bFGF2, or Ang-1 (Fig. S2A). The magnetic field exposure also did not have an effect on nhDF proliferation (Fig. S2B). No difference in sprout length was observed between spheroids exposed to the magnetic field and those not exposed (Fig. S2C).

3.2. Increasing fibril alignment via increased aspect ratio of cell-compacted fibrin gel increased EC sprout alignment

As shown with alignment mapping, fibrin fibril alignment was increased within slab constructs aligned via cell-induced gel compaction by increasing the construct aspect ratio (Fig. 2A–C,G). Alignment was stronger in the center of the slab constructs and weaker nearer the porous spacers, and quantification was thus restricted to the central region where alignment was homogenous. Fig. 2D–F displays representative images of spheroids in constructs of each aspect ratio. Again the sprouts aligned with the fibrin fibrils, as evidenced by the positive correlation of sprout anisotropy index with construct birefringence ($R^2 = 0.75$; Fig. 2H).

3.3. EC sprouts were lengthened by increasing fibrin alignment

Longer sprouts were observed when the sprouts and fibrin fibrils were more aligned, regardless of the method used to align the fibrils. Sprout length was positively correlated with sprout anisotropy index for both magnetically-aligned constructs ($R^2 = 0.71$) and cell-aligned constructs ($R^2 = 0.51$; Fig. 3). A positive correlation also existed between sprout length and construct birefringence for both magnetically-aligned ($R^2 = 0.62$) and cell-aligned constructs ($R^2 = 0.62$; data not shown). In addition, the number of cells per sprout increased with sprout length ($R^2 = 0.38$), but the average length per cell (i.e., sprout length divided by number of cells; a measure of cell elongation) did not.

3.4. Sprout orientation influenced nhDF alignment

nhDF orientation near sprouts in magnetically-aligned constructs was observed via rhodamine phalloidin and Hoescht staining (Fig. 4A–B). Within 10 μm of sprouts that grew in directions 15°–90° from the overall fibril alignment direction, approximately 50% of nhDFs were oriented more closely with the sprout than with the overall fibril alignment direction. This percentage is greater than that of nhDFs located at distances 10–50 μm and 50–100 μm of a sprout (Fig. 4D). Near sprouts that grew in directions less than 15° from the overall fibril alignment direction, a trend was observed that the anisotropy index of the nhDFs within 10 μm of the sprout was higher than that of nhDFs at distances 10–50 μm and 50–100 μm from the sprout, and that of nhDFs in regions with no sprouts (Fig. 4F).

3.5. Sprouts contained patent lumens and connected when in close proximity

Sprouts from spheroids in close proximity were able to connect (Fig. 5A,B). It is unclear whether the lumens connected as well so as to form an anastomosis. H&E staining and vWF immunostaining of histological sections of these tissues revealed the presence of lumens within the spheroids (lumen diameter was 50 ± 5.5 (s.d.) μm) and sprouts (lumen diameter was 8.5 ± 0.64 (s.d.) μm; Fig. 5CD).
4. Discussion

Previous studies of the alignment of endothelial sprouts with extracellular matrix proteins have shown correlations between fibril alignment and sprout alignment, but cause and effect relationships were not established. The method of using magnetic forces to systematically vary the alignment strength of fibrin fibrils leads to the conclusion that fibril alignment is sufficient to induce sprout alignment.

The reduction in thickness of the magnetically-aligned constructs over the culture period indicates that cell-mediated tension present within the constructs caused compaction in the Z direction. However, construct thickness near the edge of the gel was not different from that in the middle of the construct, indicating that the construct was not adherent to the Teflon wall. This observation reduces the possibility of the formation of an anisotropic tension field, which further justifies the conclusion that fibril alignment is sufficient to cause sprout alignment. In addition, the sprout alignment effect was still seen to the same extent (slope = 0.51 in the plot equivalent to Fig. 2H) in gels that had been deliberately detached from the Teflon wall. The cells producing the compaction-causing tension and alignment are likely the more numerous nhDFs, although the ECs could contribute as well. However, aside from inducing compaction in the Z-direction, the nhDFs had only a minor effect, if any, on fibril alignment. This is evident from the fact that all measurements of alignment strength,
even those taken on different days, were different between conditions. Differences did exist between days in magnetically-aligned samples, but this may have been due to any number of factors other than nhDFs.

Sprouts from EC spheroids also aligned with fibrin fibrils in the constructs that were aligned via cell-induced fibrin gel compaction. However, this method of fibril alignment has confounding factors that preclude the same conclusion that fiber alignment induces sprout alignment. For example, an anisotropic tension exists, with tension in the alignment direction, due to the anchorage of the gel to the spacers. Also, the matrix and fibroblast densities may vary with aspect ratio because of the different degrees of gel compaction, and associated gradients of fiber density could also affect sprout alignment. Additionally, the physical necking of the gel could play a role in aligning sprouts, as necking will cause the Y component of a sprout’s length to decrease. However, previous results suggest that the time scale of necking is slow enough for this effect to be neglected [37]. Rather, the results of this experiment indicate that cell-induced compaction is an effective way to align EC sprouts, a conclusion which is pertinent due to the ease of using this method for tissue engineering purposes. Cell-induced compaction and alignment of fibrin gel has been utilized to create several types of engineered tissues, including myocardium [19,38], where a functional microvasculature is critical for maintaining viability in a tissue with physiologically-relevant thickness. Therefore, the logical next step is to include ECs to form an aligned microvascular network. This work has shown that entrapping EC spheroids within aligned fibrin gels is a feasible method for achieving this goal.

Fig. 2. (A–C) Alignment maps of cell-aligned constructs. The lines represent the local direction and strength of alignment. Scalebars = 1 mm (D–F) Representative spheroids from cell-aligned gels. Scalebars = 100 μm. Aspect ratios are (A,D) 1, (B,E) 2, and (C,F) 3. (G) Birefringence of cell-aligned gels (mean ± s.e.m.). *p < 0.05 in comparison to both other groups. (H) Correlation between mean construct birefringence and mean sprout anisotropy index (R² = 0.75; slope = 0.58). #p < 0.05 for linear regression.

Fig. 3. Correlation between mean sprout anisotropy index and mean sprout length for magnetically-aligned (R² = 0.71; slope = 33) and cell-aligned constructs (R² = 0.51; slope = 7.9). #p < 0.05 for linear regression.
The mechanism by which fibril alignment leads to sprout alignment is unknown, but several mechanisms are possible [16,39]: (1) an aligned fibril matrix has pores that are elongated in the direction of fibril alignment, so there is a smaller physical barrier to sprout growth in the direction of alignment; (2) sprouts growing along fibrils encounter more adhesion sites, promoting growth in the alignment direction; and (3) the anisotropic stiffness of the matrix promotes growth in the alignment direction by reducing a sprout’s ability to contract the fibril network if it is growing in the alignment direction (high stiffness). Clearly these potential mechanisms are not mutually exclusive.

The finding that sprout length increased with alignment suggests that mimicking the native vascular structure may not be the only benefit of aligning endothelial sprouts. Longer sprouts more easily encounter a neighboring sprout, which would likely lower the spheroid density required to achieve a microvascular network. The analysis of isotropic gels either exposed to magnetic fields or not suggests that the increased sprout length was due to the fibril alignment, not to the exposure of the cells to a magnetic field. This result is not unexpected, as many groups have shown that the length of neurite outgrowth is increased when the substrate fibers are aligned [26,40–43]. The number of cells per sprout was positively correlated with sprout length and the mean length per cell was not, suggesting that either cell proliferation or recruitment of cells to the sprout was responsible for the increased sprout length. The mechanism of this increased sprout length is also unknown, but the above hypotheses may explain this effect as well [16,39].

A major difference of interest between the two methods of inducing fibril alignment is that the cell-aligned constructs aligned gradually, whereas the magnetically-aligned constructs were fully aligned at gelation. This might cause one to hypothesize that because sprouts in magnetically-aligned constructs grew in an aligned environment for the full seven days but sprouts in the cell-aligned constructs initially grew in an isotropic environment, sprouts in magnetically-aligned constructs would have a higher anisotropy index for a given day 7 birefringence level. In support of this idea, it was found that the anisotropy index of rat BOEC sprouts in cell-aligned constructs did increase gradually, mirroring the gradual increase in fibril alignment (Morin and Tranquillo, unpublished data). However, this result was not observed when comparing the relationship between birefringence and anisotropy index for different alignment methods. The linear regression equation was the same for both magnetically-aligned and cell-aligned constructs. The above logic neglects all of the confounding effects occurring in the cell-aligned constructs. Anisotropic tension could easily contribute to sprout alignment, as could gradients of fibril density. Therefore, it appears that the anisotropy index of sprouts in cell-aligned constructs reflects alignment due to both contact guidance and other factors, and the sum of all of these effects is equivalent to that of contact guidance alone when the sprouts are entrapped in a relatively unchanging aligned matrix.

Comparing the relationship between anisotropy index and sprout length for various alignment methods yields another interesting result. The values for both linear regression intercept and slope were higher for magnetically-aligned constructs than cell-aligned constructs. Here the first result is likely explained by the gradual fibril alignment in cell-aligned constructs. Initially the
sprouts grew into an isotropic matrix, in which they did not have the cue for longer growth, but as the fibrils aligned, the sprouts were able to grow longer. This contact guidance response led to longer sprouts overall in more aligned constructs, but they are shorter for a given level of sprout alignment due to the gradual fiber alignment. The difference in slope indicates that other factors besides contact guidance played a role in sprout length. Perhaps the increase in fiber or fibroblast density inhibited sprout growth in the cell-aligned constructs.

Numerous studies have detailed the ability of fibroblasts to align with extracellular matrix fibers [17,44]. However, the presence of hBOEC sprouts introduced an additional factor into the contact guidance response of the nhDFs. Instances where sprouts were not aligned with the overall fibril alignment direction were particularly interesting because there were potentially two conflicting contact guidance signals. Analysis of these cases demonstrated that the fraction of nhDFs aligned more closely with the sprout than the overall fibril alignment direction was higher for nhDFs within 10 μm of the sprout than those outside of 10 μm. These data suggest that the sprout did produce an alignment cue, but its range was limited. Evidence for this alignment cue from sprouts was also apparent when sprouts grew within 15° of the overall fibril alignment direction. Analysis of the nhDF anisotropy index indicated that nhDFs within 10 μm of a sprout tended to be more aligned than those outside of 10 μm, including those in regions where no sprouts were present. Whereas conflicting alignment cues existed when sprouts were not parallel to the overall fibril alignment direction, the two alignment cues in this case induced stronger nhDF alignment. Since entrapped nhDF were required to obtain sprouts, the interesting question of whether the contact guidance response of the nhDF to the aligned fibrils amplified the contact guidance response of the sprouts could not be directly answered by comparing the outcomes of magnetically-aligned gels with and without entrapped nhDF.

The fact that nhDFs are necessary to induce sprouting in this system raises the question of whether the mechanical or chemical signals provided by the nhDFs (or both) are required. These two effects are difficult to separate, as they are highly interconnected. For example, using an actin inhibitor would inhibit gel contraction (and consequent alignment) that occurs mainly due to the nhDFs, but this would also likely inhibit sprout growth and affect angiogenic factor production. Use of a tissue cell type with a different angiogenic factor production profile in lieu of nhDFs (e.g. pericytes) would likely result in a different gel compaction rate as well. The only technique available to separate the mechanical and chemical effects of nhDFs is siRNA or a similar technology to block the expression of specific angiogenic molecules. Because a large number of angiogenic factors would need to be blocked individually and in combination to draw conclusions, future studies will be needed to determine the answer to this interesting question.

5. Conclusions

The results presented herein that sprouts from EC spheroids align in response to fibril alignment and the evidence for connections between sprouts and lumens within sprouts indicate that contact guidance may be an effective way to generate aligned lumens within microvascular networks within engineered tissues. Such networks are vital to the culture of thick or highly metabolic engineered tissues as they provide inlet and outlet sides for network perfusion. Further research is necessary into the dependence on spheroid size and density and other factors required to form an aligned microvascular network in vitro.

![Fig. 5. (A–B) Connections of sprouts in cell-aligned gels. Scalebars = 100 μm (C–D) H&E stain of cell-aligned gel sections taken (C) parallel and (D) perpendicular to the alignment direction. In (C–D), arrowheads indicate spheroids and arrows indicate sprouts. Scalebars = 20 μm.](image-url)
Acknowledgements

The authors thank Dr. Xiaoping Wu, Dr. Julien Sein and others at the Center for Magnetic Resonance Imaging for use of the MRI equipment and the Institution for Engineering in Medicine at the University of Minnesota for funding. They also thank Dr. Robert Hebbel at the University of Minnesota for the hBOECs and Paul Carlson and Emmeline Yuan for technical assistance.

Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2011.05.018.

References


