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Biomaterial Guides for Lymphatic Endothelial Cell Alignment and Migration

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Abstract

Axillary dissection during breast cancer surgery produces extensive lymphatic vessel damage that often leads to life-long secondary lymphedema of the arm. We have developed a biodegradable material conduit for lymphatic vessel reconstruction where fibers electrospun along the conduit lumen promote endothelial cell alignment and migration, in vitro. The diameter and density of the electrospun fibers were optimized for cell migration and direction on 2-dimensional substrates by seeding human lymphatic endothelial cells (LECs) onto aligned fibers of varying diameters and densities, randomly oriented fibers, and film substrates with no fibers. We found that LECs became aligned in the fiber direction, with cells seeded on the randomly oriented fibers becoming oriented in random directions, whereas cells seeded on the highly aligned fibers became highly aligned. Cell migration was dependent upon fiber alignment and density, with optimal migration found on 1300 nm diameter aligned fibers of low density. Blood endothelial cells seeded on the fibers exhibited similar behavior as the LECs. Fiber alignment was preserved upon rolling the 2-dimensional substrate into the tubular geometry of a lymphatic vessel. The data suggest that aligned electrospun fibers may promote endothelial migration across the conduit in a manner that is independent of lymphatic growth factors.

Keywords

lymphatic regeneration; endothelial cell; aligned fibers; biodegradable materials

INTRODUCTION

Secondary lymphedema is a life-long disease of increased fluid retention, tissue swelling, and impaired immune function due to reduced lymphatic drainage from the arm that often occurs after axillary lymph node dissection to treat breast cancer [1]. In an effort to prevent tumor metastasis through the lymphatic system, the lymphatic vessels emanating from the

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arm are excised at the axilla along with their associated lymph nodes. If sentinel node dissection is used (as is quite common), then the lymphatic vessels emanating from the breast are excised instead of those from the arm. Following the surgery, the severed distal lymphatic vessels remaining in the arm are left unconnected to the severed proximal lymphatic vessels, leaving a large gap between the severed ends. In the case of a sentinel lymph node dissection, the arm lymphatics become severed as a consequence of removing the vessels and nodes draining the breast. Currently, these surgical wounds are closed to heal naturally. The ensuing fibrotic wound repair process fills the surgical space with obstructive scar tissue, which has been found to reduce recovery of lymphatic drainage [2]. Lymphedema usually develops several years after the original lymphatic-related injury has healed. Currently there are no treatments to improve lymphatic recovery following breast cancer surgery. There are subsequent non-curative treatments, such as compression garments and manual lymphatic drainage therapy, that are administered after the onset of lymphedema [3]. It is hypothesized that poor lymphatic regeneration across the obstructive scar tissue during the wound healing process may predispose the tissue to swell at a later date [4].

Because lymphatic function in the arm may be compromised by the failure of lymphatic vessels to regenerate during normal wound repair due to the abundant deposition of fibrotic tissue [4-8], in principle a pre-formed channel implanted intraoperatively may guide lymphatic vessel regeneration through the developing fibrotic tissue. Thus, a tubular implant that bridges the severed lymphatic ends may preserve lymphatic continuity and enable lymphangiogenesis while fibrosis develops in the surrounding tissue. This may prevent the onset of lymphedema prophylactically and improve patient post-surgical quality of life.

Aligned, electrospun fiber structures have been shown to direct the migration of several cell types, including myoblasts, vascular endothelial cells, periodontal ligament cells, and fibroblasts [13-17]. While biomaterial approaches to promote lymphatic migration have not been explored, lymphatic endothelial cells (LECs) have been found to respond to relatively large topographical (25-100 microns) cues on microstructured surfaces [18]. Poly-L lactic acid (PLLA) has been widely used for tissue engineering applications due to biocompatibility and biodegradability [9-12]. Here, we investigate the potential for aligned, electrospun PLLA fibers to promote a directed migration of LECs in vitro, and we optimize the material properties of these fibers for endothelial cell migration. The development of an aligned fiber scaffold as a bioconduit luminal coating represents an initial step in the long-term goal of developing suitable material conduits for reconstructing lymphatic vessels.

MATERIALS AND METHODS

Films & Fibers Solutions

PLLA films were made on 15mm × 15mm coverglass slides (Proscitech, Australia) with a 4 wt % PLLA (NatureWorks™, grade 6201D, Cargill Dow LLC, Minnetonka, MN) solution dissolved in a mixture of chloroform and dichloromethane (50:50 wt%) (Sigma-Aldrich, St. Louis, MO) at room temperature. The films were made by placing the solution on the coverslips and allowing the solvent to evaporate, leaving behind a PLLA film for fiber stabilization. Films are smooth sheets of PLLA without the fiber coating. A fiber solution of 8 wt % PLLA was made in the same way with the same solvent to electrospin the 1300 nm diameter fibers. The randomly oriented and 800 nm diameter fibers were electrospun using an 8 wt% solution of PLLA dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFP). Therefore, varying the solvent type controlled fiber diameters. All fiber types were electrospun onto a PLLA film.

Electrospinning Parameters

We utilized a process and setup described previously [19] to create highly aligned, electrospun fibers. The PLLA coated cover slips were fixed to an aluminum wheel (220 mm in diameter, 10 mm thick) with 3M VHB Tape (3M, St. Paul, MN), and a 5 mL syringe (BD Scientific, Franklin Lakes, NJ, 309603) was placed into the syringe pump (Braintree Scientific Inc., Braintree, MA). To produce the 1300 nm high density fibers, the wheel was rotated at 1500 rpm with a laboratory spinning motor (IKA Works Inc., Wilmington, NC) and the solution was pumped through an electrically insulated 22 gauge needle (Fisher Scientific, Hanover Park, IL, 14-825-15L) at $1.5 \text{ mL}\cdot\text{hr}^{-1}$ for 7.5 minutes. A voltage of 15kV was applied between the tip of the needle and the wheel (Gamma High Voltage Research, Ormond Beach, FL), with a separation distance of 5.5 cm. Low density 1300 nm fibers were produced with the same methods, however with a reduced spinning time of 1 minute. 800 nm fibers were created with the same methods and spinning time as the 1300 nm high density fibers (with a different solution, described above in *Films & Fibers Solutions*). Randomly oriented fibers were created using the same solution as the 800 nm fibers, but the film substrates were placed on a stationary aluminum collection plate and the needle to plate distance was raised to 12 cm. 15 kV was applied between the needle and the plate, while the solution was allowed to flow at $2 \text{ mL}\cdot\text{hr}^{-1}$ for 10 minutes.

Fiber Imaging

Imaging of the fiber samples was conducted using a Hitachi S-4700 field emission scanning electron microscope (FESEM) at accelerating voltages between 1 kV and 2 kV. All of the samples were coated with a 5 nm layer of gold by a Hummer 6.2 sputter coater (Anatech, Ltd. Denver, NC).

Fiber Analysis

Analysis of the fibers was conducted using NIH ImageJ software. Fiber alignment, density and diameter was manually measured and compiled. Alignment analysis was performed by measuring the angle of each fiber sample relative to a horizontal line, then using the mean of this data to normalize the angles. The normalized data was then put into angle buckets, each covering two degrees between 90 and -90 degrees. The alignment graphs were then created using the percentage of fibers in each bucket, and statistical analyses were performed on this data. 50 fibers from each sample were measured, except in the 1300 nm low density and randomly oriented specimens where 25-50 fibers were measured due to the lower densities. Fiber densities were evaluated using NIH Image-J software by drawing a line perpendicular to the fibers and counting the number of fibers present over the total length of the line. One way ANOVA tests were used to compare the diameters and densities of the fibers, with significance being designated at $p \leq 0.05$.

The packing density of the fibers was measured using a custom made Matlab program. The program crops an SEM fiber image to 780×1280 pixels to remove the SEM scale bar and SEM information at the bottom of the image. The background is then made black against white fibers. The image is then converted to binary and the ratio of the white pixels to the total number of pixels is determined, giving the packing density (fiber-filled space on the scaffold).

Cell Culture and Migration

Restricted Migration—Human Lymphatic Endothelial Cells (HLECs, Sciencell, Carlsbad, CA) were cultured until 90% confluent in endothelial cell media (Sciencell, Carlsbad, CA), containing 5% FBS, 1% endothelial cell growth supplement, and 1% penicillin/streptomycin solution. Between passages three and five, 25,000 cells were seeded

within a $\frac{1}{2}$ cm² mold on randomly oriented and aligned fibers. The mold was removed after 24 hours allowing the cells to migrate along the x but not the y direction, and the cells were cultured for an additional 24 and 120 hours. HLECs are lymph node-derived endothelial cells positive for two markers of blood vascular endothelial cells, Factor VIII and CD31, but not for the podoplanin marker of lymphatic endothelial cells. Thus the HLECs are blood endothelial cells (BECs).

Unrestricted Migration—Human Dermal Lymphatic Endothelial Cells (HDLECs, Sciencell, Carlsbad, CA) were cultured until 90% confluent in endothelial cell media (Sciencell, Carlsbad, CA) containing 5% FBS, 1% endothelial cell growth supplement, and 1% penicillin/streptomycin solution. HDLECs are dermal-derived endothelial cells positive for two markers of lymphatic endothelial cells (podoplanin and Lyve-1). Thus the HDLECs are lymphatic endothelial cells (LECs). Between passages three and four, 100 μ L drops containing 15,000 cells were placed onto the fiber samples, including aligned, randomly oriented, low density, and small diameter fibers. After 12 hours, drops were removed and fresh media deposited. Old media was substituted daily with fresh media. In contrast to the restricted migration obtained by seeding cells in a mold, cells seeded in a droplet are free to migrate in any direction. Cells were cultured for an additional 60 hours, for a total of 72 hours.

Cell Imaging

For live cell imaging, cells were incubated with a calcein AM (0.1 mg/ml in PBS) solution for 10 minutes and then imaged with an Olympus BX51 microscope. Individual images were stitched together in Adobe® Photoshop to form a montaged image of a larger region. This allowed depiction of a single image encompassing the entire surface of the coverslip in the restricted migration experiments and the entire area of cell coverage in the unrestricted migration experiments.

Quantification of the Area of Cell Coverage

Cell coverage was measured using MetaMorph® for both restricted and unrestricted migration experiments by tracing a line around the area containing cells. For cell coverage in the restricted migration experiments, the area was normalized to the initial seeding area of the mold. For unrestricted migration experiments, the area was normalized to the average 12 hour area measurements for each group. A Tukey-Kramer statistical test was used to compare the area of cell coverage.

Quantification of Cell Alignment and Extension

Cell alignment and extension was measured using ImageJ. Quadrants were overlaid onto the image, dividing the image into four sections in the unrestricted migration experiments. A grid pattern with squares having an area of 1 mm² was then overlaid on the image with the quadrant division. Two squares from each quadrant containing 30-50 cells were chosen to measure alignment and extension. Only cells whose extensions were at least equal to the size of the cell's body were measured. Measurements were taken from the ends of the longest extension and the measure function within ImageJ was used. For restricted migration experiments, the same practice was used; only a grid with areas of 2 mm² was used to account for the larger surface area of the image. Alignment data was gathered with angle function in Image J at the same time the cell extension measurements were taken, giving an angular measurement from the horizontal. 70 measurements taken from every image, randomly selected using Microsoft Excel's random number generator, were used to perform the statistical analyses for cell alignment. A Tukey-Kramer test was used to compare the extension and alignment of cells.

Experimental Design

Restricted migration experiments were performed with BECs on 1300 nm random and aligned fiber scaffolds (shown in Figure 1) to clarify the role of fiber alignment in blood endothelial cell alignment, migration, and elongation. The unrestricted migration experiments were performed with LECs on 800 nm aligned, 1300 nm aligned low density, 1300 nm aligned high density, and 2000 nm random fibers (shown in Figures 2 and 3) to determine the affect of fiber alignment (by comparing random to aligned fibers), fiber diameter (by comparing the 800 nm and 1300 nm fibers) and fiber density (by comparing the 1300 nm low and high density scaffolds) on lymphatic endothelial cell alignment, migration and elongation.

RESULTS

Fiber Characterization

Electrospun 1300 nm diameter PLLA fibers were employed to direct BEC alignment and migration in the restricted migration model. SEM images show the random and aligned fibers used in the restricted migration experiment (Figure 1A and C, respectively). The alignment analysis (Figure 1B and D) demonstrated a significant difference in fiber alignment between random and highly aligned fiber substrates. $9.36 \pm 3.2\%$ of the random fibers were oriented within 5 degrees of the parallel axis, as opposed to $85.3 \pm 3.9\%$ for the aligned fibers ($p < 0.05$ by Tukey-Kramer HSD). A high peak in the graph represents a highly aligned sample where the majority of fibers run parallel to each other, while a more even distribution signifies a relative lack of alignment. There was no difference in fiber density or diameter between groups (Figure 1E and F, respectively).

Electrospun PLLA fibers were employed to direct LEC alignment and migration in the unrestricted migration model. SEM images show the randomly oriented, 800 nm aligned high density (HD), 1300 nm aligned low density (LD), and 1300 nm aligned HD fibers used in the unrestricted migration experiments (Figure 2A, C, E, and G, respectively). Opposite each SEM image of the fiber sample is a graph depicting the fiber alignment of each sample. The alignment analysis demonstrated a significant difference in fiber alignment between the random and aligned fiber scaffolds and between the 800 nm and 1300 nm diameter fiber scaffolds ($p < 0.05$ by Tukey-Kramer HSD). $5.4 \pm 3.2\%$ of the random fibers were oriented within 5 degrees of the parallel axis, as opposed to $70.2 \pm 6.1\%$ for the 800 nm HD fibers, $93.4 \pm 7\%$ for the 1300 nm LD fibers, and $83.4 \pm 9.7\%$ for the 1300 nm HD fibers. The 800 nm fibers were not as well aligned as the 1300 nm fibers, possibly because different solvents were used to create these fibers. This effect has been shown in previous studies [19].

The diameters and densities of the fibers used in the unrestricted migration model were measured and assessed with statistical analyses (Figure 3A and B). Fiber diameter values (found to be different by Tukey-Kramer HSD, $p < 0.05$) demonstrated scaffolds with three different fiber diameters (Figure 3A). The fiber densities were all different from each other (Figure 3B), $p < 0.05$ by Tukey-Kramer HSD. The differing densities can be compared by analyzing the packing densities (fiber-filled space) of the scaffolds. The 800 nm HD and 1300 nm HD packing densities were higher than the random and 1300 nm LD fiber scaffolds (Figure 3C), $p < 0.05$ by Tukey-Kramer HSD. Although the number of 800 nm fibers per mm (fiber density) was higher than that of the 1300 nm HD fibers (Figure 3B), the packing densities (fiber-filled space) of the 800 nm HD fibers and the 1300 nm HD fibers were not statistically different (Figure 3C). The difference in fiber density was due to differences in fiber diameter (Figure 3A). The randomly oriented fibers and the 1300 nm LD fibers had similar packing densities (Figure 3C), due to the differences in fiber diameters (Figure 3A).

Cell migration restricted along the x axis is enhanced by aligned fiber scaffolds

When BECs were seeded in molds for the restricted migration model, minimal migration was observed at 48 hours on either randomly oriented or aligned fibers (Figure 4A-B). At 144 hours, cells seeded on aligned fibers experienced substantially more migration than cells seeded on the random fibers (Figure 4C-D). At 144 hours, cell migration and alignment along the aligned fibers is evident (Figure 4F & H) whereas the cells seeded on the randomly oriented fibers migrated poorly and appear randomly aligned (Figure 4E & G). Cells were observed to migrate along but not across the fibers. Cells may not have migrated well on random fibers because extensively criss-crossed fibers may serve as obstacles to migration whereas fibers that do not tend to cross one another (e.g. highly aligned fibers) may enable a relatively unimpeded migration. Measurements of cell alignment showed that cells seeded on the aligned fibers were significantly more aligned than cells seeded on the random fibers, $p < 0.05$ by Tukey-Kramer HSD. Alignment of the cells was associated with migration, with a significant increase in the cell coverage area found on the aligned fibers (Figure 5A), $p < 0.05$ by Tukey-Kramer HSD. Cells on the random fibers also exhibited shorter cell lengths whereas cells on the aligned fibers had aligned morphologies where cell processes were extended along a single axis as the cells followed the route of the fibers (Figure 5B). Differences in cell extension were significant at $p < 0.05$ by Tukey-Kramer HSD.

Unrestricted cell migration is enhanced and directed by aligned fiber scaffolds

In the restricted migration assay, cells were able to migrate only along the x axis. In the unrestricted assay, we seeded cells in droplets in order to determine how cells would migrate on the fibers when they were able to migrate freely in all directions. The random and aligned fiber samples used in the restricted migration experiment did not vary in fiber density or fiber diameter. Based on the results from the restricted migration experiment, new fiber samples with differing diameters and densities were created for the unrestricted migration experiments in order to determine whether there is a relationship between fiber diameter or density with cell alignment, migration, and cell length.

Cell migration was seen to increase on films, random fibers, and 1300 nm HD fiber scaffolds at 72 hours (Figure 6D-F, respectively) relative to 12 hours (Figure 6A-C, respectively) after seeding. Cells tended to become randomly oriented and migrated in all directions on the films (Figure 6D, G, & J), presumably because they weren't confined to the topography of the fibers. Similarly cells tended to become randomly oriented and migrated in all directions on the randomly oriented fibers, although they became less outstretched and migrated to a reduced extent relative to cells seeded on films (Figure 6E, H, & K). In contrast, cells became aligned and elongated, and migrated in a highly aligned manner when seeded on the aligned fibers (Figure 6F, I, & L). $4 \pm 2\%$ of the cells seeded on the films and $4 \pm 2\%$ of the cells seeded on the random fibers were oriented within 5 degrees of the parallel axis as opposed to $44 \pm 7\%$ of the cells seeded on the 1300 nm HD fibers, $p < 0.05$ by Tukey-Kramer HSD. Cell migration was seen to increase on 800 nm HD fibers and 1300 nm LD fibers (Figure 7C-D) at 72 hours relative to 12 hours (Figure 7A-B). Cells became aligned and elongated and migrated in the direction of the aligned fibers on both fiber scaffolds (Figure 7E-H), with no significant differences between groups. When all the scaffolds used in the unrestricted migration assay were evaluated together, it was seen that cells migrated the most on films and 1300 nm LD aligned fibers, whereas cell migration was significantly reduced on random fibers and 1300 nm HD aligned fibers (Figure 8A), $p < 0.05$ by Tukey-Kramer HSD. Cell length was found to be slightly increased on films and on 1300 nm LD fibers, $p < 0.05$ by Tukey-Kramer HSD.

Cell migration is inhibited by the randomly oriented fibers

Randomly oriented fibers were found to inhibit cell migration relative to aligned fibers and films. We compared the randomly oriented fibers from the two different migration assays to determine whether fiber density or packing density of the randomly oriented fibers may influence cell migration. We found that fiber density and packing density were significantly different between the two random fiber samples (Figure 9A & B), $p < 0.05$ by Tukey-Kramer HSD. However, these differences did not lead to a change in cell area coverage (Figure 9C). Thus, the property of randomly orienting the fibers caused the reduced cell migration irrespective of fiber density, packing density, or BEC/LEC phenotype.

DISCUSSION

Axillary dissection during surgery to treat breast cancer results in life-long secondary lymphedema of the arm in approximately 20-30% of cases [20,21], a disease for which no prophylactic treatment currently exists. In addition to the impaired lymphatic flow, lymphedema is associated with increased risk of infection due to compromised immune function. Although the painful swelling generally appears several years after the initial lymphatic-related injury has occurred [22,23], the lymphatic vessels fail to regenerate during wound repair due to the presence of obstructive scar tissue [4,8]. An improved regeneration of the lymphatic vessels during wound repair may reduce the development of lymphedema and improve immune function following breast cancer surgery, when lymph nodes and lymph vessels are removed from the axilla. In the present study, we have begun developing biodegradable material conduits for the long-term goal of bridging the severed ends of lymphatic collecting vessels and enabling their regeneration through a relatively large region of tissue undergoing fibrotic wound repair.

A novel feature of the conduit is the aligned electrospun fibers that were found to direct the migration of human lymphatic endothelial cells (LECs). It was seen with two different human endothelial cell lines that the endothelial cells were strongly influenced by the orientation of the fibers and tended to become aligned in the same direction as the fibers, irrespective of whether the fibers were random or aligned, or whether the cells were BECs or LECs, indicating that a common molecular mechanism may mediate the response of endothelial cells to nanoscale topographical cues. In previous studies, fiber alignment was also shown to correlate with cell alignment [19,24,25]. When cultured over time, cells were seen to migrate and become extended along the aligned fibers. In contrast to directed cell migration and cell extension obtained on aligned fibers, cell migration was strongly inhibited by the random fibers. An individual cell seeded on the random fiber scaffold may align and migrate on an individual fiber and then experience a reduced migration when the cell encounters an intersecting fiber. Alternatively, a cell seeded on the random fiber scaffold may attach to multiple fibers aligned in different directions; the conflicting signals may inhibit cell alignment and migration.

We found an increased migration when cells were seeded on film substrates with no fibers compared to the aligned and randomly oriented fiber substrates. Due to the absence of barriers, the cells cultured on films may have been able to move more freely and spread throughout the sample. Although the cells migrated well on the film substrates, the alignment and migrating direction was not controlled. Furthermore, cells seeded on films in the restricted migration experiments had detached by day 6 (data not shown), possibly as a consequence of the daily washing from cell culture media exchanges, signifying that these cells may not have been strongly adhered to the underlying film substrate. In contrast, cells seeded on the random and aligned fibers and exposed to the same culturing conditions were retained at day 6. The fibers may therefore serve a dual role of directing the alignment and migration of cells and of promoting cell attachment to the material by providing a three

dimensional surface. Indeed, these may be related properties, as the cell may need to attach firmly to the fibers in order to be oriented by the fibers. Thus, the alignment of cells on the fibers also provides evidence that the cells were well adherent to the fibers.

Surprisingly, cells cultured on aligned HD fiber scaffolds exhibited less migration than cells cultured on LD fibers. Although cells seeded on the 800 nm HD and 1300 nm HD fibers migrated in the fiber direction, cells seeded on the 1300 nm LD fibers migrated more extensively. A possible explanation is that the cells attached more strongly to the HD fiber scaffold due to the increased number of fibers contacting the cell, which may have made it more difficult for the cells to migrate. Thus, increased fiber density may improve cell attachment at the cost of cell mobility. The fiber densities of the 800 nm HD and 1300 nm HD fibers were slightly different, although the difference in fiber density and diameter did not affect cell migration. The slight difference in density between these fibers is due to differing fiber diameters. Although the fiber densities and diameters were different, similar packing densities between the 800 nm HD and 1300 nm HD samples lead to similar cell migration. In the case of the 1300 nm LD and 1300 nm HD fibers, differences in packing density were associated with differences in cell migration. Thus, in the case of aligned fibers, the packing density influenced cell migration. This may be the case because cells seeded on scaffolds with a lower packing density may be more likely to adopt the characteristics seen with cells seeded on films. Therefore, 1300 nm LD fibers may have promoted an optimal cell alignment and migration because cells seeded on these fibers acquired characteristics seen with fibers (attachment and alignment) and films (extended migration). Packing density was similar between the randomly oriented and 1300 nm LD samples, although cell migration was markedly different. Thus fiber alignment was a precondition for cell migration.

We have shown that LEC attachment, alignment, and migration can be controlled with topographical cues. The present biomaterial approach for promoting LEC migration does not rely upon vascular endothelial growth factor (VEGF)-C, although a natural (low) level of VEGF-C may have been present in the serum. VEGF-C promotes LEC migration and proliferation by binding to VEGF Receptors-2 and -3 on lymphatic endothelial cells [26-30] and excess (i.e. supra-physiological) VEGF-C protein delivered to lymphatic cells may increase lymphatic growth. However, in addition to enhancing lymphatic growth, excess VEGF-C unfortunately has been shown to induce lymphatic hyperplasia and diameter expansion [32,33]. This may reduce the filling capability of lymphatic capillaries [34] and produce incompetent lymphatic valves [35]. In addition to the potential harmful effects on the lymphatic system, VEGF-C can also bind receptors on the blood endothelium and increase vascular permeability [36] and fluid formation in the swollen limb. Furthermore, VEGF-C upregulation in tumors is correlated with lymphatic metastasis [37-39] and excess VEGF-C administration to cancer survivors may place them at increased risk for metastasis of recurring tumors. Therefore, VEGF-C protein that is released from an implanted conduit may render neighboring lymphatics less functional, increase the fluid drainage burden on the lymphatic system, and place women with damaged lymphatic vessels at an increased risk for secondary lymphedema. Due to the harmful side effects of excess VEGF-C it is important to develop VEGF-C-independent therapies for increasing lymphatic regeneration. We found that LECs were able to migrate on the aligned fibers in the absence of excess VEGF-C, although a natural level of VEGF-C may have been present in the serum used in the experimentation. LECs migrating on the fibers of an implanted conduit would be exposed to the natural level of lymphatic growth factors present in circulating lymph, similar to what was present in the cell culture serum. Thus, LECs may migrate across an implanted conduit with a luminal fiber coating in the absence of excess VEGF-C. The development of aligned fiber substrates that can direct LEC migration is an initial step in the development of VEGF-C-independent lymphatic growth promoting materials.

Whereas biomolecular approaches for increasing lymphatic function have been investigated over the past decade, biomaterial approaches for this purpose have not been explored. However, biomaterial approaches for reconstructing lymphatic vessels may avoid the use of excess VEGF-C along with its potentially harmful side effects. Biomaterial approaches have long been investigated for their potential to reconstruct failed or damaged arteries. However, these constructs fail primarily due to platelet activation and thrombosis [40] and high levels of mechanical stress [41]. In contrast to blood, lymph flows in a low-pressure system devoid of platelets [42]. It is therefore anticipated that materials developed for reconstructing lymphatic vessels (Figure 10) may encounter fewer challenges compared to materials that have been developed for arterial reconstruction.

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Figure 1.

Alignment, diameter, and density of 1300 nm diameter fibers used for restricted migration experiments with BECs. (A) SEM image of randomly oriented fiber sample. (B) Alignment histogram showing the degrees of fiber variation from a horizontal line. (D) SEM image of the 1300 nm aligned fiber sample. (E) Alignment histogram of the 1300 nm aligned sample. (C) Fiber densities of the samples. (F) Fiber diameters of the two sample types. Scale bar in A = 50 μ m. Asterisks in graphs denote statistical significance ($p < 0.05$). Statistical analysis was performed by ANOVA with Tukey-Kramer HSD.

Figure 2.

Fiber characterizations for unrestricted migration experiments with LECs. Depicted are randomly oriented (A), 800 nm aligned (C), 1300 nm low density (E), and 1300 nm high density (G) with their respective fiber alignment measurements shown in B, D, F, and H. Scale bar represents 50 μm . Asterisks denote statistical significance ($p \leq 0.05$) from B. Pound sign denotes significance from all other samples. Statistical analysis was performed by ANOVA with Tukey-Kramer HSD.

Figure 3.

Fiber diameter, density and packing density analysis for unrestricted migration experiments. Graph A shows the average fiber diameters, Graph B shows the average fiber density and Graph C shows the packing density. Asterisks denote statistical significance from randomly oriented samples ($p \leq 0.05$) by ANOVA with Tukey-Kramer HSD.

Figure 4.

BECs seeded on randomly oriented fibers and aligned fibers in the restricted migration model. A and B show cells after 48 hours of culturing randomly oriented fibers aligned fibers, respectively. C and D show the cells after 144 hours of culturing. White lines in A-D represent where the mold was originally placed. E and F show the migration region at 144 hours. G and H show the alignment of the cells for each situation. I shows the cell coverage area and J shows the average cell length at 144 hours. Asterisk denotes statistical significance ($p \leq 0.01$). Scale bar for A-D is 1 mm while the scale bar in E and F is 200 μm .

Figure 5. Migration and cell length analysis of BECs. Graph A shows the area fold increase for each situation normalized to the original mold area. Graph B shows end-to-end cell length measurements. Asterisks represent statistical significance ($p \leq 0.01$) by ANOVA with a Student's t-test.

Figure 6.

LECs seeded on film, randomly oriented fibers, and 1300 nm high density aligned fibers for the unrestricted migration assay. A-C show cells after 12 hours of culturing on films, randomly oriented fibers and 1300 nm high density aligned fibers, respectively. D-F show the cells after 72 hours of culturing. G-I show close-up images of the cells for each situation. J-L show the alignment of the cells for each situation. Scale bar for A-F is 500 μm . Scale bar for G-I is 100 μm . Asterisks denote statistical significance ($p \leq 0.05$) by ANOVA with Tukey-Kramer HSD.

Figure 7.

LECs seeded on variations of aligned fibers for unrestricted migration assays. A-B show cells at 12 hours of culturing on 800 nm aligned and 1300 nm low density aligned fibers, respectively. C-D represent these situations after 72 hours of culture time. E-F show close-up images of the cells in each situation. G-H show the alignment of the cells for each situation. Scale bar for A-D is 500 μm . The scale bar for E-F is 100 μm . Statistical analysis was performed by ANOVA with Tukey-Kramer HSD (no significance found).

Figure 8.

Migration and cell length analysis for LECs in unrestricted migration experiments. Graph A shows the area fold increase for each situation normalized to the 12 hour cell coverage area for the same situation. Asterisks represent statistical significance ($p \leq 0.05$) from control (film). Graph B shows cell length measurements. Values not connected by the same letter are significantly different ($p \leq 0.05$). Statistical analysis was performed by ANOVA with Tukey-Kramer HSD.

Figure 9. Packing density (A), fiber density (B), and fold increase in cell area coverage (C) for ECs seeded on the random fibers in the restricted and unrestricted cell migration models. Asterisks represent statistical significance ($p \leq 0.05$) by ANOVA with Tukey-Kramer HSD.

Figure 10.

Image of the conduit balanced on a 28 gauge needle shows biomaterial with aligned luminal fibers glued into a tubular geometry with UV adhesive (A). Open face SEM view of the conduit shows aligned, electrospun fibers coating the conduit interior (B). Scale bar in A is 500 μm and in B is 50 μm .