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Computer-Controlled Microcirculatory Support System for Endothelial Cell Culture and Shearing

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Endothelial cells (ECs) lining the inner lumen of blood vessels are continuously subjected to hemodynamic shear stress, which is known to modify EC morphology and biological activity. This paper describes a self-contained microcirculatory EC culture system that efficiently studies such effects of shear stress on EC alignment and elongation in vitro. The culture system is composed of elastomeric microfluidic cell shearing chambers interfaced with computer-controlled movement of piezoelectric pins on a refreshable Braille display. The flow rate is varied by design of channels that allow for movement of different volumes of fluid per variable-speed pump stroke. The integrated microfluidic valving and pumping system allowed primary EC seeding and differential shearing in multiple compartments to be performed on a single chip. The microfluidic flows caused ECs to align and elongate significantly in the direction of flow according to their exposed levels of shear stress. This microfluidic system overcomes the small flow rates and the inefficiencies of previously described microfluidic and macroscopic systems respectively to conveniently perform parallel studies of EC response to shear stress.

Microfluidic systems enable for a high level of fluidic control to create in vivo like microenvironments for cell culture.[1−10] Most microfluidic culture systems to date, however, have focused primarily on regulating the chemical environment and not the fluid mechanical environment of cells, such as the exposure of endothelial cells (ECs) to physiological levels of shear stress (5−20 dyn/cm^2).[11] The difficulty is due, at least in part, to the lack of integrated microfluidic pumps that can sustain high enough levels of shear stress in cell culture compatible conditions for the duration of time required (hours to days) to observe cellular effects. Here, we report the use of self-contained, computer-controlled microfluidic cell culture chamber arrays where high-velocity (max of 3 cm/s) pulsatile fluid flows inside high-resistance microchannels generate shear stress levels capable of fluid mechanical regulation of EC phenotypes.

ECs comprise the endothelium or the monolayer of cells lining the inner wall of blood vessels subjected to hemodynamic shear stress in vivo. ECs have the property to dynamically sense changes in shear stress levels present in their environment.[12] The responsiveness of ECs to changes in shear stress levels is assessed by modifications in morphology including alignment and elongation in the direction of flow,[13,14] adaptation of cytoskeleton-associated proteins,[15]fluctuations in intracellular calcium concentration involved with cell signaling,[16] secretion of factors necessary for survival,[17] and expression levels of genes.[18] A fundamental question in cardiovascular research is how the mechanical forces associated with shear stress are first sensed and then transduced by ECs into certain diseased states such as thrombosis[19] or atherosclerosis.[20] To address this question, many cardiovascular researchers model or recreate physiological flow conditions in

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vitro with macroscopic systems utilizing external pumping mechanisms, viscometers, and/or fluid reservoirs[13,14,21] Although these systems produce well-defined fluid flows to relate shear stress levels with a measurable biological response, they are limited due to their consumption of large amounts of reagents, potential for contamination, decreased portability, and inability to perform multiple experiments simultaneously. This system addresses the limitations of existing macroscopic systems by employing a self-contained, re-circulating poly(dimethylsiloxane) (PDMS) microfluidic device with parallel channels interfaced with a refreshable array of piezoelectric pins of a commercially available Braille display.[22] Each piezoelectric pin functions as an actuator for a microfluidic valve when its up-and-down movement is used to deform the elastomeric PDMS microchannels.[23] Fluid is pumped through the microchannels using a variable-speed, 3-pin, peristaltic sequence. The valving capabilities of the microarray of Braille pins enable for one-step seeding of multiple cell shearing compartments that can then be re-circulated simultaneously in parallel but under different cell shearing conditions. The system generates different shear stress levels at the same pumping frequency by varying the size of the contact area of the microchannels where the pins press in, which allows for displacement of different volumes of liquid per pump stroke. Flow rates sufficient for EC shearing are produced by maximizing the size of this contact area such that the displacement of fluid is large. This system concurrently addresses the restricted flow rates of microfluidic systems and the inefficiencies of macroscopic systems thereby opening new windows for evaluating the physiological and biological effects associated with application of shear stress to ECs in vitro.

EXPERIMENTAL SECTION

Device Fabrication. The microfluidic device (Figure 1a) was fabricated from three layers of poly(dimethylsiloxane) (PDMS) formed from prepolymer (Sylgard 184, Dow Corning) at a ratio of 1:10 base to curing agent. The top layer was formed by replica molding of the prepolymer against relief features machined from brass and steel bars (Figure 1b) to form fluid reservoirs and cured overnight at 60 °C. The middle layer was formed using soft lithography[24] to form a layer with negative relief channel features ~30 μm in height and 300 μm in width. The positive relief features of the mold were composed of SU-8 (Microchem, Newton, MA) formed on a thin glass slide (200 μm thick) using backside diffused-light photolithography.[23] The glass slide was silanized with tridecafluoro-(1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies Inc., Bristol, PA). The prepolymer was cured at 60 °C overnight, and holes were punched in it to connect channel features to the culture media reservoir. The negative relief channel features were sealed against a flat thin membrane layer formed by spin coating prepolymer onto silanized glass slides and cured at 150 °C overnight. PDMS cured at 150 °C as opposed to 60 °C demonstrates more consistent mechanical properties.[25] The three layers were sealed irreversibly by treating with plasma oxygen (SPI Supplies, West Chester, PA) for 30 s, pressing the

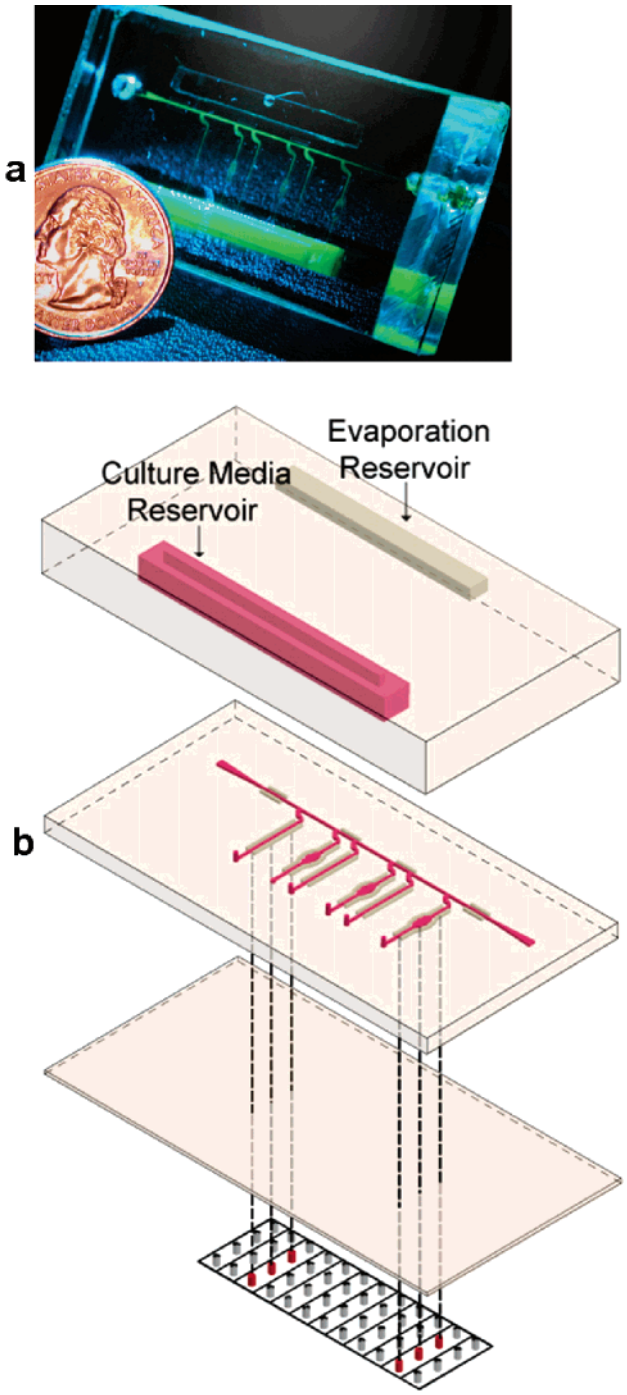

Figure 1. Microfluidic device for EC culture and shearing. (a) Photograph of the device. (b) Three-dimensional schematic depicting the three-layer device fabrication placed on top of a grid of Braille pins. The approximate thickness of the top, middle, and bottom layers are 1 cm, 1 mm, and 100−200 μm, respectively. The top layer contains two reservoirs: one to house cell culture media ("Culture Media Reservoir") to be circulated and the other containing water ("Evaporation Reservoir") to assist in preventing evaporation within the microfluidic channels. The volume of both reservoirs is ~1 mL. The bottom layer serves as a thin membrane that provides the interface between the Braille pins and the microfluidic channels of the middle layer. The pins in red and the dotted lines depict how the microfluidic channels are aligned when placing the device on top of the grid of pins.

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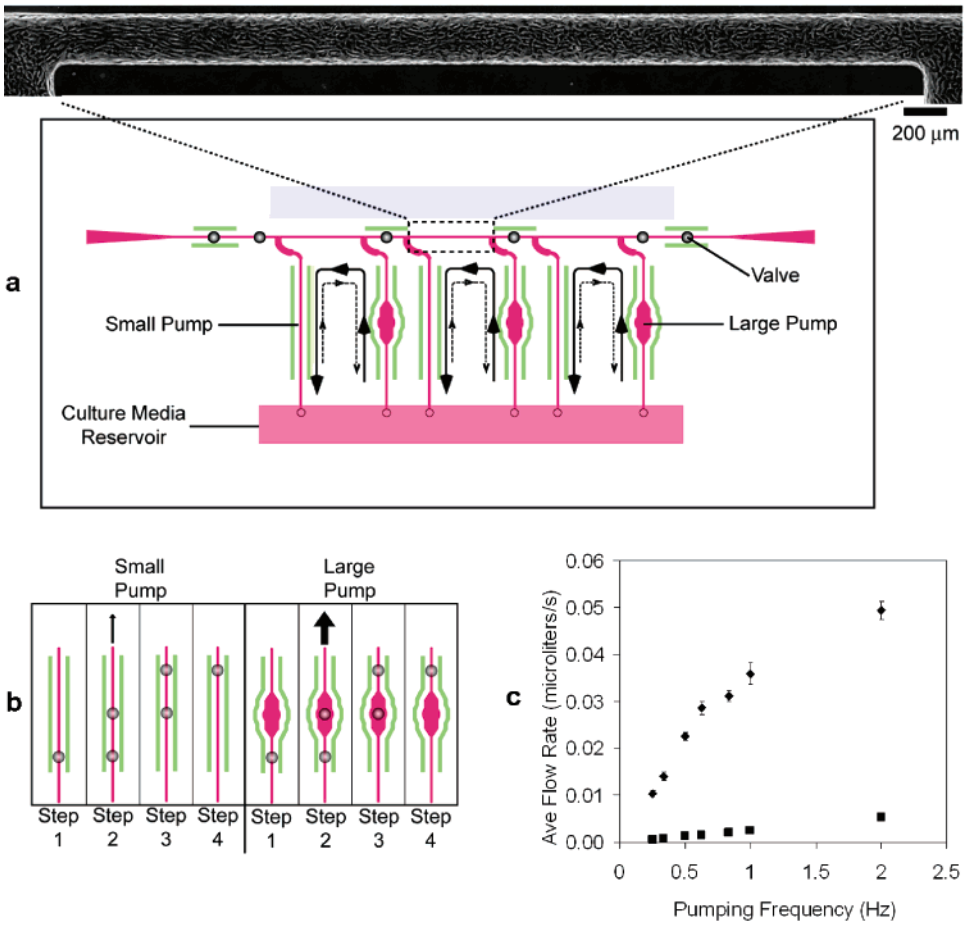

Figure 2. Demonstration of the microfluidic valves and pumps for cell culture. (a) Close-up of cells attached within an individual cellular compartment. The individual compartments are created by the Braille pins acting as valves, which are depicted by the dark circles along the horizontal region of the channel. Each compartment is circulated by flow loops that actuate fluid independently of each other. Fluid in each flow loop can be actuated either clockwise by the small pump (dashed curved line and arrows) or counterclockwise by the large pump (solid curved line and arrows). Cells shown were cultured under cell shearing conditions for 12 h. (b) Stepwise depiction of the peristaltic pumping sequence comparing the small pump and large pump. Fluid is actuated through the channels in a pulsatile nature via a 3-pin, repeating 4-step peristaltic pumping sequence.[22] In this particular pumping sequence, it is step 2 or the step where the middle pin of the 3-pin pump moves from the down to the up position that drives most of the fluid in the forward direction. The other steps in the pumping sequence prime the pump to maximize volume displacement during step 2. Increased shear stress levels are generated by the large pump by increasing the area of the channel that is positioned over the middle pin of the 3-pin pump reaching a maximum width equal to approximately the diameter of the Braille pin (1.3 mm). The pair of auxiliary dead-end channels that run parallel to fluidic channels act as void spaces to assist in deformation based actuation due to the Braille pins. These void space channels are of the same height as the fluidic channels due to them being fabricated concurrently during the backside diffused-light lithography process.[23] (c) Comparison of pumping capabilities of small pump and large pump. The average flow rate (μL/s) for the small pump (■) and the large pump (◆) were plotted against the pumping frequency (Hz). Error bars represent SEM (note: some of the values for SEM are at a value so small that the error bars do not extend beyond the boundary of the shapes at certain data points and are not visible).

flat surfaces together, and placing in 60 °C for 5−10 min. Immediately after sealing, sterile phosphate-buffered saline (PBS) was injected to maintain the hydrophilic nature of the channels. Shortly after, the device was sterilized by placing under UV light for ~30 min.

Fluid Actuation. A Braille display (DotView DV-1, KGS, Japan) provided a grid (32 × 24) of piezoelectric pins (1.3 mm in diameter) that function as an array of microfluidic valves and pumps. The microfluidic device interfaces with the Braille display by simply holding the device in place such that the channels align with the Braille pins (Figure 1b), which push upward with a force of 4 cN or approximately 1/100th of a pound force (manufacturer's specifications). The Braille pins were controlled with a computer program written in C++ that manipulates the pins to either remain in the up position and act as valves (Figure 2a) or be components of a 3-pin peristaltic pump that actuates fluid in a pulsatile nature (Figure 2b). The microfluidic channels were designed such that both low shear stress levels (maximum shear stress ~5 dyn/cm^2 per pump cycle) and high shear stress levels (maximum shear stress ~60 dyn/cm^2 per pump cycle) can be generated within the same loop by changing the volume displacement per pump stroke (Figure 2b). Flows at low and high shear levels were actuated by what will be denoted as "small pump" and "large pump", respectively. The average shear stress levels of both the small and large displacement pumps were controlled by changing the frequency of the pumping sequence.

Characterization of Fluid Flows. The 6 μm diameter fluorescent (Carnine) polystyrene microspheres (Molecular Probes, Eugene, OR) were tracked using a digital CCD camera (Hamamatsu ORCA-ER) and a fluorescence stereomicroscope (Nikon

SMZ1500). Images sequences were acquired at ~15 frames/s to determine the velocity of the microspheres that are representative of the fluid velocity and used to determine the time-varying fluid flow rate $Q(t)$. The Womersley number (α) is a dimensionless parameter used to describe the pulsatile nature of fluid flow in response to an unsteady pressure gradient[26] and is defined as:

$$\alpha = h\sqrt{\frac{\omega}{\nu}} \tag{1}$$

where h is the height of the channel (~30 μm), ω is frequency of pumping, and ν is the kinematic viscosity. For the described microfluidic device, α is small ($\ll$1), and thus the unsteady effects on shear stress levels are negligible. For fully developed, steady channel flow, the shear stress (τ_{cell}) that cells are exposed to is expressed as:

$$\tau_{cell} = \frac{6\mu Q}{h^2 w} \tag{2}$$

where μ is the dynamic viscosity, Q is the fluid flow rate, and w is the width of the channel (~300 μm). However, since the pulsatile effects on shear stress are negligible, the time-varying values of shear stress ($\tau_{cell}(t)$) can be determined by replacing Q in eq 2 with $Q(t)$.[14] The average flow rate and average shear stress levels for the given pump and pumping frequency were determined by taking the time-average of the discrete values of $Q(t)$ and $\tau_{cell}(t)$, respectively, over one wavelength multiple times and then performing statistical analysis to determine the average and standard error of measurement (SEM).

General Cell Culture. Human dermal microvascular endothelial cells (HDMECs, Cambrex, East Rutherford, NJ) passage number 7-13 were cultured in endothelial growth medium-2 (EGM-2, Cambrex) in T-75 culture flasks (Corning, Acton, MA) that were placed in a humidified 5% CO_2 cell culture incubator. The HDMECs were collected by washing and detaching with 0.25% trypsin/EDTA (Invitrogen, Carlsbad, CA). The trypsin solution was neutralized with 10% FBS in DMEM and spun down with a centrifuge (ThermoForma, Marietta, OH) for 5 min at 4 °C and 800 rpm. The supernatant was removed, and the pellet was resuspended in EGM-2. The spin and resuspension in EGM-2 were repeated to ensure removal of trypsin, which inhibits cell adhesion during seeding.

Cell Seeding and Microfluidic Cell Culture and Shearing. Prior to cell seeding, fibronectin solution (Invitrogen) at a concentration of 100 μg/mL in PBS was injected along the cell seeding channel to promote cell adhesion. The fibronectin solution was introduced into regions of the microchannels defined by the valves created by the Braille display using two 30 gauge insulin needles, one to vent and one to inject. The fibronectin solution coated the channel surface for 30 min at 25 °C and then was rinsed by circulating PBS from the reservoir for 30−60 min. PBS was then replaced in the reservoir with EGM-2 to rinse the PBS by circulating for 30−60 min. Subsequently, the cell solution (~10^6 cells/mL) was injected into the microchannels in the same manner as the fibronectin solution. After the cells were seeded, the seeding channel was valved at locations to form individual cellular compartments to be circulated with loops that are pumped independently of each other (Figure 2a). The device and Braille display were placed in a 37 °C/5% CO_2 dry incubator to allow for the cells to attach for 60−90 min. After the cells attach, culture media was circulated with the small pump at the desired pumping frequency to sustain the cells for the next 48−72 h with the culture media in the reservoir being replaced every 24 h. Once the cells reached the desired level of confluence, the culture media was replaced and remained for the duration of the subsequently described experiment.

Two experiments were conducted to compare the effects of varying levels of shear stress on cell morphology. The first experiment compared two compartments located within the same device with one circulated with the small pump and the other with the large pump and both circulated at the same frequency of 1.0 Hz. Since both compartments were located in the same device, they shared the same culture media. The time-average values of shear stress at a pumping frequency of 1.0 Hz were determined with eq 2 to be <1 dyn/cm^2 for the small pump and ~9 dyn/cm^2 for the large pump. The second experiment compared the changes in cell morphology in three compartments located within the same device that were all circulated with the large pump but at different pumping frequencies of 0.25, 0.75, and 2.0 Hz. The time-average shear stress values were determined with eq 2 to be approximately 3 dyn/cm^2 (0.25 Hz), 7 dyn/cm^2 (0.75 Hz), and 12 dyn/cm^2 (2.0 Hz).

Quantification of Cellular Alignment and Elongation. The morphological response of ECs to shear stress was measured with angle of orientation and the Shape Index (SI), which are commonly used parameters that quantify the extent that the ECs align and elongate in the direction of flow, respectively. Briefly, the angle of orientation is defined by the angle formed by the cell's major axis and the direction of flow where 0° is a cell aligned perfectly with the direction of flow and 90° is a cell aligned orthogonal to the direction of flow. The SI is a dimensionless measure of the roundness of a cell that is defined as:

$$\mathrm{SI} = \frac{4\pi A}{P^2} \tag{3}$$

where A is the area of the cell and P is the perimeter of the cell. The SI ranges from 0 to 1 where 0 is a straight line and 1 is a perfect circle. For cells in static culture, the mean angle of orientation is ~45° with a large standard of deviation and the mean SI value is about 0.8, indicating that the population of cells is randomly oriented and very round in shape.[14] However, since the cells in the described system are cultured in microchannels under flowing conditions with the small pump before they are sheared with the large pump, the typical baseline values for angle of orientation (30−40°) and SI (0.6−0.7) are slightly less and thus indicates that they are slightly more aligned and less round than cells in static culture conditions.

EC images were obtained using an inverted phase contrast microscope (Nikon TE 300) and a digital CCD camera (Hamamatsu ORCA-ER) at 10× magnification. Images were taken at the onset of circulation with the large pump and every 6−12 h thereafter. The images were analyzed with Simple PCI imaging

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software program (Compix Inc. Cranberry Township, PA) to measure the angle of orientation and the SI of individual cells. These data were then exported to an Excel spreadsheet to determine the sample average and the SEM values for angle of orientation and SI for each time frame.

Statistics. Statistical differences between experimental groups were evaluated using two-sample Student *t*-tests at a 95% confidence level assuming unequal variances.

RESULTS AND DISCUSSION

Characterization of Fluid Flows. The purpose of this study was to recreate pulsatile shear stress levels capable of remodeling ECs within a microfluidic setting. The generation of pulsatile flow is of immense physiological importance because it represents the nature of blood flow in the arterial vasculature that produces shear stress levels modifying EC morphology. Since ECs demonstrate the ability to distinguish between pulsatile versus non-pulsatile flow,[14] in order to be physiologically relevant, the described in vitro system must not only generate flow with average shear stress levels seen in vivo (5−20 dyn/cm^2)[11] but deliver it in a pulsatile nature as well.

The pulsatile flow was characterized by relating the average flow rate to the pumping frequency (Figure 2c). For the small pump, the average flow rate increased linearly ($R^2 = 0.99$) with pumping frequency and the maximum average flow rate achieved was 5.3×10^{-3} μL/s at a pumping frequency of 2.0 Hz. The maximum pumping frequency applied for the small pump as well as the large pump was 2.0 Hz because pumping frequencies above 2.0 Hz are not commonly present within the blood circulation in vivo.[27] As seen with the small pump, the average flow rate for the large pump increased linearly with smaller values of pumping frequency. However, unlike the small pump, the average flow rate for the large pump plateaus for pumping frequencies larger than ~0.75 Hz (Figure 2c) reaching a maximum average flow rate of 4.9×10^{-2} μL/s at a pumping frequency of 2.0 Hz. For smaller values of pumping frequencies (<0.75 Hz), the average flow rate for the large pump was ~20 times larger than the average flow rate for the small pump. At a pumping frequency of 2.0 Hz, the average flow rate for the large pump was ~10 times larger than the average flow rate for the small pump exhibiting a significant plateau effect for increased pumping frequency with the large pump.

The plateau effect was only seen with the large pump and indicates reduced efficiency in actuating fluid in the forward direction for pumping frequencies above ~0.75 Hz. This plateau effect is most likely due to one of the steps in the pumping sequence becoming rate-limiting with increased pumping frequency.

EC Morphology Response to Cell Shearing Conditions. The responsiveness of ECs to different levels of shear stress was evaluated in terms of changes in morphology. Figure 3a is a time-lapse comparison between the changes in EC morphology due to the small pump (average shear stress <1 dyn/cm^2) versus the large pump (average shear stress ~9 dyn/cm^2), both circulated at a pumping frequency of 1.0 Hz. The images suggest that the morphology of the cells circulated by the small pump at 1.0 Hz remain random in orientation and relatively round in shape whereas the morphology of the cells circulated by the large pump at 1.0 Hz progressively align and elongate in the direction of flow with time. Alignment and elongation were quantified in terms of EC angle of orientation (Figure 3b) and SI (Figure 3c), respectively. For the small pump, angle of orientation decreased 10° (values given for changes in angle of orientation and SI are approximate) from $T = 0$ h and $T = 6$ h but did not change significantly after $T = 6$ h ($p > 0.05$). The decrease in angle of orientation from $T = 0$ h to $T = 6$ h was not expected and is considered a measurement artifact based on results from previous experiments. Thus, for the small pump, neither the angle of orientation nor the SI for the ECs changed significantly from $T = 0$ h to $T = 24$ h ($p > 0.05$).

For the large pump, both angle of orientation and SI decreased significantly ($p < 0.0001$) from $T = 0$ to $T = 24$ h. The ECs

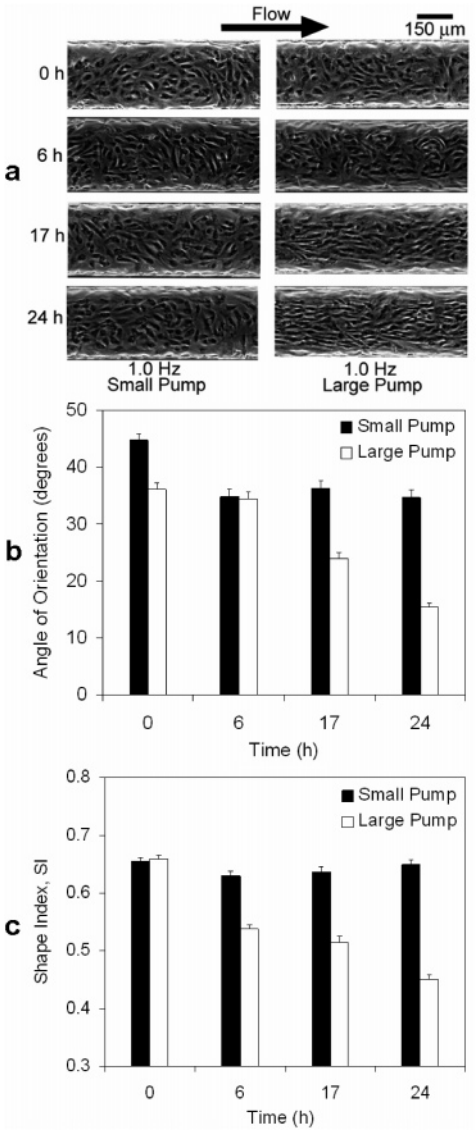

Figure 3. Cell morphology response to cell shearing conditions. (a) Time-lapse images comparing changes in cell morphology between small and large pumps circulated at the same pumping frequency (1.0 Hz). Quantification of cell alignment (b) and elongation (c) comparing the EC response due to circulation in one loop by the small pump and the other loop by the large pump both circulated at a pumping frequency of 1.0 Hz.

(27) Wégria, R.; Frank, C. W.; Wang, H.; Lammerant, J. *Circ. Res.* **1958**, *6*, 624−632.

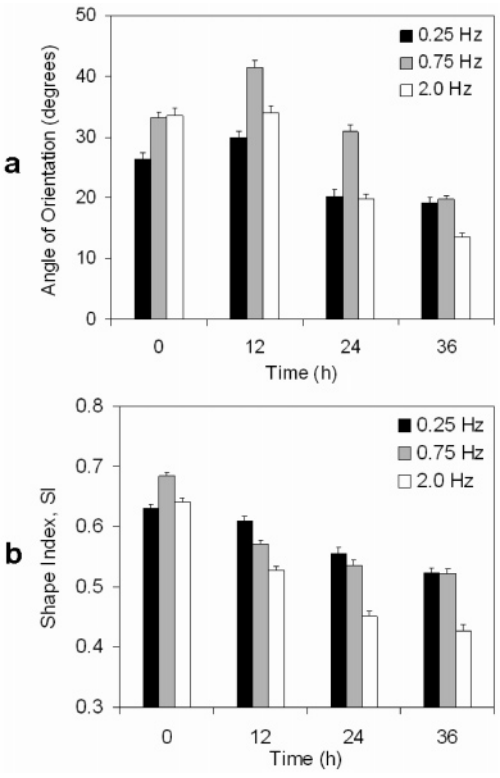

Figure 4. Morphological response of cells subjected to different levels of cell shearing conditions. Quantification of cell alignment (a) and elongation (b) comparing EC response in three loops due to circulation by the large pump circulated at frequencies of 0.25, 0.75, and 2.0 Hz respectively.

cultured with the large pump exhibited a decrease in angle of orientation by 20° and a decrease in SI by 0.21 from $T = 0$ to $T = 24$ h. The percent decrease from $T = 0$ to $T = 24$ h for angle of orientation and SI was 57% and 32%, respectively. There was also a substantial decrease in the standard of deviation of the angle of orientation from $T = 0$ to $T = 24$ h, which is characteristic of EC alignment in the direction of flow.[14] Furthermore, the angle of orientation and the SI values were significantly different than the values for the small pump at $T = 24$ h ($p < 0.0001$). In agreement with previous studies, EC elongation occurred more rapidly than alignment under cell shearing conditions (Figure 3b,c).[28] In addition, the cell density remained steady between 400 and 550 cells/cm^2 for both experiments for the duration of the experiment demonstrating that cell detachment was not an issue for the given pumping conditions (data not shown).

The morphological response of the ECs validate that this in vitro model system is capable of generating shear stress levels sufficient enough to modify EC phenotypes. The average shear stress levels were altered by changing the amount of fluid displacement per pump stroke at a constant pumping frequency. In addition, it is possible to vary the average shear stress levels by changing the pumping frequency while keeping the amount of fluid displacement per pump stroke constant. Figure 4 shows results from a single chip three-loop experiment where each loop of ECs was exposed to flow generated by large pumps, but the average shear stress was varied by changing the pumping frequency. Average shear stress levels ranging from ~2.5 to 12 dyn/cm^2 were generated by varying the pumping frequency between 0.25 and 2 Hz. All flow conditions exhibited the ability to significantly modify EC morphology in terms of alignment and elongation ($p < 0.0001$). It has been shown previously that it is the average shear stress that is the primary factor that regulates the relative timing of EC alignment and elongation.[21] The results of the multiple cell shearing experiment support those previous outcomes.

The described microfluidic system has five characteristics that make it advantageous over existing macroscopic systems used to study EC response to shear stress. (i) One-step seeding of cells into multiple compartments. (ii) Re-circulation of cell culture media from a single reservoir only ~1 mL in volume. (iii) Cells and reagents once placed within the device remain there indefinitely. (iv) Multiple culture loops whose pulsatile fluid flows are actuated independently of each other. (v) System remains portable enough to be placed entirely within a cell culture incubator. The combination of these characteristics address the limitations of macroscopic systems such as consumption of large amounts of cells and reagents, potential for contamination, decreased portability, and inability to efficiently perform multiple pulsatile flow experiments in parallel. This current device is restricted in its range of average shear stress levels that it can generate (up to ~12 dyn/cm^2), which does not encompass the entire range of average shear stress levels seen physiologically (5−20 dyn/cm^2).[11] The system, however, to our knowledge is the first one of its kind that produces shear stress levels that align and elongate ECs with pulsatile fluid flow and hence demonstrates the ability to create an arterial-like microenvironment within a self-contained, reconfigurable microfluidic device.

CONCLUSIONS

We present the foundation for an in vitro microfluidic cell culture system that recreates physiological conditions present in the EC environment in vivo in terms of shear stress levels and pulsatile flow patterns. Pulsatile flow is essential for this system to be physiologically relevant because ECs have the marked ability to discriminate between pulsatile and non-pulsatile flow.[14] The generation of pulsatile flow was accomplished by integrating the elastomeric channels of the microfluidic device with an array of Braille pin actuators to create a 3-pin peristaltic pump. Previously described microfluidic systems[3,22] are limited in their capacity in generating high enough levels of shear stress necessary for EC remodeling. This system overcomes these limitations by designing the microfluidic channels to maximize the volume displacement per Braille pin actuation. Efficiency not present in macroscopic systems is intrinsic to this design because the microarray of pin actuators coupled with elastomeric channels enables one-step seeding of multiple cell shearing chambers, followed by compartmentalization of the chambers into separate circulation loops, and simultaneous culture of ECs in the different compartments under different shear stress conditions. Furthermore, the flexibility of the design should allow for ready incorporation of additional analytical components. This marks a significant step in creating a fully integrated microfluidic device capable of providing greater insight into the mechanisms involved with mechanotransduction of signals associated with shear stress that regulate EC phenotype.

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