31st Annual International Conference of the IEEE EMBS
Minneapolis, Minnesota, USA, September 2-6, 2009

Upstream Mechanotaxis Behavior of Endothelial Cells

Sukhyun Song, Mina Kim, and Jennifer H. Shin, *Member, IEEE*

Abstract— **Vascular endothelial cell migration, which plays an important role in vascular remodeling, is known to be regulated by hemodynamic forces in the blood vessels. When shear stress is applied on mouse microvessel endothelial cells (bEnd.3) *in vitro*, cells exhibit upstream migration behavior with respect to the direction of the flow. To determine how shear stress magnitude influences mechanotaxis of the cells, endothelial cells were exposed to different magnitudes of unidirectional shear stress. While a higher flow rate reduces the speed of the motility, the horizontal component of the velocity parallel to the flow increases with the flow rate, indicating the higher alignment of cells in the direction parallel to the flow at a higher level of shear stress. In addition, cells seeded on softer substrate, whose elastic modulus is comparable to that of the blood vessels, show enhanced directional persistence when compared to those seeded on a stiffer substrate. The higher directionality accompanies increased stress fiber formation and focal adhesion turn-over, exhibiting higher mechanotaxis behavior. Therefore, the increased stiffness in the vessel may hinder the mechano-sensing mechanism of the endothelial cells, resulting in reduced mechanotaxis in response to hemodynamic shear stress. This substrate stiffness-dependent migration behavior can further elucidate the endothelial cell remodeling and wound healing in pathologically hardened vessels as well as re-endothelialization of vascular stents and grafted tissues.**

I. Introduction

THE vascular endothelium forms a continuous monolayer lining of the luminal surface in the cardiovascular system, providing the structural and communicational interface between the circulating blood and underlying tissues. Vascular endothelial cells (ECs) are continuously exposed to hemodynamic forces such as hydraulic pressure and tensile stress induced by blood vessel contraction and dilatation. ECs are also exposed to shear stress induced by blood flow along with biochemical stimuli from the neighboring cells and blood cells [1]. Recent research shows that shear stress, in particular, can modulate gene and protein expressions, which leads to changes in the cytoskeleton, proliferation rate, cell migration, and apoptosis [2]. EC, thus, is largely sensitive to conditions of the shear stresses and proper shear condition is essential for EC to keep their innate characteristics expressed in its morphological and physiological behavior. Cell motility and proliferation play a vital role in vascular remodeling during angiogenesis as well as wound healing following the surgical procedures such as angioplasty, stent placement or bypass surgery.

Like that of other cell types, EC migration is a mechanically integrated dynamic molecular process that involves regulation of spatial and temporal changes in cell adhesions, cytoskeletal organization, and signal transduction [3]. The migration process includes the protrusion of the leading edge, the formation of new adhesions at the front, the contraction of the cell, and the release of adhesions at the rear, each step of which may be modulated by fluid shear stress [4, 5]. ECM type, specificity, and density-dependent chemo- and hapto-tactic migration behavior has been reported in various cell types. Recently, the mechanical force-driven mechanotaxis [6] has been added to a manipulation tool to modulate cell behavior. Once cells sense mechanical cues from an extracellular microenvironment, whether they are in the form of force or rigidity of the matrix, a complex yet balanced array of signaling network passes the directional cues onto the intracellular structures to induce migration. *In vitro*, laminar shear stress is known to enhance ECs migration preferentially in a direction parallel to that of shear stress [7, 8].

In this work, we report the upstream migration behavior of bEnd.3 mouse microvessel endothelial cells under shear stress whose directional persistence and acceleration increase with the magnitude of the shear stress. This upstream migration behavior is further enhanced when the stiffness of the substrate is lowered. These results may shine light on better understanding of the mechanism of EC mechanotaxis behavior, providing a rational base for developing strategies to control the EC migration on pathologically hardened vessels as well as the re-endothelialization of stents and grafted tissues.

II. MATERIALS AND METHODS

A. Cell Culture

For the shear stress experiments, b.End3 cells (Mus musculus brain cerebral cortex endothelium, CRL-2299 ATCC) were cultured in Dulbecco's Modified Eagle Medium (12800-017, DMEM Gibco), supplemented with 10% fetal bovine serum (26140-079, Gibco) and 1% antibiotics (15240-096, Gibco) containing penicillin and streptomycin to prevent bacterial and microbial contaminations. Before cell

Manuscript received April 7, 2009. This work was supported in part by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2006-311-D01003), and the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (No. R01-2007-000-20959-0).

Sukhyun Song is with the Department of Mechanical Engineering, Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea (e-mail: ssh0505@kaist.ac.kr).

Mina Kim is with the Department of Mechanical Engineering, Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea (e-mail: bellflower-7@kaist.ac.kr)

Jennifer H. Shin is with the Department of Mechanical Engineering, Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea (corresponding author phone:+82-42-350-3232; fax:+82-42-350-3210; e-mail: j_shin@kaist.ac.kr).

978-1-4244-3296-7/09/$25.00 ©2009 IEEE

seeding, the glass slides were sterilized by an autoclave and coated with 10 μg/ml fibronectin (F1141-1MG, Sigma-Aldrich) for 1 hour at room temperature.

B. Substrate Preparation

In order to investigate the effects of the substrate stiffness on the migration behavior of endothelial cells, we prepared a poly(dimethylsiloxane) (PDMS) substrate at a 10:1 and 30:1 (base:curing agent) ratio. The silicone elastomer base and crosslinker were mixed thoroughly. After removing bubbles under vacuum for 30 minutes, PDMS was cured at 60°C for 18 hours. Young's modulus of PDMS was determined by a tensile test method described previously [9]. The Young's moduli of 10:1 and 30:1 ratio PDMS are measured as 1.783 MPa and 0.259 MPa, respectively, which are comparable to that of a blood vessel at 2.68±1.81 MPa [10, 9].

C. Shear Stress Stimulation

We developed a parallel plate flow chamber which allows for the application of desired shear stress on the cultured EC [11]. It is designed based on three-dimensional computational fluid dynamics (3D-CFD) analysis. To apply the desired shear stress on the VEC monolayer, the flow chamber system employs a damper and dual pump heads to minimize undesired pulsation due to peristaltic pumping. In addition, the essential cell culture conditions (5% CO_2 and 95% air) are carefully controlled and monitored throughout the experiments.

D. Imaging

Expression and localization of F-actin, paxillin were assessed by immunofluoresence. The cells were fixed with 3.7% formaldehyde (533998, Sigma-Aldrich) for 20 minutes at 37oC and then permeablized for 30 minutes in 0.2% Triton X-100 (T8787, Sigma-Aldrich) at room temperature, followed by rinsing three times in PBS. The cells were blocked with 3% bovine serum albumin (0332, Amresco) in PBS (14200-075, Gibco) for 60 minutes and then incubated with an anti-paxillin primary antibody (1:200, 610052, BD) overnight at 4oC. The cells were then labeled with a secondary antibody conjugated with Alexa Fluor 488 (1:200, A11001, Molecular Probes) for 30 minutes at room temperature, followed by brief rinsing in PBS. After completing the secondary antibody conjugation for paxillin, the cells were stained for F-actin using Alexa Fluor-568 phalloidin (1:50, A12380, Molecular Probes) for 20 minutes at room temperature. After rinsing, the nuclei of the cells were labeled with DAPI (300 nM, D1306, Molecular Probes), and then they were finally mounted in a Vectashield (H-1000, Vector Laboratories) to minimize photobleaching. The cells were imaged using a Zeiss fluorescence microscope (Axiovert-200M, Zeiss) equipped with a CCD camera (Axiocam HSm, Zeiss) and image analysis software (Axiovision, Zeiss).

The dynamic responses of the cells were monitored by time-lapse images using the Zeiss inverted microscope system described above. The phase contrast images were captured at 30-minute intervals for 16 hours. Cell migration was quantified by analyzing the cell speed and direction. The positions of individual cells were traced by tracking the location of the nuclei every 30 minutes using the Manual Tracking Module in the ImageJ software (NIH).

E. Analysis of cell migration

The following components of the ensemble averaged velocity were analyzed as a function of time: The instantaneous velocity projected on the x-axis tangential to the flow, <*Vx*>, the instantaneous velocity projected on the y-axis perpendicular to the flow, <*Vy*>, and the magnitude of the velocity <*S*> [12].

$$< Vx \geq \frac{1}{N}\sum_{i=1}^{N}\left(\frac{x_i - x_{i-1}}{\Delta t}\right)$$

$$< Vy >= \frac{1}{N}\sum_{i=1}^{N}\left(\frac{y_i - y_{i-1}}{\Delta t}\right)$$

$$< S >= \frac{1}{N}\sum_{i=1}^{N}\sqrt{\left(\frac{x_i - x_{i-1}}{\Delta t}\right)^2 + \left(\frac{y_i - y_{i-1}}{\Delta t}\right)^2}$$

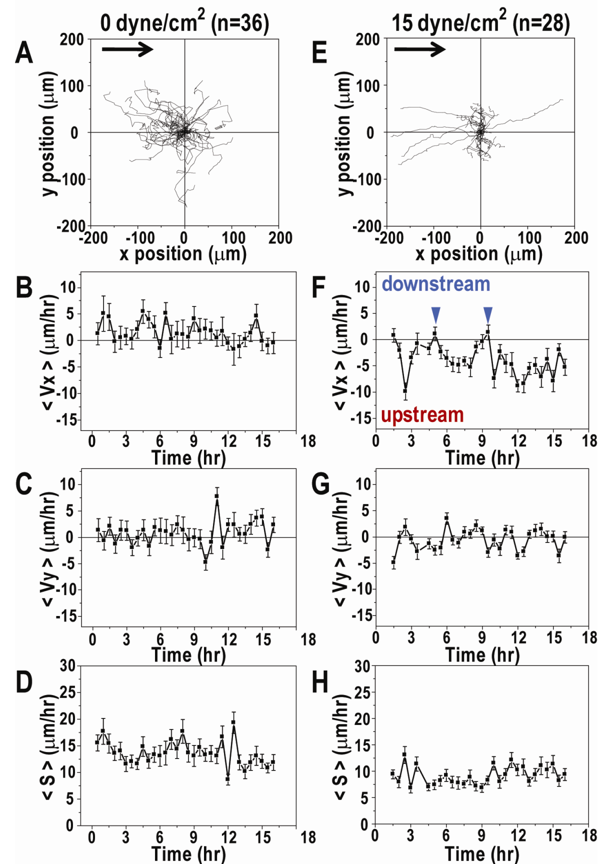

Fig. 1 (A, E) Migration tracks are shown for 10 representative b.End3 endothelial cells (ECs) in a confluent monolayer after flow onset in no flow (A) and 15 dynes/cm^2 flow condition (E). The cells are pre-conditioned in a flow chamber for 2 hrs before the flow started. EC positions were measured every 30 minutes for 16 hours. Flow direction is from left to right. For *n* different cells in the field, the mean velocity projected onto the horizontal axis <*Vx*> (B, F), the mean velocity projected onto the vertical axis <*Vy*> (C, G), and the mean migration speed <*S*> (D, H) are plotted after (time > 0 h) onset of shear stress (Error bars are standard error).

To analyze the directional motion of the cell quantitatively, directional persistence (DP) was calculated by dividing the shortest distance traveled (displacement) by the contour length of its path [12]. The cells with DP=1 represent perfect directional persistence in response to the stimulus whereas the cells with DP=0 show perfectly random motion.

$$\text{Directional Persistence (DP)} = \frac{\text{Displacement}}{\text{Contour Length}}$$

III. RESULTS

We examined the effects of shear stress on the motility of bEnd.3 mouse brain microvessel endothelial cells for 16h after the onset of unidirectional, steady laminar flow with the wall shear stress of 15 dynes/cm^2 and then compared these cells to those of control cells with no flow. Individual migration tracks of cells under no flow condition extend out randomly with similar magnitude in all directions (Fig. 1(A)). Unlike previous reports by Lin and Helmke [12], we did not observe any long-term adaptation to the shear stress within 16h of the experiment. The migration tracks were generally biased towards the upstream direction during the entire observation period.

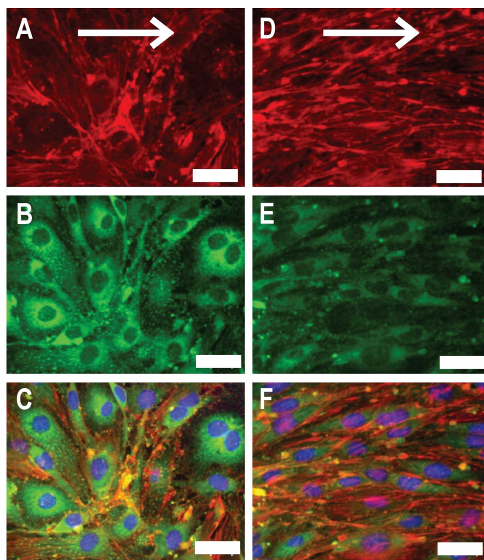

Fig. 2. Morphological changes in b,End3 cells under no flow (A-C) and 15 dyne/cm^2 (D-F). Red:Actin, Green: Paxillin, Blue: Nucleus. Cells become more elongated and oriented in the direction parallel to the flow when shear stress is applied for 16h. While actin stressfibers become thicker and stronger under flow condition, the focal adhesion represented by paxillin becomes smaller and weaker. Scale bar =20μm

Moreover, instead of the tri-phase migration behavior mentioned by Lin and Helmke, involving the first 4h of upstream and 4h of random followed by the last 4-8h of downstream migration [12], we observed a different type of tri-phase migration behavior: the transient fluctuation period (0-4.5h), the steady migration in the upstream direction (4.5-9h), and lastly the dynamic migration whose mean value of horizontal velocity points toward the upstream direction with fluctuating magnitudes (9-16h). Despite individual variations in the field, the ensemble-averaged velocity and the migration speed reflect the changes in their overall behavior. We observe rather abrupt loss of directionality (with the mean <*Vx*>~0) or change in directionality marked with arrow heads at the end of each phase described above (Fig. 1(F)). However, the overall directionality of the ensemble-averaged cells is found in the upstream flow direction with an increasing horizontal directional displacement represented by the area under the <*Vx*> versus time curve (Fig. 1(F)). In the meanwhile, the average migration velocity projected onto the y-axis (Fig. 1(C,G)) fluctuated around 0 μm/hr after 2h with a slight bias in negative direction, indicates that the number of cells migrating in opposite directions were approximately equal. During the 1-1.5h interval, the ensemble-averaged cells show transient migration perpendicular to the flow, which probably represents the period of orienting toward the upstream direction. Overall, this data suggest that bEnd.3 cells in a confluent monolayer have a tendency of migrating in the upstream direction under the influence of shear stress. These differences in motility behavior between cells under no-flow condition and 15 dyne/cm^2 are also reflected in the fixed fluorescence images of cells after 16h of incubation with no-flow and flow, respectively (Fig. 2). As shown in Fig. 1(A-H), cells that are subject to 15 dyne/cm^2 shear stress exhibit more controlled behavior than those under no-flow condition. Cells under shear are less dynamic with lower average speed (Fig. 3(D)) and higher directional persistence (DP) than those under no-flow condition (Fig. 3(A)).

The directional persistence (DP) and migration speed is further enhanced by using a polymer substrate of lower

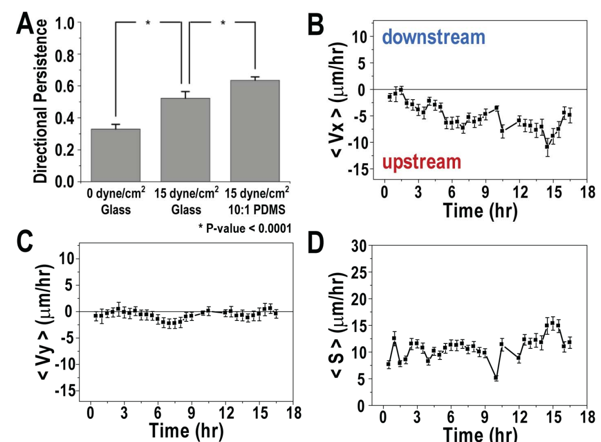

Fig. 3. (A) Directional persistence (DP) calculated by dividing the shortest distance traveled by the contour length is plotted for cells under no-flow condition on glass, 15 dyne/cm^2 on glass, and 15 dyne/cm^2 on 10:1 PDMS. The DP value of 1 represents a perfectly persistent directional motility whereas 0 represents the perfect randomness in motion on 10:1 PDMS. (B-D) The cells are pre-conditioned in a flow chamber for 2 hours before the flow started. Control data in no-flow condition were performed for 4h on a separate experiment and patched in open circle (o) symbols to provide a baseline. EC positions were measured every 30 minutes for 16 hours from the start of the flow. For *n=76* different cells in the field, the mean velocity projected onto the horizontal axis <*Vx*> (B), the mean velocity projected onto the vertical axis <*Vy*> (C), and the mean migration speed <*S*> (D) are plotted (Error bars are standard error).

Young's modulus (Fig. 3(A)). Unlike the cells on glass, cells on a softer PDMS surface show persistent and accelerating migration behavior as evidenced by smoother and higher slope in the $\langle Vx \rangle$ vs. time curve shown in Fig. 3(B). Moreover, after ~11 h of shear stress, migration speed showed the increasing tendency relative to the initial values (Fig. 3(B)). $\langle Vy \rangle$ showed little change at roughly 0 μm/hr, suggesting no directional biases in migration rates perpendicular to the flow direction (Fig. 3(C)). Since the observed difference between glass (68 GPa) and the PDMS may have arisen from the difference in material properties or even in fibronectin coating efficiency between two different materials, we also tested a softer PDMS substrate at a 30:1 (base:curing agent) ratio whose Young's modulus is 0.26 MPa. In the softer substrate, cells show 1.96 times higher directional persistence and 1.67 times higher acceleration in upstream *x*-axis parallel to the flow [13]. This confirms that cells on a softer substrate exhibit higher directional persistence and motility. From these experimental results, we learned that the mechanical stimulus mediated by flow and substrate stiffness can affect the migration behaviors of endothelial cells.

IV. DISCUSSIONS AND CONCLUSION

While many researchers had reported downstream migration of endothelial cells under laminar shear stress [6, 14-16], the anti-parallel upstream migration of bovine aortic endothelial cells has been recently reported by Lin and Helmke [12]. Unlike our sustained upstream directional motility, the upstream migration of bovine aortic endothelial cells lasted for only about 4 hours after the onset of the shear stress, and the authors speculated that this was caused by the effects of transient defect formations in cell-cell junctions. This difference may arise from the fact that b.End3 is a tumorigenic microvessel endothelial cell derived from a mouse. Further investigation must be conducted to determine how b.End3 cells respond differently, compared to other previously reported normal endothelial cells.

The directional migration of cells under shear stress, called mechanotaxis, may be explained by polarized remodeling in the mechano-sensitive pathway. Song Li proposed a model to explain the downstream migration of endothelial cells with a balanced signal pathway related to the cytoskeleton, including focal adhesion kinase and the Rho family small GTPase of RhoA, CDC42, and Rac [17]. Since our experiments showed opposite directional effects of the flow, it remains unclear which of the components proposed in this model may or may not contribute to the upstream migration behavior.

There are two main puzzles remained to be solved in this mechanotaxis modulation by shear or ECM stiffness: One is the mechanism by which the sustained upstream migration is controlled and the other is the reasoning behind the cells' enhanced motility and directional persistence on softer substrates. Our recent work with cells treated with 10 μM ROCK inhibitor, Y27632 (688000, Calbiochem), for 18 h showed enhanced motility with higher velocity and acceleration in an upstream direction, yet with a lower directional persistence accompanied by a large fluctuation perpendicular to the flow direction as well [13]. These results imply that RhoA controls the motility and the persistence of the cells but not the upstream migration behavior. Depletion of external calcium from the cell culture medium, on the other hand, caused cells to lose directionality with no directional bias in its motility, suggesting the signaling pathway involving external calcium influx as a potential key player in the directional migration of the cells [13]. In this regard, the polarization of cavelolae on the EC membrane under the shear condition, which contains active calcium channels and is also related to calcium signal in the trailing edge of the migrating cells [18], may be a critical factor in controlling the migration behavior of bEnd.3 cells. In addition, since confluency of the monolayer is known to influence the migration behavior of cells, we also examined the sub-confluent group of cells and again observed the upstream directional migration [13]. We will have to further investigate how shear stress may modify the activity of the migration machineries found in the EC and its interaction with ECM, thereby modulating cell migration directionality and persistence as well as motility represented by speed. A better understanding of the mechanism of EC migration may provide a rational base for developing strategies to control EC migration under pathologically hardened vessels as well as re-endothelialization of stents and grafted tissues.

ACKNOWLEDGMENT

Authors thank Sunhee Kim, Wootaek Lee, Hana Han, and Jung Woo Hong for technical help in preparation for the experiments.

REFERENCES

[1] T. Ohashi and M. Sato, "Remodeling of vascular endothelial cells exposed to fluid shear stress: Experimental and numerical approach," Fluid Dyn. Res. 37 (1-2) pp. 40-59, 2005

[2] S. Chien, "Molecular and mechanical bases of focal lipid accumulation in arterial wall," Prog. Biophys. Mol. Biol. 83 (2) pp. 131-51, 2003

[3] A. J. Ridley, M. A. Schwartz, K. Burridge, R. A. Firtel, M. H. Ginsberg, G. Borisy, J. T. Parsons and A. R. Horwitz, "Cell migration: Integrating signals from front to back," Science. 302 (5651) pp. 1704-9, 2003

[4] D. A. Lauffenburger and A. F. Horwitz, "Cell migration: A physically integrated molecular process," Cell. 84 (3) pp. 359-69, 1996

[5] M. P. Sheetz, D. Felsenfeld, C. G. Galbraith and D. Choquet, "Cell migration as a five-step cycle," Biochem. Soc. Symp. (65) pp. 233-43, 1999

[6] S. Li, P. Butler, Y. X. Wang, Y. L. Hu, D. C. Han, S. Usami, J. L. Guan and S. Chien, "The role of the dynamics of focal adhesion kinase in the mechanotaxis of endothelial cells," Proc. Natl. Acad. Sci. U.S.A. 99 (6) pp. 3546-51, 2002

[7] P. P. Hsu, S. Li, Y. S. Li, S. Usami, A. Ratcliffe, X. Wang and S. Chien, "Effects of flow patterns on endothelial cell migration into a zone of mechanical denudation," Biochem. Biophys. Res. Commun. 285 (3) pp. 751-9, 2001

[8] M. Masuda and K. Fujiwara, "The biased lamellipodium development and microtubule-organizing center position in vascular endothelial-cells migrating under the influence of fluid-flow," Biol. Cell. 77 (3) pp. 237-45, 1993

[9] X. Q. Brown, K. Ookawa, and J. Y. Wong, "Evaluation of polydimethylsiloxane scaffolds with physiologically-relevant elastic

moduli: interplay of substrate mechanics and surface chemistry effects on vascular smooth muscle cell response," Biomaterials 26 (16) pp. 3123-9, 2005.
[10] Laurent, X. Girerd, J. J. Mourad, P. Lacolley, L. Beck, P. Boutouyrie, J. P. Mignot and M. Safar, "Elastic modulus of the radial artery wall material is not increased in patients with essential hypertension," Arterioscler. Thromb. Vasc. Biol. 14 (7) pp. 1223-31, 1994
[11] S. Song, M. Kim, and J. H. Shin "Shear stress system for quantitative analysis of long-term cellular dynamic responses," ASME, J. Biomech. Eng., submitted for publication.
[12] X. F. Lin and B. P. Helmke, "Micropatterned structural control suppresses mechanotaxis of endothelial cells," Biophys. J. 95 (6) pp. 3066-78, 2008 S.
[13] S. Song and J.H. Shin, "Dynamic behavior of bEnd.3," unpublished.
[14] P. F. Davies, A. Robotewskyj and M. L. Griem, "Quantitative studies of endothelial-cell adhesion - directional remodeling of focal adhesion sites in response to flow forces," J. Clin. Invest. 93 (5) pp. 2031-8, 1994
[15] P. P. Hsu, S. Li, Y. S. Li, S. Usami, A. Ratcliffe, X. Wang and S. Chien, "Effects of flow patterns on endothelial cell migration into a zone of mechanical denudation," Biochem. Biophys. Res. Commun. 285 (3) pp. 751-9, 2001
[16] Y. L. Hu, S. Li, H. Miao, T. C. Tsou, M. A. del Pozo and S. Chien, "Roles of microtubule dynamics and small gtpase rac in endothelial cell migration and lamellipodium formation under flow," J. Vasc. Res. 39 (6) pp. 465-76, 2002
[17] S. Li, N. F. Huang and S. Hsu, "Mechanotransduction in endothelial cell migration," J. Cell. Biochem. 96 (6) pp. 1110-26, 2005
[18] M. O. Parat, B. Anand-Apte and P. L. Fox, "Differential caveolin-1 polarization in endothelial cells during migration in two and three dimensions," Mol. Biol. Cell. 14 (8) pp. 3156-68, 2003