A Mechanostimulation System for Revealing Intercellular Calcium Communication in HUVEC Networks

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***Abstract* — This paper reports a mechanostimulation system for studying mechanically induced intercellular calcium signaling in networks of human umbilical vein endothelial cells (HUVECs). By incorporating a capacitive (comb drive) force probe and plasma lithography cell patterning, the roles of biophysical factors, including force, duration, and network architecture, in calcium intercellular communication can be investigated systematically. Particularly, we observed cancellation of calcium waves in linear networks and bi-directional splitting in cross junctions. The effects of key biophysical factors on intercellular calcium wave propagation were studied. These results demonstrate the applicability of the mechanostimulation system in studying intercellular calcium signaling and reveal the robustness of calcium signaling in HUVEC networks, which mimics the vasculature.**

Index Terms*—*Calcium signaling, Endothelial networks, Intercellular Communications, HUVEC

I. Introduction

Many essential functions of the vasculature are known to be regulated by intracellular calcium signaling [1]. To allow proper physiological functions, cytosolic calcium is tightly controlled in endothelial cells by multiple intracellular and transplasmalemmal calcium regulatory mechanisms [2]. Under resting conditions, free calcium is maintained at a low concentration. The endoplasmic reticulum (ER), which contains numerous calcium binding proteins, is a major intracellular calcium store for endothelial cells [3]. The ER accounts for ~75% of the total intracellular calcium reserve while the majority of the remaining portion is stored in the mitochondria. The release of ER calcium to the cytoplasm can be controlled by calcium release channels, such as inositol 1,4,5-triphosphate (IP_3) and ryanodine receptors, on the ER and can also be spontaneously released through luminal calcium leakage. Calcium mobilization can be triggered by agonists, e.g., IP-3 and ryanodine, which bind to their specific receptors and modulate the calcium release properties of these channels. Remarkably, calcium can triggers calcium release resulting in calcium induced calcium release (CICR) in an autocatalytic manner. To avoid cytotoxicity due to high concentration of calcium, the calcium release channels terminate after a short duration despite the presence of the agonists. At the same time, the cytosolic calcium is resequestered inside the ER as well as pumped outside of the cell through transmembrane ATPases, ATP-dependent calcium pumps, which continuously take up calcium from the cytosol. This resets the cytosolic calcium to a resting condition (~100 nM) and allows stimulation again after a refractory time period [1].

Physiologically, cells move calcium not only between cellular compartments and the exterior of a single cell, but also amongst neighboring cells. These connections are made by gap junctions, which connect vascular as well as many other cell types and allow moving not only calcium ions but also transfer of other molecules and small proteins between cells [4, 5]. These junctions consist of connexin proteins which form pores between cells allowing exchange of the various substances to pass through them. In the case of endothelial cells, several types of gap junction connexin proteins including connexin 40, 43 and 37 are relevant to calcium signaling [5]. Gap junction directly links the cytoplasms of cells and allows exchange of ions and secondary messengers, including calcium and IP_3. Furthermore, many cell types are known to communicate by releasing diffusible factors into the microenvironment. As a result, once calcium release in a cell has been stimulated, the signal can be transferred to neighboring cells via gap junction intercellular communication (GJIC), and extracellular diffusion, even though they are not affected by the stimulus themselves. The transfer of the calcium signal results in a spatiotemporal propagation of intercellular calcium wave communicating a signal between neighboring cells. This calcium wave propagation is known to occur in numerous cells including endothelial cells.

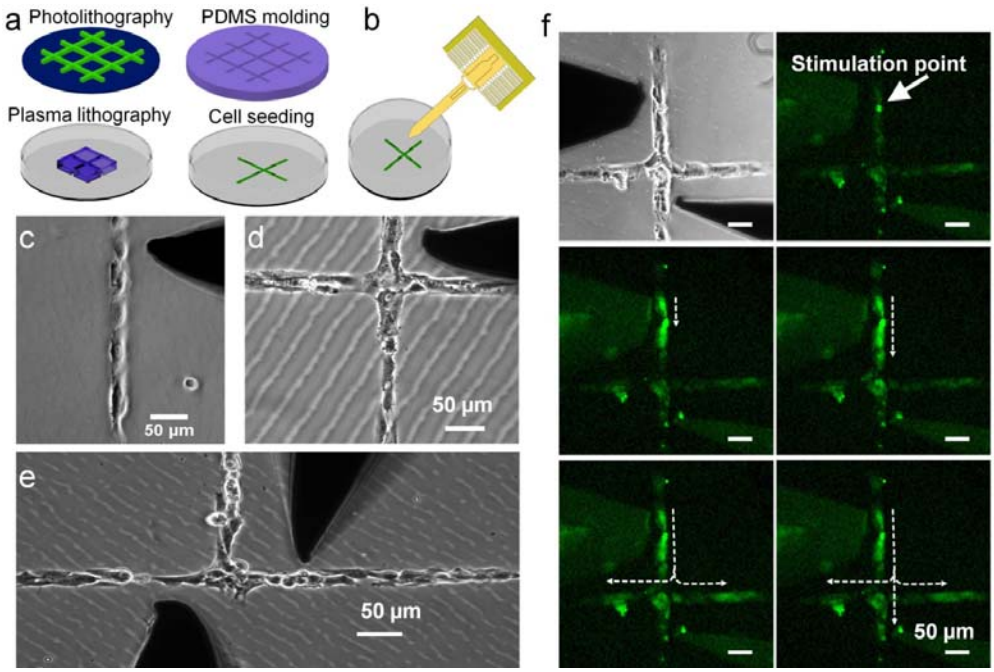

Fig. 1. A mechanostimulation platform for studying the architecture dependence of intercellular calcium communication. (a) Plasma lithography for cell patterning. A PDMS mold is fabricated by molding photolithography patterned structures. The PDMS mold is used to physical shield selected areas of the substrate during plasma surface functionalization to create patterns for cell adhesion. (b) A comb-drive based force probe can be applied to physically stimulate the patterned cells with real-time force monitoring. (c-e) HUVEC structures patterned by plasma lithography. Dark shadows are the physical probe for single cell simulation. (f) A video time series showing a single cell (white arrow) was stimulated mechanically to create a calcium wave. The wave was then propagating along the branch and split at the junction. The duration of the experiment is 50 sec. Scale bars represent 50 µm.

To serve as an effective cell-cell communication mechanism, intercellular calcium signaling must be robust against functional and operational conditions in vascular structures. These involve various topologies and continuous exposure to numerous biomechanical and biochemical stimuli in the cellular microenvironment. Despite the fact that extensive efforts have been devoted to elucidate the molecular mechanisms responsible for the regulation of cytosolic calcium, there is a lack of understanding in the implication of the local calcium regulation in the global characteristics of intercellular calcium communication. Herein, we investigate the functional characteristics of intercellular calcium signaling of networks of mechanically stimulated human endothelial cells. The endothelial structures are confined using a plasma lithography cell patterning technique, which allows systematic control of the network topology and architecture [6-10].
Materials and Methods

A. Plasma lithography cell patterning

In this study, geometric confinement of cells was achieved by plasma lithography, which creates spatial templates of cell adhesive surface chemistry on polystyrene substrates [6-10]. Plasma lithography applies selective shielding of plasma via a flexible polydimethylsiloxane (PDMS) mold to produce a chemical template on the substrate. The PDMS molds used to produce the selective plasma shielding were created via standard soft lithography [11], which replicated shapes by means of molding from master patterns.

B. Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from American Type Culture Collection (ATCC CRL-1730). HUVEC were cultured in F-12K Medium (ATCC) supplemented with 20% screened FBS (Gemini Bio-Products), 0.035 mg/ml endothelial cell growth supplement (Sigma-Aldrich), 0.1 mg/ml heparin (Sigma-Aldrich), and 0.1% gentamycin (GIBCO). HUVEC were used from passages three to six in the experiments.

C. Real-time intracellular calcium imaging

To perform real-time imaging of intercellular wave propagation, a calcium-sensitive dye (Fluro-3AM (Invitrogen) dissolved in DMSO (Fisher)) was first introduced inside the cells with 10 mg/ml of Pluronic® F-127 (Invitrogen). The dye was incubated inside the cells for 35 minutes for esterase cleavage activation. The dye then became fluorescent when bound to calcium thereby allowing visualization of intracellular calcium ion concentrations and movement. For fluorescence observation, endothelial cells were maintained on a microscope stage top hotplate at 37°C with Hank's buffered salt solution (HyClone). The buffer normally contained calcium, except for experiments exploring the signaling mechanisms without extracellular calcium which it was removed from the medium. The microscope hotplate was placed onto an epi-fluorescence microscope (Nikon TE2000-U) equipped with a CCD camera (Cooke SensiCam) for real-time fluorescence imaging.

D. Cell Stimulation

To mechanically stimulate calcium release at the single cell level, individual endothelial cells were probed with a comb-drive based force probe (FemtoTools Instruments, FT-S540) or a 30 gage syringe needle (VWR). The comb-drive (capacitive) probe allows time-resolved measurement of force applied to the cell during stimulation, while the syringe needle allows improved observation of cells due to the size of the probe, which is significantly smaller than the force probe. To control the location of mechanical stimulation, the probes were mounted to a custom three-axis translational stage. In our setup, two probes can be controlled simultaneously to provide mechanical stimulation to cells in the network independently. At the beginning of each experiment, the probes were brought close to the cells before stimulation and a bright field image was obtained to monitor the position of the cells. A fluorescence image was also gathered for background estimation in the image analysis. Real-time fluorescence imaging was then captured to study calcium wave propagation after mechanical stimulation. All images were captured within ~25 minutes of dye loading.

II. Result and Discussion

A. Calcium wave propagation in HUVEC networks

HUVEC networks were organized via plasma lithography to create cell structures consisting of desired topologies (Fig. 1a). Individual HUVECs could then be mechanically stimulated with a force probe or a needle (Fig. 1b). Several structures, including monolayers, linear patterns, and cell junctions, were designed to explore the architecture dependence on calcium wave propagation in HUVECs (Fig. 1c-e). Upon mechanical stimulation, the cells displayed an increase in calcium in the cytoplasm and the increase in calcium was observed to pass onto neighboring cells in monolayers and networks of HUVECs (Fig. 1f).

To study the nature of calcium communication in HUVEC networks, calcium release and propagation were observed under several experimental conditions (Fig. 2a). Cellular calcium release can be triggered by both mechanical probing and ATP loading consistent with previous reports [12, 13]. To determine the calcium source, the endothelial cells were probed in the absence of calcium in the buffer by using a calcium free buffer with additional calcium chelator, ethylene glycol tetraacetic acid (EGTA). Under this condition, calcium wave propagation could still be observed suggesting that extracellular calcium is not a necessary condition in the mechanotransduciton of calcium wave in HUVEC. The same observation was also reported in monolayers of bovine aortic endothelial cells [12]. The increase in calcium is, therefore, likely contributed from intracellular store, such as from the ER. The involvement of the ER is further studied by blocking calcium uptake in the ER pharmacologically. With thapsigargin, an ER calcium pump inhibitor [14, 15] that depletes intracellular calcium store, calcium signaling was not provoked either mechanical stimulation nor by addition of ATP. These observations further support that the primary source of calcium released in calcium wave propagation is from intracellular stores.

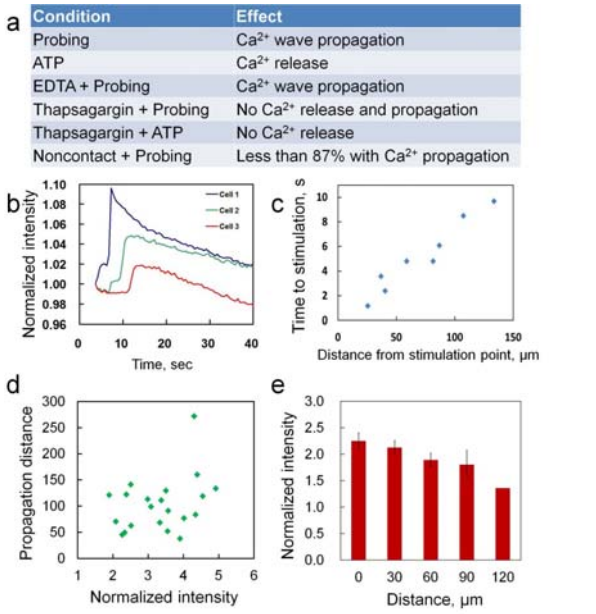

Fig. 2. Characteristics of calcium wave propagation in linear chains of HUVECs. (a) Behaviors of calcium release and propagation under different conditions. (b) Propagation of calcium wave in endothelial cells patterned in a linear cell chain. (c) A representative plot of the propagation distance and propagation time in a linear cell chain. (d) The dependence of the cell intensity on the propagation distance. (e) The average cell intensity as a function of distance from the stimulation point.

Intracellular calcium signaling can be mediated through extracellular diffusion and GJIC. Extracellular diffusion of messager, such as ATP, could contribute to the intracellular calcium communication for several cell types [16-18]. To test the role ATP in calcium signaling of HUVECs, ATP was added to the extracellular space via a pipette. The addition of ATP gently, but not cell media, provokes calcium release in cells within the field of view of the image. This suggests calcium signaling in HUVEC can be initiated by diffusible factors, such as ATP. This is consistent with previous studies that release of intercellular contents can induce calcium signaling and initiate injury response in pulmonary endothelial cells [19]. In fact, addition of extracellular ATP has also shown to be the dominant mode of communication in networks of osteoblasts [17, 20, 21]. To examine the importance of GJIC in mechanically stimulated calcium signaling of the HUVEC network, individual cells were poked gently and the calcium levels of nearby, non-contacting cells were observed. In the majority cases (87%), nearby cells did not increase in the fluorescence intensity despite the calcium signal was provoked in the stimulated cells. These results indicate that direct cell-cell contact is required for calcium signaling in mechanically stimulated HUVEC. GJIC is conceivably the primary mechanism for mechanotransduction of HUVEC networks without injury, which is the focus of this study.

B. Physical characteristics of the calcium propagation

We further investigated the characteristics of calcium propagation in linear HUVEC structures. Fig. 2b shows the calcium levels of three cells patterned in a linear chain. Examining the fluorescence intensity indicates that the free cytosolic calcium level of the stimulated cell undergoes a rapid increase followed by a slower decay, eventually returning to the resting level. A small duration (~1-6 seconds) is typically required to pass the signal between adjacent cells. A representative plot of the propagation distance versus arrival time behavior is shown in Fig. 2c, which shows an approximately linear relationship with an average speed of ~12 μm/s. In our experiment, the calcium signal generally propagated for 4-6 cells (~120 μm). These observations are in good agreement with previous investigation in monolayers of bovine aortic endothelial cells [12]. Remarkably, the intensity of the stimulated cells shows only weak correlation with the propagation distance and the calcium wave propagates a similar distance independent of the initial signal amplitude (Fig. 2d). We observed a large variation in the amplitude among cells in the networks; nevertheless, the average amplitude of the calcium wave appeared to decrease slightly along the propagation direction (Fig. 2e). These observations further support that the calcium level in the HUVEC networks is originated by a local regenerative event, such as CICR, in contrast to diffusive processes from the point of stimulation.

III. Conclusions

Overall, the signaling behavior which was observed implies that the body incorporates a calcium release mechanism which provides for fairly regular behavior in terms of the amount of calcium released as well as signal transmission distance and speed in relation to mechanical inputs. This is in line with the tight control that is usually observed for cellular calcium handling. This in turn is usually believed to be related to the many possible actions of free calcium which require tight control for proper cell function. Furthermore, our study demonstrates the technological platform combining the cell patterning and mechanostimulation technologies for systematic investigation of the architecture dependence of intercellular calcium communication.

Acknowledgements

This work is supported by the James S. McDonnell Foundation 21st Century Science Initiative in Studying Complex Systems, NSF (0855890), and NIH Director's New Innovator Award (1DP2OD007161-01).

References

[1] D. E. Clapham, "Calcium Signaling," *Cell,* vol. 131, pp. 1047-1058, 2007.

[2] Q. K. Trana, *et al.*, "Calcium signalling in endothelial cells," *Cardiovasc Res,* vol. 48, pp. 13-22, 2000.

[3] C. Booth and G. L. Koch, "Perturbation of cellular calcium induces secretion of luminal ER proteins," *Cell,* vol. 59, pp. 729-37, Nov 17 1989.

[4] D. A. Goodenough, *et al.*, "Connexins, connexons, and intercellular communication," *Annual Review of Biochemistry,* vol. 65, pp. 475-502, 1996.

[5] G. Sohl and K. Willecke, "Gap junctions and the connexin protein family," *Cardiovasc Res,* vol. 62, pp. 228-32, May 1 2004.

[6] Y. L. Yang, *et al.*, "Comparative assembly of colloidal quantum dots on surface templates patterned by plasma lithography," *Soft Matter,* vol. 7, pp. 10085-10090, 2011.

[7] M. Junkin and P. K. Wong, "Probing cell migration in confined enivironments by plasma lithography," *Biomaterials,* vol. 32, pp. 1848-1855, 2011.

[8] M. Junkin, *et al.*, "Plasma lithography surface patterning for creation of cell networks," *Journal of Visualized Experiments,* vol. 52, 2011.

[9] M. Junkin, *et al.*, "Cellular self-organization by autocatalytic alignment feedback," *Journal of Cell Science,* vol. 124, pp. 4213-4220, Dec 15 2011.

[10] M. Junkin, *et al.*, "Template-guided self-assembly of colloidal quantum dots using plasma lithography," *Advanced Materials,* vol. 21, pp. 1247-1251, 2009.

[11] G. M. Whitesides, *et al.*, "Soft lithography and nanofabrication," *Abstr Pap Am Chem S,* vol. 229, pp. U936-U936, 2005.

[12] L. L. Demer, *et al.*, "Mechanical stimulation induces intercellular calcium signaling in bovine aortic endothelial cells," *Am J Physiol Heart Circ Physiol,* vol. 264, pp. H2094-2102, 1993.

[13] D. Pubill, *et al.*, "ATP induces intracellular calcium increases and actin cytoskeleton disaggregation via P2x receptors," *Cell Calcium,* vol. 29, pp. 299-309, May 2001.

[14] N. Nejime, *et al.*, "Effect of P2 receptor on the intracellular calcium increase by cancer cells in human umbilical vein endothelial cells," *Naunyn-Schmiedeberg's Archives of Pharmacology,* vol. 377, pp. 429-436, 2008.

[15] B. Huo, *et al.*, "Intercellular calcium wave propagation in linear and circuit-like bone cell networks," *Phil. Trans. R. Soc. A,* pp. 617-633, 2010.

[16] N. Nejime, *et al.*, "Effect of P2 receptor on the intracellular calcium increase by cancer cells in human umbilical vein endothelial cells," *Naunyn Schmiedebergs Arch Pharmacol,* vol. 377, pp. 429-36, Jun 2008.

[17] Z. Henriksen, *et al.*, "The predominant mechanism of intercellular calcium wave propagation changes during long-term culture of human osteoblast-like cells," *Cell Calcium,* vol. 39, pp. 435-44, May 2006.

[18] V. E. Klepeis, *et al.*, "P2Y receptors play a critical role in epithelial cell communication and migration," *J Cell Biochem,* vol. 93, pp. 1115-33, Dec 15 2004.

[19] P. J. Sammak, *et al.*, "How do injured cells communicate with the surviving cell monolayer?," *Journal of Cell Science,* vol. 110, pp. 465-475, Feb 1997.

[20] L. M. Godin, *et al.*, "Mechanically induced intracellular calcium waves in osteoblasts demonstrate calcium fingerprints in bone cell mechanotransduction," *Biomechanics and Modeling in Mechanobiology,* vol. 6, pp. 391-398, Nov 2007.

[21] B. Huo, *et al.*, "Fluid Flow Induced Calcium Response in Bone Cell Network," *Cellular and Molecular Bioengineering,* vol. 1, pp. 58-66, Mar 2008.