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Integration of Biochemical and Biomechanical Signals Regulating Endothelial Barrier Function

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Abstract: Endothelial barrier function is critical for tissue homeostasis throughout the body. Disruption of the endothelial monolayer leads to edema, vascular diseases and even cancer metastasis among other pathological conditions. Breakdown of the endothelial barrier integrity triggered by cytokines (e.g. IL-8, IL-1β) and growth factors (e.g. VEGF) is well documented. However, endothelial cells are subject to major biomechanical forces that affect their behavior. Due to their unique location at the interface between circulating blood and surrounding tissues, endothelial cells experience shear stress, strain and contraction forces. More than three decades ago, it was already appreciated that shear flow caused endothelial cells alignment in the direction of the flow. After that observation, it took around 20 years to begin to uncover some of the mechanisms used by the cells for mechanotransduction. In this review, we describe mechanosensors on the endothelium identified to date and the associated signaling pathways that integrate biochemical and biomechanical inputs into biological responses and how they modulate the integrity of the endothelial barrier.

Keywords: Endothelial barrier, gap formation, shear flow, mechanotransduction, cytokines, growth factors.

1 Introduction

Endothelial cells line the interior of the circulatory system, including arteries, veins, capillaries, lymph vessels and the heart. The main function of the endothelium is to maintain tissue homeostasis [Neubrander and Helmke (2015)]. It provides a route for the body to distribute nutrients and oxygen necessary for cells physiologic activities and to collect metabolic waste products. Located at the interface between blood and surrounding tissues, the endothelium experiences multiple biochemical and biomechanical stimuli that modulate its behavior. From the biomechanical perspective, depending on the location within the vascular tree, endothelial cells are subject to different types of fluid flow on the luminal side (Fig. 1). At straight sections of arteries, endothelial cells can experience laminar and unidirectional flow while in bifurcations the flow pattern can turn irregular or multidirectional. In lymph vessels, the flow can reverse directions and be oscillatory

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[Baeyens and Schwartz (2016)]. All these patterns result in shear stress of different magnitudes and directions that ultimately affect protein expression and influence endothelial cell behavior and barrier integrity. Another major biophysical event taking place on the luminal side of endothelial cells is the transmigration of cells. Leukocytes, attracted through biochemical signals, sense the mechanical properties of the surrounding microenvironment and exert traction forces on the endothelium and the basal lamina [Stroka (2015)]. From the biochemical perspective, multiple studies show that cytokines, especially the ones associated to the inflammatory response, and receptor-receptor interactions between immune cells or cancer cells and the endothelium affect the integrity of the endothelial barrier [Aragon-Sanabria, Pohler, Eswar et al. (2017); Weidert, Pohler, Gomez et al. (2014)].

On the abluminal or basal side, endothelial cells also experience a wide range of biomechanical forces. Expansion of blood vessels in response to increased blood flow results in endothelial cell stretching along the vessel wall [Neubrander and Helmke (2015)]. Again, depending on the location within the vascular tree, the wall strain varies. Near bifurcations, cells experience strain in 2-dimensions while in straight segments of arteries, the strain is mainly uniaxial (Fig. 1) [Neubrander and Helmke (2015)].

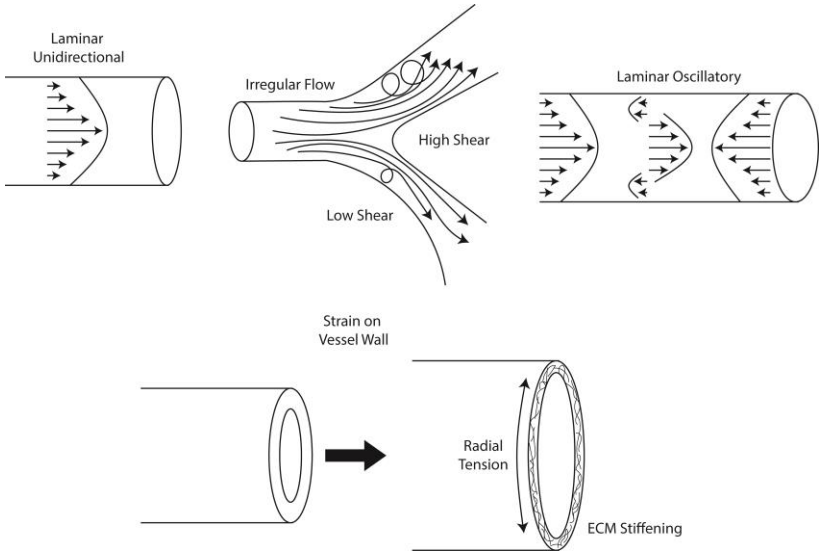

Figure 1: Biomechanical stresses acting on the endothelium. Top-different types of fluid flow patterns throughout the vascular tree. Bottom-strain on the vessel wall due to expansion of the blood vessel

These biomechanical signals are translated by mechanosensors on endothelial cells and integrated to generate a cellular response. Some of these biomechanical cues promote endothelial barrier function while others promote endothelial cell proliferation or migration. Mounting evidence shows that these biomechanical stimuli are involved in proper physiological functioning as well as in the development of vascular diseases and even cancer progression [Baeyens and Schwartz (2016); Mierke (2012)]. Recent technological advances have provided new and more accurate tools for the study of the interplay between

mechanical forces and biological processes. Here we examine the connections between major biophysical forces and biochemical cues affecting endothelial barrier function and focus on the proposed sensors and associated signaling pathways where these signals converge.

2 Endothelial barrier regulation from the luminal or apical side

The endothelium is the main tissue in the body that is under constant fluid shear stress due to blood and lymph in circulation (Fig. 1). On the luminal or apical side, biomechanical forces arising from the fluid shear stress have been widely recognized as a regulator of endothelial barrier integrity. The frictional force due to fluid flow that is parallel to the endothelium was shown to align cells in the direction of the flow around 3 decades ago [Dewey, Bussolari, Gimbrone et al. (1981)]. Cell alignment was observed only under laminar flow, which occurs in straight sections of vessels and is characterized by high shear stress on the vessel wall. In bifurcations and bends, the fluid pattern turns non-uniform or turbulent leading to low shear stress on the vessel wall (Fig. 1) [Chiu and Chien (2011)]. Evidence from in vitro models that compare laminar *vs.* turbulent flow patterns show that gene expression in endothelial cells changes depending on the flow condition. Dai and colleagues showed that genes upregulated under a turbulent flow pattern are involved in inflammation (e.g. IL-8, CXCR4) and angiogenesis (e.g. PGF, CTGF), while genes upregulated under laminar flow have been implicated in endothelial maintenance and homeostasis (e.g. CNP) [Dai, Kaazempur-Mofrad, Natarajan et al. (2004)]. In addition, their results suggest that the protective effect of the laminar flow can even counteract endothelial activation triggered by IL-1β, a cytokine that promotes expression of e-selectin and VCAM-1 during inflammation, cancer and other pathological conditions. Expression of VCAM-1 on the surface of endothelial cells has been shown to increase endothelial gap formation followed by cancer cell extravasation through VLA-4/VCAM-1 interactions [Aragon-Sanabria, Pohler, Eswar et al. (2017)]. In addition, previous studies from our group highlighted the role of shear rate in facilitating interactions between neutrophils and cancer cells in circulation that result in cell arrest on the endothelium, barrier disruption and subsequent extravasation [Liang, Hoskins, Khanna et al. (2008)]. A parallel study determined that subsequent binding of neutrophils and cancer cell aggregates to the endothelium are mostly shear rate and not shear stress dependent [Liang, Slattery, Wagner et al. (2008)] (Fig. 2). Fluid flow has been shown to be very important for leukocyte arrest on the endothelium. In fact, rolling leukocytes detach from the endothelium when flow is stopped which suggests that binding of l- and p-selectin require shear stress [Ley, Laudanna, Cybulsky et al. (2007)].

The luminal side of the endothelium is covered by a layer of "sugar" molecules called the glycocalyx. First observed with the invention of the electron microscope about 50 years ago [Luft (1966)], the glycocalyx is mainly composed of carbohydrate molecules, glycoproteins (selectins, integrins, immunoglobulins and cadherins) and proteoglycans (glycosaminoglycans -GAGs-, consisting of variable backbone proteins and multiple combinations of 5 major side chains: heparin sulfate, chondroitin sulfate, dermatan sulfate, hyaluronan and keratin sulfate). These proteins form a mesh on the surface of endothelial cells where soluble components from the plasma or proteins and cytokines secreted by the endothelium are entrapped [Reitsma, Slaaf, Vink et al. (2007)]. Also known as the

endothelial surface layer (ESL), the glycocalyx is very dynamic due to its constant remodeling; it can be degraded enzymatically or shed due to fluid shear stress [Lipowsky (2005)]. One of the main functional attributes of the glycocalyx is to promote endothelial barrier integrity. Due to steric and charge effects, this layer restricts access to the surface of endothelial cells by cells in circulation and soluble molecules [van Haaren, Van Bavel, Vink et al. (2003)]. Multiple studies have shown that endothelial barrier function is impaired when the glycocalyx is removed [Dull, Dinavahi, Schwartz et al. (2003); Jacob, Bruegger, Rehm et al. (2006); Rehm, Zahler, Lötsch et al. (2004)]. There is some evidence that the degradation of the glycocalyx is part of the early steps in the development of cardiovascular disease, edema and infectious pathologies [Curry and Adamson (2012)].

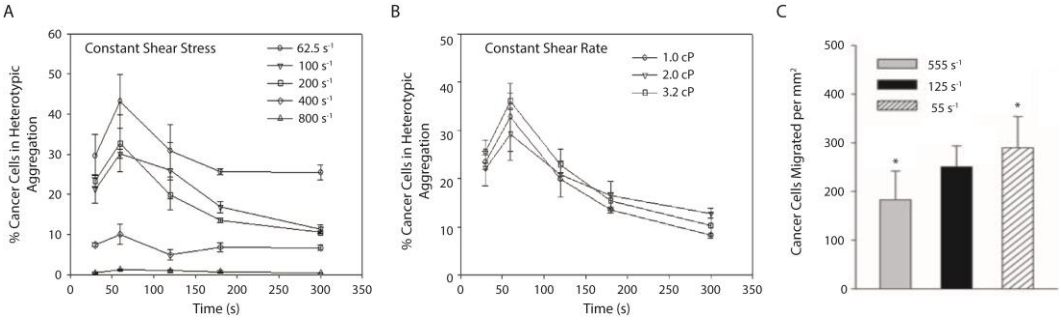

Figure 2: Effects of shear rate and shear stress on cell-cell heterotypic aggregation in circulation and extravasation. A. Effect of shear rate on cancer cell and neutrophil heterotypic cell-cell aggregation. B. Effect of shear stress on cancer cell and neutrophil heterotypic cell-cell aggregation. C. Effect of shear stress on cancer cell extravasation across the endothelial barrier [Liang, Hoskins, Khanna et al. (2008); Liang, Slattery, Wagner et al. (2008)]

It is recognized that major components of the glycocalyx attached to the surface of endothelial cells are responsible for mechanotransduction initiated by flow shear stress in circulation. A model proposed by Weinbaum and colleagues (Fig. 3) (Weinbaum, Zhang, Han et al. (2003)], based on observations by Squire et al. predicts that a collection of core proteins in the glycocalyx will experience a drag force of ~1.9×10^{-2} pN under a shear stress of 10 dyn/cm^2, which results in a lateral displacement of 6 nm in an adjacent actin filament and cause further deformation of the cortical cytoskeleton and intracellular signaling [Squire, Chew, Nneji et al. (2001); Weinbaum, Zhang, Han et al. (2003)]. In this model, it was assumed that the thickness of the glycocalyx layer is between 150 nm and 400 nm. However, this is not always the case, estimates of the thickness of the glycocalyx suggest that it can range from a few tens nm to a few microns. When the thickness is larger, the drag force can dissipate along the layer and the surface of the endothelial cells sense an effective shear force of zero and no deformation takes place [Secomb, Hsu and Pries (2001)]. On most theoretical and microfluidic models, the shear force experienced by the surface of endothelial cells depends primarily on the thickness of the glycocalyx layer and the hydraulic resistance [Battiato, Tartakovsky, Cabrales et al. (2017); Secomb, Hsu, Pries et al. (2001); Tarbell and Shi (2013)]. Using experimental values of the velocity profile of fluorescent microparticles moving along blood vessels with intact and degraded glycolayx,

the model by Battiato et al. demonstrates that the shear stress on the endothelial wall decreases as the permeability of the glycocalyx decreases [Battiato, Tartakovsky, Cabrales et al. (2017)].

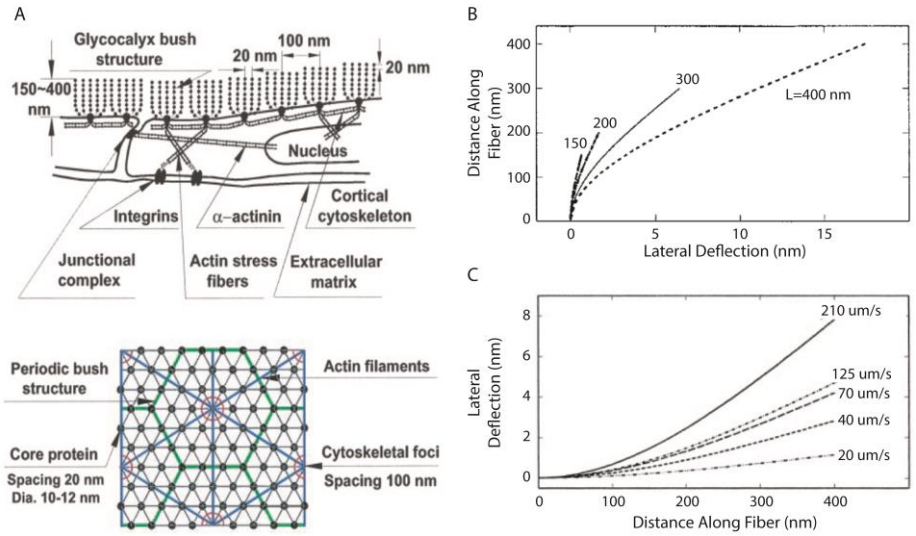

Figure 3: Model of the glycocalyx and analysis of protein deflection within the glycocalyx. A. Sketch of the glycocalyx showing core proteins and the connection to internal filament actin (top) and the proposed model with hexagonal arrangement of actin lattices (bottom). B. Predictions using this model for lateral deflection of core proteins of different sizes under a constant fluid shear stress of 10 dyn/cm^2 at the edge of the glycocalyx. C. Predictions of lateral deflection of proteins in the glycocalyx underneath red cells moving at different velocities [Weinbaum, Zhang, Han et al. (2003)].

Shear stress has also been shown to regulate nitric oxide (NO) production in the endothelium through heparin sulfate proteoglycan [Florian, Kosky, Ainslie et al. (2003)]. NO is a vasodilator responsible for modulating vessel tone by inducing relaxation on smooth muscle cells and inhibiting their proliferation; it is produced by the enzyme nitric oxide synthase (eNOS) [Hsieh, Liu, Huang et al. (2014)]. Florian et al. [Florian, Kosky, Ainslie et al. (2003)] conducted experiments comparing NO production in endothelial cells in response to steady and oscillatory flow patterns. Steady flow is laminar and parallel to the major axis in elongated endothelial cells; oscillatory flow is also parallel and laminar but it temporarily changes flow to an antiparallel direction. Their results show that both, steady and oscillatory flow induced NO production only when heparin sulfate proteoglycan was intact; when the cells were pretreated with heparinase, NO production was significantly decreased. Interestingly, when NO production was induced by Bradykinin, a potent peptide vasodilator that activates NO production through Ca^{2+} -dependent eNOS activation, degradation of heparin sulfate did not decrease NO production, highlighting its role as a mechanosenor for shear stress. NO production is a good example of a biological response that can be triggered either through biomechanical or biochemical stimuli (Fig. 4). NO has been shown to regulate endothelial barrier function by modulating cytoskeletal reorganization through a Rho GTPase-dependent pathway [Di Lorenzo, Lin, Murata et al.

(2013)]. Using eNOS siRNA, Di Lorenzo and colleagues showed impaired actin stress fiber formation upon stimulation of vascular endothelial growth factor (VEGF) on vascular endothelial eNOS depleted cells. VEGF stimulation of endothelial cells results in activation of vascular endothelial growth factor receptor-2 (VEGFR-2), followed by activation of mitogen activated protein kinase (MAPK)-3, MAPK-1 and phosphorylation of eNOS in residue S1177. Endothelial barrier integrity was assessed by TEER measurements and the results showed a transient decrease in TEER measurements upon VEGF addition in control cells that was attenuated in eNOS siRNA treated cells [Di Lorenzo, Lin, Murata et al. (2013)]. These results suggest that NO mediates VEGF-induced endothelial barrier disruption and promotes gap formation and explains why eNOS deficient mice show decreased vascular leakage. However, NO has also been shown to prevent leukocyte binding to the endothelium by suppressing expression of ICAM-1 and VCAM-1 [De Caterina, Libby, Peng et al. (1995)]. More recent evidence suggests increased binding of leukocytes to endothelial cells under reduced NO levels is mediated not through increased protein synthesis of adhesion molecules but through Src-mediated phosphorylation of ICAM-1, which increases its adhesiveness [Gao, Lucke-Wold, Li et al. (2018)]. Once the endothelium is activated, which is characterized by elevated expression of adhesion molecules, our studies show that the integrity of the endothelial barrier is compromised; interactions between VCAM-1 and VLA-4 receptors are sufficient to form intercellular gaps [Aragon-Sanabria, Pohler, Eswar et al. (2017)].

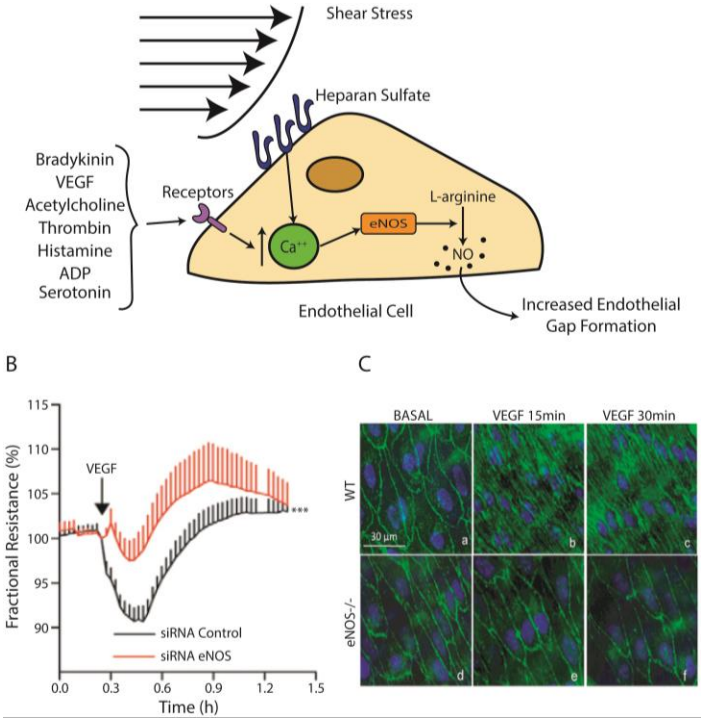

Figure 4: Stimulation of NO production via biochemical and biomechanical signals. A. NO production on endothelial cells can be stimulated either by shear stress or by biological molecules. B. HDEMCs transfected with either control or eNOS siRNA and cultured to

post-confluency to measure TEER after treatment with VEGF (100 ng/ml). C. Intrapulmonary arteries isolated from WT and eNOS-/- mice and stimulated with VEGF for either 15 min or 30 min, fixed and stained for F-actin. B-C modified from Di Lorenzo et al. [Di Lorenzo, Lin, Murata et al. (2013)]

Another important regulator of endothelial barrier integrity is vascular endothelial (VE)-cadherin. This cadherin is specific for endothelial cells and maintains barrier function by dimeric association on the plasma membrane of adjacent cells. VE-cadherin was shown to play a role in shear flow induced response in endothelial cells. Using VE-cad$^{-/-}$ cells transfected with a lentivirus coding for either VE-cadherin or N-cadherin (a close paralogue also mediating endothelial cell-cell adhesion), Coon et al. [Coon, Baeyens, Han et al. (2015)] showed that only the cells transfected with VE-cadherin aligned in the direction of the shear force after 18 h of laminar flow at 12 dyn/cm^2, cells transfected with N-cadherin remained unresponsive to shear stress. In addition, they identified the transmembrane domain of VE-cadherin as the main modulator of this response. By using VE-cadherin/N-Cadherin chimeras and immunoprecipitation assays, this study showed that the VE-cad transmembrane domain is directly responsible for VE-cadherin association with VEGFR-2 and VEGFR-3 [Coon, Baeyens, Han et al. (2015)]. A recent study identified Y658 as the residue responsible for modulating this response. Comparing wild type endothelial cells with VE-cadherin deficient cells and VE-cadherin Y658F mutated cells, Conway [Conway, Coon, Budatha et al. (2017)] and colleagues found that only the wild type endothelial cells elongated and aligned in the direction of the flow, while the other cells remained unresponsive. In our recent study, tyrosine phosphorylation of VE-cadherin in Y658 or Y731 was associated with disruption of the endothelial barrier, intercellular gap formation and increased cancer cell extravasation across the endothelium [Aragon-Sanabria, Pohler, Eswar et al. (2017)]. However, our experimental setup was under static conditions, no shear flow was involved. Thus, VE-cadherin is another example of a transmembrane protein that mediates biological responses triggered by biomechanical and biochemical signals.

Integrins, a glycoprotein present on the surface of endothelial cells, are also responsible for mechanotransduction from shear flow [Gulino-Debrac (2013)]. Initially, it was considered that integrin expression on endothelial cells was mainly located on the basal side, anchoring the cells to the basal membrane. However, evidence suggests that β1 integrins are also located on the luminal side and act as mechanosensors to shear flow. Experiments in vitro showed that laminar shear flow promoted activation of β1 integrins on the apical side of endothelial cells and that activation was not dependent on cytoskeletal reorganization [Yang and Rizzo (2013)]. Integrins are linked to the actin cytoskeleton and can be activated through an inside-out mechanism. However, in this case, pretreatment of endothelial cells with cytochalasin D, a major actin filament disruptor agent, did not prevent β1 integrin activation under shear flow. Another perspective on integrin activation induced by shear flow is on the basal side of endothelial cells, the integrins binding to the basal lamina, in an inside-out fashion [Shyy and Chien (2002)]. Tzima and colleagues showed that platelet endothelial cell adhesion molecule (PECAM)-1, VE-cadherin and VEGFR-2 form a mechanosensory complex responsible for modulating responses to shear stress in endothelial cells [Tzima, Irani-Tehrani, Kiosses et al. (2005)]. The proposed mechanism

explains that shear stress triggers changes in the tension experienced by PECAM-1 molecules on the cell membrane. The cytoplasmic tail of PECAM-1 then binds to Src, which is upstream of phosphoinositide-3-kinase (PI3-K)-dependent integrin activation. VE-cad serves as an adaptor protein and VEGFR-2 directly interacts with and activates PI3-K [Conway, Breckenridge, Hinde et al. (2013)]. A follow-up study showed that p120-catenin and polarity protein LGN compete for binding to VE-cadherin. Thus, VE-cadherin phosphorylation on Y658 disrupts binding to p120-catenin and increases interactions with LGN. Furthermore, LGN binding to VE-cadherin is required for endothelial cell alignment in response to shear stress [Conway, Coon, Budatha et al. (2017)].

3 Endothelial barrier regulation from the basal side

Endothelial cells lining the interior of blood vessels are subject to elongation by circumferential stress resisting blood pressure (Fig. 1) [Tarbell and Pahakis (2006)]. Depending on the location within the vascular tree, these cells are more or less affected by cyclic strain; high blood pressure in the arteries and respiration in the lungs result in large mechanical stretching forces on the endothelium [Zebda, Dubrovskyi and Birukov (2012)]. Uniaxial cyclic stretch causes endothelial cell alignment that is perpendicular to the direction of the loading axis and increases cell stiffness [Hatami, Tafazzoli-Shadpour, Haghighipour et al. (2013)]. Using a silicone membrane to culture monolayers of endothelial cells coupled to a cyclic stretching device and a micropipette aspirator to measure whole body elastomeric properties of cells, Hatami and colleagues compared endothelial cells cultured under static conditions with cells subjected to cyclic mechanical stretching. Their results showed that after 3 h of cyclic stretch, actin stress fibers aligned in the direction of minimal deformation and increased cell stiffness. They also showed that the effect is dependent on the magnitude of the stretch. Earlier, Huh et al. [Huh, Matthews, Mammoto et al. (2010)] showed that cyclic stretching alone did not affect endothelial barrier integrity, but when endothelial cells were stimulated with interleukin-2 (IL-2) in addition to a 10% cyclic strain, barrier permeability increased [Huh, Leslie, Matthews et al. (2012)]. In addition, cyclic stretch has been shown to increase FAK phosphorylation [Yano, Geibel and Sumpio (1996); Zebda, Dubrovskyi, Birukov et al. (2012)], which ultimately leads to increased permeability of the endothelium.

The role of substrate stiffness in modulating cell behavior has been intensely investigated in the last decade [Trichet, Le Digabel, Hawkins et al. (2012)]. The development of new technologies led to a better understanding of the mechanisms governing cell-ECM and cell-cell interactions and the mechanical forces involved [Liu, Sniadecki and Chen (2010)]. In the case of the endothelium, proper barrier function depends on the collective behavior of endothelial cells; coordination between cell-cell and cell-ECM adhesions is critical for the integrity of the dynamic barrier. Evidence suggests that endothelial barrier function is affected by the rigidity of the ECM [Urbano, Furia, Basehore et al. (2017)]. The stiffness of the vessel wall can increase due to type II diabetes [Oxlund, Rasmussen, Andreassen et al. (1989)], atherosclerosis [Smilde, van den Berkmortel, Wollersheim et al. (2000)] and other pathological conditions [Mattace-Raso, Van Der Cammen, Hofman et al. (2006)]. Human pulmonary artery endothelial cells (HPAEC) cultured on soft (0.55 kPa), medium (8.6 kPa) and hard (42 kPa) polyacrylamide substrates were shown to differentially form actin filaments in response to thrombin [Birukova, Tian, Cokic et al. (2013)]. Comparing cells without stimulation cultured on soft *vs.* hard substrates revealed that harder substrates alone increased actin stress fiber formation. The effect

was replicated after stimulation; cells on soft substrates did not show a significant increase in F-actin polymerization after addition of thrombin. In contrast, F-actin stain in cells grown on hard substrates displayed more than a two-fold difference compared to unstimulated cells cultured on the same substrate. This evidence suggests that the rigidity of the substrate not only affects cell behavior but also how cells respond to certain stimuli. Another example of the effect of substrate stiffness on cell behavior is that cell contractility of endothelial cells significantly increases on hard substrates compared to soft substrates. Traction forces of human umbilical vein endothelial cells (HUVEC) were measured using micropattern techniques. Cells were grown in small groups (around 10 cells per group) in substrates of varying stiffness and stimulated with thrombin. Consistent with higher F-actin polymerization, cells on stiffer substrates showed larger traction forces than cells on softer substrates. In this case, contractility forces were large enough to disrupt the endothelial monolayers and cause gap formation only on hard but not on soft substrates (Fig. 5) [Krishnan, Klumpers, Park et al. (2010)]. Interestingly, it was observed that most traction forces located on the edge of the micropatterns, not on the inside of the monolayer, suggesting that cells within the monolayer are mostly attached to neighboring cells while cells with free edges attach to the substrate. This is important because it points to a connection between forces on cell-ECM and forces on cell-cell adhesions to maintain the integrity of the monolayer. Consistent with clinical observations of increased vascular leakage related to age-related stiffening of blood vessels, recent evidence shows that rigid substrates amplify the disruptive effect triggered by mechanical forces on endothelial monolayers [Eguiluz, Kaylan, Underhill et al. (2017)]. Using micropatterned substrates of different stiffness, Andresen et al. [Eguiluz, Kaylan, Underhill et al. (2017)] showed that stiff substrates promote heterogeneity in the distribution of forces on endothelial monolayers in response to mechanical stimuli and this leads to increased gap formation and endothelial barrier disruption.

Endothelial cells cultured on stiffer substrates and stimulated by mechanically pulling VE-cadherin exhibit more and larger focal adhesions (FA) compared to cells on softer substrates [Eguiluz, Kaylan, Underhill et al. (2017)]. This effect is accompanied by increased cell contractility and gap formation. Further evidence shows a crosstalk between FA and adherens junctions (AJ) that is essential for the maintenance of the endothelial barrier function, however the relationship is complex [Quadri (2012)]. FA are the main contact points between cells and the ECM and AJ are one type of the main connections in-between adjacent cells. The cytoplasmic part of mature FA consist of multiple proteins of which focal adhesion kinase (FAK), Src and F-actin networks are the most prominent, and talin and vinculin are the key mechanosensing adapters [Wehrle-Haller (2012)]. FAK, the main modulator of FA assembly, and Src have been shown to translocate from FA to AJ and to integrate multiple signaling pathways that ultimately affect endothelial permeability [Jean, Chen, Nam et al. (2014)]. Recent evidence shows that phosphorylation of FAK at Y576, a marker for FAK activation, increases VE-cadherin phosphorylation at Y658 which results in AJ disassembly and decreased barrier function [Jean, Chen, Nam et al. (2014)]. The study of FAK *in vivo* is challenging because generation of FAK knockout mice results in embryonic lethality. However, Chen et al. [Chen, Nam, Jean et al. (2012)] overcame this difficulty by creating an inducible knock-in hemizygous mouse model that expresses a kinase-dead FAK mutant. Using this tool, they were able to show that inhibition of FAK activity results in lower VE-cadherin phosphorylation in response to stimulation with

VEGF and stabilization of AJ in the endothelium. On the other hand, Src, a non-receptor tyrosine kinase involved in cell adhesion, migration and differentiation, was recently shown to increase phosphorylation of VE-cadherin in the endothelium [Aragon-Sanabria, Pohler, Eswar et al. (2017)]. Using a Förster Resonance Energy Transfer (FRET) sensor to monitor Src activation and western blot to determine VE-cadherin phosphorylation, we reported an increase in Src activity during cancer cell extravasation that resulted in an increase of VE-cadherin phosphorylation, disruption of AJ and higher endothelial barrier permeability (Fig. 6). Previously, Src was shown to mediate endothelial barrier disruption by promoting FAK phosphorylation and translocation to focal contacts leading to association with αvβ5 integrins [Eliceiri, Puente, Hood et al. (2002)]. Experiments using $pp60^{c\text{-}src}$ -deficient mice showed significantly decreased FAK/αvβ5 complex formation compared to wild type mice. Src-deficient mice also showed decreased vascular permeability upon stimulation with VEGF.

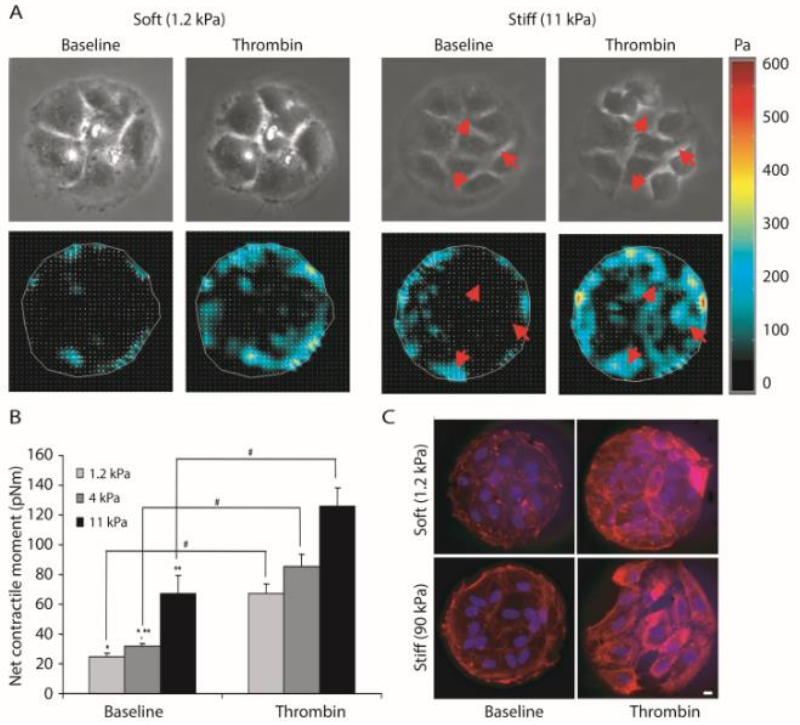

Figure 5: Substrate stiffening promotes endothelial barrier disruption and increases cell contractility. A. Traction forces of endothelial cells cultured on PA gels micropatterned with collagen. Top-phase contrast images, Bottom-Traction force maps. B Forces of endothelial cells represented by net contractile moment. Values are means +/- SE, *p<0.05, 1.2 *vs.* 4 kPa. **p<0.05, 4 *vs.* 11 kPa, #p<0.05 basal forces *vs.* thrombin. C. F-actin staining on 1.2 kPa and 90 kPa gels. Scale bar=10 μm (Krishnan, Klumpers, Park et al. (2010)]

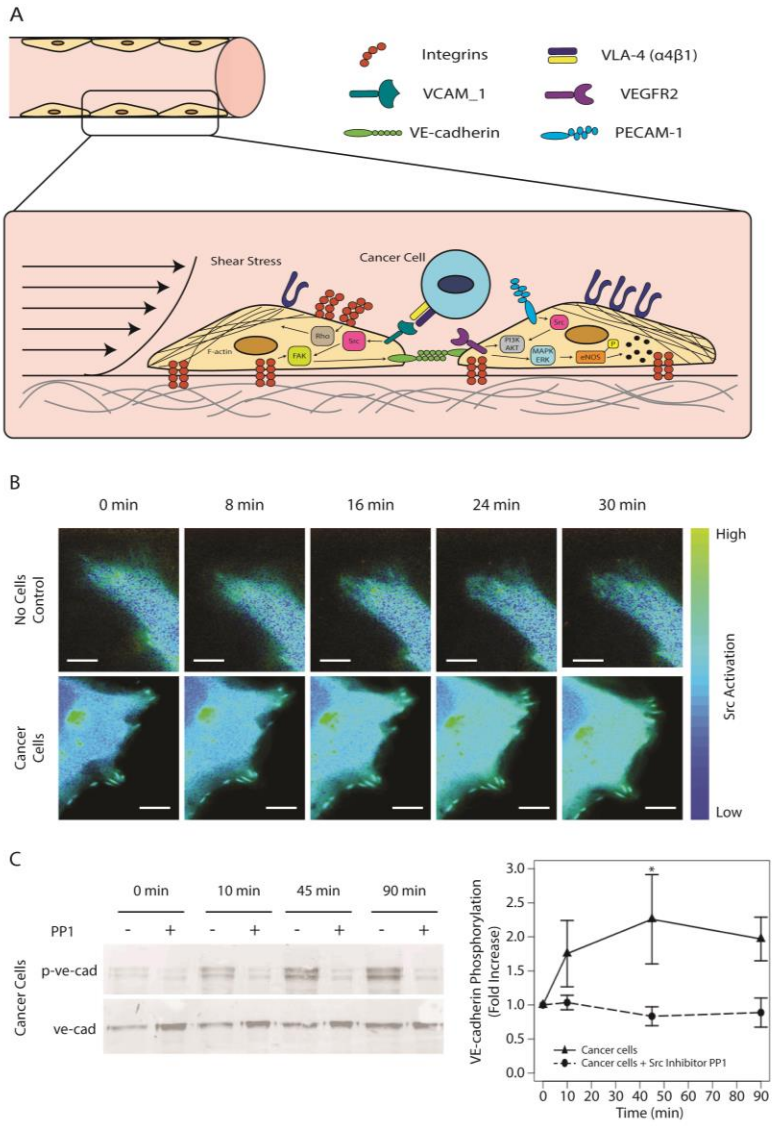

Figure 6: Src activation in the endothelium in response to cell-cell contact with cancer cells is correlated with phosphorylation of VE-cadherin and intercellular gap formation. A. Phosphorylation of VE-cadherin in endothelial cells is triggered by cancer cells in part through VLA-4 and VCAM-1 interactions which results in intercellular gap formation. B. FRET signal indicating Src activation increases in endothelial cells when they are in contact with cancer cells. C. Correlation of Src activation and phosphorylation of VE-cadherin in endothelial cells in contact with cancer cells. B-C modified from Aragon-Sanabria et al. [Aragon-Sanabria, Pohler, Eswar et al. (2017)]

Table 1: Summary of biochemical and biomechanical stimuli that affect the endothelial barrier

Stimulus	Mechanism	Effect on the endothelial barrier	Reference
Biochemical			
VLA-4	-VCAM-1/VLA-4 interactions increase Src activation that results in phosphorylation of VE-cadherin and gap formation	-Disruption	[Aragon-Sanabria, Pohler, Eswar et al. (2017)]
IL-8	-CXCR1/2 receptor activation by IL-8 increases Src activation that results in phosphorylation of VE-cadherin and gap formation	-Disruption	[Aragon-Sanabria, Pohler, Eswar et al. (2017)]
VEGF	-VEGF disrupts binding between VE-cadherin and β-catenin through Src activation	-Disruption	[Weis, Cui, Barnes et al. (2004)]
	-Activation of VEGFR2 promotes endocytosis of VE-cadherin via clathrin coated vesicles		[Gavard and Gutkind (2006)]
	-Activation of VEGFR2, MAPK1 and MAPK3 results in phosphorylation of eNOS and increased NO production		[Di Lorenzo, Lin, Murata et al. (2013)]
β2 integrin	-β2 binding to ICAM-1 increases cancer cell extravasation	-Not assessed directly but increased cancer cell transendothelial migration suggests that the endothelial barrier is disrupted	[Liang, Slattery, Dong (2005); Liang, Hoskins, Khanna et al. (2008)]
Soluble cationic ligands, L-arginine and L-lysine peptides	-Heparan sulfate proteoglycans induce cytoskeletal reorganization	-Disruption	[Dull, Dinavahi, Schwartz et al. (2003); Rehm, Zahler, Lötsch et al. (2004)]
Thrombin	-RhoA activation increases endothelial cell contractility and increases phosphorylation of MLC2	-Disruption	[Nieuw and Helmke (2008)]
Histamine	-Activation of PKC via H1 receptor leads to activation of	-Disruption	[Ashina,Tsubosaka, Nakamura et al. (2015)]

	ROCK and NO-dependent vascular dilation		
Biomechanical			
High shear stress	-Increased tension in PECAM-1 and association with Vimentin -Association with vimentin is necessary for endothelial cells to align in the direction of the flow	-Not assessed directly but increased cell alignment has been linked with endothelial barrier enhancement	[Conway, Breckenridge, Hinde et al. (2013); Tzima, Irani-Tehrani, Kiosses et al. (2005); Dai, Kaazempur-Mofrad, Natarajan et al. (2004)]
	-Promotes cytoskeletal alignment -Counters endothelial cell activation (VCAM-1 expression) by IL-1β		
	-Heparan sulfate increases NO production	-Not assessed directly but increased NO production has been linked with endothelial barrier disruption	[Florian, Kosky, Ainslie et al. (2003)]
Low shear stress	-Phosphorylation of VE-cadherin through Src activation	-Not sufficient to increase barrier permeability	[Orsenigo, Giampietro, Ferrari et al. (2012)]
	-Several genes related to inflammation and angiogenesis are upregulated	-Not assessed directly but inflammation and angiogenesis have been linked with endothelial barrier disruption	[Dai, Kaazempur-Mofrad, Natarajan et al. (2004)]
Oscillatory and low shear stress	-Oscillatory and low shear flow show increased VE-cadherin phosphorylation compared to high shear stress -Low shear stress promotes expression of ICAM-1 and VCAM-1	-Not assessed directly but increased phosphorylation of VE-cadherin has been linked with endothelial barrier disruption	[Conway, Coon, Budatha et al. (2017)]
Increased substrate stiffness	-Amplifies the disruptive effect of pulling on VE-cadherin	-Disruption	[Eguiluz, Kaylan, Underhill et al. (2017)]

	-Increased endothelial cell contractility		[Birukova, Tian, Cokic et al. (2013)]
	-Increased Rho kinase activity that leads to increased endothelial cell contractility		[Krishnan, Klumpers, Park et al. (2010)]
Cyclic stretch	-Induces actin stress fiber alignment in the direction perpendicular to the stretch	-Disruption only when combined with IL-2	[Huh, Leslie, Matthews et al. (2012)]
	-Increased phosphorylation and reorganization of FAK	-Not assessed directly but increased endothelial cell migration is linked with disruption of the endothelial barrier	[Yano, Geibel, Sumpio et al. (1996)]

4 Future perspectives

Endothelial barrier function is critical for multiple physiological processes. Increasing evidence shows that proper function of the endothelial barrier is not only affected by biochemical but also by biomechanical signals. The combination of these two aspects ultimately determines the biological response and status of the endothelium, healthy vs. compromised. Integration of biomechanical and biochemical information is key in the development and assessment of potential therapies for vascular diseases or other pathologies that involve the deterioration of the endothelial barrier. Most studies analyze the behavior of endothelial cells stimulated either through cytokines/growth factors, shear flow or changes in the stiffness of substrates and all of them show that these factors have a role in the function of the endothelial barrier. However, in reality, all these factors act simultaneously, and we still do not understand the importance of each variable relative to the others. Hopefully with the introduction of new microfluidic technologies that allow manipulation of flow, substrate stiffness and active molecules, we will better understand the interplay between these cues.

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