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Forces and mechanotransduction in 3D vascular biology

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Abstract

The effects of hemodynamic and interstitial mechanical forces on endothelial biology *in vivo* have been appreciated for over half a century, regulating vessel network development, homeostatic function, and progression of vascular disease. Investigations using cultures of endothelial cells on two-dimensional (2D) substrates have elucidated important mechanisms by which microenvironmental stresses are sensed and transduced into chemical signaling responses. However recent studies *in vivo* and in three-dimensional (3D) *in vitro* models of vascular beds have enabled the investigation of forces and cellular behaviors previously not possible in traditional 2D culture systems. These studies support a developing paradigm that the 3D chemo-mechanical architecture of the vascular niche impacts how endothelial cells both sense and respond to microenvironmental forces. We present evolving concepts in endothelial force sensing and mechanical signaling and highlight recent insights gained from *in vivo* and 3D *in vitro* vascular models.

INTRODUCTION

Dynamic cellular response to mechanical forces is fundamental to vascular biology, regulating the development of the vascular plexus [1], vessel morphogenesis and sprouting [2,3], vessel barrier function [4], inflammatory signaling [5], gene transcription, and arteriosclerosis [6,7]. These mechanical forces are comprised of both extrinsic stresses, from blood flow-driven shear stress and circumferential stretch, extracellular matrix (ECM) ligation, and interstitial pressure, and intrinsic stresses from applied cellular tractions through cell-cell and cell-ECM adhesions. Precise sensing and integration of these stresses maintain vascular homeostasis and, when dysregulated, drive pathological progression [8,9].

Initial investigations of cells cultured on flat (2D) surfaces have identified key cellular structures and molecular machinery that sense and transduce forces of fluid shear and matrix stretch in endothelial cells and these observations continue to provide the scientific foundation for current studies. However, 2D endothelial cultures are inherently limited to recapitulating only a subset of relevant mechanical forces, such as interstitial flow, and

cannot appropriately model some of the endothelial behaviors observed *in vivo,* such as angiogenic sprouting. Recent advances across scientific disciplines including tissue engineering, materials sciences, molecular sensors, mechanics, and computational methods have permitted the inclusion and tunability of distinct 3D hemodynamic and interstitial forces in both *in vivo* and 3D *in vitro* vascular models. Observations of dynamic endothelial behaviors in response to mechanical force stimuli in such systems have revealed that the distinct 3D chemo-mechanical architecture of the vascular microenvironment critically influences the endothelial response to force.

In this commentary, we first briefly provide a historical context of studies in endothelial mechanotransduction. We then will focus on recently identified molecular mechanisms of endothelial mechanotransduction, with highlights of conceptual advances derived from 3D *in vivo* and *in vitro* vascular models. We will explore the forces influencing vascular biology in microvasculature models, but allude to other vessel classes when appropriate.

EARLY INVESTIGATIONS OF ENDOTHELIAL MECHANOTRANSDUCTION

The field of endothelial mechanotransduction arose from the early observations in the arterial circulation that areas of disturbed blood flow were a critical determinant for where the early pathologic changes of atherosclerosis were initiated. Shortly thereafter, the ability of the endothelium to actively sense and respond to fluid shear stress was demonstrated by varying the viscosity of perfused medium in isolated arterial preparations [10]. Initial investigations into endothelial mechanotransduction thus focused on the mechanical stresses resulting from hemodynamic flow, which manifests as shear stress (σ_{ss}), the frictional drag force per unit area from blood flow parallel to the vessel wall, and luminal blood pressure (P_{ves}) which acts normal to the vessel wall to induce circumferential stretching (Figure 1A).

To study endothelial behaviors in response to shear stress, 2D parallel-plate flow chambers and cone-and-plate chambers were utilized to expose monolayers of endothelial cells to defined flow profiles. These seminal studies revealed that applied shear stress initiated mechanical changes within the cell, inducing cellular and cytoskeletal alignment in the direction of flow and the strengthening of cell-cell adherens junction complexes [11,12]. Fluid shear stress was further demonstrated to directly regulate endothelial cell proliferation, gene expression, lipid composition and metabolism, and inflammation [7,8,13]. Pulsatile or pathological changes in blood pressure can create an acute or chronic mechanical stimulus in the form of circumferential stretch. Early investigations into endothelial stretch sensing were conducted by culturing cell monolayers on deformable 2D silicone rubber membranes that were subjected to defined stretch. Endothelial cells were observed to remodel and orient their actin stress fibers perpendicular to the axis of stretch to bear less tension and thus minimize stretch-induced increases in intracellular mechanical energy [14]. These early shear and stretch studies demonstrated that hemodynamic mechanical forces directly modulate the structure and function of endothelial cells, providing the validation to explore further the forces and molecular mechanisms that influence endothelial mechanotransduction.

MOLECULAR FORCE SENSING AND MECHANOTRANSDUCTION

Despite a developed understanding of the importance of mechanical force on endothelial biology, surprisingly little is known about how endothelial cells sense force and transduce it into chemical signaling. This is due to the conundrum that, unlike chemical receptor-ligand signaling, mechano-receptors and transducers have no inherent “ligand” and researchers are thus forced to broadly probe cellular states before and after mechanical stimulus [15]. Many candidate mechanosensors have been proposed to function in endothelial force sensing, including the glycocalyx [16,17], plasma membrane fluidity [18], ion channels [19,20], primary cilia [21], nuclei [22], integrin-based focal adhesions [23-25], cell-cell adherens junctions, intermediate filament and actin networks [26,27], G proteins [28], and caveolae [29] (Figure 1B). In all likelihood each of these mechanically-sensitive structures acts in concert, or contextually, with the others to define a multimeric, force-sensitive network. However, we lack a precise molecular understanding of how, and in what context, these various elements conduct their signaling response to mechanical force.

Identified endothelial mechanosensors, transducers, and associated signaling molecules are summarized in Table 1. Non-homogenous remodeling of cytoskeletal networks at lateral and basal structures in response to external stresses implicate cell-cell and cell-matrix adhesions as primary endothelial mechanotransducers [30]. At the apicolateral membrane, endothelial cells form mechanical connections to neighboring cells through a form of cell-cell adhesion termed adherens junctions (AJs). AJs resist dissociating forces, transmit forces to adjacent cells, and are remodeled in response to changes in internal and external tension. Within AJs is the shear stress-responsive complex of PECAM-1, VE-Cadherin, and VEGFR2/3, a complex that is both necessary and sufficient to impart flow-responsiveness [31], directs cellular alignments to flow, and is instrumental in promoting pro-atherosclerotic states. FRET-based molecular tension sensors elegantly demonstrated that shear stress triggers an increase in tension across junctional PECAM-1, but a decrease in tension across VE-Cadherin and cell-cell junctions [32]. Src-mediated phosphorylation of PECAM-1 was one of the first identified molecular modifications in endothelial cells in response to a variety of mechanical stimuli. PECAM-1 phosphorylation promotes Erk signaling, activation of VEGFR2, and production of nitric oxide in response to flow [15]. Despite changes in molecular tension, VE-Cadherin is not a direct mechanotransducer, rather a scaffolding molecule. Recent studies have articulated that the transmembrane domain of VE-cadherin facilitates the association of PECAM-1 and VEGFR2/3, and is required for the downstream activity of VEGFR2 in response to mechanical activation of PECAM-1 [31,33]. VEGFR2 undergoes ligand-independent phosphorylation in response to shear stress, leading to activation of MAPK, Akt, and PI3K pathways among others [34]. Cyclic strain has been demonstrated to trigger VEGFR2 dissociation from VE-cadherin at AJs and increase vascular permeability [35], implicating the complex in the transduction of mechanical stretch. VEGFR3 was recently identified as another member of this shear-responsive complex, signaling similarly to VEGFR2 and also dependent on VE-Cadherin transmembrane scaffolding [33].

At the basal interface, endothelial cells interact with basement membrane and interstitial ECM proteins through integrin-based focal adhesions. PI3K signaling downstream of the AJ

mechanotransduction complex leads to the conformational activation of integrins and changes in cell-ECM stresses at the basal interface [23,25]. Integrin activation and ligation to ECM is required for endothelial adaptation to shear stress through the activation of the Rho family GTPases Rac1, RhoA, and Cdc42 [36,37], which coordinate the cytoskeletal rearrangements required for cellular alignment to flow, inflammatory signaling, and presentation of apical cell-adhesion receptors [38]. Rac1 activity also serves to promote VE-Cadherin stability by locally counteracting actomysoin force on VE-cadherin trans-dimers [39]. Recent evidence suggests that the AJ mechanotransduction complex may be able to spatially control the activation of Rac1 through the local recruitment of the GEF Trio to VE-cadherin [40].

Circumferential strains and changes in ECM stiffness modulate cell-ECM adhesion traction stresses (σ_{ecm}) through conformational changes in integrin activation that manifest as alterations in endothelial phenotype, global cytoskeletal organization, and luminal shear responsiveness (Figure 1A) [6,41]. Endothelial cells plated on 2D polyacrylamide gels show that increased substrate stiffness alters the shear stress threshold required to induce morphological changes and alignment [41], ECM stiffening enhances VE-cadherin-mediated forces [42], and evidence suggests similar mechanisms exist in 3D vessels [43]. Rearrangements of actin stress fibers in response to cyclic stretch are also dependent on integrin activation and ECM ligation [8]. While the mechanisms of integrin force mechanotransduction have been reviewed in detail [44], in endothelial cells the downstream cytoskeletal changes in response to altered cell-ECM stresses are orchestrated primarily through the Rho GTPase RhoA, Src, and FAK kinases. Additionally, *in vivo* observations have shown that mural cells, pericytes and smooth muscle cells, can contribute to the basal contractile traction stresses translated to the endothelium.

The concept of decentralized endothelial mechanotransduction across a cellular continuum of force-sensitive molecules, adaptive structural units, and signal transmission elements is not novel [7,9] (Figure 1b), yet its relevance persists as our molecular understanding of endothelial mechanical signaling deepens. With the discoveries of new mechanosensors, transducers, and their associated signaling, and crosstalk mechanisms, it will be important to understand not only how these molecules function individually, but also how they mechanically integrate into this continuum. With advances in tunable biomaterials and 3D *in* vitro microfluidic platforms, it will be of interest to test the roles of identified endothelial force sensors under newly testable mechanical settings, such as 3D interstitial flow and pressure, which have been demonstrated to dictate 3D cancer cell migration and tumor phenotype [45,46].

FORCES AND 3D ENDOTHELIAL BEHAVIOR

While historic significance has been placed on correlations between anatomical vascular architectures, blood flow profiles, and sites of vascular pathogenesis, studies using *in vivo* vascular models have directly demonstrated the regulatory role of mechanical forces during vascular morphogenesis. Using a combination of genetic and mechanical manipulation, *in vivo* reduction of shear stress impaired developmental vascular remodeling at the onset of blood flow in the developing yolk sac [1]. In a recent follow-up study, Udan et al. elegantly

demonstrated that vessel diameter changes during embryonic remodeling occurred via both vessel fusions events and directed endothelial migration. These dynamic endothelial behaviors were observed to be mechanically-dependent, dictated by local flow profiles and restricted to specific vessel classes [47]. Further, *in vivo* platforms permit the study of distinct force types, such as interstitial pressure (Figure 1A), on endothelial behavior. During tumor progression, local interstitial fluid accumulation due to lymphatic dysfunction, increased vascular permeability, and altered oncotic gradients elevate interstitial pressures. Resulting pressure gradients generate transmural flows which impair vascular transport and apply transmural shear stresses which influences endothelial function [48,49].

Intrinsically, force generation through RhoA-mediated contractility is critical for proper vessel architecture during *in vivo* angiogenesis [50]. Soluble morphogenic factors such as VEGF and sphingosine-1-phosphate, which stimulate angiogenic events *in vivo*, directly modulate applied cellular traction stresses through the RhoA-ROCK signaling axis [51]. Intriguingly, a novel antagonistic regulatory mechanism was recently identified between Notch and VEGFR signaling pathways during angiogenesis. Fluctuations in Notch/VEGFR signaling result in differential VE-cadherin dynamics, cell-cell adhesion phenotype, and endothelial cell migration during angiogenic sprouting [52]. These findings identify the regulation of cell-cell adhesions through intrinsic force-generation in response to chemical factors as critical for *in vivo* angiogenesis.

While these *in vivo* studies demonstrate elegantly how complex force fields appear to drive changes in vascular morphogenesis, our inability to model these processes in traditional 2D *in vitro* systems has limited detailed molecular mechanistic characterization of the mechanotransduction processes. Recently, engineering investigators have begun to develop 3D in vitro microfluidic devices in which one can seed endothelial cells to form perfusable vascular networks. The inherent similarity (vessel geometry, ECM composition and dimensionality, cellular architectures, flow profiles) between *in vivo* microvascular networks and these 3D *in vitro* vascular models provides an attractive approach to begin to investigate these questions. The first studies using these 3D *in vitro* microfluidic vessel models have focused on confirming many force-driven cell behavior observations made in *in vivo* and 2D culture: increased barrier function and junctional reorganization in response to elevated shear stress [53], changes in transmural pressure affect vessel permeability, sprouting, and monolayer integrity [54-56], and alterations in ECM stiffness dictate flow responsiveness and vessel barrier function [41,43]. Studies in 3D *ex vivo* and *in vitro* vascular beds have demonstrated that application of bulk tensile stress to alter 3D ECM mechanical properties can regulate neovessel sprouting, elongation during angiogenesis, and vascular network organization [57,58].

However the allure of *in vitro* microfluidic vessel platforms is identifying novel cellular behaviors and molecular functions that are uniquely observable in controlled 3D *in vitro* settings. Recently, our laboratory identified a previously uncharacterized endothelial response to shear stress using 3D *in vitro* microvascular vessel models. For both luminal and transmural flows there exists a shear stress threshold that, when surpassed, triggers local angiogenic sprouting. While matrix metalloproteinase 1 (MMP1) was identified as the dominant downstream effector regulating branching initiation [3], it remains unclear what

shear-sensitive molecules initiate this response and whether similar processes govern luminal versus transmural shear sensing. Of interest will be the systematic combination of synthetic biomaterials that allow independently tunable ECM mechanical properties [59] and microfluidically controlled flow profiles in 3D *in vitro* vascular models to dissect the relative contributions of mechanical force stimuli and associated molecular mechnotransducers during dynamic endothelial force-responsive behaviors.

FUTURE OUTLOOK

Mechanical signaling in endothelial cells is at a compelling juncture. While numerous putative sensors and transducers of mechanical force have been identified, undoubtedly more remain undiscovered. Discoveries of candidate mechanosensors are confounded by an emerging theme that all proteins that are force-responsive also have secondary, non-mechanical functions. This is illustrated by the recently identified shear-responsiveness of the ion channel Piezo1 [19], the transmembrane proteoglycan Syndecan-4 [60], and the PECAM-1, VE-Cadherin, VEGFR2/3 complex [31,33]. While the emergence of CRISPR/Cas9 genome editing technology should facilitate high throughput screening for potential mechanical sensors and transducers, new discoveries will be driven by the combination of creative molecular approaches and engineered assays in response to this mechanical challenge. 3D microfluidic *in vitro* vascular models provide a platform to not only investigate novel influences of force on endothelial behavior, but also elucidate the contextual relevance and coordination of the growing list of mechanically-sensitive proteins. Advances in synthetic biomaterials compatible with microfluidic implementation will allow for the independent tuning of mechanical properties in the 3D perivascular environment. Combining these approaches with cellular and molecular engineering will begin to provide a framework for understanding how individual mechanical signaling elements contribute to a larger endothelial mechanosensitive continuum.

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HIGHLIGHTS

- Environmental and cell-generated forces profoundly influence endothelial behavior.
- 3D *in vitro* models allow the recapitulation of *in vivo* force-driven endothelial behaviors.
- Novel mechanosensory mechanisms provide insight into an endothelial force-sensitive continuum.

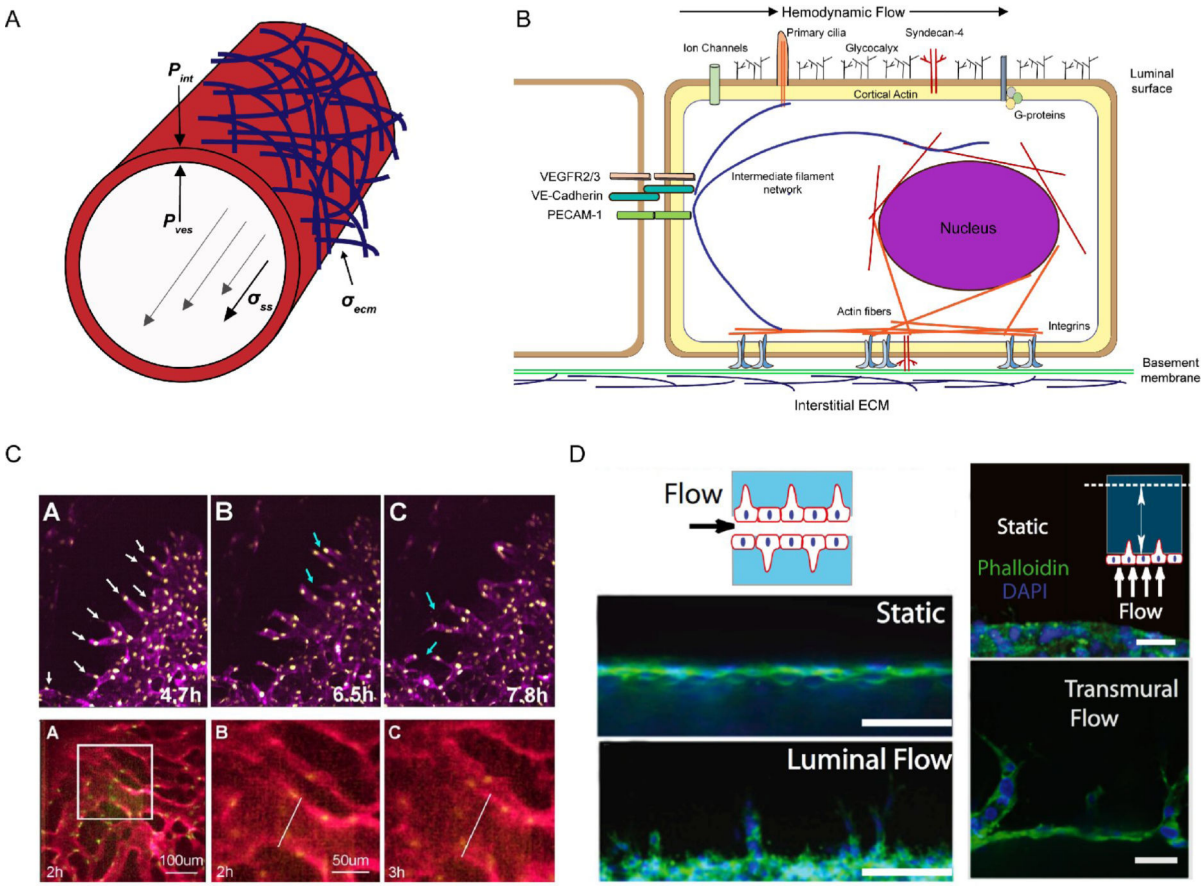

Figure 1.

A) Force diagram of a microvessel under flow. Hemodynamic flow (gray arrows) exerts frictional shear stress σ_{ss} parallel to the vessel wall, and pressure P_{ves} normal to the vessel wall. At the basal interface, cell-ECM stresses σ_{ecm} are driven by integrin ligation to basement membrane and interstitial ECMs. Interstitial fluid accumulation increases interstitial pressures P_{int} that act on the outer vessel membrane. Transmural pressure P_{TM} is defined by the difference vessel and interstitial pressures. B) Representative diagram of the intracellular localization of mechanosensors and transducers during endothelial exposure to flow, noting how individual elements are integrated in a force-sensitive continuum. Adapted from [6]. C) (top) Timelapse images of sprouting angiogenesis (white arrows) and anastomosis events (blue arrows) in E8.5 yolk sacs from [64]. (bottom) Timelapse images of flow-driven vessel arterial fusion events in yolk sacs from [47]. D) Using 3D *in vitro* vessel models, a shear stress threshold was identified for both luminal (left) and transmural (right) flows that drives vascular sprouting. Scale bars 50 and 100 microns. [3].

Table 1

Identified mechanical sensors and transducers in endothelial cells

Structural mechanosensors		
	Cellular localization	**Mechanical activation**
Stretch-induced ion channels [19,20]	Apical membrane	Fluid shear stress, circumferential strain
Membrane fluidity/lipid composition [18,61]	Apical membrane	Fluid shear stress, circumferential strain
Primary cilia [21]	Apical membrane	Fluid shear stress
Glycocalyx [16,17]	Apical membrane	Fluid shear stress
Caveole [29]	Internal plasma membrane	Fluid shear stress
Nucleus [22]	Cytoplasm	Fluid shear stress, circumferential strain, cell-ECM stress
Focal adhesions [25]	Basal ECM interface	Fluid shear stress, circumferential strain, cell-ECM stress
Heterotrimeric G-proteins [28]	Apical/basal membrane	Fluid shear stress, cell-ECM stress
Adherens junctions [31]	Apical/lateral membrane	Fluid shear stress, circumferential strain

Molecular mechanosensors, transducers, and associated signaling		
	Intracellular localization	**Relevant function**
PECAM-1	Adherens junctions, Apicolateral membrane	Phosphorylated in response to mechanical stimuli, transactivates VEGFR2/3 [15].
VE-Cadherin	Adherens junctions	Transmembrane scaffolding of PECAM-1 and VEGFR2/3 [31].
VEGFR2	Adherens junctions, Apical membrane	Ligand-independent phosphorylation in response to shear stress, stretch, activates PI3K/Akt [33].
VEGFR3	Adherens junctions, Apical membrane	Ligand-independent phosphorylation in response to shear stress, activates PI3K/Akt [33].
Cholesterol	Apical membrane	Composition in membrane alters bilayer viscosity, depletion abolishes shear responses [61].
Piezo1	Apical membrane	Shear regulation of Piezo1 ion channel currents during developmental vascular remodeling [19].
Syndecan-4	Apical and basal membranes	Shear-driven cell alignment independent of VEGFR2 pathway [60].
α5, β1, αVβ3 integrins	Basal adhesion complexes	Activation by PI3K downstream of shear stress to controls cell alignment. Application and sensing of cell-ECM stresses [6,25].
FAK, Src kinases	Focal adhesions, cortical membrane	Shear stress increases phosphorylation and associated signaling [62].
Actin and intermediate filament cytoskeletons	Cortical plasma membrane, cytoplasmic, perinuclear	Fluid shear stress drives non-homogenous filament deformations. Inhibition blocks many responses to flow/ECM stress [26,30].
Rap1b	Internal plasma membrane/cytoplasm	Activated by shear stress, promotes formation of PECAM-1, VE-Cadherin, VEGFR2 complex [63].
Rho GTPases (RhoA, Cdc42, Rac1)	Internal plasma membrane/cytoplasm	Activity increased in response to shear-driven integrin activation to orchestrate cytoskeletal, junctional, and morphology dynamics [38,39].