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## Synergistic Regulation of Angiogenic Sprouting by Biochemical Factors and Wall Shear Stress

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### Abstract

The process of sprouting angiogenesis involves activating endothelial cells in a quiescent monolayer of an existing vessel to degrade and migrate into the underlying matrix to form new blood vessels. While the roles of biochemical factors in angiogenic sprouting have been well characterized, the roles of fluid forces have received much less attention. This review summarizes results that support a role for wall shear stress in post-capillary venules as a mechanical factor capable of synergizing with biochemical factors to stimulate pro-angiogenic signaling in endothelial cells and promote sprout formation.

### Keywords

angiogenesis; extracellular matrix; mechanotransduction; sphingosine-1-phosphate; sprout formation

## INTRODUCTION

The growth of new blood vessels from pre-existing vessels is necessary to support the perfusion of blood into new tissues during formation of the vascular system in the developing fetus. In adults, angiogenesis also occurs during menstruation, ovulation, wound healing, and in certain diseases (e.g. tumor growth, diabetic retinopathy). The viability of engineered tissues depends on the ability to transport nutrients and oxygen to cells that cannot be accessed through simple diffusion, thus providing a strong motivation for understanding the mechanisms involved in controlling angiogenesis.

As depicted in Figure 1, sprouting angiogenesis is a complex, multi-step process (reviewed in 32). First, one or more cells in the endothelium of a pre-existing blood vessel must be induced to enzymatically digest the underlying extracellular matrix to generate a space for subsequent migration into the matrix. These tip cells are then followed by other endothelial cells (ECs) to form the stalk of the nascent sprout (9). A lumen is created through formation and fusion of vacuoles within the sprouting structures (53). Tip cells from neighboring sprouts eventually join to form a continuous conduit for blood flow that is eventually stabilized by pericytes and smooth muscle cells.

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Sprouting angiogenesis is stimulated by growth factors (32). In addition, ECs are exquisitely sensitive to fluid wall shear stress (WSS). Much of the EC flow mechanotransduction literature has been motivated by a need to understand the role of fluid forces in atherosclerosis. These studies largely indicate that arterial levels of WSS ( $>15$  dyn/cm<sup>2</sup>) promote a quiescent cell phenotype, while low and/or reversing WSS stimulates a pro-atherogenic phenotype (69). Fluid forces also play a role in sprouting angiogenesis, as was first reported 80 years ago (21). Sprouting primarily occurs in the post-capillary venules, where WSS is estimated to range from 1 to 8 dyn/cm<sup>2</sup> (51, 56, 58). In this review, we shall briefly review studies that have investigated the role of flow in sprouting angiogenesis and associated signal transduction. These studies provide evidence indicating that blood flow within venules provides an optimal WSS stimulus for sprouting angiogenesis.

### Roles of Biochemical Factors and Fluid Forces

Of the many extracellular signals that are capable of inducing the transition of a quiescent EC into an invading phenotype, biochemical signals have been the most studied by far. Vascular endothelial growth factor A (VEGF-A) is generally regarded as the master regulator of angiogenesis. Secretion of VEGF-A from cells in an avascular region creates a concentration gradient that provides a chemoattractive cue for stimulating and directing angiogenic sprouting from nearby blood vessels. Other growth factors, including acidic- and basic-fibroblast growth factors (aFGF and bFGF), platelet-derived growth factor (PDGF), stromal-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ), and placental growth factor (PIGF) are also potent pro-angiogenic factors with similar influences on sprouting. With the exception of SDF-1 $\alpha$ , which signals through a G-protein coupled receptor, CXCR4, these growth factors bind to their cognate tyrosine kinase receptors to elicit effects on EC function via intracellular signals. Sphingosine 1-phosphate (S1P) is a lysosphingolipid that acts as both an intracellular signaling molecule and an extracellular factor deposited by activated platelets during wound healing (17, 47). Sphingosine kinase 1 (SPHK1) and SPHK2 phosphorylate the sphingosine backbone to produce S1P. S1P binds one of five G-protein coupled receptors (S1P1-S1P5). ECs express S1P1 and S1P3 receptors and activate phosphatidylinositol 3-kinase (PI3K). Exogenous S1P administration or endogenous S1P production by SPHK1 over-expression promotes post-ischemic angiogenesis and blood flow recovery in mouse ischemic hind limb models (82).

Only a few studies have directly evaluated the role of WSS in angiogenesis *in vivo*. Zhou et al. (118) reported angiogenesis occurring through internal capillary division in response to elevated flow in rat skeletal muscle in response to chronic vasodilation with the  $\alpha$ 1-antagonist prazosin. Ichioka et al. (51) reported that increasing WSS from 3.7 to 5.3 dyn/cm<sup>2</sup> using prazosin increased the rate of microvascular ingrowth through sprout formation in a rabbit ear chamber. While enhanced flow due to prazosin treatment may have additional pro-angiogenic effects other than changes in WSS, these studies suggest that flow promotes angiogenesis *in vivo*. Dickinson and colleagues (66) demonstrated that shear forces stimulate endothelial nitric oxide synthase (eNOS) expression and are crucial for vascular remodeling during development in the murine yolk sac. Altogether, these studies indicate mechanical forces contribute to proper vascular remodeling and angiogenesis.

### In Vitro Systems for Studying Sprouting Angiogenesis

Multiple approaches have been developed that recapitulate the process of angiogenesis in cell culture. These approaches can be separately categorized as 2-D and 3-D systems.

**2-D systems**—Unlike the EC monolayers formed on tissue culture plastic, ECs cultured on surfaces coated with Matrigel create cord-like networks of cells (Fig. 2A). Although widely-used to test activity of many pro- and anti-angiogenic compounds, some caution

should be used in interpreting those results, because many non-vascular cell types such as fibroblasts, tumor cells and kidney cells also form cords when cultured on Matrigel (11, 31, 33, 108). Cord formation is inhibited by reducing the thickness of the matrix, by including fibrillar type I collagen in the matrix, or by disrupting cytoskeletal microfilaments and microtubules (108). These results suggest that cord formation on Matrigel may be caused by intracellular tension generation rather than by specific differentiation of ECs into capillary-like structures.

The effects of pro-angiogenic stimuli on cell migration have been characterized by quantifying cell motility along a 2-D surface (3). A drawback to this assay is that the cells do not need to circumnavigate a dense matrix. Boyden chambers or Transwell migration assays (Fig. 2B) are a convenient method for quantifying migration orthogonal to the cell monolayer. In this assay, cells in a monolayer on the top face of a porous substrate are driven to migrate through the pores to the opposite face due to the presence of a chemotactic factor (e.g. VEGF-A) in the lower media chamber. The substrate can be coated with matrix proteins to occlude the pores, thus requiring the cells to degrade the matrix to transmigrate.

**3-D systems**—Standard 2-D cell culture systems cannot replicate all the steps involved in sprouting angiogenesis, including EC activation, basement membrane degradation, invasion and proliferation of ECs, lumen formation, and stabilization. Three-dimensional extracellular matrices (ECM), typically composed of fibrillar collagen I, fibrin, or basement membrane components, provide an environment in which some or all of these steps can be observed. As summarized in Figure 2 and described below, there are a number of ways to introduce ECs into these matrices.

**Vasculogenesis model**—During vasculogenesis, individual foci of ECs assemble into vascular structures, thus distinguishing this process from angiogenesis, where new blood vessels originate from pre-existing vessels. Individual ECs can be suspended in collagen and fibrin matrices prior to polymerization (7, 26). In these systems, ECs interconnect to form tubes through sprout extension and the formation and coalescence of vacuoles and lumens. Davis and colleagues have utilized this model extensively to study molecular signals that drive lumen formation events (27). These studies illustrate the utility of 3-D systems for better understanding various steps of vasculogenesis, and angiogenesis, many of which are likely to overlap.

**Blood vessel explant assays**—Another commonly used assay to quantify angiogenesis is the *ex vivo* aortic ring assay (Fig. 2C), where a segment of rat or mouse aorta is embedded within an ECM. ECs, primarily from the vessel intima, sprout into the matrix. In this system, key steps of angiogenesis (cf. Fig. 1) are represented, and native cells (including pericytes, fibroblasts and monocytes) are available to contribute to stimulate growth and stabilize the sprouts. A disadvantage of this system is the variability introduced by incomplete removal of connective tissue surrounding the aortas and variability in the aorta sources themselves.

Angiogenesis typically occurs in microvessels, but not major vessels such as the aorta. Microvascular fragments isolated from adipose tissue can be implanted in collagen matrices (59) to provide ECs for sprouting angiogenesis (Fig. 2D). These sprouts are then able to intersect and fuse with other nascent microvessels, yielding complex 3-D networks. Like the aortic ring preparation, this model incorporates multiple cell types. Disadvantages are the heterogeneity in microvessel fragments and the lack of a homogenous imaging plane to perform quantification studies.

**EC spheroids and EC-coated microbeads**—ECs cultured with water-soluble methylcellulose polymers spontaneously form cell spheroids (Fig. 2E). These spheroids

have been implanted into collagen matrices to induce sprouting (57). In separate assays, microbeads coated with ECs prior to embedding in fibrin matrices recapitulates EC sprouting, migration, proliferation, lumen formation and anastomosis (77, 79). In these experiments, fibroblasts are cultured on the surface of the fibrin matrix and secrete factors that complement exogenous VEGF and bFGF. This model has the advantage of studying the contribution of fibroblasts, but does not incorporate a polarized layer of ECs forming an interface between the underlying ECM and luminal compartment.

**EC monolayer invasion assay**—Bayless and Davis (5) developed a model of sprouting angiogenesis where an EC monolayer is seeded on the surface of a 3-D collagen matrix and stimulated to invade into the underlying matrix (Fig. 2F). Unlike other 3-D systems described above, this is the only system which begins with a monolayer of ECs seeded on a basal ECM with an apical fluid-filled compartment. EC sprouting responses stimulated by S1P, VEGF, and bFGF occur overnight in the absence of serum and are readily quantifiable (6).

**Systems for studying the role of WSS on angiogenesis**—The assays above are generally used to study the effects of biochemicals (e.g. growth factors) on angiogenic processes in static cultures. Many of these assays are not amenable to studying the role of fluid forces. Initial approaches involved first applying WSS to cell monolayers and then performing angiogenic assays afterwards using the presheared cells. Gloe et al. (38) presheared ECs on laminin-coated plates at 16 dyn/cm<sup>2</sup> for 6h, then further cultivated the cells under static conditions to observe tubulogenesis. Preshearing caused cord formation in a manner dependent on WSS-induced bFGF secretion. After applying WSS to ECs for 16h, Cullen et al. (22) trypsinized and transferred presheared cells to new chambers to quantify cord formation on Matrigel and migration through Transwell filters. Both migration and cord network formation increased monotonically with increasing preshearing magnitude from 1 to 20 dyn/cm<sup>2</sup> in a manner dependent on G-protein signaling. Using a similar approach, Tressel et al. (105) compared the effects of preshearing cells with either steady (5 and 15 dyn/cm<sup>2</sup>) or oscillatory WSS (0±5 and 0±15 dyn/cm<sup>2</sup>) on cord formation on Matrigel. They found that steady, but not oscillatory, WSS significantly inhibited network formation (relative to static cells) and this correlated with downregulation of Ang-2 production by steady WSS. Though the results of these studies are not consistent with each other, the results do indicate that angiogenic responses are highly dependent on the WSS magnitude and pattern. A drawback to these approaches is that the effects of WSS must be “remembered” by the cells during the angiogenesis assay.

We and others (54, 106) have applied WSS to endothelial monolayers in parallel-plate flow chambers while the ECs simultaneously invaded into the underlying 3-D collagen matrix. These two studies demonstrated that 3 dyn/cm<sup>2</sup> WSS promoted sprouting. By performing our experiment using defined culture media, we found that S1P must be present within the collagen matrix for WSS to induce sprouting into the matrix (54). Apparently, the S1P serves as a chemoattractant to stimulate the cells to move deeper into the matrix. The synergy between WSS and S1P is similar to that observed between VEGF/bFGF and S1P (54, 98). Furthermore, the number of sprouts was highly dependent on WSS magnitude, with the greatest number of sprouts occurring at an intermediate WSS of 5.3 dyn/cm<sup>2</sup> (54). As illustrated in Figures 3A–C for human dermal microvascular ECs, the 3-D collagen invasion assay provides information about the morphological characteristics of the resulting sprouts using primary ECs. At 0.12 dyn/cm<sup>2</sup> WSS, some cell invasion was observed (Figure 3A), while at 5.3 dyn/cm<sup>2</sup> WSS, many more cells invaded (Figure 3B). At 12 dyn/cm<sup>2</sup> WSS, very few cells invaded (Figure 3C). Quantification of human dermal (Figure 3D) and retinal (Figure 3E) microvascular EC, and HUVEC (Figure 3F) invasion reveals the same dependence on WSS magnitude we previously reported (54).

While WSS experiments with 2-D EC monolayers are comparatively simple, a number of unique issues must be considered when performing WSS experiments on a 3-D collagen matrix. Wells containing collagen matrices must be fabricated within one of the plates of a parallel-plate system so that the gels are flush with the plate surface. Our system involves a simple modification of a typical parallel-plate flow chamber with the addition of a customized silicone rubber gasket to form the wells. The collagen density must be sufficiently high to provide the mechanical resilience required to keep the collagen matrix flat during exposure to WSS. In our experience, WSS alone does not induce invasion; hence, a chemoattractive agent (e.g. S1P) must also be incorporated into the collagen matrix. After gaining experience with this system, we have found that EC invasion in our system is reproducible. Under these conditions, we have documented a role for activated Akt and metalloproteinases in sprouting responses induced by WSS and S1P (54). In addition, we have recently shown calpains regulate localization of a key metalloproteinase to facilitate sprouting events (Kang et al., in revision). Thus, this system incorporates application of controlled levels of WSS to an endothelial monolayer, which is seeded on an underlying 3-D matrix. This design is amenable to varying WSS and S1P levels, as well as separation of biochemical extracts to better understand how S1P and WSS combine to direct sprouting behavior.

### Angiogenic Signaling Regulated by Biochemical Signals and Fluid Forces

In addition to the handful of studies that have directly quantified the effects of WSS on angiogenic events, a number of studies have been performed using ECs cultured on 2-D substrates to investigate flow mechanotransduction relevant to angiogenesis. Several of these studies have reported changes in angiogenic signaling induced by flow in a manner that is highly dependent on WSS magnitude. These findings are summarized in Table 1 and discussed below.

**Cell surface receptors**—Like VEGF receptors, the Tie receptors (Tyr kinase with Ig and EGF homology domains) represent a class of receptor tyrosine kinases specific to vascular cells that are important contributors to angiogenesis (101). The two known Tie receptors, TIE1 and TIE2, bind to angiopoietins, Ang-1 and Ang-2, and phosphorylate tyrosine residues to mediate intracellular signaling. Ang-1 and Ang-2 are agonistic and antagonistic TIE2 ligands, respectively. Ang-TIE signaling is closely associated with the stabilization of new microvessels. Mice deficient in Ang-1 or TIE2 expression fail to develop their cardiovascular system beyond the primary capillary plexus (101). Overexpression of Ang-2, on the other hand, disrupts blood vessel formation (68). In contrast to TIE2, TIE1 signal transduction is poorly understood. Goettsch et al. (40) demonstrated that Ang-2 mRNA, protein expression and release from HUVECs was upregulated by 1 dyn/cm<sup>2</sup> WSS, but downregulated by 30 dyn/cm<sup>2</sup> WSS. VEGF-A expression was upregulated at both levels of WSS, while vascular endothelial growth factor 2 (VEGFR-2) inhibition attenuated the effects of low WSS on Ang-2. Interestingly, high WSS blocked the ability of VEGF to upregulate Ang-2 expression. Thus, VEGF and WSS have a complex interactive effect on signaling via the Tie receptors.

Ephrin is a transmembrane signaling molecule involved in cell-cell communication during angiogenesis. Ephrin-B2 is capable of binding to several different transmembrane Eph receptor tyrosine kinases. While the Ephrin-B2 receptor EphB4 is primarily expressed in veins, it is also observed in retinal capillaries and sprouts. Ephrin-B2 is essential for angiogenesis and is upregulated during both physiological and pathological angiogenesis in adults (93). Recently, Ephrin-B2 has been reported to regulate sprouting through regulation of VEGF internalization and signaling (92, 112). HUVECs and human coronary arterial ECs each express Ephrin-B2 in static culture, but the level of expression decreases significantly

in response to 24h of high WSS (30–50 dyn/cm<sup>2</sup>), but not for low WSS (1–10 dyn/cm<sup>2</sup>) (39). Hu and Chien (50) reported that 20 dyn/cm<sup>2</sup> WSS induced increased Protein Kinase C immunostaining in HUVECs. Consistent with a role for WSS induction of PKC in mediating Ephrin-B2 suppression, the downregulation of Ephrin-B2 expression by high WSS was blocked by an inhibitor of Protein Kinase C (39). The expression of the receptor EphB4 was not significantly changed by any WSS in the range of 1–50 dyn/cm<sup>2</sup> (39). Since Ephrin-B2 is critical to angiogenesis, these results are consistent with high WSS having a negative impact on cell invasion.

Only a fraction of the cells in the endothelium of a vessel are induced to invade, and this decision is regulated by the Notch pathway. Delta-like 4 (Dll4) and Jagged1 are each proangiogenic transmembrane ligands for the Notch receptor (8). Tip cells express relatively high levels of Dll4, which binds Notch receptors on adjacent stalk cells leading to downregulation of VEGF receptors. With relatively high levels of VEGF receptors, tip cells are thought to be more responsive to VEGF gradients to induce their migration. Stalk cells primarily express Jagged1, which antagonizes Dll4-Notch signaling and thereby upregulate the number of tip cells and sprouts. Inhibition of Notch signaling leads to enhanced sprouting through increased tip cell proliferation in 3-D fibrin (89) and collagen (98) matrices. The effects of WSS on Notch signaling has not been investigated in ECs, though experiments performed using murine embryonic stem cells (mESC) positively expressing VEGF receptor 2 (VEGFR2) may provide insight (70). WSS induced an increase in Ephrin-B2 expression in VEGFR2-positive mESCs in a magnitude-dependent manner for WSS magnitudes of 5 to 20 dyn/cm<sup>2</sup>, while 10 dyn/cm<sup>2</sup> WSS decreased EphB4 expression. The levels of Notch ligand cleavage and Ephrin-B2 expression induced by 10 dyn/cm<sup>2</sup> WSS was equivalent to that induced by VEGF. The expression of the Notch signaling proteins Notch1, Notch4, Dll4 and Jagged1 were each increased in a WSS magnitude-dependent manner for WSS applied over a range of 1.5 to 10 dyn/cm<sup>2</sup>. Further, the upregulation of Ephrin-B2 by WSS was abolished upon inhibition of Notch signaling using inhibitors of  $\gamma$ -secretase, as well as by a VEGF receptor tyrosine kinase inhibitor. There is still much to learn regarding the role of WSS on Notch signaling.

**Intracellular signaling**—Akt is a serine/threonine kinase that plays a major role in EC invasion and sprouting. Akt1 knockout mice exhibit a 40% reduction in the average length of sprouts compared to wild-type mice in *ex vivo* aortic ring assays (115), which is consistent with the proposed role of Akt in vascular maturation and angiogenesis during wound healing (97). Multiple angiogenic events are mediated by Akt, including EC migration, survival, and tube formation (95). WSS, growth factors (VEGF-A and bFGF), and S1P induce dose-dependent Akt phosphorylation and correlate with tubulogenesis and endothelial sprouting (29–30, 52, 54, 60, 75). Dimmler et al. (28) demonstrated that Akt phosphorylation increases monotonically with increasing WSS ranging from 0 to 45 dyn/cm<sup>2</sup> in 2-D cultures. We found S1P and WSS-induced Akt phosphorylation at Ser473 in ECs cultured on 3-D collagen matrices was maximal at 5.3 dyn/cm<sup>2</sup> WSS and decreased at 12 dyn/cm<sup>2</sup> WSS (54). Furthermore, Akt inhibition diminished EC invasion responses induced by the combined application of WSS and S1P (54). S1P<sub>1</sub> receptor activation in ECs stimulates Akt activation via pertussis toxin-sensitive G<sub>i</sub> proteins (41, 64), and S1P-induced migration (64) and 3-D invasion (5) are blocked by pertussis toxin. Interestingly, EC migration and tubulogenesis following preshearing are also attenuated by pertussis toxin (22). Akt has several downstream targets implicated in angiogenesis, including endothelial nitric oxide synthase (eNOS) (29), hypoxia-inducible factor (42), and the transcription factor FOXO (19). WSS regulation of eNOS activity has an important role in the regulation of vascular remodeling and angiogenesis. Dickinson and colleagues (66) reported that WSS is critical for vessel remodeling and eNOS expression in mouse embryos. WSS induces Akt phosphorylation at Ser473, which then mediates eNOS activation and subsequent NO

production (29). S1P and VEGF are also reported to stimulate the phosphoinositide 3-kinase (PI3K)/Akt pathway to induce eNOS activation (19, 29). WSS (12 dyn/cm<sup>2</sup>) induces tyrosine phosphorylation of the junctional protein PECAM-1 in ECs, and PECAM-1 expression is necessary for shear-induced Akt and eNOS phosphorylation, suggesting that PECAM-1 may act as the mechanosensor for this pathway (35).

**Transcriptional regulation**—An important component of homeostatic mechanisms linking vascular oxygen supply with demand is the regulation of angiogenesis by hypoxia. Hypoxia increases the expression of VEGF and PDGF in ECs and this is mediated by activation of hypoxia-inducible transcription factors (HIF) (19, 29). HIF is a heterodimer composed of  $\alpha$  and  $\beta$  subunits. HIF-1 $\alpha$  is stabilized by hypoxia, while HIF-1 $\beta$  is constitutively expressed in the nucleus. Activated HIF-1 $\alpha$  and HIF-2 $\alpha$  bind to hypoxia response elements (HREs) to increase promoter activity, while HIF-3 $\alpha$  appears to negatively regulate transcription. After 24 h of 16 dyn/cm<sup>2</sup> WSS, HIF-1 $\alpha$  and HIF-2 $\alpha$  expression were reduced significantly relative to static controls and HIF- $\alpha$  proteins were undetectable (71). Consistent with the regulation of VEGF-A by both HIF isoforms, VEGF-A expression was significantly reduced by 16 dyn/cm<sup>2</sup> WSS (71). These results suggest long-term application of laminar WSS promotes vascular stabilization by downregulating HIF and VEGF production. Interestingly, NO can either drive HIF-1 $\alpha$  stabilization under normoxia or degradation under hypoxia (4).

The O subgroup of forkhead transcription factor (FoxO) proteins are involved in vascular development and maturation. Loss of FoxO1 expression results in embryonic lethality caused by severe defects in vascular development (37). The FoxO protein family is regulated primarily by posttranslational modifications, including phosphorylation, acetylation, and ubiquitination, which control protein levels, subcellular localization, and transcriptional activity (25, 90). FoxO factors are negatively regulated by Akt; dephosphorylated FoxO1 localizes to the nucleus and exhibits transcriptional activity, whereas Akt-dependent phosphorylation of FoxO1 induces nuclear export and allows proteasomal degradation (2, 10, 16). Activation of the FoxO pathway increases expression of Angiopoietin-1, which promotes vascular stability, and (although it is not well-understood) also increases expression of its antagonist, angiopoietin-2 (24, 40). FoxO1 and FoxO3a may elicit anti-angiogenic effects in part by repressing eNOS (84). Haas and colleagues demonstrated that chronic ischemia reduced Akt signaling, and enhanced FoxO1 levels, which ultimately suppressed angiogenic responses (73). FoxO1 binding activity and nuclear translocation were also reduced by 30 dyn/cm<sup>2</sup> WSS, but were not affected by 1 dyn/cm<sup>2</sup> WSS. Chlench et al. (19) reported that 6 dyn/cm<sup>2</sup> WSS downregulates FoxO1 expression and increases Foxo-1 exclusion from the nucleus. Meanwhile, WSS at 6 dyn/cm<sup>2</sup> (vs. static) had no effect on Ang-1 expression. Together, these studies indicate that Ang-2 induction via Foxo-1 is inversely related to the magnitude of applied WSS. Whether or not these signals are affected by the S1P signaling pathway in either 2-D or 3-D settings remains to be investigated.

**Matrix proteolysis**—Matrix metalloproteinases (MMPs) are zinc-dependent enzymes that can cleave extracellular matrix proteins including collagen type I and fibrin to control tissue morphogenesis (76, 113) and vascularization (62) by regulating many biological processes including liberation of growth factors (55, 76). A link between mechanotransduction and MMPs has been extensively reviewed (23). Various studies have linked MMPs and their endogenous inhibitors to regulating angiogenic sprouting and invasion events (1, 62, 102–103, 110). The membrane-type matrix metalloproteinases (MT-MMPs) have been shown to play a critical role in invasion of 3-D matrices by degrading ECM proteins at the cell surface-ECM interface (5, 91). MT-MMPs allow highly-regulated proteolysis to occur at the cell surface, while maintaining the integrity of the supporting ECM scaffold (46). MT-

MMPs coordinate with integrins and growth factors to direct angiogenic sprouting and lumen formation (5, 88), and mice lacking MT1-MMP, (but not MMP-2 or MMP-9), exhibit defective sprouting responses (20). Thus, membrane-associated metalloproteinases are critical in mediating successful angiogenic responses. Using gelatin zymography, we measured increases in pro and active forms of MMP-2 in the perfusate that was highest at 5.3 dyn/cm<sup>2</sup> WSS and significantly lower when WSS was either 0.12 or 12 dyn/cm<sup>2</sup> (54). Active MT1-MMP cleaves pro-MMP-2, and in HUVEC, MMP-2 activation is a reliable indicator of MT1-MMP activity (63). In addition, a number of studies have investigated the effects of WSS on MMP activity and expression. Microvascular ECs produced more MMP-2 mRNA and protein levels when subjected to 5 dyn/cm<sup>2</sup> WSS than to 16 dyn/cm<sup>2</sup> WSS (72). MMP-2 secretion is downregulated by 30 dyn/cm<sup>2</sup> WSS in bovine aortic ECs (BAECs) (116), while MMP-2 secretion was upregulated by 8 dyn/cm<sup>2</sup> WSS in human saphenous vein ECs (HSVEC) (99). Furthermore, MMP-9 expression and activation increased in HSVEC and HUVEC subjected to WSS in the range of 4 to 8 dyn/cm<sup>2</sup> (99). These results are consistent with MMP activity being increased by WSS at intermediate, but not high, magnitudes.

A Disintegrin and Metalloproteinases (ADAMs) are membrane-associated proteinases that contain N-terminal zinc-dependent metalloproteinase, disintegrin, transmembrane and cytoplasmic tail domains. Like MT-MMPs, anchoring at the plasma membrane renders these molecules perfectly poised to mediate shedding events. ADAM17 was originally identified as tumor necrosis factor (TNF)-alpha-converting-enzyme, or TACE, because of its ability to mediate TNF- $\alpha$  release (12). Other substrates for ADAMs include adhesion molecules, surface receptors and members of the epidermal growth factor family (13–14). Like MT1-MMP, ADAMs also co-localize with integrins (34). Functional ADAM17 appears to be required for normal embryonic development and most transgenic animals lacking the ADAM17 metalloproteinase domain die between embryonic day 17.5 and birth (83). Regarding angiogenesis, pathological retinal neovascularization and growth of heterotopically injected tumor cells was reduced in mice lacking ADAM17 expression in ECs. Moreover, silencing of ADAM17 in ECs decreased endothelial sprouting responses (61) and cord formation on Matrigel (114). Mice lacking expression of ADAM15, ADAM8 and ADAM9 exhibit reduced neovascularization responses in a model of retinopathy of prematurity (43–44, 49), and ADAM10 null mice die at day 9.5 of embryogenesis, a critical time at which the vasculature is forming (45). Recently, ADAM17 has been implicated in mechanotransduction by compressive stress in airway epithelial cells (96). Whether the ADAMs family of metalloproteinases is modulated by mechanical signals remains to be demonstrated. Clearly, ADAM proteinases regulate vasculogenesis and angiogenesis *in vivo* and *in vitro*. Recombinant, soluble ADAMTS1 inhibited angiogenesis in corneal pocket and chick chorioallantoic angiogenic assays (107). In rat mesentery, Hohberg et al. (48) demonstrated positive immunostaining for ADAMTS1 in microvessels experiencing flow, while there was a lack of ADAMTS1 staining in unperfused sprouts. Hohberg et al. also reported that ADAMTS1 expression and liberation of TSP fragments in HUVECs increased monotonically with WSS in the range of 1 to 20 dyn/cm<sup>2</sup> (28). These results are consistent with an anti-angiogenic role for ADAMTS1, where ADAMTS1 expression is high in stabilized, non-angiogenic vessels experiencing high WSS, but is downregulated in sprouting structures where WSS is low. In contrast, ADAMTS1 is expressed in the highly vascularized tissue during ovulation (15, 74, 86), suggesting a positive effect on angiogenesis. In our 3-D EC invasion assay, increased expression of full length, cell-associated ADAMTS1 correlated with endothelial sprouting responses, and ADAMTS1 silencing inhibited sprout formation (94). ADAMTS1 can degrade collagen (85), consistent with our data showing that decreasing ADAMTS1 expression levels resulted in decreased endothelial sprout density and length (94). ADAMTS1 can cleave and release anti-angiogenic polypeptides from matrix-bound Thrombospondin 1 and 2 (TSP1 and TSP2),



and TSP1 is critical for the anti-angiogenic response mediated by soluble ADAMTS1 (65). The C-terminus of ADAMTS1 can be cleaved to release the last two TSP domains and liberate ADAMTS1 from the cell surface. The cleavage event requires metalloproteinases, which may include MMP-2, MMP-8, and MMP-15 (87). In our 3-D invasion system, ADAMTS1 remains associated with the endothelial surface (98), suggesting a more limited ability to release the TSP domains within ADAMTS1. Altogether, these data indicate that the ability of ADAMTS1 to promote angiogenesis depends on the solubility state of ADAMTS1, as well as other local factors, including TSP and MMPs, which must be tested and considered carefully.

### Clinical Relevance of WSS Effects on Angiogenesis

Angiogenesis plays a critical role in wound healing and tumor vascularization (36, 104). Experimental and computational studies indicate that WSS and S1P modulate microvascular growth during these pathogenic events. During wound healing, the levels of blood flow and cytokines such as S1P provided by activated platelets are increased in the wound area (47). Local hemodynamics have been implicated in microvascular growth within tumors (78). *In silico* models incorporating WSS-dependent microvascular growth rules have been used to describe angiogenic network formation during tumor growth and wound healing (18, 67, 81). Quantitative relationships between WSS and angiogenic sprouting responses can be used to inform such models and may potentially lead to a more accurate description of angiogenic network formation.

## CONCLUSIONS

Post-capillary venules have relatively thin walls that are particularly permissive to angiogenic sprouting. The WSS at these locations appears to also prime ECs to respond to pro-angiogenic signals such as S1P. It remains unclear how cells perceive the magnitude of WSS to regulate an angiogenic response. While it is tempting to draw upon the vast literature of WSS mechanotransduction studies, results obtained from flow experiments performed on 2-D substrates do not always translate directly to endothelial mechanoresponses on 3-D matrices. For example, Akt activation increases monotonically in response to WSS for ECs on 2-D substrates (28), but Akt shows maximal activation at an intermediate WSS of 5.3 dyn/cm<sup>2</sup> for ECs on 3-D collagen matrices (54). ECs are sensitive to matrix stiffness (117), so it is very possible that the endothelial response to WSS on pliable collagen matrices may differ significantly from that of ECs on stiff matrix-coated glass slides.

This review focused on the effects of luminal WSS on angiogenesis, but ECs are also subjected to fluid forces generated by fluid flow through the matrix, i.e. interstitial flow. There is strong evidence that interstitial flow has profound effects on endothelial network morphology in 3-D matrices (80, 109). Cell culture systems capable of controlling both tangential WSS and interstitial flow are expected to provide even greater insight into the roles of fluid forces on sprouting angiogenesis.

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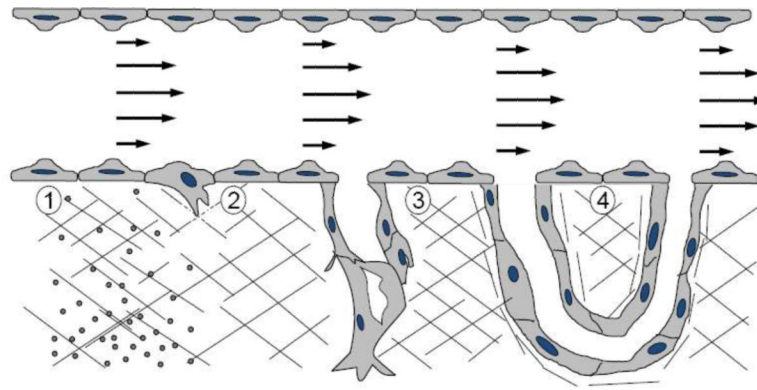
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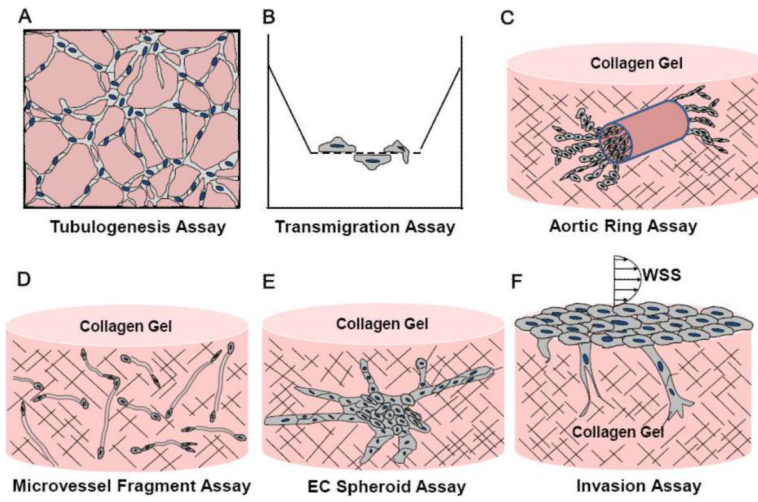
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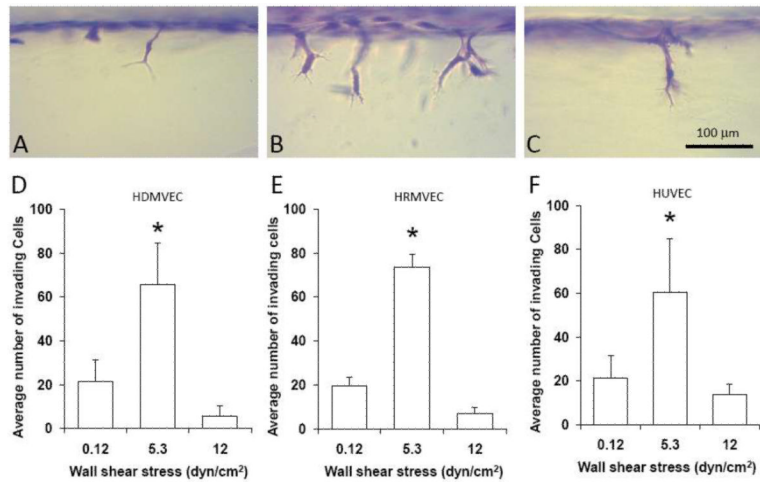


**Figure 1.**

The process of angiogenesis occurs as an orderly series of events. 1) Fluid WSS and a chemotactic concentration gradient of biochemical agonists synergistically activate ECs lining a pre-existing blood vessel. 2) Activated ECs begin to cleave the underlying extracellular matrix through enzymatic proteolysis and migrate into newly-formed passages. 3) A lumen forms while additional ECs migrate and proliferate behind the tip cell to form the stalk. 4) Sprouts join at their tips, anastomosing with existing vessels, to form a new path for blood flow.



**Figure 2.**  
Experimental models for studying angiogenic events.



**Figure 3.**

EC invasion depends on the magnitude of WSS. Human dermal microvascular ECs (HDMVECs) on 3-D collagen matrices containing 1 μM S1P were subjected to 24h of 0.12 (A), 5.3 (B) and 12 dyn/cm<sup>2</sup> WSS (C). The cultures were fixed and stained with toluidine blue to identify invading cells. Scale bar, 100 μm. Quantification of HDMVECs (D), human retinal MVECs (E) and HUVECs (F) invasion into 3-D collagen matrices containing 1 μM S1P subjected to 22h of WSS ranging from 0.12 to 12 dyn/cm<sup>2</sup>. Cultures were fixed, stained with toluidine blue for morphometric analysis and analyzed for invasion density. The density of invading ECs are plotted as a function of WSS magnitude (mean ±SD). \* indicates the invasion density at 5.3 dyn/cm<sup>2</sup> is significantly different from the values at the other WSS magnitudes (ANOVA followed by Student-Newman-Keuls post-hoc test; P < 0.01).

**Table 1**

Effects of WSS magnitude on angiogenic events and signaling. Experiments were performed using HUVECs unless otherwise stated.

	<2 dyn/cm <sup>2</sup>	3–8 dyn/cm <sup>2</sup>	>10 dyn/cm <sup>2</sup>	References
3-D invasion assay				
invasion density	↓	↑	↓	(54)
invasion depth		↑	↑	(54)
2-D Matrigel tubule formation assay	↑	↑↑	↑↑↑	(22)
Transwell cell migration rate	-	↑	↑↑	(22)
Ang-2 expression	↑	↓	↓	(40)
		↓		(19)*
TIE-2 expression		↑	↑	(19)*
	-	↑	↑	(40)
VEGF-A expression (in skeletal muscle microvascular EC)	↑		↑↑	(40)
			↑	(71)*
Ephrin-B2 expression	↑	↓	↓	(39)
Ephrin-B2 expression (in VEGFR+ ESC)		↑	↑	(70)
Ephrin-B4 expression (in VEGFR+ ESC)			↓	(70)
Notch			↑	(111)
Notch1 and Notch 4 expression (in VEGFR2+ ESC)		↓	↑	(70)
Akt Phosphorylation	↓	↑	↓	(54)
		↑		(19)*
		↑	↑↑	(29)
eNOS phosphorylation			↑	(29)
HIF-1 $\alpha$ , HIF-2 $\alpha$ expression (in skeletal muscle microvascular EC)			↓	(71)*
Foxo-1 binding activity	-		↓	(40)
Foxo-1 expression		↓		(19)*
Foxo-1 phosphorylation and nuclear exclusion		↑		(19)*
MMP-2 secretion	↓	↑	↓	(54)
		-	↓	(72)
			↓	(116)
		↑		(99)*
MMP-9 secretion		↑		(100)*
		↑		(99)*

	<2 dyn/cm <sup>2</sup>	3–8 dyn/cm <sup>2</sup>	>10 dyn/cm <sup>2</sup>	References
ADAMTS1 expression	-	↑	↑↑	(48)

\* vs. static condition