Endothelial Cell Migration During Angiogenesis

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Abstract—Endothelial cell migration is essential to angiogenesis. This motile process is directionally regulated by chemotactic, haptotactic, and mechanotactic stimuli and further involves degradation of the extracellular matrix to enable progression of the migrating cells. It requires the activation of several signaling pathways that converge on cytoskeletal remodeling. Then, it follows a series of events in which the endothelial cells extend, contract, and throw their rear toward the front and progress forward. The aim of this review is to give an integrative view of the signaling mechanisms that govern endothelial cell migration in the context of angiogenesis. (Circ Res. 2007;100:782-794.)

Key Words: endothelial cells ■ chemotaxis ■ haptotaxis ■ mechanotaxis ■ migration ■ angiogenesis ■ VEGF ■ integrins ■ actin ■ remodeling

Endothelial cells derive from the successive differentiation of mesodermal cells into hemangioblasts, which leads to the formation of the first vascular structures that are called primitive blood islands. The hemangioblasts from the center of the islands give rise to the hematopoietic stem cells, whereas the peripheral hemangioblasts differentiate into angioblasts, the precursors of mature endothelial cells. Under the influence of vascular endothelial growth factor (VEGF), the angioblasts and newly formed endothelial cells migrate on a matrix constituted mainly of collagen and hyaluronan, allowing the fusion of the blood islands, their remodeling into tubular structures, and the formation of the first primitive vascular plexus. These tubules remodel through vasculogenesis into larger vessels, leading to vascularization of the embryo.1 In contrast to vasculogenesis, angiogenesis refers to the formation of new blood vessels from preexisting ones (Figure 1). It is required in many physiological and pathological conditions, including embryonic development, wound healing, tissue regeneration, and tumor growth. The process is regulated by a tight balance between pro- and antiangiogenic agents and involves a cascade of events of which migration of capillary endothelial cells is an essential component.2

The basic concepts of the cellular and molecular machinery underlying endothelial cell migration have been obtained mostly from observations of cell culture systems (see the online data supplement, available at http://circres.ahajournals.org). However, the 3D environment within a whole organism is far more complex, and the cells must move between and among other cells while they interpret attractive and repulsive cues to choose their path. They must integrate and coordinate their adhesion with their surroundings and sense when to start and stop moving. Genetic dissection using in particular zebrafish3,4 and mouse embryo5 now offers the possibility to understand endothelial cell migration in a physiological context. The aim of this review is to make an integrative update on the signaling and physiological aspects of endothelial cell migration in angiogenesis.

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Actin remodeling at the heart of endothelial cell migration: An overview

Actin is a major cytoskeletal component of endothelial cells. It is composed of 43-kDa monomeric globular subunits (G-actin) that polymerize into helical filaments (F-actin). The assembly of F-actin is tightly associated with the hydrolysis of ATP by its intrinsic ATPase activity. Mg\(^{2+}\)/ATP-bound monomeric G-actin is incorporated into growing filaments at the barbed end. ATP-actin is then converted into ADP-actin, as actin monomers are shifted along the filaments toward the pointed end. The constant remodeling of the actin cytoskeleton into filopodia, lamellipodia, and stress fibers (Figure 2) is essential for cell migration, as briefly overviewed below and discussed in detail by K. Mizumo and colleagues in a forthcoming article within this thematic review series.

Filopodia are membrane projections that contain long parallel actin filaments arranged in tight bundles. These particular structures act as sensors of motile stimuli. Classically, the formation of filopodia is regulated by activation of the small GTPase Cdc42 that associates with Wiskott–Aldrich syndrome proteins (WASPs). Lamellipodia are cytoplasmic protrusions that form at the leading edge of spreading or migrating cells. These protrusions are approximately 1 to 5 \(\mu\)m wide and approximately 2 \(\mu\)m thick. The formation of lamellipodia is associated with important actin polymerization involving Rac and Arp2/3 complex. Stress fibers are actin filaments of inverted polarity linked by \(\alpha\)-actinin and myosin and distributed along contractile fibers. All 3 structures are essential to drive the several steps of actin-based endothelial cell motility as shown in (Figure 3A): (1) sensing of the motogenic signal by filopodia; (2) formation and protrusion of lamellipodia and pseudopodia-like forward extension; (3) attachment of the protrusions to the extracellular matrix (ECM); (4) stress fiber-mediated contraction of the cell body to allow forward progress; (5) rear release; and (6) recycling of adhesive and signaling materials.

Endothelial cell migration during angiogenesis

Endothelial cell migration involves 3 major mechanisms, namely chemotaxis, the directional migration toward a gradient of soluble chemoattractants; haptotaxis, the directional migration toward a gradient of immobilized ligands; and mechanotaxis, the directional migration generated by mechanical forces. Endothelial cell migration during angiogenesis is the integrated resultant of these 3 mechanisms. Typically, chemotaxis of endothelial cells is driven by growth factors such as VEGF and basic fibroblast growth factor (bFGF), whereas haptotaxis is associated with increased endothelial cell migration activated in response to integrins binding to ECM component. Because of their location at the inner face of blood vessels, endothelial cells are constantly in contact with shear stress, which contributes to activate migratory pathways. In fact, there is now accumulating evidence that fluid shear stress initiates mechanotaxis and modulates the various steps of migration including extension at the leading edge, adhesion to the matrix, and
release of adhesions at the rear. Hence, endothelial cell migration is a mechanically integrated molecular process that involves dynamic, coordinate changes in cell adhesion, signal transduction, and cytoskeletal dynamics and organization.

**Chemotactic Endothelial Cell Migration**

Many different cytokines are involved in the regulation of chemotactic endothelial cell migration during angiogenesis. The 3 major promoters of this type of actin-based motility are VEGF, bFGF, and angiopoietins. Other contributing cytokines include: FGF-2, hepatocyte growth factor, platelet-derived growth factor, epidermal growth factor, transforming growth factor-β, interleukins, tumor necrosis factor-α, platelet-activating factor, ephrins, soluble adhesion molecules, endoglin, and angiogenin. For the sake of integration and understanding, we have concentrated our review on VEGF and angiopoietins.

**Vascular Endothelial Growth Factors and Their Receptors**

VEGFs encompass a family of structurally related proteins that include placental-derived growth factor, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E.51 Human VEGF-A monomers exist as 5 different isoforms, of which VEGF 

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**Figure 2.** Actin structures involved in endothelial cell migration. HUVECs treated with VEGF form actin structures involved in endothelial cell migration (Houle François and J.H., unpublished observations, 2006). A, The filopodia are filamentous membrane projections that contain long parallel actin filaments arrange in tight bundles. These structures act as sensors of motile stimuli. B, The lamellipodia are cytoplasmic protrusions that contain a thick cortical network of actin filaments. They are found at the leading edge of migrating cells allowing their swimming-like motility. C, Stress fibers are bundles of actin filaments associated with myosin II and accessory proteins such as tropomyosins. These structures are anchored at focal adhesions and are required for the traction of the rear of the cells toward the leading edge during migration.

**Vascular Endothelial Growth Factor Receptor-2 in Chemotactic Endothelial Cell Migration**

**Role of the Rho Small GTPases**

The activation of the small GTPases of the Rho family is centrally involved in regulating endothelial cell migration in response to activation of VEGFR-2. In particular, Cdc42 is involved in the formation of filopodia; these structures that act as sensors, underlying the “guidance migratory mecha-
nism” recently shown in early postnatal angiogenesis in the retina. The concept of angiogenic guidance emerged by analogy with other guidance processes involving tip structures that contain sensor cells such as the growth cone in axonal guidance and tracheal tip cells during tracheal branching in Drosophila. In both cases, the sensor cells use Cdc42-induced formation of dynamic filopodia to sense guidance cues and to migrate coordinately. Interestingly, endothelial sprouts also extend multiple filopodia at their distal tips, indicating that growing vascular sprouts are endowed with specialized tip structures with potential functions in guidance and migration in response to a VEGF gradient. The activation of Cdc42 downstream of VEGFR-2 is also involved in the formation of stress fibers by contributing to activate the p38 pathway. On the other hand, VEGFR-2–mediated activation of Rac1 in concert with the activation of WAVE2 leads to the formation of lamellipodia assuring the swimming movement of the endothelial cells.

RhoA, by contributing to phosphorylation of VEGFR-2 and activation of phosphatidylinositol 3-kinase (PI3K), is also an important determinant of endothelial cell migration. The RhoA-mediated activation of PI3K regulates cell motility by generating phosphoinositides, like phosphoinositol3–5 triphosphate, that influence various downstream motogenic events. In particular, the PI3K-derived inositol phosphates, in conjunction with calcium influx generated by phospholipase Cγ, regulate the function of a number of actin-regulating protein, such as profilin, cofilin and α-actinin. PI3K also contributes to the activation of 3-phosphoinositide-dependent protein kinase-1 (PDK1), which leads to the activation of Akt/PKB (Akt/protein kinase B) and of a number of kinases of the AGC family potentially involved in cell migration. Notably, as described below, eNOS is activated by Akt/PKB and, by producing NO, plays a role in endothelial cell migration and angiogenesis. In addition to regulate endothelial cell migration via activation of the PI3K/NO pathway, the molecular link between VEGFR-2 and RhoA further involves activation of the Rho-associated kinase (ROCK) and focal adhesion kinase (FAK) phosphorylation on Ser732. This elicits an unfolding of FAK that unmask Tyr407 making it accessible to Pyk2, also activated by VEGF downstream of α,β integrins. This aspect will be addressed in more details in the section on “haptotactic cell migration.”

Role of NO
The role of NO as a major regulator of cell migration and angiogenesis is suggested by the observation that it is quickly produced by eNOS following its activation downstream of the VEGFR-2/PI3K/Akt-PKB axis in endothelial cells activated by VEGF. It is further supported by the finding that knockout mice for eNOS show impaired angiogenesis in response to ischemia. Moreover, inhibition of NO production blocks the chemotactic actions of VEGF, and NO transduces the increase of migration that results from VEGF exposure of bovine lung microvascular endothelial cells expressing an activated form of PKB. Interestingly, eNOS is located in caveolae, a subset of lipid rafts that are prevalent on the plasma membrane of endothelial cells. This particular localization is of utmost importance in modulating the
motogenic property of eNOS and NO. In particular, caveolin-1, a typical resident protein of caveolae, interacts with eNOS and negatively regulates its activity. Given that caveolin-1 is also associated with VEGFR-2, and that the complex is dissociated by VEGF, an attractive possibility is that VEGF can contribute to activate eNOS and NO production by dissociating eNOS from caveolin-1. Along these lines, eNOS cannot be properly activated in endothelial cells devoid of caveolae and the cells cannot migrate. Together, these findings indicate that NO is an important messenger of VEGFR-2. It presumably modulates angiogenesis by inducing a vasodilatation-associated expansion of endothelial cell surface that enables a more proper response of the endothelium to angiogenic and promigratory agents.

Role of Other Reactive Oxygen Species

The influence of ROS in regulating endothelial cell migration is not restricted to NO. In fact, ROS produced via activation of NADPH oxidase stimulate diverse redox signaling pathways leading to angiogenic responses including endothelial cell migration. In particular, VEGF stimulation increases ROS production via activation of Rac1-dependent NADPH oxidase and, thereafter, ROS are involved in VEGF-induced autophosphorylation of VEGFR-2. The signaling properties of ROS are caused by the activation of several kinases including Src kinases and to the reversible oxidation of redox-sensitive protein tyrosine phosphatases (PTPs) and lipid phosphatase (PTEN). In fact, several PTPs including SHP-1 and SHP-2 associate with VEGFR-2 in response to VEGF. Their activation by ROS might contribute to stop the VEGF signals by dephosphorylating VEGFR-2. These specific aspects of VEGF signaling upstream of endothelial cell migration have recently been covered in an excellent review and will not be further developed here.

Role of Nck

In response to VEGF, the increased actin polymerization required to trigger actin-based motility involves the recruitment of Nck to VEGFR-2. At least 2 converging signaling mechanisms were identified in endothelial cells. A first signal that emanates from VEGFR-2/Nck is associated with the recruitment of Nck to phospho-Tyr1214 within VEGFR-2 and it mediates actin polymerization and formation of stress fibers downstream from sequential activation of Fyn, Cdc42, MKK3 (mitogen-activated protein kinase kinase 3), SAPK2p38a (stress-activated protein kinase 2/p38a), and MAPKAP K2 (mitogen-activated protein kinase activated protein kinase 2) and phosphorylation of heat shock protein 27 (HSP27). HSP27 is an actin-capping protein, and its phosphorylation has been proposed to release it from actin filaments, thus allowing addition of actin monomers and elongation of the filaments. A second mechanism downstream of Nck recruitment to VEGFR-2 in endothelial cells involves the nucleation factor N-WASP (neuronal WASP), which is relocated at the cell surface by Nck. Then, N-WASP activates the Arp2/3 complex, a key regulator of actin nucleation and stress fibers in motile cells. Interestingly, Nck recruitment to VEGFR-2 triggers the assembly of focal adhesions via PAK activation, which may also contribute to enable the bundling of actin filaments into stress fibers. On the other hand, the activity of LIM kinase also regulates actin remodeling and formation of stress fibers downstream of PI3K/ROCK and p38/MAPKAP K2 activation by VEGF. In turn, activated LIM kinase leads to phosphorylation of cofilin impairing actin depolymerization and thereby potentiating the VEGF-induced actin reorganization into stress fibers and endothelial cell migration (Figure 4A).

Intriguingly, the residue Y1214 within VEGFR-2 is phosphorylated in all endothelial cell types, whereas phosphorylation of Y951 is restricted to certain endothelial cells that belong to immature vessels devoid of pericytes. The adapter molecule TSAd/VRAP is recruited to phospho-Y951 within VEGFR-2, where it is tyrosine phosphorylated and forms a complex with Src to regulate stress fiber formation and endothelial cell migration. In turn, this contributes to increase endothelial cell migration during pathological angiogenesis and may explain why the recruitment of TSAd/VRAP is associated with cancer angiogenesis.

Overall, these findings indicate that endothelial cell migration induced by VEGF results from several signaling pathways downstream of VEGFR-2 (Figures 3A and 3B and 4). Notably, the complementary role of signaling through p38 (actin polymerization) and FAK (focal adhesion turnover) in contributing to the formation of stress fibers emerges as an integrating concept given that these structures generate the contraction force required to pull the back of the cell and allow directed migration (Figure 4A and 4B).

Angiopoietins As Inducers of Endothelial Cell and Pericyte Migration and Their Roles in Vascular Stability

Angiopoietins (Ang) 1 to 4 are proangiogenic growth factors that specifically activate endothelial cells in a paracrine manner. Their action depends mainly on their binding to the tyrosine kinase receptor Tie-2, whereas the ligand for the related Tie-1 receptor remains to be identified. Ang1 is an activator of Tie-2 and Ang2 is known to antagonize the binding of Ang1 to Tie-2. Nonetheless, Ang2 also activates Tie-2 in a context-specific manner. Similar phenotypes are observed in knockout mice for Ang1 or Tie-2. The primary vasculature is normal, but the mice die at embryonic day 12.5 from endocardial and myocardial defects. A role for Ang3 and Ang4 is less defined, and knockout-mouse experiments have yet to be done. However, it seems that Ang4, but not Ang3, is capable of binding and activating Tie-2. Ang2 is found at high levels at sites of vascular remodeling in adults. Ang3 expression is high in most tissues, whereas Ang4 expression seems to be restricted to the lung. These 2 members of the family are considered as interspecies orthologs (Ang3, mouse; Ang4, human).

Angiopoietins have low mitogenic or proliferative activity for endothelial cells. Nevertheless, Ang1 promotes in vivo angiogenesis in a Matrigel plug assay and both Ang1 and Ang2 increase endothelial cell migration and sprouting. In particular, Ang1 induces polarized lamellipodia formation that is associated with the spatial redistribution of RhoA and Rac1, which may modulate their migratory functions.
Moreover, binding of Ang1 to Tie-2 initiates the recruitment of Dok-R to Tie-2, which elicits the activation of a signaling cascade that involves Nck and Crk, upstream of actin remodeling and cell migration.68 Cell migration in response to Ang1 may also result from the activation of PI3K downstream of VEGFR-2, which contributes to the formation of stress fibers enabling cell migration by allowing endothelial cell contraction. The activation of PI3K downstream of VEGFR-2 activates Akt/PKB and eNOS. The subsequent production of NO is required for vasodilatation and endothelial cell migration. B, Actin stress fibers are anchored at focal adhesions, as shown in the colocalization of actin and vinculin, in porcine endothelial cells treated with VEGF. Ang2 induces endothelial cell migration, proliferation, and sprouting, as well as an increase in the diameter of capillaries. In contrast, in the absence of VEGF, Ang2 does not promote cell migration; rather, it induces apoptosis of endothelial cells and regression of blood vessel.70 Ang1 alone and Ang2 in the presence of VEGF are thus positive regulators of angiogen
esis by a direct action on endothelial cell migration. Interestingly, hypoxia induces the expression of the angiopoietin-like family member ANGPTL4, which is followed by its storing in the surrounding ECM. In turn, immobilized ANGPTL4 limits the formation of focal adhesions by endothelial cells and thereby negatively regulates endothelial cell migration, angiogenesis as well as metastasis.71,72 These new findings are important because they highlight other functions for angiopoietins in regulating endothelial cell migration and angiogenesis and because ANGPTL4 may reveal to be a useful new target to design antiangiogenic therapeutics in cancer.

In addition to their roles in regulating endothelial cell migration, another major contribution of angiopoietins to angiogenesis relies on their essential role in vessel stabilization and remodeling in vivo. The vessel stabilization induced by Ang1 may result directly from an inhibition of endothelial permeability or indirectly through stimulation of endothelial cell-dependent release of attractants like TGF-β and platelet-derived growth factor-B, which leads to an increased migration of pericytes and their recruitment to the nascent vessel.73 The angiopoietin-mediated recruitment of pericytes to the growing vessels is further regulated by FGF-4-induced expression of VEGF, which contributes to increase the levels of matrix metalloprotease-1 (MMP-1) and inhibits tissue inhibitor of metalloprotease-1 (TIMP-1), thus facilitating pericyte invasion via ECM degradation.74,75 Following their recruitment to the nascent vessels, pericytes play a major role in vascular stability by inducing the deposit of a matrix and initiating signals that allows endothelial cell differentiation and quiescence.76

In sum, angiopoietins are major regulators of angiogenesis either by a direct modulation of endothelial functions such as actin remodeling and migration or by an indirect effect that is mediated through cytokines released by activated endothelial cells, which in turn activates pericytes via a paracrine pathway.

**Haptotactic Endothelial Cell Migration**

Gradients of immobilized ECM components such as collagen drive endothelial cell migration independently of chemotactic cytokines.77 This type of migration is called haptotaxis, and it may be defined as directed cell crawling toward ECM proteins. The adhesive interactions that trigger haptotaxis of endothelial cells are governed mainly by components of ECM and integrins.

**Role of ECM in Haptotactic Endothelial Cell Migration**

The vascular endothelium is supported by an ECM that is assembled by endothelial cells, pericytes, and supporting smooth muscle cells. This ECM is critical for endothelial cell migration. In fact, depending on its nature and the cellular context, it may promote or inhibit endothelial cell migration. In the absence of angiogenic stimulus, the ECM contributes to maintain the endothelial cells in a quiescent state. However, during the early steps of angiogenesis, the ECM is broken down by metalloproteases, which initiates motogenic signals generated either by the proteolytic fragments or the release of embedded angiogenic stimuli (VEGF, bFGF).78 We review here the role of ECM in initiating and sustaining endothelial cell migration.

Vascular endothelial cells should adhere to ECM to migrate either dependently or independently of chemoattractants. In that regard, many of the interstitial and provisional ECM components encountered during angiogenesis, such as fibrin and collagen I, are capable of supporting chemotactic migration.79 On the other hand, several studies have shown that gradients of ECM components such as fibronectin can, by themselves, guide and regulate the speed of haptotactic cell migration.80,81 Although the significance of haptotaxis in vivo has been proven for several types of cells including T cells, its role is still debated in the case of endothelial cells.77,82 Nevertheless, given that interstitial collagen has marked haptotactic properties in vitro, it is plausible that the high concentrations of interstitial collagen encountered by endothelial cells during sprouting may drive haptotactic cell migration in vivo.83 Haptotaxis may be especially important in driving endothelial cell migration during repair of large vessel.84

The individual importance of specific ECM proteins in supporting haptotactic and chemotactic endothelial cell migration is still unclear because of signaling and functional overlap. For example, cytokine-dependent chemotactic cell migration may be supported by functional converging signaling initiated by the attachment to 2 different ECM. Moreover, a given integrin can bind to similar sites (eg, RGD peptide) on 2 different ECM to promote similar haptotactic signals. Furthermore, a variety of ECM components provide sufficient support for endothelial cell migration, although not with equal potency. In addition, there is evidence indicating that components of ECM function cooperatively.85 For example, laminins 8 and 10 have complementary effects on human skin dermal microvascular endothelial cells migration and tube formation.86 Interestingly, as we reported above for ANGPTL4, ECM proteins or their breakdown products may act as scaffolds that sequester cytokines that may either initiate or inhibit endothelial cell migration. These ECM/cytokine scaffolds may provide determinant cues to guide endothelial cell migration and sprout formation. In particular, collagenous matrices may function as release carriers of bFGF and VEGF. This feature is clinically promising and offers the possibility to generate a scaffold able to control angiogenesis and tissue regeneration.87,88

Endothelial cells are connected to ECM at focal adhesions. These latter are sites of tight adhesion between the membrane and the ECM on one hand, and the membrane and the cytoskeleton on the other. They are assembled following the recruitment of signaling molecules such as FAK and paxillin and of structural and membrane actin-anchoring proteins such as talin, vinculin, tensin, and α-actinin, which links the microfilament network to the adhesive molecules integrins at their sites of clustering.89,90 These proteins provide a structural link allowing the anchorage of stress fibers to the membrane and to integrins. In migrating endothelial cells, focal adhesions and actin stress fibers are aligned in the direction of migration, supporting their participation in the process of actin-based motility (Figure 4B).91 Moreover, in migrating endothelial cells, the forward movement is tightly
associated with the rapid assembly/disassembly of the focal adhesions, which allows the adhesion/de-adhesion processes inherent to migration.

**Integrins in Haptotactic Cell Migration**

Integrins are a family of heterodimeric transmembrane adhesion receptors that contains 16 α and 8 β subunits that associate to form 24 different receptors that bind to ECM with distinct yet often overlapping specificity. Several studies pointed to αβ integrin, a receptor for both fibronectin and vitronectin, and αβ integrin, a vitronectin receptor, as major players in blood vessel formation. Indeed, the blockade of both αβ and or αβ integrins disrupts tumor and experimental angiogenesis. For example, patients with melanoma benefit from Vitaxin, a α integrin antagonist. On the other hand, genetic experiments showed that successful vasculogenesis and angiogenesis depend on fibronectin and αβ integrin rather than on α integrins. Accordingly, antagonists of integrin αβ block tumor angiogenesis and have entered clinical trials. The apparent discrepancy between these results is difficult to explain but likely results from cellular context specificity. Nevertheless, they all point to the crucial role played by integrins in angiogenesis.

Integrins modulate angiogenic migration by enabling endothelial cell to adhere to ECM (Figure 5). Notably, the activation of integrins allows the functional connection between focal adhesions and actin cytoskeleton that is required to drive cell migration. Moreover, integrins are active in initiating and regulating angiogenic signaling. Signaling from integrins requires their oligomerization and engagement with their ligands and it starts from focal adhesions. Activation of integrins at focal adhesions may be triggered by a higher density of ECM, which in turn stimulates Rac and Cdc42 to induce actin remodeling, presumably through activation of the Arp2/3 complex and induction of actin filament via WASP. Alternatively, the Rho GTPases also activate PAK, which enhances the level of polymerized actin by activating LIM kinase. Interestingly, Rac and Cdc42, via an inside-out mechanism, may recruit more integrins at the cell protrusions thus forming a positive-feedback loop to amplify the haptotactic signal that originates from the concentration gradient of ECM. Possible elements upstream of the GTPases, especially Rac, may involve tyrosine phosphorylation of the docking protein Cas (Crk-associated substrate) and the recruitment and binding of Crk (CT10 regulator of kinase) and the activation of the GTPase-activating proteins DOCK180 and ELMO. The Cas/Crk scaffold module is present in endothelial cells and its activation is essential for integrin-directed cell migration. In particular, the activation of this pathway is induced following the activation of integrin αβ and Rac by laminin-10/11, which triggers the migration of endothelial cells. On the other hand, integrin-induced activation of RhoA mediates the assembly and contraction of the actomyosin fibers, which in turn contributes to pulling the trailing edge forward during migration. The cascade downstream of RhoA involves a cooperative interaction between mDia and ROCK to induce the assembly of actomyosin fibers. In that regard, activation of ROCK inhibits myosin light chain (MLC) phosphorylation, thus increasing the level of phospho-MLC and contraction of the acto-
myosin fibers.98 Phosphorylation of MLC during collagen-mediated haptotaxis may also be induced via an ERK-dependent activation of MLCK.10

Crosstalk Between Integrins and Growth Factors
There is a wealth of evidence that supports that integrins and growth factor signaling pathways interact to co-coordinately integrate the message initiated by both types of receptors. In particular, VEGF and bFGF enhance the expression and the activation of several integrins, such as \( \alpha_5\beta_1 \), \( \alpha_6\beta_1 \) and \( \alpha_5\beta_1 \), that are involved in angiogenesis.103 Conversely, several endogenous inhibitors of angiogenesis such as endostatin exert their functions by blocking integrins.99 Other crosstalk mechanisms involve interaction at the receptors level. For example, \( \alpha_5\beta_1 \) integrin mediates fibronectin-induced epithelial cell proliferation through activation of the EGF receptors.104 Similarly, \( \alpha_5\beta_1 \) integrin associates with platelet-derived growth factor-\( \beta \) and VEGF-2 to potentiate their activity.105–107 Little is known, however, about how the signals initiated by growth factor/integrin receptor complexes are integrated by the cells to activate the appropriate targets. Integrin-linked kinase integrates the insulin and fibropectin-dependent signals and FAK integrates the signal generated by integrins and the EGF and platelet-derived growth factor receptors.108,109 FAK is a converging signaling point between VEGFR-2 and integrin \( \alpha_5\beta_1 \) and it controls the assembly/disassembly of focal adhesions that is necessary with regulation of actin polymerization for endothelial cell migration. More recently, as mentioned previously, we found that activation of VEGFR-2 initiates the phosphorylation of Ser732 within FAK, which triggers a conformational change within the FAK structure, thereby unmasking Tyr407 making it accessible to direct phosphorylation by Pyk2, downstream of integrin \( \beta_3 \).17 Interestingly, FAK is also central to the regulation of endothelial cell migration by the VEGFR-2/\( \alpha_5\beta_3 \) complex.110–111 Some findings indicate that Src kinase can coordinate specific growth factor and ECM inputs by recruiting integrin \( \alpha_5\beta_3 \) into a FAK-containing signaling complex during growth factor-mediated biological responses.112 Thus, it seems that integrins \( \alpha_5\beta_3 \) and \( \alpha_6\beta_1 \) can induce endothelial cell migration in response to VEGF by cooperating with VEGFR-2. Nevertheless, if evidence exist that signals from these integrins transit through similar molecules such as Src and FAK, it is clear that both integrins have different implications in angiogenesis.113 Notably, integrin \( \alpha_5\beta_3 \) but not \( \alpha_5\beta_1 \) requires insulin-like growth factor stimulation for integrin-mediated cell migration in vitro and metastasis in vivo.113

Several studies have shown that cell–cell adhesions inhibit cell migration and that they should be quickly broken to allow migration. In that regard, VEGF is very efficient in disrupting endothelial cell–cell contacts by dissociating the VE-cadherin/\( \beta \)-catenin complex at adherens junctions.114,115 Interestingly, recent studies indicate that VEGFR-2–mediated angiogenic signaling is amplified by its association with VE-cadherin. Given that \( \alpha_5\beta_1 \) integrin–mediated adhesion on type I collagen decreases the localization of E-cadherin and \( \beta \)-catenin in cell–cell contacts, this finding raises the possibility that integrins may regulate VEGF signaling by modulating the VE-cadherin/VEGFR-2 interaction.116,117 These recent discoveries are important because they highlight that the VEGF-productive signaling does not only require crosstalk with integrins but also with cadherins.

Overall, the crosstalk between integrins and growth factors receptors enhances the individual potency of both receptors in signaling to cell migration. Nevertheless, much remains to be done to fully understand the mechanisms involved and how signals from both receptors contribute to coordinately integrate mototaxis and haptotaxis.

Mechanotactic Endothelial Cell Migration
Given that endothelial cells line the interior of the blood vessels, they are in constant contact with fluid shear stress, the tangential component of hemodynamic stress.9 Shear stress is a friction exerted on the endothelium by the blood flow. If uniformly applied over the entire surface of the endothelium, the force exerted by shear stress is of 1 pN/\( \mu \)m², which is roughly 1000 to 5000 times weaker than the traction forces of focal adhesions.119 However, shear stress regulates the migration of endothelial cells, presumably more so than does chemotaxis in large vessels where convection is stronger.9 In the microcirculation, shear stress may guide endothelial cells along the interstitial tunnels during wound healing. In that case, it promotes the formation of lamellipodia and endothelial cell migration in the flow direction. This suggests that the cytoskeleton and the cell–ECM interaction are polarized under flow. Shear stress influences each step of cell migration, as described in Figure 6, and it influences both chemotactic and haptotactic cell migration. The endothelial cells response to shear stress involves dissociation of the cell–cell contacts and the activation of several signaling pathways that remodel the actin cytoskeleton to trigger endothelial cell migration in a polarized manner.9

There is evidence indicating that the extracellular mechanical force generated by shear stress is sensed by integrins or by the cytoskeleton itself. The integrins lie on the basal surface of the cells, whereas blood flow exerts its pressure on the apical surface of the endothelium. A layer of glyocalyx protects the endothelium and the embedded glycoproteins, such as syndecan-4, transmit the forces generated by shear stress to integrins and to cell–cell adhesion components, via signaling to the cytoskeleton.118 Integrins are thus activated by shear stress, which in turn contributes to activate outside-in and inside-out motogenic signaling pathways involving particularly the Rho GTPases.

The small Rho-family GTPases are crucial regulators of actin dynamics in response to shear-stress. In fact, shear stress differentially regulates Rho GTPases at different location inside endothelial cells, presumably through regulating microtubules dynamics. In particular shear stress may induce microtubule elongation in the flow direction, which in turn activates Rac to promote actin polymerization and thus lamellipodia protrusion in the flow direction.9 On the other hand, Rac promotes microtubule elongation through PAK, thus forming a positive feedback loop for microtubule elongation and Rac activation at the cell front. Meanwhile, Rac inhibits the activation of RhoA in the lamellipodia so that RhoA activity is restricted to the back of the cell, generating...
the contraction required for rear detachment and migration.119 Alternatively, mechanotransduction through cell–ECM adhesions, cell–cell junctions, and endothelial cell plasma membranes may be involved in the shear stress activation of Rho GTPases. In particular, by increasing membrane fluidity, shear stress initiates signaling cascades from caveolae, which regulates the activity of specific Rho GTPases, especially RhoA, and eNOS.9,3,120,121 In turn, this will increase the contractility of stress fibers at the rear of endothelial cells. In addition, shear stress, by regulating the Ca2+ exchange from the caveolae, may enhance the activity of ROCK increasing actin/myosin contraction and promoting detachment at the rear of migrating endothelial cells.

Hence, shear stress is a continuous stimulus for endothelial cells and, by mechanically influencing the response of endothelial cells to haptotactic and chemotactic signals, it importantly contributes to angiogenic cell migration. A very nice and complete review on the topic has recently been published by the Li et al.9

Concluding Remarks

Endothelial cell migration is an essential component of angiogenesis that requires a tight regulation of the contractile and noncontractile states of the cell. These processes require the integration of signals elicited by chemotactic, haptotactic, and mechanotactic stimuli. In turn, this is associated with the activation of intracellular pathways that converge on cytoskeleton remodeling. This includes the pathways involving activation of the small GTPases of the Rho family, PI3K and eNOS; SAPK2/p38; and phosphorylation of FAK. A critical question for future studies will be to determine how these pathways interact and how their timing is adjusted in the cell to generate appropriate cell migration. Future studies should also attempt to integrate linear data into a 4D model defining the spatiotemporal parameters that regulate the motile potential of a moving cell. Another major challenge for the future is to better understand tensity and especially how actin, microtubules, and intermediate filaments cooperate to coordinate regulation of cell migration, as previously proposed.122–124 This will yield major breakthroughs in understanding cell migration and angiogenesis and will hopefully identify new targets for chemotherapeutic interventions.

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Experimental models to study endothelial cell migration

A number of *in vitro* and in vivo assays have been developed for studying angiogenesis and endothelial cell migration.

**Wound healing assay**

In this assay, endothelial cells are cultivated as a confluent monolayer and the layer is scraped with a rubber policeman or the pointed end of a sterile tip to create a cell-free zone. Thereafter, cells are washed with medium and stimulated with pro-angiogenic agents. Over time, the cells migrate to close the wound and the process is monitored under phase contrast microscopy. Endothelial cell migration is quantified by measuring the width of the cell-free zone (distance between the edges of the injured monolayer) before and after different exposures after stimulation at 5 distinct positions (every 5 mm). ¹

**Modified Boyden chambers assay**

The Boyden chamber consists of an upper chamber that is separated from a lower chamber by a porous membrane coated with an appropriate matrix. The endothelial cells are seeded on the top of the membrane and the lower chamber is filled with medium containing chemotactic pro-angiogenic agent. The cells sense and migrate toward the source of the chemo-attractant. After four hours, the cells that have crossed the membrane are stained and counted under the microscope. This approach is useful for the statistical quantification of the chemotactic migration process *in vitro*.²⁻⁴
**Tube formation in Matrigel**

This assay is somehow at the frontier between *in vitro* and *in vivo* models. Matrigel is liquid as 4°C but becomes a solid gel at 37°C. Endothelial cells are embedded in the gel and cell migration is evaluated by determining the formation of tubular structures. This type of assay provides the advantage of studying endothelial cell migration and tubule formation in the presence of surrounding non-endothelial cells, thereby mimicking the *in vivo* situation. Alternatively, a Matrigel plug containing cells or an angiogenic factor can be implanted subcutaneously in mice to test the extent of CD31-positive vessels formed in the plug.5, 6.

**Chick chorioallantoic membrane**

The chick chorioallantoic membrane (CAM) allows performing relatively inexpensive large scale *in vivo* studies. A test substance is prepared on a slow-release pellet or disc and implanted on the CAM through a window in the eggshell. The number of blood vessels can be counted using a stereomicroscope.7 This type of assay is widely used and allows for the study of pro- or anti-angiogenic molecules.

**Zebrafish**

The zebrafish was brought to the scientific scene in the lasts years as a model of choice for studying vasculature, vessel functions, blood flow and developmental angiogenesis. The zebrafish embryos are transparent and therefore allow the observation of the vasculature with a great optical clarity.7 The zebrafish model is used as a primary screening approach to assess compounds that affect angiogenesis.
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