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# Mechanical and Chemical Signaling in Angiogenesis

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# Contents

# Mechanical and Chemical Regulation of Arterial and Venous Specification

**Thomas N. Sato**

**Abstract** The fact that blood circulates through vessels was realized by William Harvey in the early seventeenth century. The blood flows away from the heart via arteries delivering oxygen and nutrients to peripheral organs and return to the heart via veins. Until late the 1990s, the distinction between artery and vein was recognized solely based on anatomical and function differences, basis, and it had been believed that arterial and venous specific characteristics are controlled by the respective hemodynamic forces that they are exposed to. However, in the past 15 years or so, it has become clear that they are also distinguished by the molecules that they express. Furthermore, their phenotypes are also regulated by genetic, hence, molecular (chemical), programs. In this chapter, I will summarize historical perspectives of the recognition of arteries and veins, and will review recent advance in our understating of mechanisms underlying arterial and venous specification mediated by mechanical and chemical signals. I conclude this chapter by proposing three models, morphogenetic, habituation, and integrative models, explaining how these two classes of signals become integrated to specify arteries and veins.

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## 1 Introduction

Blood circulates throughout the body. This was first clearly shown by a series of simple, but ingenious, experiments performed by William Harvey in the early seventeenth century [1]. This finding has lead to the establishment of the modern concept of "artery and vein".

The presence of the vascular system and its relationship to a variety of organs had already been documented by careful and impressive histological analyses of developing embryos by Aristotle in 300s BC. He had already speculated inter-dependence of the vascular system and organs in their establishment. However, he believed that pulsations in the vascular system were due to respiration, and the heart simply moved as a result of the same breathing process.

In the Middle Ages and early Renaissance, the concept based on Galenic physiology that had begun in the second century remained prevalent. According to this "ancient" understanding of the arteries and veins, all veins emanate from the liver and deliver fluids (blood) that maintain and nourish the body, and all the arteries from the heart disseminated vitality in the form of spiritus throughout the body.

By the end of fifteenth century, the Italian physician Jacopo Berengario da Carpi proposed a putative cross-talk between arteries and veins. He stated that "no artery without its vein to accompany it. Thus the artery may keep the vein alive, and the vein may give blood to the artery in its needs, the blood by which the vital spirit is made and the artery itself is nourished." This notion was followed by Leonardo da Vinci who stated: "All the veins and arteries arise from the heart. The reason for this is that the maximum thickness found in the veins and the arteries occurs at the junction which they make with the heart. The more removed they are from the heart, the thinner they become and divide into smaller branches".

By early sixteenth century, many anatomists already knew that the walls of the arteries are thicker than those of veins, but they attributed this difference to the nature of the substances that passed through each of them. Therefore, arteries and veins were still treated as two separate systems.

However, in the early seventeenth century, William Harvey unambiguously showed that the blood is delivered to the organ and then return to the heart, thus the establishment of the concept that arteries and veins belong to the single circulatory system [1]. He defined artery as the "vessel" that delivers the blood from the heart to peripheral organs, and vein as the "channel" through which the blood returns from the periphery to the heart.

## 2 Mechanical Basis of Arterial and Venous Specification (Specification by Mechanical Signals)

Due to their locations relative to the heart, arterial and venous lumens are exposed to differential hemodynamic forces. Two classes of hemodynamic forces are known to modulate structure and function of blood vessels: shear stress and pressure.

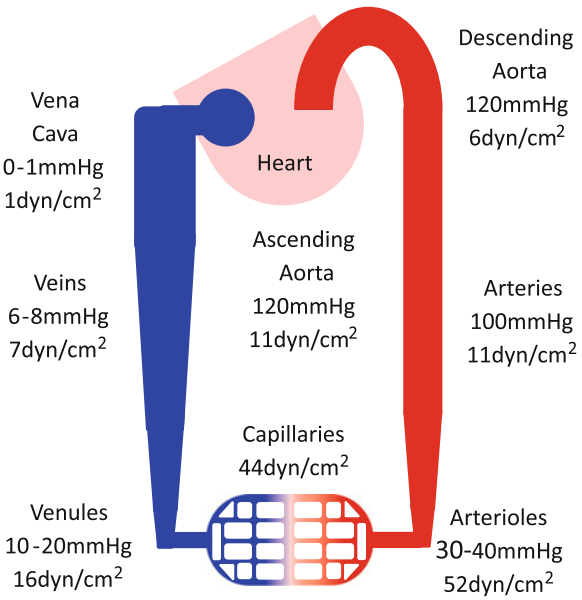


**Fig. 1** Two types of hemodynamic forces, shear stress and blood pressure, of various vascular beds. Shear stress (dyn/cm$^2$) and blood pressure (mmHg) of various arterial and venous vascular beds are indicated

These hemodynamic forces elicit significant impacts on vessel remodeling, vessel lumen size, morphology, orientation, cytoskeletal organization, ion channel activities and gene expression in endothelial cells that are under direct influences of blood flow [2–8].

The differences in the hemodynamic forces of arteries and veins play critical roles in their specification during vascular development and maintenance of their differential structure and function in adults.

## *2.1 Hemodynamic Differences among Different Vascular Beds*

The hemodynamic forces include both shear stress and pressure. Various vascular beds are exposed to differential shear stress due to the differences in flow speeds and lumen sizes [9]. As examples, the shear stresses of ascending aorta, descending aorta, conduit arteries, arterioles, capillaries, venule, large veins and vena cava are approximately 11, 6, 11, 52, 44, 16, 7, 1 dyn/cm$^2$, respectively [9] (Fig. 1).

Arteries and veins are also under differential blood pressure. The blood pressures of large arteries, arterioles, venule and large veins are approximately 100, 30–40, 10–20, and 6–8 mmHg, respectively [9]. Although such large differences in blood pressure are present between arteries and veins in adult, significantly smaller differences (1–2 mmHg) are present among embryonic vessels.

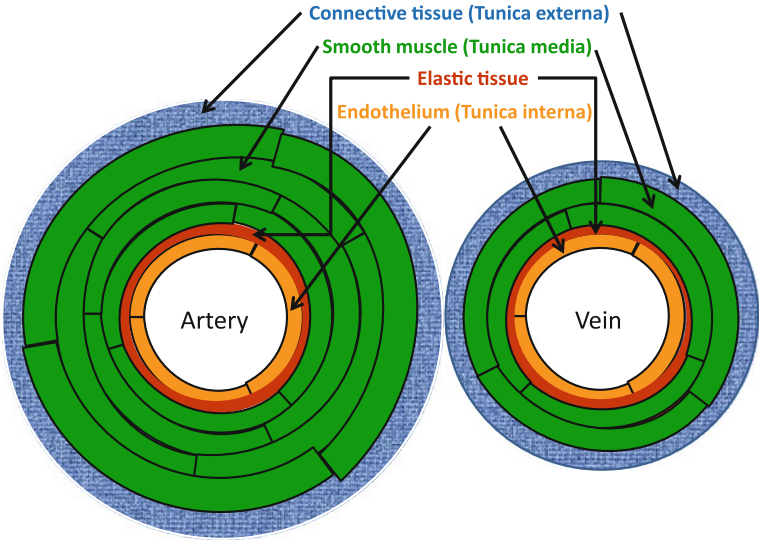


**Fig. 2** Distinctive anatomical structure of artery and vein. Each vessel consists of connective tissue (tunica externa), smooth muscle (tunica media), elastic tissue and endothelium (tunica interna) layers. Artery exhibits thicker smooth muscle layer (tunica media)

## *2.2 The Effects of Hemodynamic Forces on Anatomical Structure of Vasculature*

Blood vessels are formed by layer(s) of endothelial cells with elastic tissues and perivascular smooth muscle cells (Fig. 2). Arteries are supported by thick elastic smooth muscle layers so that they can withstand higher pressure. In contrast, veins contain thinner elastic smooth muscle layers with semilunar valves that prevent blood backflow. Therefore, arteries and veins adapt to differential hemodynamic forces that they are respectively exposed to.

## *2.3 The Effects of Hemodynamic Forces on Vascular Development*

It has been reported that blood flow cast major impacts on vascular network remodeling and vascular gene expression. However, its role in specification and/or maintenance of arteries and veins remains ill-defined.

In one study, no-flow mouse embryos were generated by snipping the inlets to the heart at both sides of the heart of 3- to 4-somite stage embryos [10]. In these no-flow embryos, as compared to normal embryos, no significant differences in the expression

of arterial markers such as Cx40, EphrinB2, and Dll4 were found. Furthermore, no differences in venous markers such as EphB4 and Nrp2 were detected.

In another study where embryonic explants and Rasip1$^{-/-}$ embryos were assessed as no-flow models, it was found that the expression of an arterial marker, Cx40, is significantly downregulated [11]. However, the expression of another arterial marker, Dll4, was found to be unaffected in these no-flow embryo models. This study, therefore, suggested that the blood flow is required to maintain the expression of certain arterial markers such as Cx40, but not others such as Dll4.

These two independent studies appear to contradict each other with respect to the putative role of blood flow in maintaining the expression of an arterial marker, Cx40. However, it was also suggested that some pulsative pressure from the beating heart to the aortae persisted in the "no-flow" model in the first study and was sufficient to generate the flow necessary to maintain the normal level of Cx40 expression [11].

In a third study, using time-lapse video microscopy, it was shown that a posterior arterial and an anterior venous pole in the yolk sac of developing chick embryos connect to each other by *cis–cis* endothelial interactions [12]. In contrast, the paired and interlaced arterial-venous pattern of the mature yolk sac was found to form by initially pinching off of the arterial domain of small caliber vessels from the growing arterial tree and by subsequently reconnecting to the venous system. This observation implies that arterial endothelial cells contribute to the formation of venous vessels, thus indicating the presence of phenotypic plasticity of endothelial cells in developing yolk sac vascular system. In this study, it was further reported that arteriovenous differentiation in developing yolk sac is dependent on blood flow. A no-flow model was generated by either performing longitudinal incision through all germ layers on the left side of yolk sac of embryos from the head to the tail or by ligating the right vitelline artery. In both models, the expression of the arterial markers, Nrp1 and EphrinB2 are significantly downregulated which was accompanied by concurrent upregulation of venous markers such as Nrp2 and Tie2. These results indicated that flow-dependent plasticity of endothelial cell phenotype (i.e. arterial vs. venous) is necessary to fashion arteriovenous patterning of developing vessels.

A variety of evidence reported in these studies appears to be in support of the idea that hemodynamic force is critical for the specification and/or maintenance of arterial and/or venous phenotypes. However, generating the no-flow model where nothing else but only blood-flow (i.e., mechanical signal) itself is "completely" abrogated is extremely difficult to accomplish in vivo. Thus, further studies are necessary to determine the definitive role of hemodynamic forces in arteriovenous specification and/or patterning.

## *2.4 The Effects of Hemodynamic Forces in Adult*

In contrast to the study of the flow-regulated arteriovenous specification in development, investigation on the role of hemodynamic forces in the arteriovenous plasticity in adult is lagging behind. In the adult vascular system, the identities of

endothelial cells as artery or vein have already been established. However, the hypothesis that these endothelial cells possess flow-dependent phenotypic plasticity has not been rigorously tested [13, 14]. Although it has been long known that venous grafts into the coronary artery (sometimes performed as a surgical procedure for heart diseases) induce changes in the graft morphology such as enlargement of grafted vessel diameter, the endothelial phenotypes are not thoroughly characterized at the molecular level.

## 3 Genetic Basis of Arterial and Venous Specification (Specification by Chemical Signals)

As discussed in the previous sections, it has been long believed that arteriovenous differentiation and maintenance of endothelial phenotypic identities are primarily determined by the differential hemodynamic forces that they are exposed to. However, more rigorous analyses are necessary to crystalize the hypothesis that blood flow modulates the arteriovenous phenotypes of endothelial cells. In contrast, the genetic basis of arteriovenous identity has been more rigorously studied, even though this idea emerged within less than 15 years ago.

It all began when a single serendipitous finding was made in 1998 by Wang et al [15]. They had been studying the role of the EphrinB2-EphB4 ligand-receptor system in nervous system development. They generated EphrinB2-deficient mice by introducing a lacZ reporter into the ephrinB2 gene locus (this mouse line is referred to as EphrinB2: lacZ). This genetic manipulation system was commonly used to disrupt a gene and at the same time it highlights the site(s) of expression of the disrupted gene in vivo.

By investigating this mouse line, they found that EphrinB2 is expressed only in a subset of the vascular system. Furthermore, the expression of a receptor for EphrinB2 ligand, EphB4, exhibited a perfect complimentary pattern to that of EphrinB2 in the developing vascular system. Surprisingly, this complimentary expression pattern of EphrinB2 and EphB4 was nearly the perfect mirror image of the arterial and venous vascular system, respectively, that can be found in all histology and embryology textbooks [15]. More surprisingly, it was discovered that the segregation of EphrinB2 and EphB4 expression to the arteries and veins, respectively, is established among nearly all vascular beds prior to the initiation of blood circulation. This finding casts doubt on the longstanding belief that blood circulation precedes the establishment of arteriovenous specification, and provided hard evidence for the possibility that the arteriovenous distinction is established prior to the onset of circulation. This single discovery resulted in a major shift in our understanding of how arteriovenous specification and patterning is established. This breakthrough finding was to be followed by numerous investigations which culminated in our current view of the genetic and molecular mechanisms underlying the differentiation and formation of arteries and veins as discussed in the following sections.

### 3.1 Notch Signaling in Arterial Vessel Specification

The first insight into the mechanism underlying arterial specification came from a study of a mutant zebrafish in which an arterial vessel is specifically missing [16, 17]. Zebrafish have served as a unique genetic model system in deciphering molecular mechanisms underlying a variety of processes in development and disease. This organism is a particularly powerful model system in studying the developing vascular system. In other higher vertebrate models such as mice, malformation or deficiency of the vascular system results in early embryonic lethality, thus they are unsuitable for identifying genes involved in the formation of the early vascular system. However, early development of zebrafish embryos is not dependent on the formation of the vascular system (i.e., early stage zebrafish embryos can survive without circulation), thus they provide a unique genetic system for identifying genes that are critically involved in the differentiation and formation of the vascular system.

Through the screening for a mutant that is defective in vascular development in zebrafish embryos, *Gridlock* (*grl*) was identified [18]. In this mutant embryo, the assembly of the first arterial vessel, the aorta, is specifically perturbed. A gene that is responsible for this specific mutant phenotype was subsequently identified to encode a basic-helix-loop-helix (bHLH) transcription factor belonging to the Hairy/Enhancer of the split family of bHLH proteins, Hey2 [17]. The *grl/Hey* gene is expressed in lateral plate mesoderm that is destined to become angioblast, precursor cells for vascular endothelial cells. Its expression subsequently becomes restricted to aorta (an artery) but undetectable in veins. Other studies by the same and another groups indicated that grl/Hey2 is a downstream target of Notch signaling by showing that the expression of grl/Hey2 can be induced by activating Notch signaling and can be inhibited by inhibitors of this signaling pathway [16].

The critical involvement of Notch signaling in arterial specification was further indicated by another zebrafish study where it was shown that the inhibition of Notch signaling by either the injection of a dominant negative Su (H) (suppressor of hairless) results in the failure of arterial marker, EphrinB2, expression and the concomitant ectopic expression of venous markers, EphB4 and Flt4, a phenotype that is also detected in a notch-deficient mutant, *mindbomb* (*mib*) [19]. Furthermore, the constitutive activation of Notch signaling by the forced expression of NICD (Notch intracellular domain) resulted in reduced venous marker expression.

Several studies with mice also indicate an importance of Notch signaling in arterial fate specification. The Notch1, Notch4, and the Notch ligands, Jag1, Jag2 and Dll4 are all expressed in arterial, but not in venous, endothelial cells [20–29]. Furthermore, Dll4 heterozygous mutant mice exhibit reduced expression of EphrinB2 and increased expression of EphB4, a phenotype that is also observed in Notch-deficient Rbpj (the recombination signal-binding protein for immunoglobulin-$\kappa$ J region) and *Mib* mutant mice and *Hey1/Hey2* double mutants [30–33]. These results are also consistent with the notion that Notch signaling is critical for arterial differentiation.

### 3.2 VEGF Signaling in Arterial Vessel Specification

In 2002, discoveries by three independent studies unveiled a surprising mechanism underlying arterial specification. In studying a transgenic mouse line where VEGF-A was over expressed in the heart, it was found that nearly all cardiac endothelial cells become EphrinB2$^{+}$, indicating that VEGF-A can transdifferentiate venous endothelial cells (EphB4$^{+}$) to arterial type (EphrinB2$^{+}$) [34]. Interestingly, this VEGF-mediated arterial transdifferentiation was found to be inhibited by angiopoietin-1 [34]. Another angiopoietin, angiopoietin-2, also inhibited this arterial transdifferentiation, but this inhibition caused the endothelial cells to assume EphrinB2$^{-}$/EphB4$^{-}$ phenotype indicating that they are neither arterial nor venous types [34].

In another study, cultured endothelial cells can be induced to express EphrinB2 upon treatment with VEGF-A164 [35]. In this study, it was further shown that arteries, but not veins, specifically align with peripheral sensory neurons, and VEGF-A164 is expressed by these neurons and Schwann cells [35]. In the mutant mice where peripheral neurons or Schwann cells are eliminated, arteriogenesis is disrupted. However, in the mutant with disorganized neurons, arteries remain aligned with misrouted neurons. Another report by the same group has shown that the deficiency of VEGF-A expression in sensory neurons, motor neurons or Schwann cells results in the elimination of arterial marker expression, while the alignment of the vessels with sensory neurons remained intact [36]. In this study, it was further reported that the arterial differentiation is mediated by endothelial expression of Nrp1 (an artery-specific VEGF co-receptor) which is induced by VEGF derived from peripheral sensory neurons and Schwann cells [36]. These results indicate that peripheral nerve derived VEGF induce arterial specification via Nrp1 mediated positive-feedback.

In a third study, it was reported that VEGF-A is expressed by somite of developing zebrafish embryo in notochords-derived Shh-dependent manner [37]. In this report, it was also shown that loss of VEGF or Shh results in failure of arterial specification [37]. Furthermore, it was shown that exogenous overexpression of VEGF or Shh causes ectopic expression of arterial markers. Expression of VEGF in the embryos lacking Shh signaling rescues the defects in arterial differentiation [37]. These results indicate that notochord-derived Shh promotes VEGF expression by somite, which induces arterial specification.

Based on these three independent studies, two major conclusions can be drawn. VEGF-A is a major inducer of arterial endothelial specification during development. Furthermore, these studies indicate an unexpected plasticity of an endothelial phenotype: Venous endothelial cells can be converted to arterial type by VEGF-A.

### 3.3 Functional Interaction of Notch and VEGF Signaling in Arterial Vessel Specification

As discussed in the previous sections, the VEGF-Notch axis is a central pathway in specifying arterial fate. However, it had not been clear how VEGF signaling is transduced into a Notch signal. This problem was addressed by a series of studies using zebrafish models. As described in a Sect. 3.1, *grl* is a zebrafish mutant where the aorta is perturbed due to a mutation in a gene encoding a Notch signal target transcription factor, Hey2. Thus, it is a suitable genetic mutation for studying arterial specification. In a suppressor chemical screening using the *grl* mutant zebrafish, two classes of small-molecule chemicals were identified [38]. One is GS4012, a chemical that was found to interfere with extracellular signal-regulated kinase (ERK). GS4012 was found to activate VEGFR by inhibiting ERK signaling. The other class is PI3K/Akt inhibitors such as G4898, LY294002 and wartmannin. GS4898 inhibits Akt, a downstream target of PI3K signaling. LY294002 and wartmannin are chemical inhibitors of PI3K. These chemicals were able to rescue *grl* phenotype by partial inhibition of PI3K which leads to activation of ERK [39]. Furthermore, it was shown that activated phosphorylated ERK is preferentially expressed in arterial endothelial cells [39]. Inhibition of mitogen-activated or extracellular signal-related protein kinase kinase (MEK), an upstream activator of ERK, by either SL327 or U0126, results in loss of arterial endothelial cells and improper aorta formation [39]. Inhibition of the VEGF signal pathway with a VEGFR inhibitor 676475 leads to an overall reduction in the ERK activation and defective arterial endothelial differentiation [39].

In another study, forward genetic screens have identified a zebrafish Plc-$\gamma$ (phospholipase C-$\gamma$) mutant that exhibits defects in the formation of arteries, but not veins [40]. In this study, it was also shown that Plc-$\gamma$ mutant zebrafish embryos failed to respond to exogenous VEGF expression. Injection of VEGF121 mRNA into wild type zebrafish embryos resulted in ectopic expression of EphrinB2 in veins, a Notch signal-dependent arterial marker. However, Plc-$\gamma$ mutant zebrafish embryos injected with VEGF121 mRNA displayed either normal or reduced EphrinB2 expression. In an independent cell culture study, it was reported that PLC-$\gamma$ pathway activates ERK in the VEGF/VEGFR signal transduction cascade [41]. These results indicate that VEGF/VEGFR induces arterial specification via PLC-$\gamma$/ERK signaling pathway which resides upstream of Notch signaling.

In contrast, the role of PI3K/Akt signaling in arterial fate determination requires some caution. As discussed above, the arterial defect in *grl* mutant can be rescued by the inhibition of PI3K signaling by small molecules [38, 39]. Arterial specification can be promoted by overexpression of dominant-negative Akt which results in the inhibition of Akt, a downstream mediator of PI3K signaling [39]. Furthermore, constitutively active Akt induces venous endothelial fate [39]. These results suggest that PI3K/Akt signaling inhibits arterial specification by interfering with ERK activation, thus leading to venous cell fate. However, it is also reported that PI3K signaling acts to induce Notch and Dll4 activation, the pathways for arterial

specification [42, 43]. Therefore, further studies are necessary to delineate the exact role of PI3K/Akt in arterial cell fate determination.

### 3.4 Forkhead Box (Fox) Transcription Factors in Arterial Specification

Two family members of the Foxc subclass of Fox transcription factors, Foxc1 and Foxc2, are indicated for their role in arterial specification [44, 45]. Targeted disruption of *Foxc1* and *Foxc2* in mice results in arteriovenous malformation which is accompanied by aberrant expression of several arterial markers such as Dll4 and Notch1. It is also reported that there are Foxc-binding sites in the upstream promoter region of the Dll4 gene, supporting the idea that Foxc transcription factors activate the Dll4 gene which induces Notch signaling, a primary signaling pathway that is required for arterial specification.

### 3.5 Venous Identity and Coup-TFII

It had been believed that venous identify of endothelial cells is a default state of these cells and arterial fate is an acquired state by the activation of Notch signaling [46]. However, it was later reported that the venous state of endothelial differentiation requires the suppression of Notch signaling by a transcription factor, COUP-TFII (chicken ovalbumin upstream promoter-transcription factor II) [47]. COUP-TFII is a member of the orphan nuclear receptor superfamily and is specifically expressed in venous, but not in arterial, endothelial cells [47, 48]. Conditional ablation of the COUP-TFII gene in endothelial cells in mice results in the acquisition of an arterial phenotype in veins manifested by the expression of arterial markers, such as EphrinB2, Npr1, and Notch-signaling molecules [47]. Furthermore, these mutant veins that acquired arterial marker expression are able to form haematopoietic cell clusters and recruit smooth-muscle cells, two functional features of normal arteries [47]. These results indicate that COUP-TFII deficient venous endothelial cells not only acquire arterial markers, but also function as arteries.

In addition, it was shown that ectopic expression of COUP-TFII in endothelial cells in mice results in an abnormal fusion of arteries and veins, a phenotype that is a mirror image of Npr1 or Notch1 mutant mice [47]. In this study, it was also shown that the ectopic expression of COUP-TFII leads to a loss of expression of arterial markers, including Nrp1 and the Notch signaling molecules. All of these results are in support of the idea that COUP-TFII expression in veins suppresses the Notch signaling, thus leading to the maintenance of the venous phenotype of endothelium.

### 3.6 Other Signaling Pathways Involved in Arterial Vessel Specification

In addition to the above discussed chemical factors that contribute to arteriovenous specification, there are several other chemical factors that are somewhat less characterized for their roles in this process.

The G protein-coupled receptor APJ is preferentially expressed in venous endothelial cells [49, 50]. Morpholino knockdown of APJ and its ligand, Apelin, in *Xenopus* results in defects in intersomitic vessel development [51]. However, no obvious vascular defects are detectable in APJ null mice [52].

Members of the Sry-related HMG box (Sox) family of transcription factors have also been indicated for their roles in arteriovenous differentiation [53–55]. It has been reported that Sox7 and Sox18 are both expressed in developing vasculature and their double knockdown results in arteriovenous malformation.

A member of the sucrose non fermenting kinase family (snrk-1) has been implicated to act downstream of or in parallel to Notch signaling and function upstream of *grl* [56]. Calcitonin receptor like receptor (crlr) expressed by developing somites is a receptor for adrenomedullin and suggested to be under the regulation of shh and to control arterial differentiation as an upstream regulator of VEGF [57]. However, the exact roles of these factors in controlling arteriovenous differentiation remains unknown.

## 4 Integrated Mechanisms Underlying Arteriovenous Specification by Chemical Signals

As discussed above, in less than the last 15 years, extraordinary advances have been made in our understanding of the detailed molecular basis of the arterial and venous specification (Fig. 3). It seems clear that activation of Notch signaling is the central pathway in arterial specification. This signaling is activated by multiple inputs such as those that lead to the activation of VEGF, Foxc1/2, and Plc-$\gamma$/PKC/MEK/ERK signals. Ectopic activation of any of these signals in venous endothelial cells can override the genetic program determining their phenotype, and they acquire the arterial phenotype. This plasticity of arterial-venous phenotypes persists even in adult endothelial cells.

In contrast, the mechanism underlying venous specification is less understood. Activation of a transcription factor, COUP-TFII, in venous endothelial cells is required for retaining their phenotype. It is also reported that the activation of PI3K/Akt signaling is involved in venous specification, even though its site of action remains unclear. Although COUP-TFII and PI3K/Akt signaling are involved in venous specification, their primary function appears to be the suppression of arterial phenotype, rather than the induction of an venous phenotype. Furthermore, rendering a venous phenotype in arterial endothelial cells by

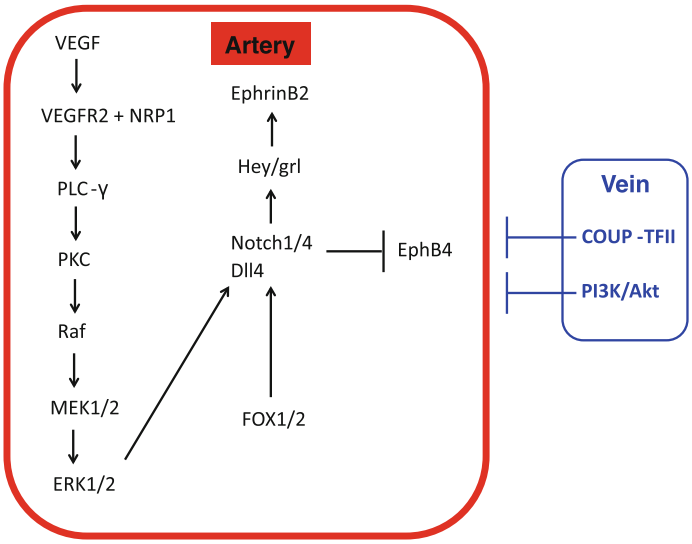


**Fig. 3** Signaling mechanisms underlying arterial and venous specification. See text for details

experimental manipulation has not been reported. Moreover, upstream regulators that induce the activation of COUP-TFII or PI3K/Akt signaling in endothelial cells are unknown. Therefore, it is not clear if there are any inductive processes involved in venous fate determination.

One hallmark of veins is the presence of valves. However, no molecular signals have been uncovered that are involved in the formation of venous valves. Furthermore, it remains unknown how the formation of valves is suppressed in arteries.

As early as the fifteenth century, it was already proposed that there may be cross-talk between arterial and venous vessels by Jacopo Berengario da Carpi based on his anatomical observation. However, there is currently no evidence for the existence of paracrine factors that mediate a putative cross-checking of the phenotypes by each other.

## 5 Speculative Remarks

Although significant advances have been made in understanding mechanical and chemical mechanisms underlying arterial and venous phenotype and function, it is far from a complete understanding how this regulatory system works. Thus, I end my chapter with "speculative remarks" instead of customary concluding remarks.

Based on what we currently know about this subject, I speculate on three hypothetical models (Fig. 4): Morphogenetic, habituation, and integrative models.

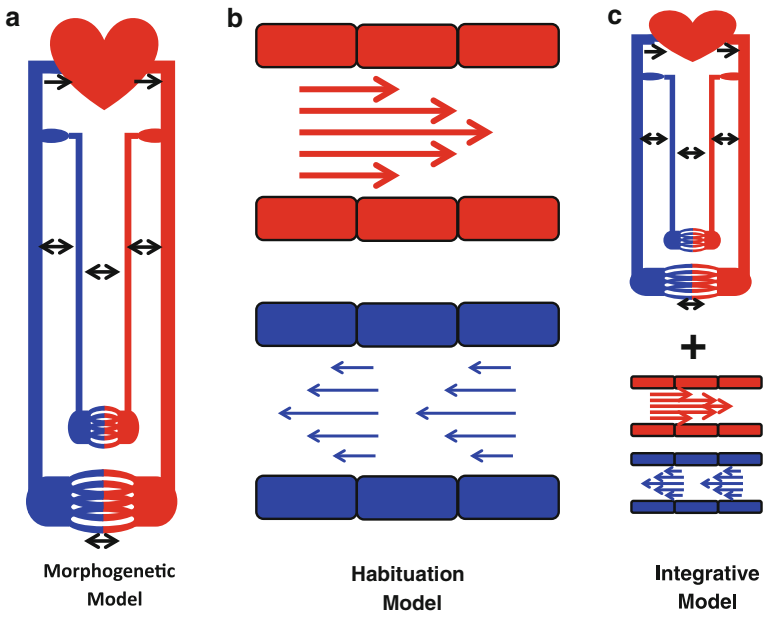


**Fig. 4** Three speculative models explaining how mechanical and chemical signals become integrated in specifying artery and vein. Morphogenetic, Habituation, Integrative models are proposed

## *5.1 Morphogenetic Model*

Based on this model, genetic regulatory mechanisms that determine arterial and venous phenotype provide information necessary for each endothelial cell type to lay themselves in specific spatial positions (Fig. 4a). Such mechanisms allow them to be positioned at the right place relative to each other and among arteries and veins, thus leading to the establishment of specific geometrical arrangements of the vascular system consisting of arterial and venous wiring.

According to this model, endothelial cells sense chemical and mechanical cues in their microenvironment. Upon sensing these signals, endothelial cells respond by expressing arterial and venous genes that code proteins necessary for establishing their spatial organization. Both arterial and venous endothelial cells are to be laid down correctly among themselves and also relative to each other. Their position in the body relative to the heart is also critical for their required physiological functions. Furthermore, their positions relative to body axis (left-right, anteroposterior, dorsoventral, etc.), as well as those within each organ, are also essential for the whole body and organ functions and development [58, 59].

In fact, it has been implicated that EphrinB2 and EphB4, respective arterial and venous markers, function as a ligand-receptor system that mediates *cis*- and *trans*-interactions necessary for establishing normal arteriovenous architecture of the vascular system [60].

### 5.2 Habituation Model

This model explains arterial and venous specific phenotypes as seeds and states that are required for them to correctly respond to mechanical forces generated by fluid dynamics in arteries and veins, respectively (Fig. 4b). According to this model, chemical cues in the microenvironment in each endothelial cell induce genetic and morphogenic changes that "habituate" them for the mechanical signals, to which they are to be exposed. Therefore, one consequence of this model is that endothelial cells fail to adapt and/or respond to specific mechanical forces generated by circulation unless they are specified either to arterial or venous types by chemical and genetic programs. Furthermore, endothelial cells of aberrant phenotypes with regard to arterial or venous types may be malfunctional or dysfunctional under the normal circulation.

It has been long known that venous grafts in arterial vessels for cardiac bypass surgery fail to adapt to arterial circulation. This may support the idea that arterial and venous specific endothelial cell phenotypes established by genetic programs are prerequisite for them to adapt and respond to their respective circulation.

### 5.3 Integrative Model

The third model is the one that integrates both morphogenetic and habituation models. Certain genetic programs and chemical cues in the microenvironment establish arterial and venous specific architecture of the vascular network. However, other genetic programs and chemical signals are to be used to build a chemical signaling network in arterial and venous endothelial cells that allows them to adapt and respond to specific mechanical forces generated by the respective circulation.

These three models are distinguishable and are experimentally testable. In the past 15 or so years, we gained increasing knowledge on genetic and chemical regulation of arterial and venous specification. With further identification of arterial and venous specific genetic codes and formulation of testable models such as those discussed in this section, it is anticipated that we are nearing towards the complete understanding of the mechanisms underlying arteriovenous specification.

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# Mechanosensory Pathways in Angiocrine Mediated Tissue Regeneration

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**Abstract** Endothelial cells not only form the vascular networks that deliver nutrients and oxygen throughout the body, they also establish instructive niches that stimulate organ regeneration through elaboration of paracrine trophogens. Priming of the vascular niche promotes repair and regeneration of damaged tissues by establishing an inductive vascular network that temporally precedes new tissue formation. This induction of endothelial cells provides a platform for essential instructive cues. Tissue regeneration in certain organs such as the liver, involves cell mitosis and expansion, which is orchestrated by a dynamic interplay between cytokines, growth factors, and metabolic pathways. Although the intrinsic events of cell mitosis have been thoroughly studied, the extrinsic triggers for initiation and termination of liver regeneration, especially the set points rendered by the original liver size, are unknown. Furthermore, the gatekeepers that control organ size remain unidentified. The prevailing dogma states that liver regeneration involves the proliferation of parenchymal hepatocytes and nonparenchymal cells such as biliary epithelial cells. However, recent findings also implicate hepatic sinusoidal endothelial cells (SECs) as drivers of this process. In the classic liver regeneration model, in which 70 % partial hepatectomy induces regeneration, the abrupt increase in blood flow into the sinusoidal vasculature of the liver's remaining lobes correlates with initiation of the regeneration cascade. As such, the shear stress and mechanical stretch exerted on the endothelial cells may activate mechanosensory mediated molecular programs, and may be involved in the elaboration of endothelial cell-derived angiocrine growth cues that support

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hepatocyte proliferation. Physiological liver regeneration would therefore depend on the proper inductive and proliferative functioning of liver SECs. Thus, uncovering the cellular mechanisms by which organisms recognize and respond to tissue damage remains an important step towards developing therapeutic strategies to promote organ regeneration. In this chapter, we demonstrate the mechanism by which tissue-specific subsets of endothelial cells promote organ regeneration, and further discuss the roles of physical forces and molecular signals in initiating and terminating angiocrine-mediated tissue regeneration.

## 1 Introduction

Mammalian cells are able to sense, decode, and respond to physical stimuli in a manner specific to their type. Physical stimuli play a role in virtually all stages of cell differentiation, as early as embryogenesis—where stiffness and contractility provide mechanosensory cues to dictate cell proliferation and differentiation (reviewed in [1])—to post-natal cranial development [2]. They also play an integral role in tissue engineering [3, 4] and regeneration [5, 6]. For instance, frogs (*Xenopus laevis)* during development can only undergo gastrulation, convergence and extension movements if the mesoderm and notochord maintain their stiffness and avoid buckling [7, 8]. Throughout this rearrangement, the marginal zone becomes stiffer in order to avoid tissue deformation and collapse during gastrulation [9]. This increased stiffness further provides the mechanical strength that initiates the following developmental events. Whether these changes also serve as cues within the mechanosensory pathway to trigger other related cellular processes is unknown.

Cell contractility is another significant source of mechanical cues. Contraction of the actomyosin cytoskeleton triggers cellular responses in the contracting cell as well as external forces on the surrounding cells. The embryogenesis of *Xenopus laevis* also appropriately illustrates cellular responses to cell-generated contractile forces. It has been shown that during gastrulation, cultured explants of the dorsal involuting and non-involuting marginal zones of frog embryo, neither of which are subjected to external forces, converge and expand. This study suggests that tissue movement related to gastrulation is dictated by the tissue itself, rather than by surrounding external forces [10].

Mechanotransduction plays an important role in the signaling of endothelial cells because of their unique position in the blood vessel lumen. Endothelial cells have direct contact with blood and so can first sense changes in shear stress caused by alterations in flow rate. It is well known that endothelial cells perceive and respond to blood flow by undergoing a series of molecular changes, including expression of various transcription factors. These molecular alterations guide cell differentiation and may drive tissue regeneration. Nevertheless, the role of biomechanical forces in orchestration of tissue regeneration and subsequent

restoration of the function of damaged or diseased organs remains to be elucidated. This chapter focuses on the role of paracrine (angiocrine) factors derived from sinusoidal endothelial cells (SECs) on initiating hepatic regeneration, and the impending role of biomechanical forces in triggering this angiocrine-mediated regeneration process.

## 2 Organ Regeneration

Restoration of tissue upon injury or physical loss occurs through the processes of repair, remodeling, and regeneration. While repair and remodeling generally restore tissue continuity by synthesizing extracellular matrix (ECM) proteins and forming scar tissue, regeneration resolves the same issue by recovering the missing organ mass at the original anatomical site.

Three different strategies potentially lead to organ regeneration. Non-dividing cells from the injured organ begin to multiply and grow to resynthesize the lost tissue. Specialized cells dedifferentiate into multipotent cells that replicate, re-specialize and replace the missing tissue. Lastly, pools of stem cells can divide and regenerate the lost tissue. The molecular and cellular pathways that regulate regeneration of adult organs remain largely unknown.

### *2.1 Liver Regeneration*

The liver has a remarkable regenerative capacity and is able to rapidly restore its original weight and size after surgical resection. Liver regeneration is orchestrated by a complex interplay of cytokines, growth factors, and metabolic pathways (reviewed by [11, 12]). The "blood flow" theory, proposed over 50 years ago, suggested increased mechanosensory cues caused by increased blood flow to the liver post-partial hepatectomy (PH) triggered liver regeneration [13].

Another theory suggests that following liver damage factors such as Lipopolysaccharides (LPSs) are produced by the gut and delivered to the liver via the portal vein. LPS then activates the Complement system, leading to the production of anaphylatoxins, complement component 3 (C3a), and complement component 5 (C5a). Both C3a and C5a play a central role in the activation of the Complement system, a part of the innate immune system, which aids antibodies and phagocytic cells in clearing pathogens. LPS, C3a, and C5a all participate in the activation of Kupffer cells, mononuclear phagocytic cells found on the hepatic sinusoids, through Toll-like receptor 4 (TLR4), C3a receptor (C3aR), and C5a receptor (C5aR). The activation of Kupffer cells is indispensable in directing liver regeneration, namely through the production of TNF-$\alpha$ and IL-6, both of which are involved in priming the hepatocytes from the quiescent G0 to the G1 phase [14]. The transition of hepatocytes from G1 to M phases of the cell cycle is promoted by

other factors, such as insulin, growth hormone (GH), bile acids, vasopressin (AVP), platelet-derived serotonin, and endothelial growth factor (EGF) from the blood. These factors cooperate with prostaglandins, heparin-binding EGF-like growth factor, hepatic growth factor, as well as insulin-like growth factor-1. Tumor growth factor and amphiregulin from different hepatic cells block the G1 to M transition of the cell cycle.

Hepatic Stem Cells (HSCs) have the ability to inhibit G1 to S transition [15, 16]. TGF-beta signaling is thus blocked during cell proliferation. Pro-Nerve Growth Factor (Pro-NGF) and NGF, produced by regenerating hepatocytes, are able to trigger apoptosis in activated TGF-producing HSCs. Once the liver has reached its original mass, several factors (including activin A) are responsible for triggering the end of liver regeneration.

The liver sinusoidal vasculature is lined with endothelial cells (SECs) that distribute hepatic blood flow into each lobe. It is well known that occlusion of one particular branch of the portal vein system results in an atrophy of its corresponding lobes and a compensatory hypertrophy of the remaining liver tissue [17]. Given that hepatocytes make up most of the liver volume, this observed atrophy and loss of volume is mainly caused by apoptosis and necrosis of hepatocytes [18]. The exact mechanism of hypertrophy unique to liver regeneration is not completely known. However, considering the speed of regeneration, a very early trigger—possibly an abrupt change in a physiological parameter—may be responsible.

Similar to blood flow changes upon portal vein occlusion, one of the most obvious events in liver regeneration following partial hepatectomy is the subsequent increase in portal blood flow to the remaining lobes. Shear stress resulting from such increased blood flow on vascular lining could represent a first-line trigger for liver regeneration after PH. Cell location dictates the specific cells involved in mechanotransduction of hemodynamic forces in liver vasculature.

## *2.2 Sensing and Regulation of Blood Flow by the Vasculature*

The endothelium is the innermost layer of blood vessels, made up of cells that had largely been assumed to function primarily as a delivery conduit for oxygen and nutrients. It has been increasingly appreciated that endothelial cells not only form vascular networks that deliver nutrients and oxygen throughout the body, but also establish instructive niches that stimulate organ regeneration through elaboration of paracrine trophogens. Endothelial cells distributed in different organs exhibit various morphological and functional attributes. Arteries and veins have distinct structures, with arteries comprised of three layers (tunica intima, tunica media and tunica adventitia), while veins consist of only two. The tunica intima is a single layer of simple squamous endothelium attached to a sub-endothelial connective tissue layer; the tunica media, mostly present in arteries, contains circularly arranged elastic fiber, connective tissue, and polysaccharides; the tunica adventitia is made of connective tissue and nerves. In the case of arteries, the tunica media

layer is rich in vascular smooth muscle, which serves to control the caliber of the vessel. Taken together, these cells experience cyclic stretch when they serve to modulate vasodilation and vasoconstriction as well as control blood rheology [19].

By virtue of its location, the endothelium has an important role as the primary interface between circulating blood and the vascular wall. Endothelial cells in particular sense blood flow and shear stress. The role of biomechanical forces in the ability to regenerate tissues and thus restore the function of damaged or diseased organs requires clarification.

## 3 Biomechanical Forces

Traditionally, it has been thought that biochemical signals serve as the main method in which signaling pathways are activated in endothelial cells. However, new research has determined that mechanical forces experienced by endothelial cells have a significant impact on cell phenotype and function. Biomechanical forces play essential roles in tissue and organ development [20]. Recent studies have demonstrated that mechanical forces can regulate endothelial cell proliferation, survival, migration [21], and ECM remodeling, and hence influence angiogenesis [22]. Mechanosensitivity describes the ability of cells to perceive mechanical stimuli and interpret them into biological signals at the molecular level [23]. As such, mechanotransduction is a set of fundamental physiological mechanisms that allow cells to react to physical forces. Mechanical stretch resulting from pulsatile blood flow can modulate differentiation, proliferation and production of paracrine (angiocrine) factors of vascular cell. In this mechanotransduction-based response of the arterial system, numerous endothelial cellular components such as proteoglycans have a crucial role [24, 25].

### *3.1 Effect of Mechanical Stress on Endothelial Cells*

Cells in the vascular system are subjected to mechanical forces due to the pulsatile nature of blood flow. Endothelial cells and smooth muscle cells in the vessel wall experience these mechanical forces the most. Mechanical stretch, resulting from changes in blood pressure, can modulate several different cellular functions in vascular smooth muscle cells. Our data provides a new framework in which to consider how physical forces and molecular signals synchronize during the program of liver regeneration (Fig. 1).

The effect of shear stress on vascular endothelial cells has been extensively analyzed. There is a large body of evidence showing that it directly influences the expression of many adhesive molecules like VCAM-1 [30] and ICAM-1 [31] and mediator systems such as TGF-$\beta$ [32] and nitric oxide synthase [33]. Several groups of molecules have been shown to play a role in the detection of flow shear

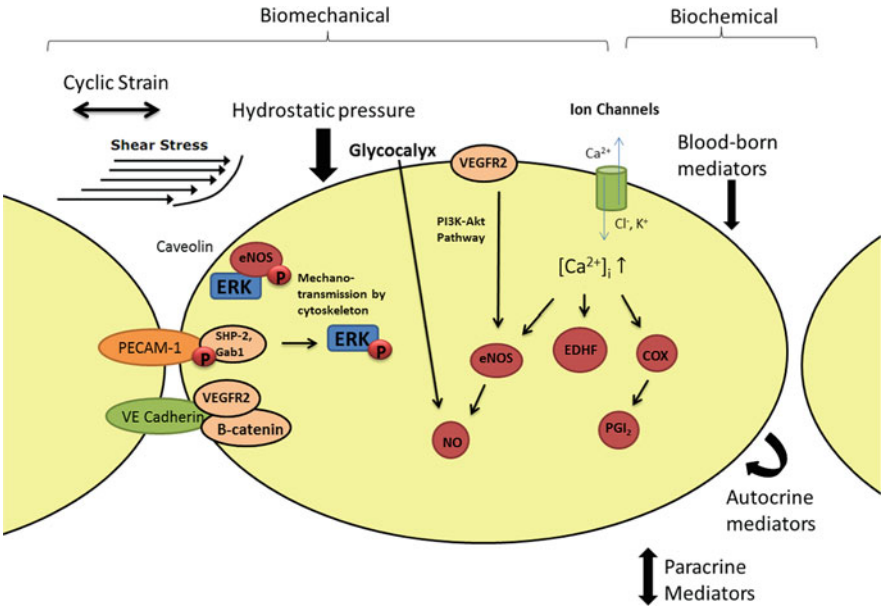


**Fig. 1** Early molecular response of endothelial cells to shear stress. Endothelial phenotype is constantly modulated by both biomechanical and biochemical stimuli. Biochemical stimuli include hormones, growth factors, cytokines, etc. that are delivered to the cell via autocrine or paracrine mechanisms. Hemodynamic forces result in hydrostatic pressure, cyclic strain, and shear stress. Different mechanosensors play specialized roles in interpreting this stress in endothelial cells. These sensors include ion channels (TRP, P2X4, $K^+$, $Cl^-$ channels) [26], integrins, platelet endothelial cell adhesion molecule-1 (*PECAM*-1), VE-cadherin, caveolae, G proteins, glycocalyx, and the endothelial cytoskeleton [27]. The activity of ion channels leads to an influx in $Ca^{2+}$, which results in vasodilator production (namely nitric oxide), prostacyclin, and Endothelium-Derived Hyperpolarizing Factor (*EDHF*). Shear stress travels through the cytoskeleton to the endothelial surface, where either integrins or PECAM-1 and VE-cadherin are activated sequentially. Activated integrins bind to adaptor protein Shc as well as numerous kinases, including focal adhesion kinase (*FAK*). Shear stress also triggers tyrosine phosphorylation of PECAM-1 and localization of SHP-2 and Gab1 near the cell junction. This will subsequently results in ERK phosphorylation. Shear activation of (VEGF)-A receptor-2 (VEGFR2) at the luminal surface leads to the recruitment of phosphatidylinositol 3 kinase (*PI3 K*), which itself leads to the activation of Akt and eNOS. The activation of ERK and the phosphorylation of serine 1179 of eNOS at caveolae by shear have also been documented. Shear stress is rapidly followed by G protein activation and results in the activation of Ras and extracellular signal-regulated kinases-1/2 (ERK1/2). Deformation of the luminal surface will result in direct shear stress-induced signaling through the glycocalyx (via NO production) (adapted from [28, 29])

force sensation, namely ion channels, cell–matrix and cell–cell junction molecules, Tyrosine Kinase Receptors [(VEGF)-A receptor-2 (VEGFR2)], caveolae, G-protein coupled receptors/G-proteins, glycocalyx, and the endothelial cytoskeleton (reviewed in [20, 27]). Mechanotransduction of hemodynamic forces plays a central role in regulation of arterial diameter. This process is orchestrated

by the production and release of autacoids by the endothelial cells affected. Nitric oxide (NO), prostacyclin (PGI2), and endothelin-1 (ET-1) are the best-characterized endothelium-derived autacoids, the first two being vasodilators, and the last being a vasoconstrictor. Two other factors have been suggested to play a significant role in vasodilation: endothelium-derived hyperpolarizing factor (EDHF) and reactive oxygen species (ROS) [27, 34].

### *3.2 Integrin-Mediated Mechanotransduction*

Integrins, as the main receptors that mediate the connection of the cytoskeleton to the ECM, have been implicated in sensing mechanical forces [35]. When the apical surface of an endothelial cell experiences shear stress due to blood flow, the tension experienced by the cell is transferred to transmembrane integrins, which undergo conformational changes and alter the phenotype of the cell. In order to maximize adhesion, the cell needs to increase its surface area in contact with the ECM so it flattens in a manner consistent with the blood flow. Tension at the upstream end of the cell with then be greater than the tension at the downstream end [35]. In integrin-mediated cell binding to the ECM, proteins in the ECM bind to integrins attached to the cell's cytoskeleton. This linkage bears much of the mechanical force inflicted on the cells, and as such may serve as a candidate mechanotransducer.

Glycoproteins within the extracellular matrix can be displaced by both cyclic stretching and shear stress. These interact with integrins that transmit the mechanical signal intracellularly. Shear stress can cause endothelial cell integrins to form clusters [36], associate with adaptor proteins such as Shc [37], and bind to WOW-1, an antibody that specifically binds to a particular activated intergrin [38].

The cytoplasmic domains of integrins are often linked to intracellular proteins that constitute the cytoskeleton as well as kinases, including focal adhesion kinase (FAK), a key regulator of biochemical cascades triggered by mechanical forces. Therefore, integrins serve as a signal transmitter between the cell and the ECM [27].

Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein and a sub-family of the cysteine-knot growth factor superfamily, a platelet-derived growth factor family [39, 40]. They have profound involvement in signaling for both vasculogenesis (the de novo formation of blood vessels) and angiogenesis (the growth of blood vessels from pre-existing vasculature). VEGF promotes vasculogenesis during embryonic development, angiogenesis after injury and within muscles following exercise, as well as new vessels that serve to bypass older, blocked vessels.

Overexpression of VEGF can contribute to disease. Solid tumors require sufficient blood supply after they reach a certain size. Tumor cells that have the ability to express VEGF can therefore further expand and metastasize. VEGF overexpression may also cause vascular diseases of the retina. Anti-VEGF therapies are used to control or slow down the progression of diseases facilitated by VEGF

**Table 1** Vascular endothelial growth factor function

| Receptor | Ligand | Function |
|---|---|---|
| VEGFR1 (Flt-1) | VEGF-A, VEGF-B | Decoy receptor of VEGF-R2, unknown |
| VEGFR2 (KDR/Flk-1) | VEGF-A,VEGF-C, VEGF-E, VEGF-D | Varied |
| VEGFR3 | VEGF-C, VEGF-D | Lymphangiogenesis |

overexpression. An outline of VEGF isomers and receptors they interact with is outlined in Table 1.

VEGF-A binds to VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1). VEGFR2 seems to mediate most of the known cellular responses to VEGF [41]. The function of VEGFR1 is less well-defined; it is thought to modulate VEGFR2 signaling. VEGFR1 may also act as a decoy receptor, sequestering VEGF from VEGFR2 binding (particularly during embryonic vasculogenesis). VEGF-C and VEGF-D are ligands for VEGFR3, which plays a role in lymphangiogenesis.

### *3.3 Parallel-Plate Flow System*

To understand how shear stress is measured in vivo in small vessels, it is first necessary to have an understanding of how shear stress can be measured in vitro. The parallel-plate flow chamber is a commonly used platform for subjecting monolayers of anchorage-dependent cells, such as endothelial cells, to relatively uniform laminar shear stress. This approach has allowed scientists to recreate the in vivo environment in which endothelial cells are exposed to shear stresses due to blood flow.

A cell monolayer attached to one internal plate surface is subjected to fluid flow by creating a pressure gradient across the chamber. In order to calculate the shear stress on the cells, a Newtonian fluid is assumed. For steady flow between infinitely wide parallel plates, shear stress is determined according to fluid flow, viscosity of the media, and the depth of the chamber.

There are many advantages to using parallel-plate flow chambers. They promote uniform shear stress in a controlled environment. They can also simulate a wide variety of flow rates, which associatively varies shear stress levels. This enables simulation of blood vessels of many different sizes.

A parallel-plate flow chamber with a channel height of 1 mm was used to subject Human Umbilical Vein Endothelial Cells (HUVECs) to shear stress (Stovall Life Sciences, Inc). This chamber is optically transparent, allowing for direct visualization of the cell behavior under flow on a microscope stage and real-time monitoring. A high-precision peristaltic pump (Ismatec IPC, Model #ISM931C) is connected with tygon tubing (1/16 in ID) to the flow cell. The pump can impart a range of fluid shear stress by varying the flow rate. Cells are plated

into the flow cell 24 h before the experiment to allow the cells to grow to confluence. The experiment is performed at 37 °C and 5 % $CO_2$. The peristaltic pump is placed inside the incubator to minimize the tubing length and as a result the flow path is considerably reduced. At the completion of the experiment, the flow cell can be disassembled by stopping the pump and fixing the cells for visualization or lysing for assaying.

As fluid flows through the parallel-plate flow chamber there are several variables that are used to calculate the shear stress imparted on the cell monolayer on the internal plate of the chamber. The magnitude of shear stress ($\tau$) can be determined by quantifying the following variables: fluid viscosity ($\mu$), chamber width ($b$), distance between plates ($h$), and volumetric flow rate ($Q$). The shear stress acting on the cell monolayer adhering to the plate is expressed in the following mathematical form:

$$\tau_w = \frac{6\mu Q}{bh^2} \tag{1}$$

In order to derive the shear stress model, the Navier–Stokes equations can be used. In the following Navier–Stokes equation for a fluid, $\rho$ is density, $u$ is fluid velocity, $\mu$ is viscosity, $P$ is pressure, and $t$ is time.

$$\rho\left(\frac{\partial u}{\partial t} + u \bullet \nabla u\right) = -\nabla P + \mu\nabla^2 u \tag{2}$$

Since it is assumed that the flow is fully developed, there is no change in flow velocity with respect to time, and as a result, the entire left side of the equation is neglected.

$$0 = -\nabla P + \mu\nabla^2 u \tag{3}$$

Rearranging and simplifying the terms, the equation shows the relation between the pressure gradient in the flow direction and the change in shear stress in the direction normal to the plate:

$$\frac{d^2u}{dy^2} = \frac{1}{\mu}\frac{dP}{dx} \tag{4}$$

Since it is assumed that the no-slip boundary condition applies, the following boundary conditions exist for this second order differential equation, where $h =$ the height of flow chamber.

(1) $\frac{du}{dy} = 0$ at $y = 0$ due to the shear rate being zero at the axis of symmetry
(2) $u = 0$ at $y = \pm\frac{h}{2}$ due to the no-slip condition at the top and bottom

integrating Eq. (4) once gives:

$$\frac{du}{dy} = \left(\frac{1}{\mu}\frac{dP}{dx}\right)y + c_1 \tag{5}$$

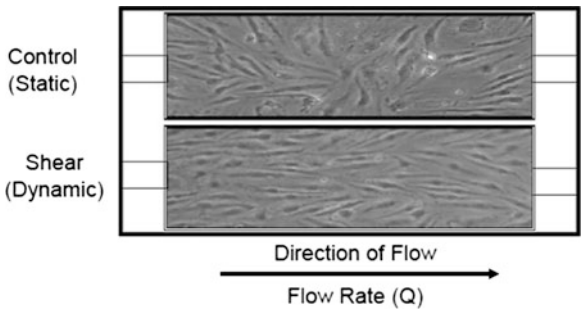


**Fig. 2** Transformation of Endothelial Cell Morphology by Shear Stress. Alignment of endothelial cells by shear stress in a parallel-plate flow chamber. HUVECs were exposed to physiological stress of approximately 6 dynes/cm$^2$ for 12 h aligned with the directions of blood flow

Apply the first boundary condition to eliminate c, and then integrate again, when the second boundary condition is used, the result is:

$$u = \frac{1}{2\mu}\left(\frac{-dP}{dx}\right)\left[\left(\frac{h}{2}\right)^2 - y^2\right] \tag{6}$$

By integrating Eq. (6) over the height, the volumetric flow rate per unit width can be solved.

$$q = \frac{1}{2\mu}\left(\frac{-dP}{dx}\right)\left(\frac{h^3}{6}\right) = \frac{-h^3}{12\mu}\frac{dP}{dx} \tag{7}$$

Assuming two-dimensional flow (since the side walls are much further apart than the parallel plates, or $b \gg h$):

$$Q = -\frac{h^3 b}{12\mu}\left(\frac{dP}{dx}\right) \tag{8}$$

An expression for shear stress can be obtained by using Eq. (5) with $c_1 = 0$, noting that $\tau = [\mu du/dy]_{y=-h/2}$, and substituting for dP/dx from Eq. (8)

$$\tau = -\frac{dP}{dx}\frac{h}{2} = \frac{6\mu Q}{bh^2} \tag{9}$$

It is assumed that flow is laminar, which is true for the low Reynolds number flow being investigated. This laminar flow is also steady state, which helps to simplify the modeling of the shear stress. Another assumption is that the fluid is Newtonian and incompressible, which for the case of blood flow is acceptable (Fig. 2).

## *3.4 VEGFR2 and Mechanotransduction*

VEGFR2 is important for normal endothelial cell proliferation, migration, and angiogenesis. VEGFR2 was established as having a role in the mechanosensory steps preceding integrin activation in integrin-mediated mechanotransduction [42].

VEGFR2 is part of a mechanosensory complex, comprised of Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1) (which transmits mechanical force), vascular endothelial cell cadherin (VE-cad), beta-catenin (which acts as an adaptor), and VEGFR2 (which activates phosphatidylinositol-3-OH kinase) [42]. This complex is sufficient to confer responsiveness to shear stress induced by flow in heterologous cells. Mechanical activation of VEGFR2 enables it to recruit PI3-kinase, and therefore mediates the activation of Akt and endothelial nitric oxide synthase (eNOS). Blocking the receptor activity in these complexes leads to a significantly diminished response to mechanical stress in endothelial cells. Therefore, a correctly assembled complex is indispensable to form a sensing mechanotransduction mechanism.

### *3.5 Regulation of MicroRNA's by Biomechanical Forces*

MicroRNAs (miRNAs) are small noncoding RNA molecules (21–25 nucleotides) that function as post-transcriptional regulators by binding to complementary sequences on target mRNA transcripts [43, 44]. This usually results in translational repression, target degradation, or gene silencing [45]. Approximately 30 % of human protein coding genes are estimated to be regulated by miRNAs. The role of miRNAs in various biological processes, in both health and disease, is becoming more evident. These processes include cell proliferation and differentiation, angiogenesis [46–48], and cardiovascular homeostasis [49, 50]. The role of miRNA in endothelial cell (EC) biology was shown by studying the various knockdowns of key molecules in miRNA biogenesis (such as Dicer, and Drosha). EC Dicer knockouts studied in vitro showed changes in the expression of genes involved in EC biology, as well as reduced EC proliferation and angiogenesis [51]. ECs with knockdown of both Dicer and Drosha had significant reduction in capillary sprouting and tube forming activity [52]. Therefore, an overall reduction of miRNAs (via knockdown of Dicer and/or Drosha) significantly affects EC functions in vitro and in vivo, which indicates miRNAs might have an important role in regulating angiogenesis and vascular function [43, 44, 52, 53]. EC gene expression in response to pulsatile shear flow indicated that miRNAs (specifically miR-12b) play an important regulatory role in the inhibition of EC proliferation by pulsatile shear stress [54]. Additionally, when the influence of shear stress on aortic arch vessels was investigated, it was found that a flow-induced genetic pathway was necessary for angiogenic sprouting. This pathway requires the production of mechanosensitive zinc finger transcription factor (klf2a) that induces an EC-specific miRNA (mir-126) to activate VEGFR signaling [55]. This is yet another example of miRNAs facilitating integration of a physiological stimulus using growth factor signaling in ECs to guide angiogenesis.

# 4 Hemodynamics and the Liver

## *4.1 Blood Flow in the Liver*

The liver is the body's most important metabolic organ and plays a central role in detoxifying blood-transported components. The liver has a unique dual circulation system, receiving 75 % of its oxygenated blood from the hepatic portal vein and 25 % from the hepatic artery. Each of these two vessels provides about half of the liver's oxygen requirement. The hepatic artery provides arterial blood, whereas the hepatic portal vein is responsible for transporting the venous blood from the spleen and the gastrointestinal tract, and its associated organs. In humans, both hepatic lobes are composed of about a million small lobules that are a maximum of 1–2 mm in size. Blood entering the liver flows through morphologically sinusoidal microvessels surrounded by hepatocytes and drains into a centrally located vein. This exceptional architecture ensures that the blood is brought into best possible contact with the hepatocytes when it flows through the organ. These veins then merge into the hepatic vein, which drains the blood out of the liver.

Blood flow subjects the endothelial cells to two major complex hemodynamic forces. The cyclic strain that results from mechanical distortion and tension of a vessel's wall, caused by the pumping action of the heart, affects the vascular cells. The shear stress, due to the frictional force that results from viscous blood flow over the vessel lumen, is imparted onto the endothelial cells lining the vessel's lumen. Blood pressure is the major determinant of cyclic stretch, while shear stress is determined by the blood flow, viscosity, and the diameter of the blood vessel. The liver's capacity to withstand vascular stress is illustrated through its management of blood flow following PH. Following a 2/3 PH, all blood provided by the hepatic portal vein is shunted to the remaining one-third of the liver, effectively tripling the incoming blood flow rate and volume, causing acute portal hypertension. This increase in blood flow is achieved by circumferential vessel wall expansion resulting in the stretching of endothelial cells and other cell types in the vessel wall. The liver lacks the ability to control the volume of portal blood flow despite it comprising up to 75 % of total liver intake. It is reasonable to postulate that the increase in blood volume entering the liver following partial hepatectomy may play a role in triggering the rapid regeneration of the liver. The cellular response to this stress may be mediated through mechanotransduction. Interestingly, when portal blood flow is experimentally deprived, a compensatory increase of arterial blood supply, known as the hepatic arterial buffer response (HABR), occurs. This phenomenon implicates that the abrupt shift in blood flow distribution after partial hepatectomy is a first-line trigger to provoke the regeneration process, while regeneration is halted following the gradually normalized blood flow.

### *4.2 Liver Regeneration*

Liver regeneration has also been linked to the proliferation of hepatocytes post partial hepatectomy, which coincides with the secretion of a variety of growth factors and cytokines, such as interleukin-6 (IL-6), hepatocyte growth factor (HGF), and tumor necrosis factor (TNF). It has previously been shown that growth factors and cytokines produced by hepatocytes play an important role in orchestrating the process of liver regeneration. In fact, it seems both parenchymal cells (particularly hepatocytes) and nonparenchymal cells (Kupffer cells, SECs, hepatic stellate cells, and billiary epithelial cells) are involved in producing cytokines for liver regeneration [56]. Hepatic SECs are the second most common liver cells after hepatocytes [57]. They compose a functionally unique capillary network that vascularizes organs such as bone marrow and liver. Hepatic SECs play a vital role in providing nutrients and growth factors to proliferating hepatocytes via the formation of new vessels during regeneration. Bone marrow SECs in adult mice support hematopoietic regeneration through expression of specific angiocrine trophogens, such as Notch ligands [58, 59]. The hepatic circulation is also largely lined by LSECs [60, 61], with each hepatocyte dwelling in cellular proximity to ECs. However, the lack of a suitable definition of liver ECs and scarcity of relevant mouse angiogenic genetic models [14, 16] have hampered studies of the role of LSECs in regulation of hepatic regeneration [62–64].

As early as 1965, portal venous blood flow has been identified as an important factor in liver regeneration [13]. Liver mass is significantly reduced post-PH (due to liver resection), and the remaining hepatic segments are subjected to increased portal blood flow and pressure. The earliest and most notable change following PH is the drastic increase in liver blood flow, with an increase in liver blood flow of up to 10-fold. Considering that hepatic SECs respond to shear stress and radical increases in blood flow, hepatic SECs must play a role in the initiation of liver regeneration.

## 5 Partial Hepatectomy Model

Recently, we have used a PH model to examine the instructive role of LSECs in hepatic regeneration (Fig. 3) [65]. In this model, resection of 70 % of the liver mass without perturbing the integrity of the residual liver vasculature activates hepatocyte regeneration [11, 63, 66]. This method is in contrast to the administration of hepatotoxic chemicals, which impair the organization of LSECs and causes tissue hypoxia, cell death, and inflammation [16, 60, 67]. This approach offers an instructive model for examining the role of structurally and functionally intact LSECs in supporting liver regeneration.

Growth factors such as those in the VEGF family play a significant role in the regeneration of bone marrow SECs [68]. As such, we hypothesized that two VEGF

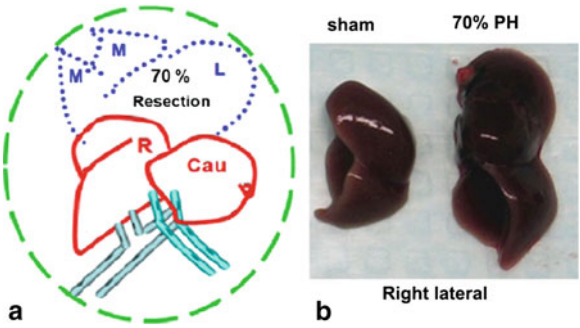


**Fig. 3** Liver Hepatectomy. **a** Schematic diagram of partial hepatectomy model in mouse. **b** Picture of sham and 70 % partial hepatectomy (*PH*)

receptors, VEGFR2 or VEGFR3, also modulate liver SEC function. Using VEGFR2-GFP mice in which the expression of green fluorescent protein (GFP) is driven by the native promoter of VEGFR2, we have demonstrated that VEGFR2 and VEGFR3 are exclusively expressed in the liver endothelial cells but not in other liver cell types, including hepatocyte nuclear factor 4α $(HNF4A)^+$ hepatocytes (Fig. 4a). As shown in Fig. 4b, the distribution of VEGFR3 expression is restricted to $VEGFR2^+$ LSECs that branch out from $CD34^+VEGFR3^-$ large vessels.

## 5.1 Flow Cytometry Analysis

Flow cytometric analysis on non-parenchymal cells demonstrates the expression of endothelial-specific marker VE-cadherin on non-hematopoietic $VEGFR3^+$ $VEGFR2^+CD45^-$ LSECs, 97.6 % of which are non-lymphatic endothelial cells expressing coagulation factor VIII (Fig. 5a, b). We have designated a unique phenotypic and operational signature for LSECs and other cells of adult mice as shown in Table 2. Identification of LSECs as $VEGFR3^+CD34^-$ and non-sinusoidal endothelial cells such as $VEGFR3^-CD34^+$ is sufficient for quantification, purification, and molecular profiling of LSECs.

In order to determine the mechanism by which LSECs regulate hepatic proliferation, we examined the regenerative kinetics of hepatocytes and LSECs after partial hepatectomy. Two days after partial hepatectomy, staining with VE-cadherin, hepatocyte marker epithelial (E)-cadherin, and mitotic marker phosphorylated-histone-3 (P-H3) revealed that $P\text{-}H3^+E\text{-}cadherin^+$ mitotic hepatocytes were positioned in the proximity of non-proliferating LSECs (Fig. 5c).

Proliferation of LSECs starts at day 4 and plateaus by day 8 after PH (Fig. 6a). In comparison, quantification of $P\text{-}H3^+HNF4A^+$ hepatocytes showed that the rate of hepatocyte proliferation peaks during the first four days, leveling off by day 8 (Fig. 6b). The roles of the inductive signals in liver regeneration is schematized (Fig. 6c).

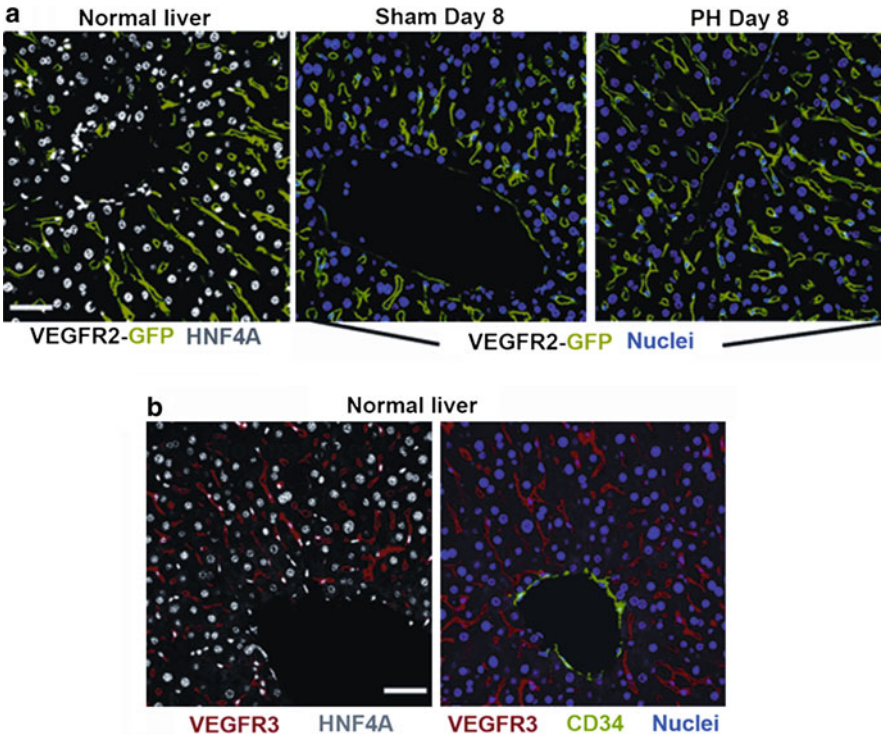


**Fig. 4** Phenotypic signature and contribution of LSECs to physiological liver regeneration induced by 70 % partial hepatectomy. **a** Liver sections obtained from VEGFR2-GFP reporter mice. During liver regeneration VEGFR2 is exclusively expressed on the liver endothelial cells. **b** Restricted expression of VEGFR3 on LSECs, but not $CD34^+$ large vessels or hepatocytes. Scale bars, 50 μm (Adapted from [65])

**Table 2** Signatures of various cell types in adult liver mice

| Cell Type | Markers |
|---|---|
| Liver-sinusoidal ECs | **$VEGFR3^+CD34^-$**$VEGFR2^+$VE-cadherin$^+$Factor$VIII^+$Prox-$1^-CD45^-$ |
| Non-sinusoidal ECs | **$VEGFR3^-CD34^+$**$VEGFR2^+$VE-cadherin$^+$ $CD45^-$ |
| Lymphatic ECs | $VEGFR3^+CD34^+$Prox- $1^+$Factor$VIII^-CD45^-$ |

These results suggest a two-step contribution of LSECs in mediating hepatic reconstitution. At the early phases of PH (days 1–3 after partial hepatectomy), inductive angiogenesis in the non-proliferative LSECs stimulates hepatic regeneration, possibly by releasing angiocrine factors. In contrast, 4 days after partial hepatectomy, the increased demand of blood supply for the regenerating liver is met by proliferative angiogenesis of LSECs.

To investigate the significance of VEGF receptors during liver SEC driven hepatic regeneration, the VEGFR2 gene was conditionally deleted by crossing $VEGFR2^{loxP/loxP}$ mice with ROSA-$CreER^{T2}$ mice, generating inducible VEGFR2-

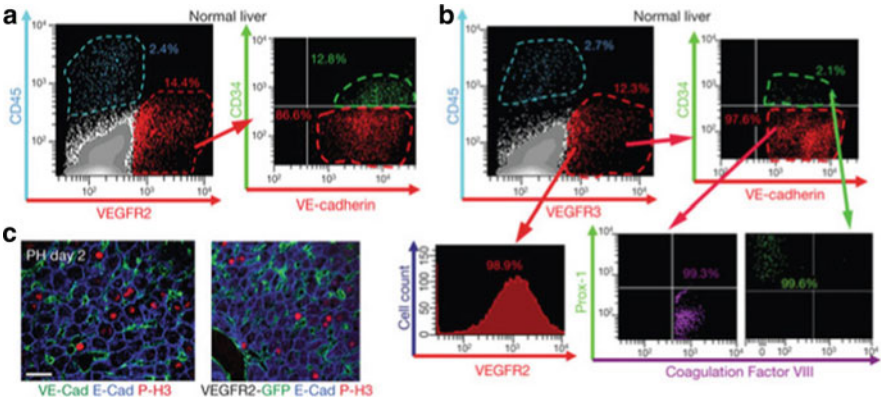


**Fig. 5** **a** Flow cytometric analysis of the liver nonparenchymal cells. VEGFR2$^{+}$ cells that are CD45$^{-}$, express endothelial-specific VE-cadherin. **b** Specific expression of VEGFR3 on VEGFR2$^{+}$VE-cadherin$^{+}$CD45$^{-}$ LSECs, with a predominant fraction being CD34$^{-}$ Factor-VIII$^{+}$Prox-1$^{-}$. **c** Forty-eight hours after partial hepatectomy, E-cadherin$^{+}$P-H3$^{+}$ mitotic hepatocytes are localized adjacent to VE-cadherin$^{+}$ and VEGFR2$^{+}$ endothelial cells. Scale bars, 50 μm (Adapted from [65])

deficient, VEGFR2$^{flox/flox}$ (VEGFR2$^{fl/fl}$) mice [68]. Owing to the endothelial-cell specific expression of VEGFR2 in the liver, in VEGFR2$^{fl/fl}$ mice only liver ECs, but not non-endothelial cells, will manifest functional defects. As control, we used mice with heterozygous deletion of the VEGFR2 gene (VEGFR2$^{fl/+}$). Forty-eight hours after partial hepatectomy, bromodeoxyuridine$^{+}$ hepatocyte proliferation (BrdU$^{+}$HNF4A$^{+}$ cell number) was decreased by 67 % in VEGFR2$^{fl/fl}$ mice (Fig. 7a, b). Despite the patency of the VE-cadherin$^{+}$isolectin$^{+}$ perfused vessels at this early phase, the regeneration of liver mass was attenuated in VEGFR2$^{fl/fl}$ mice (Fig. 7c). The result demonstrates that in the early phases (PH days 1–3) of liver regeneration, targeting VEGFR2 primarily impairs the effect of endothelial-derived angiocrine factors to induce hepatocyte regeneration, but not vascular perfusion capacity.

However, in VEGFR2$^{fl/fl}$ mice at the later stages of liver regeneration (PH days 4–8), proliferative angiogenesis was also defective (Fig. 7c), interfering with the assembly of patent VE-cadherin$^{+}$isolectin$^{+}$ vasculature (Fig. 7d, e), thereby diminishing restoration of the liver mass for at least 28 days. Furthermore, in VEGFR2$^{fl/fl}$ mice, liver function after PH was abnormal, as manifested by elevated plasma bilirubin levels. To confirm the endothelial-specific VEGFR2 function in mediating liver regeneration, VEGFR2$^{loxP/loxP}$ mice were also crossed with VE-cadherin-CreER$^{T2}$ mice to induce endothelial-selective deletion of VEGFR2.

Both the liver mass and formation of perfused vessels in the VE-cadherin-CreER$^{T2}$VEGFR2$^{fl/fl}$ mice were decreased after PH, which emphasizes the significance of VEGFR2 in mediating liver regeneration. In fact, if the VEGF-A/VEGFR2 pathway supports SEC-driven hepatic regeneration, then VEGF-A should enhance liver regeneration. We compared the effect of VEGF-A$_{164}$ with

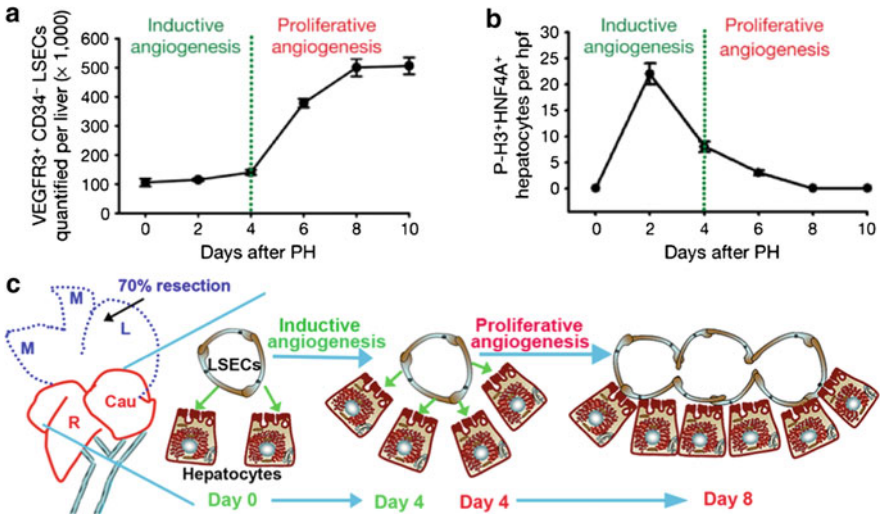


**Fig. 6** **a** Kinetics of liver SEC expansion and **b** hepatocyte mitosis during liver regeneration ($n = 4$). **c** Proposed schema describing the roles of SECs in liver regeneration. Upon partial hepatectomy (day 0), LSECs initiate the proliferation of proximal hepatocytes by exerting "inductive signals", possibly by releasing angiocrine factors. Day 4 post-PH, there is a significant liver SEC expansion (proliferative angiogenesis) to sustain hepatic reconstitution. Scale bars, 50 μm. Error bars, SEM

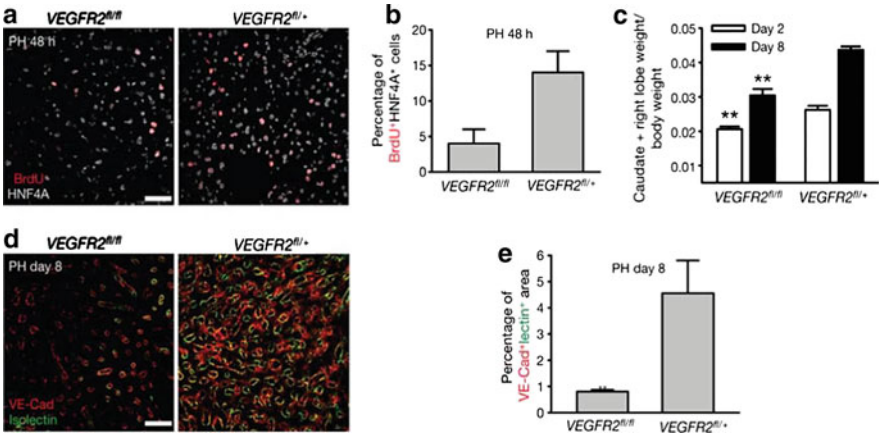


**Fig. 7** Partial hepatectomy induced VEGFR2-Id1 activation in LSECs mediates liver regeneration. **a**, **b** Hepatocyte proliferation after partial hepatectomy is impaired in VEGFR2$^{fl/fl}$ mice ($n = 5$). **c**–**e** Inhibition of liver mass regeneration **c** and functional VE-cadherin$^{+}$isolectin$^{+}$ vessel formation **d**, **e** in VEGFR2$^{fl/fl}$ mice after partial hepatectomy ($n = 4$–6). $^{**}P < 0.001$. Scale bar, 50 μm. Error bars, SEM (Adapted from [65])

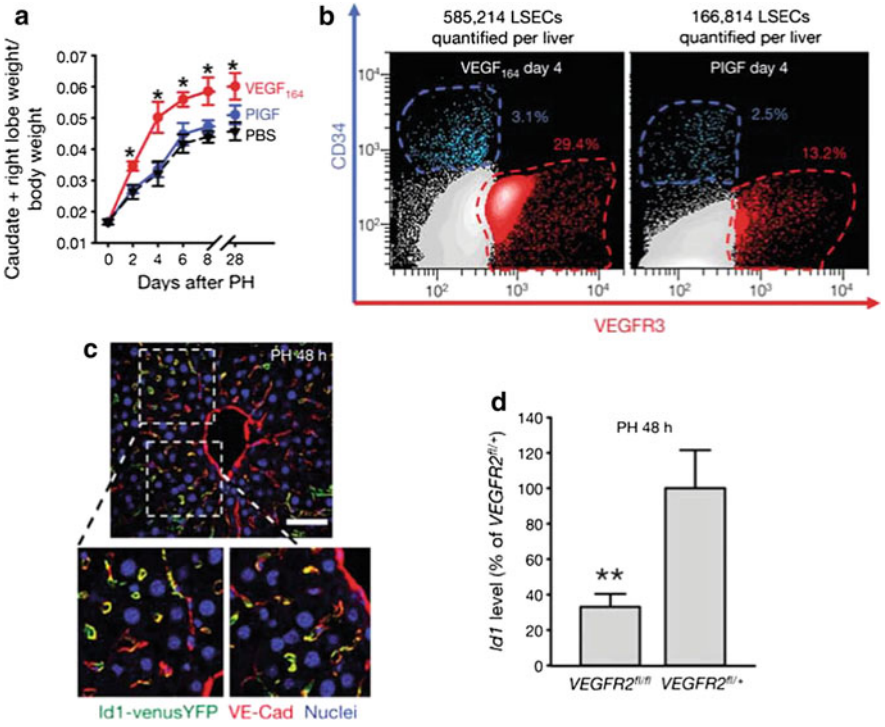


**Fig. 8** Injection of VEGF-$A_{164}$, but not VEGFR1-specific ligand PlGF, accelerates the regeneration of liver mass (**a**), associated with an incremental increase in $VEGFR^{+}CD34^{-}$ liver SEC number (**b**) (n = 4). **c** Regenerative liver section of $Id1^{VenusYFP}$ mouse. Id1 is selectively upregulated by partial hepatectomy in VE-cadherin$^{+}$ vessels. **d** VEGFR2 deletion diminishes Id1 upregulation in the regenerative liver **d** ($n = 5$).; $^{*}P < 0.05$, $^{**}P < 0.01$. Scale bars, 50 μm

placental growth factor (PlGF), as the latter selectively activates VEGFR1 [69]. After partial PH, $VEGF_{164}$, but not PlGF, accelerated the regeneration of both liver mass and the number of $VEGFR3^{+}CD34^{-}$ LSECs, which were sustained for at least 28 days (Fig. 8a, b). Therefore, after partial hepatectomy, the activation of VEGF-A/VEGFR2, but not PlGF/VEGFR1, is crucial for priming LSECs to initiate and maintain hepatic proliferation.

## 5.2 Microarray Analysis

To identify the angiocrine signals that stimulate liver regeneration, we used microarray analysis (Fig. 9). Among the endothelial-specific genes, the transcription factor Id1 was specifically upregulated in the endothelial cells activated by PH [70]. Using $Id1^{venusYFP}$ reporter mice in which the venus YFP expression is

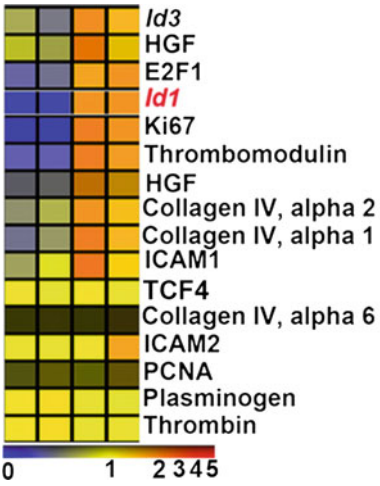


**Fig. 9** Heatmap comparing transcriptional profiles. Tree view of the representative Affymetrix gene expression in the regenerative liver 48 h after partial hepatectomy (PH 48 h) compared to that of sham-operated mice (Sham 48 h). Note that the upregulation of angiogenic transcription factor Id1 is comparable to Ki67 and higher than E2F1, both of which are closely associated with hepatocyte proliferation

driven by the Id1 promoter [71], we found Id1 upregulation in LSECs 48 h after PH (Fig. 8c) which was significantly blunted in VEGFR2$^{fl/fl}$ mice (Fig. 8d).

The liver mass recovery in Id1-deficient (Id1$^{-/-}$) mice after PH was impaired for 28 days and remained unchanged upon VEGF-$A_{164}$ administration (Fig. 10a). Furthermore, after partial hepatectomy, Id1$^{-/-}$ mice exhibited significant decrease in mitotic BrdU$^{+}$HNF4A$^{+}$ hepatocyte number, disrupted formation of functional VE-cadherin$^{+}$isolectin$^{+}$ vessels, diminished proliferation of VEGFR3$^{+}$CD34$^{-}$ LSECs, and abnormal liver function, as evidenced by an increase in plasma bilirubin levels. Thus activation of the VEGF-A/VEGFR2 pathway through upregulation of Id1 drives liver regeneration [65].

The role of Id1 upregulation in mediating the angiocrine function of LSECs on hepatocyte proliferation was also examined by a liver SEC–hepatocyte co-culture system. Co-incubation of isolated hepatocytes with primary LSECs led to a ninefold increase in hepatocyte number, which was selectively inhibited by knockdown of Id1 in LSECs (Fig. 10b). Conditioned medium from LSECs failed to support hepatocyte growth, underlining the importance of cell–cell contact in liver SEC-derived angiocrine function. Therefore, lack of Id1 results in defective inductive function of LSECs, impairing hepatocyte regeneration.

To determine whether in vivo angiocrine effects of Id1$^{+/+}$ LSECs could initiate hepatocyte regeneration in Id1$^{-/-}$ mice, we used the intra-splenic transplantation approach on day 2 after PH to engraft LSECs into the Id1$^{-/-}$ liver vasculature [72].

GFP-marked Id1$^{+/+}$ LSECs selectively incorporated into the VEGFR3$^{+}$sinusoidal vascular lumen and restored the regeneration of liver mass and SEC expansion (Fig. 10c). In contrast, the transplanted Id1$^{+/+}$ LSECs failed to restore the regeneration of the Id$^{+/+}$ liver. Partial vascular chimaerism afforded by the incorporation of Id1-competent LSECs generates sufficient endothelial cell-derived inductive signals to initiate hepatic proliferation in the Id1$^{+/+}$ liver.

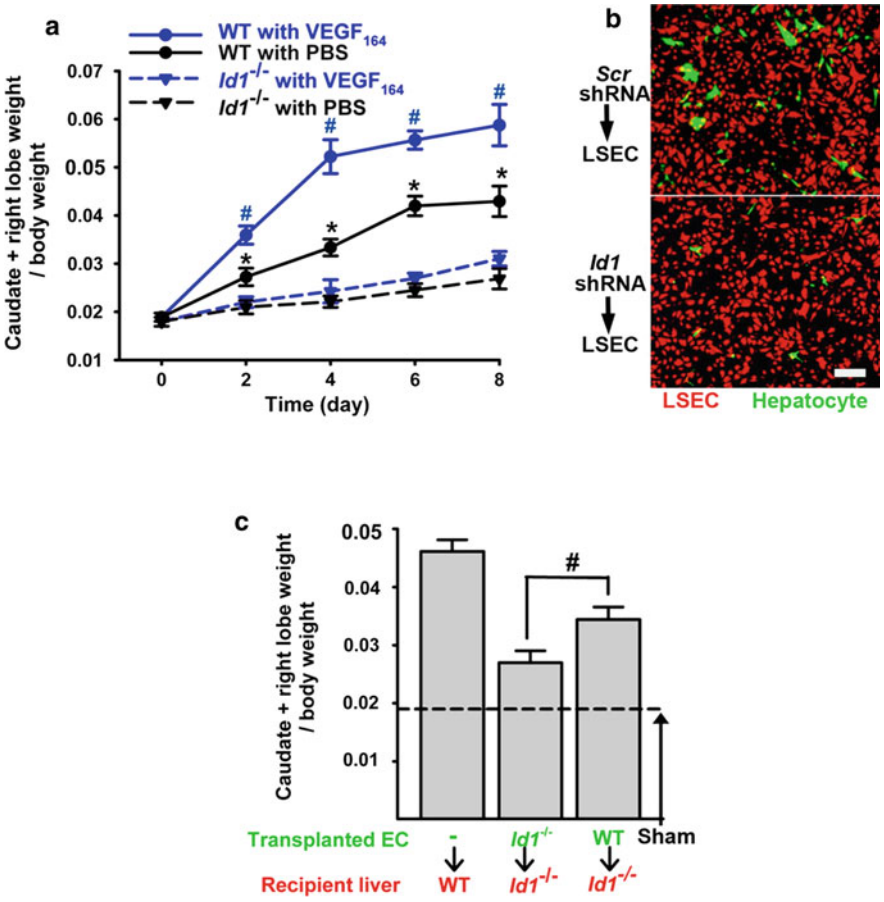


**Fig. 10** Id1 upregulation in LSECs is essential for liver regeneration. **a** Compared with their wild-type (*WT*) littermates, $Id1^{-/-}$ mice manifest impaired regeneration in liver mass, which fails to be rescued by VEGF-$A_{164}$ administration ($n = 5$). **b** The liver SEC-dependent stimulation of hepatocyte proliferation was specifically inhibited by Id1 gene knockdown. Scr, scrambled liver SEC-conditioned medium ($n = 4$). **c** Transplantation of $Id1^{+/+}$ LSECs restores the regeneration of mass in the $Id^{-/-}$ liver ($n = 4$). *Dashed line*, level of $Id1^{-/-}$ liver without endothelial cell transplantation. *P,0.05, versus $Id1^{-/-}$ **a**; **P,0.01, versus $Id1^{-/-}$ with $VEGF_{164}$ **a**. Scale bars, 50 mm **b** and 20 mm. Error bars, SEM

To identify endothelial-derived angiocrine factors that induce liver regeneration, we analyzed LSECs purified from the wild-type and $Id1^{+/+}$ mice 48 h after partial hepatectomy. The expression of Wnt2 and HGF, but not other hepatic trophogens expressed by LSECs, such as Wnt9B and thrombomodulin, was drastically diminished in $Id^{+/+}$ LSECs (Fig. 11a). These results suggest that Id1 upregulation in LSECs initiates hepatocyte proliferation through inducing Wnt2 and HGF expression. To test this hypothesis, on day 2 after partial hepatectomy, we engrafted $Id1^{-/-}$ LSECs transduced with Wnt2, HGF or both into the $Id1^{-/-}$

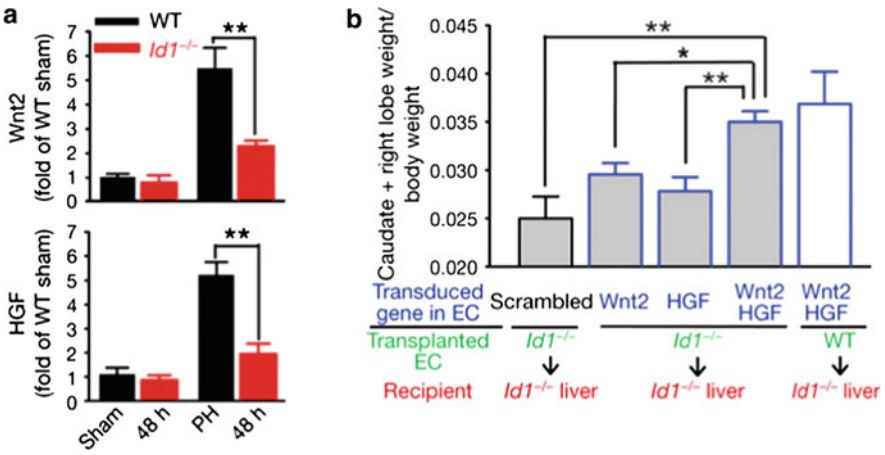


**Fig. 11** Id-1-mediated induction of Wnt2 and HGF in LSECs stimulates hepatic regeneration. **a** Upregulation of HGF and Wnt2 is impaired in $Id1^{-/-}$ LSECs after partial hepatectomy ($n = 5$). **b** Intrasplenic transplantation of GFP-marked $Id1^{-/-}$ LSECs carrying both Wnt2 and HGF ($Id1^{-/-}Wnt2^{+}HGF^{+}GFP^{+}$) rescues the regeneration of $Id1^{-/-}$ liver mass ($n = 4$). $^{*}P < 0.05$; $^{**}P < 0.01$. Error bars, SEM

liver vasculature by intra-splenic transplantation. Only $Id1^{-/-}$ LSECs carrying both Wnt2 and HGF ($Id1^{-/-}Wnt2^{+}HGF^{+}$) restored the regeneration of mass and liver SEC expansion in the $Id1^{-/-}$ liver (Fig. 11b), which suggests a collaborative effect between HGF and Wnt2. Therefore, Id1-activated LSECs through Wnt2 and HGF synthesis induce proliferation of juxtaposed hepatocytes (Fig. 12).

In the PH study, we have used conditional VEGFR2 knockout, $Id1^{-/-}$ mice, and an endothelial cell transplantation model to recognize the essential angiocrine role of a specialized organ-specific vascular niche cell in orchestrating physiological liver regeneration. Similar to upregulation of Id1 in the angiogenic tumor vessels, Id1 expression is minimal in normal LSECs, but after activation of VEGFR2 induces exclusive upregulation of Id1 in the angiogenic LSECs. In the first 3 days after partial hepatectomy, activation of the VEGFR2-Id1 pathway switches on an inductive angiogenesis program in non-proliferative $VEGFR3^{+}CD34^{-}VEGFR2^{+}Id1^{+}$ LSECs. Through production of angiocrine factors Wnt2 and HGF, this program provokes hepatic proliferation. Subsequently, as the regenerating liver demands additional blood supply, VEGFR2-Id1-mediated proliferative angiogenesis of LSECs reconstitutes hepato-vascular mass. Therefore, we introduce the concept that SECs support liver regeneration through a biphasic mechanism: at the early phase immediately after partial hepatectomy, inductive angiogenic LSECs promote organogenesis through release of angiocrine factors, whereas proliferative angiogenic LSECs vascularize and sustain the expanding liver mass.

Our earlier work has shown that transplantation of the $Id1^{-/-}Wnt2^{+}HGF^{+}$ LSECs into $Id1^{-/-}$ mice initiates and restores liver regeneration. These findings, and the observation that hepatic proliferation is severely reduced in the VEGFR2

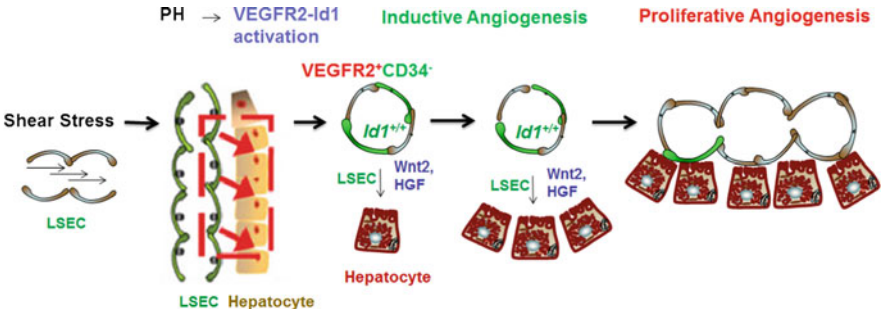


**Fig. 12** Requirement for VEGFR2-Id1 pathway in liver SEC-mediated liver regeneration. Intrasplenic transplantation of $Id1^{+/+}$ LSECs into the $Id1^{-/-}$ liver sinusoids restores hepatic-vascular regeneration. Transplanted $Id1^{+/+}$ *or* $Id1^{-/-}Wnt2^{+}HGF^{+}GFP^{+}$ LSECs localize to the vicinity of hepatocytes, promoting inductive and proliferative angiogenesis thereby sustaining physiological liver regeneration

and Id1-deficient mice, suggest that LSECs are responsible for establishing an inductive vascular niche to initiate hepatic proliferation [65].

## 6 Discussion

Regenerative medicine promises to improve health by repairing or regenerating cells, tissues or organs under different sets of circumstances including acute chemical injury, inflammation, surgical resection, remodeling, and age-related organ degeneration. Developmental biologists have tried to identify extrinsic and intrinsic factors that may potentially act as master regulators of embryonic organ formation. The biomechanical microenvironment also induces ingrowth and differentiation of tissue. For instance, injectable polymer systems have been shown to control the spatiotemporal dynamics of morphogens and in situ programming of cells at sites of injury [73].

Regeneration is a natural process in which the body can restore and repair damage to an existing cell, tissue, or organ. Scientists are interested in organ regeneration due to the possible medical applications of further advances in this field. The liver is one organ that naturally exhibits this ability, which even enables it to regenerate itself after the majority of the organ has been removed. Given that the liver already receives approximately 25 % of an organism's cardiac output, one theory is that an increased blood flow to the liver recorded immediately after PH leads to increased shear stress. Mechanotransduction, the conversion of mechanical forces acting upon a cell into biochemical activity, activates the signaling pathways leading to organ regeneration [74]. Further study of this dynamic between liver blood flow and regeneration could lead to important advances in the field of organ regeneration.

Integrins are receptors that help mediate the connection between the cell and the surrounding matrix. One way in which mechanotransduction is thought to take place is through integrins, which are regarded as mechanosensors due to the conformational changes they adopt when affected by external forces. Two integrins that have been found to exhibit this behavior are the $\alpha 5\beta 1$ and the $\alpha L\beta 2$ integrins [75]. When the apical surface of an endothelial cell experiences shear stress due to the blood flow acting on it, the tension experienced by the cell is transferred to integrins. These integrins, now activated, increase adhesion to the ECM, which alters the phenotype of the cell. In order to maximize adhesion, the cell needs to increase the surface area in contact with the ECM so it flattens in a fashion consistent with the blood flow. This allows the side of the cell facing the oncoming blood flow to resist more tension, while the opposite end of the cell will experience less tension [35]. This would increase the endothelial cell's chance of survival when under the influence of high rates of blood flow and the consequent shear stress.

Human liver can regenerate its original functional mass after hepatectomy and stops this process once the structure of the organ is rebuilt and its normal size restored. The developmental signaling mechanism that serves as the basis for this phenomena requires understanding in order to apply similar regenerative capacity in other organs. Identifying inductive factors that guide stem cells to differentiate into various cell types within different organs can be used as part of a therapeutic strategy.

Interestingly, we have recently shown that after unilateral pneumonectomy (PNX), activation of capillary endothelial cells plays a critical role in initiating and sustaining the regeneration of the lung, another organ constantly subjected to both cell cyclic stretch and changing blood flow [76]. It is plausible that the altered blood flow inside pulmonary vasculature post PNX ignites the activation of VEGFR2, which in turn stimulates the expansion of epithelial progenitor cells. In addition, it is tempting to postulate that changing pulmonary mechanics also plays an essential role in activating epithelial progenitor cells inside alveoli.

Induction of (organ-specific) stem cell differentiation alone may not be sufficient to complete the regeneration process, and creation of the proper local environmental cues and architectural arrangements inherent to the dynamic in vivo setting has been shown to play an integral role. To this end, the physical interactions between cells, ECM, and blood flow, as well as the molecular cross-talk between these constituents play a significant role in tissue regeneration. For example, transplanting hepatocytes in patients with end-stage liver disease results in suboptimal outcomes. With the identified inductive function of LSECs in both initiating and supporting hepatocyte amplification, co-transplantation of properly primed LSECs or pre-conditioning of the host's vasculature would significantly enhance hepatocyte reconstitution, as well as facilitate the engraftment of injected functional hepatocytes via optimized cellular interactions.

The self-regenerative capacity of the liver is frequently hampered by detrimental factors such as extensive resection, hepatotoxins, aging, and tumor metastasis. Thus, end-stage liver disease often requires transplantation therapy. However, lack of available liver tissue consistently curbs successful liver

engraftment in many patients. Development of strategies to expand functional transplantable hepatocytes will significantly increase the success of liver regeneration. We have shown that durable hepatic reconstitution requires cellular interaction between hepatocytes and the liver vascular niche demarcated by specialized liver SECs [65]. Production of liver SECs-derived paracrine growth factors, which we define as "angiocrine factors", has been shown to initiate and maintain hepatocyte regeneration, underscoring the urgency to develop cell therapy approaches to rebuild not only hepatic but also vascular function for liver diseases.

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# Microfluidic Devices for Quantifying the Role of Soluble Gradients in Early Angiogenesis

**Patrick Benitez and Sarah Heilshorn**

**Abstract** Early angiogenesis, as defined by endothelial polarization and directional sprouting, is regulated by gradients of soluble factors in addition to a multitude of other anisotropic cues including interstitial flow, insoluble gradients, and topography of the extracellular matrix (ECM). Adding to this complexity, other microenvironmental inputs, such as matrix density and rigidity, are known to modulate the extent to which vascular endothelial cells react to these anisotropic cues. Given this complexity, novel platforms are needed to decouple and systematically assess signals regulating early angiogenesis. To this end, we discuss a microfluidic device that achieves stable, matrix-independent soluble gradients via passive diffusion, which shields the culture chamber from shear-induced anisotropy. These devices enable direct time-lapse imaging of single cell and collective cell phenomena within both two-dimensional (2D) and three-dimensional (3D) cultures. These experimental platforms have been used to quantify the growth factor concentration requirements that induce endothelial cell chemotaxis, to identify previously unknown regulators of brain angiogenesis, to screen biomaterials for their angiogenic potential, and to investigate the navigational ability of nascent sprouts.

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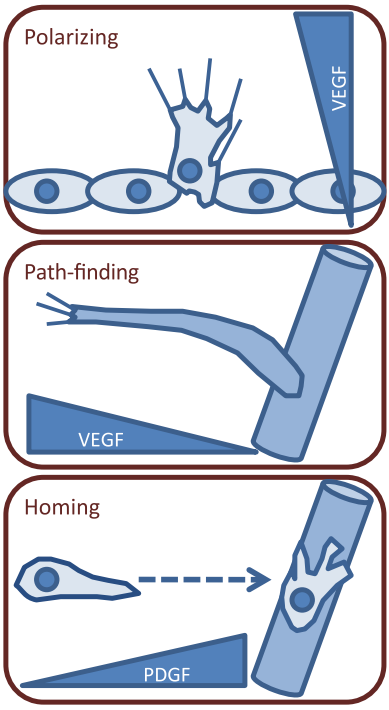


**Fig. 1** Schematic of three physiological processes that can be modeled using microfluidic devices. When exposed to a soluble gradient for a period of days or weeks, vascular endothelial cells polarize, angiogenic sprouts pathfind, and pericytes home. Spatiotemporally consistent, quantitatively predictable gradients within 3D culture matrices are required to reproducibly recapitulate these processes in vitro

## 1 Motivation: Understanding Vascular Development

In the cellular microenvironment, a soluble gradient is a vector quantity whose direction and magnitude reflect the greatest spatial rate of change in a soluble factor's concentration. Vascular endothelial cells respond to three aspects of the concentration field: the gradient direction, the gradient steepness, and the magnitude of concentration. These multiple inputs play an essential role in regulating angiogenesis, enabling the development of functional microvasculature by polarizing endothelial cells [1], guiding sprouts [2], and recruiting perivascular cells [3] (Fig. 1). Strategies to engineer blood vessels, including controlled delivery of soluble factors [4] and use of micro-patterned [5] and multivariate biomaterials [6], demand a quantitative, multifactorial understanding of signaling by soluble gradients.

Microfluidic devices that support specified, substrate-independent, and stable soluble gradients meet this critical need. First, the user's ability to specify a given concentration profile enables quantitative screening of chemotactic factors and decoupling of concentration magnitude from gradient steepness, parameters that can elicit distinct cell behaviors (Fig. 2) [7, 8]. Second, the gradient's substrate independence allows for reductive studies on the role of matrix properties in chemotaxis. The extracellular matrix affects cellular morphology [9], proliferation [10], migration [11], and specialization [12], impacting a cell's capacity to

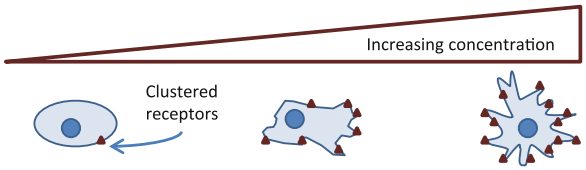


**Fig. 2** Schematic showing that cellular response to a chemotactic gradient depends not only on gradient steepness but also on concentration magnitude, which is a function of the cellular position along the concentration profile. There is an optimal concentration (*center*) for chemotaxis at which signaling by motility factors (a function of concentration magnitude) and directional bias of receptor clustering (a function of the gradient steepness) are sufficient to induce chemotaxis. Clustered receptors (*triangles*) drive extensions from the cell membrane. At concentrations below the activation threshold (*left*), signaling from bound receptors is insufficient to induce migration. At concentrations above the saturation threshold (*right*), receptors are saturated on all sides of the cell, resulting in no sustained directional bias in receptor binding

integrate and act on soluble gradients. Substrate independence also allows for three-dimensional (3D) culture under controlled gradients, which is important because native vasculature is inherently 3D. Third, reductive experiments on the time scales, i.e., days or weeks, necessary to quantify the role of soluble gradients in collective behaviors such as path-finding [13], inosculation [14], and maturation [15] of nascent sprouts are only possible under stable concentration profiles. With microfluidic devices that apply specified, substrate-independent, stable gradients, we can quantitatively and multifactorially analyze how soluble gradients and other inputs contribute to angiogenesis.

## 2 Background: Gradient-Generator Devices for Cell Culture Studies

Previous devices to study soluble gradients, such as transwell (*aka* Dunn) assays [16] and Zigmond chambers [17], are ill-suited to developing a quantitative, reductive understanding of the microenvironmental regulation of angiogenesis. These devices expose cells to transient gradients by permitting diffusion from a factor-rich to a factor-empty chamber. The transwell assay, for example, separates the chambers with a porous membrane; the Zigmond chamber separates them with a narrow intermediate chamber. For these "no-flow devices" (Table 1), as concentrations in the chambers equalize, cells are exposed to an attenuating gradient that may be completely absent in only 2 h [18]. Chemotaxis is analyzed by determining the fraction of cells that transit to the source. Because the gradient is transient, sprouting morphogenesis, which takes several hours to days [13], cannot be assessed. Recent improvements upon Zigmond chamber technology, such as the microslide device for chemotaxis [19], can maintain a gradient for up to 2 days (Fig. 3a). Likewise, transwell assays have been modified to support 3D culture [20]. The time scale of the

**Table 1** Comparative advantages of published microfluidic devices for studying the role of soluble gradients in angiogenesis

| | No-flow | Cell-chamber-flow | Restricted-flow |
|---|---|---|---|
| Steady-state gradient | No | Yes | Yes |
| Commercially available | Yes | Yes | By custom order |
| Minimal shear in culture chamber | Yes | No | Yes |
| Quantitative specification of a gradient | No | Yes | Yes |
| Fully substrate-independent | No | No | Yes |
| Compatible with 3D matrices | Yes | No | Yes |
| Representative examples | Refs. [16–20] | Refs. [23, 24] | Refs. [27–30] |

transient gradient, though, is still insufficient for studying relevant processes in angiogenesis. The initial concentration in the source chamber also determines both the concentration magnitude and the gradient steepness as detected by cells at a given time during the evolution of the concentration profile. Growth factor decay, moreover, is a problem for static devices, i.e., those without input of fresh reagent; for example, vascular endothelial growth factor's (VEGF) active half-life is 50 min under standard culture conditions [21]. Due to these limitations, novel microfluidic devices are needed to create controlled gradients of active factors that operate at steady-state and, therefore, are suited to long-term culture.

Limitations of traditional assays have led to the development of several microfluidic devices that use continuous flow to generate stable gradients. By employing steady-state flows to maintain a gradient, these devices enable long-term culture and the study of emergent properties of angiogenic sprouts. These microfluidic devices can be separated into two broad categories based on whether the fluid stream flows directly over the cells, i.e., "cell-chamber-flow devices", or through channels adjacent to the cells, i.e., "restricted-flow devices", (Table 1). As a consequence of direct contact with fluid flow, cells in cell-chamber-flow devices experience significant shear stresses. For example, early microfluidic devices, such as the 'Y' chamber [22], create a gradient at steady-state by merging a source and sink flow over the cell culture chamber. Other devices, which use a serpentine network to merge flows [23, 24], have the additional advantage of being able to create arbitrary concentration profiles (Fig. 3b). These devices mimic key aspects of in vivo vasculature, where blood flow and interstitial flow are important anisotropic signals for sprouting angiogenesis [25]. However, in these devices, the direction of shear with respect to the gradient cannot be varied: they are always perpendicular. This coupling of shear and gradient limits these devices as platforms for developing a systematic understanding of the role of soluble gradients. Furthermore, studies have demonstrated that cell migration can be significantly altered by the presence of shear alone [26]. Reliance on flow through the chamber also precludes the use of 3D matrices as culture scaffolds, which greatly restricts their use in the evaluation of biomaterials and cell-extracellular matrix (ECM) interactions.

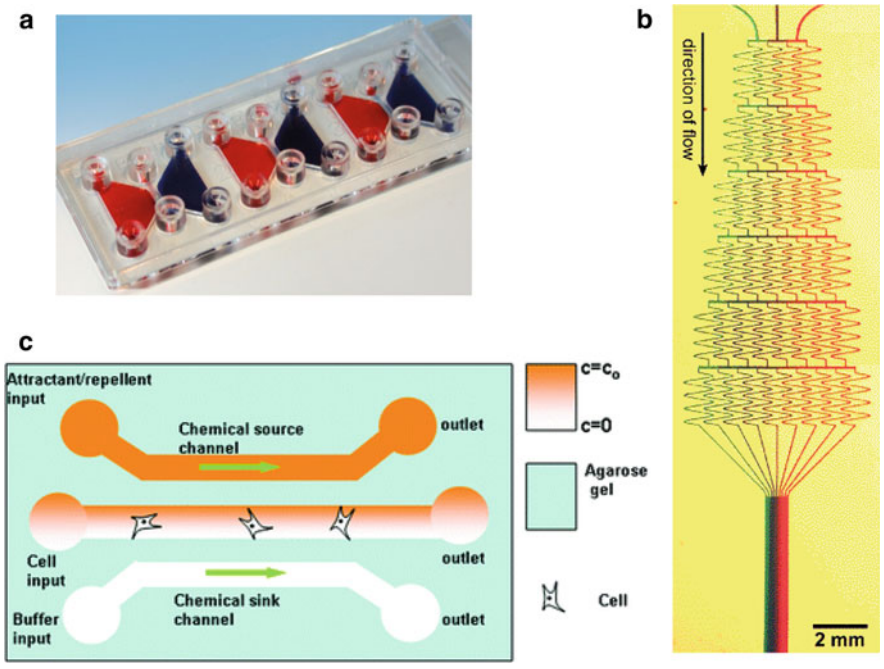


**Fig. 3** Representative examples of the three different types of microfluidic devices for studying soluble gradients. **a** The commercially available microslide is a static device that can maintain a gradient for 2 days within a small channel for cell culture. **b** A serpentine network device can create an arbitrary concentration profile at steady-state. Cells are directly exposed to flow [24]. Reprinted with permission from [24]. Copyright 2001 American Chemical Society. **c** A restricted-flow device that uses an agarose hydrogel to shield a culture chamber from source and sink flows, between which a linear concentration profile forms [29], originally published in [29]. *Reproduced by permission of The Royal Society of Chemistry,* http://dx.doi.org/10.1039/B618463D

To address these limitations, several research groups have reported the development of shear-minimized, or "restricted-flow" devices (Table 1, Fig. 3c) [27–29]. In one example, Heilshorn et al. have developed a shear-minimized microfluidic device that enables controlled, substrate-independent control of capillary-mediated soluble gradients for use in endothelial cell chemotaxis and sprouting morphogenesis assays [30]. In this device, stable gradients are formed by exposing a culture chamber to two flowing reagent channels; one channel is a source of fresh VEGF and the second a sink (Fig. 4). Micro-capillaries, which allow Fickian diffusion, inhibit convective fluid transport, thereby minimizing shear stress on cells and substrate. The gradient that arises at steady-state depends only on the concentration of VEGF in the source and the sink channels. The fabricated culture chamber is further able to accommodate either an adsorbed surface coating or an injectable 3D matrix. Therefore, dimensional context of the culture can be varied between 2D and 3D culturing platforms to match the

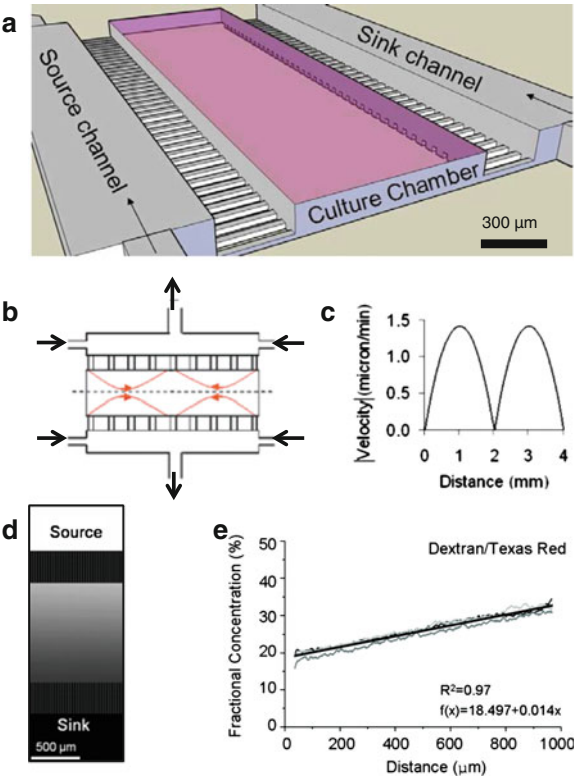


**Fig. 4** Design and characterization of a "restricted-flow" microfluidic device. **a** Schematic of the device. Finite element simulation, at a flow rate of 8 nL/min, of **b** fluid flow streamlines, with flows at inlets and outlets marked by *black arrows*, and **c** absolute value of fluid velocity along the *dashed line* shown in **b**. **d** Validation of the generated gradient using FITC-labeled dextran ($Mw = 20$ kDa, similar to VEGF-165), originally published in [30]. *Reproduced by permission of The Royal Society of Chemistry,* http://dx.doi.org/10.1039/C005069E. **e** The concentration profile of Texas red-labeled dextran across the culture chamber. Originally published in [70]

physiological environment of the cultured cells. This microfluidic device enables application of quantitatively specified linear gradients to study (1) sprouting over a period of days, (2) the contribution of matrix properties, and (3) interactions between concentration magnitude, gradient steepness, and other inputs.

## 3 Background: Soluble Gradients in Angiogenesis

Soluble gradients impart directional signals that are critical to the formation of functional blood vessels during wound healing and development. Soluble gradients enable specialization of tip cells, pathfinding of sprouts to hypoxic tissue, and vessel maturation via sprout inosculation and recruitment of pericytes. To elaborate on the importance of gradient-mediated anisotropy to sprouting angiogenesis, we briefly summarize the current knowledge about how gradients of VEGF and other soluble factors regulate sprouting angiogenesis.

Soluble gradients arise and impart directionality during angiogenesis through various biochemical and biophysical mechanisms. Upon hypoxic stress, cells down-regulate the degradation of hypoxia inducible factors (HIFs), transcription

factors that trigger expression of a portfolio of pro-survival and angiogenic cues. Under normoxia, cells hydroxylate HIFs, marking them for degradation [31]. While HIFs are active, modified gene expression leads to secretion of VEGF, a chemotactic agent of sub-nanomolar potency [32], and formation of a paracrine diffusion gradient. Moreover, interaction between protease secretion and interstitial flow intensifies the gradient [33]. VEGF stimulates human microvascular endothelial cells (hMVECs) to secrete proteases such as matrix metalloproteinase-9 (MMP9) [34] and urokinase plasminogen activator [35], which cleave the extracellular matrix and release sequestered VEGF [36], adding to the already present paracrine VEGF. Interstitial flow, which is increased during sprouting angiogenesis due to loosening of intercellular junctions in the sprouting vessel [37], adds directionality to protease-released VEGF, creating a gradient as it sweeps growth factor and protease away from the sprout's origin [38]. Cells sense these soluble gradients through the spatial mechanism of competitive receptor clustering (Fig. 1) [39]. As activated receptors cluster, the pool of potentially active receptors is depleted. Since clusters will form more quickly, on average, on the side of the cell facing the higher concentration, the distribution of receptor signaling complexes acquires a spatial bias in response to a gradient. Overall, hypoxic cells biophysically communicate their need for perfusion by establishing self-reinforcing soluble gradients of VEGF that impart directionality to targeted hMVECs.

Among the soluble factors involved in sprouting angiogenesis, VEGF is the most well-known for its capacity to elicit sprouts from existing blood vessels. Upon stimulation by a soluble VEGF gradient, quiescent hMVECs polarize and transition to a migratory phenotype, as marked by lamellipod extension [40] and cytoskeletal polarization [41]. In addition, soluble VEGF gradients induce asymmetry in the distribution of caveolin-1, partitioning it to the side of the cell facing the higher concentration [42]. Caveolin-1 anchors caveolae, protein- and cholesterol-rich invaginations in the plasma membrane, to the cytoskeleton. Organization of caveolae facilitates integrin turnover [43] and migration [44]. In addition to caveolae, actin-anchored filopodia segregate in the direction of the gradient in activated hMVECs. Filopodia enhance path-finding by presentation of receptors [45] and membrane-bound proteases [46]. Protease activity in response to a soluble gradient can amplify directional signaling by selectively degrading the basal lamina in addition to releasing sequestered VEGF. ECM components of the basal lamina signal for vascular quiescence [47], so their removal may facilitate proliferation and migration. The overall result is that soluble VEGF gradients polarize hMVECs and contribute to their phenotypic transition from quiescent to invasive during sprouting angiogenesis.

To resolve hypoxia, the VEGF gradient continues to impart directionality to hMVECs as they form sprouts, tubular structures that precede nascent vessels, and undergo columnar migration. After detecting a gradient and transitioning to a migratory phenotype, activated hMVECs, known as tip cells, suppress tip specialization and encourage stalk specialization in neighboring cells [48]. Tip cells remodel the ECM and chemotax, while the main activity of stalk cells is

proliferation [49, 50]. These identities are fluid and result from dynamic VEGF receptor-2 (VEGFR2) and Notch signaling [51]. VEGFR2, when potentiated by VEGF, signals for migration and matrix degradation. Delta-like ligand-4, which is up-regulated by VEGFR2 signaling, is a cell-displayed Notch ligand that triggers cell proliferation and expression of VEGF receptor-1 (VEGFR1) in neighboring cells, i.e., in the stalk [52]. These proliferating cells ignore the VEGF gradient as VEGFR1 sequesters VEGF from migration-inducing VEGFR2 [53]. Stalk cells eventually form a perfusible lumen by merging pinocytic vacuoles [54]. This division of labor, with the proliferating stalk cells and migratory tip cells, means that gradients not only regulate path-finding but also stalk morphology. Steeper VEGF gradients increase the velocity of the tip cell as it travels through the ECM by statistically increasing the directional bias of the cell's random walk. The stalk cells, meanwhile, proliferate along the path of least resistance, which is the trail blazed by the tip cells though the mechanically cell-restrictive ECM. It has been hypothesized that faster migration results in thinner sprouts; the proliferation of the stalk can only marginally keep up with the tip [55]. Slower migration, i.e., shallower gradients, results in thicker spouts, since cells must proliferate outward if there is insufficient space along the forward path. Through these mechanisms, VEGF gradients instruct sprouts to grow in the appropriate direction and with the appropriate morphology.

Although VEGF is the primary soluble factor that initiates sprouting angiogenesis, gradients of other factors play crucial roles in maturation of nascent sprouts. Tip cells secrete angiopoietin-2 (Ang-2) [56], destabilizing nearby mature vessels and potentially eliciting a sympathy sprout with which to anastomose. Ang-2 is sensed by the receptor "tyrosine kinase with immunoglobulin-like and EGF-like domains-2" (TIE-2) [57], which triggers directed release of proteases such as MMP-9 [58]. Digestion of the local ECM results in increased interstitial flow and a gradient of liberated VEGF, potentially leading to sympathetic sprouting. Merger of the initial sprout with a perfusable network is essential to flow, without which nascent vessels revert [59]. Meanwhile, up-regulated expression of autocrine angiopoietin-1 (Ang-1), a TIE-2 antagonist, protects the proliferating stalk cells from Ang-2-mediated destabilization [60]. As an example of another maturation process, pericytes, cells that reside in the perivascular niche surrounding the endothelial sprout, detect soluble gradients and chemotax to nascent blood vessels where they deposit basal lamina [61] and help to re-establish quiescence [62]. Gradients of platelet-derived growth factor (PDGF), which induce directionally-biased clustering of PDGF receptors, can induce chemotaxis of pericytes to nascent vessels [63]. Chemotactic PDGF gradients enable recruitment and proliferation of pericytes from distant tissues, including bone marrow [64]. In summary, while VEGF gradients are sufficient to induce the beginning stages of sprouting morphogenesis, soluble gradients of other factors are required for the maturation of nascent blood vessels.

## 4 Recent Results and Quantitative Studies

Our laboratory recently reported the development of microfluidic devices that enable the culture of cells in a low-shear, stable soluble gradient environment for mechanistic studies of sprouting morphogenesis. These devices allow decoupling of the role of soluble gradients from other environmental cues, such as signals from the ECM or biomechanical forces. To design such devices, we first simulated several configurations *in silico* [30]. For each simulation, the diffusion and flows were modeled using the Navier–Stokes equations, solved numerically by a finite element method (Fig. 4b, c). Depending on the diffusivity of the gradient molecules (which is related to the molecular weight of the diffusing solute and the medium present in the cell culture chamber), devices with either two or four inlets were selected (Fig. 4a). Soluble factors diffuse through micro-capillaries that connect the reagent channels to the cell culture chamber and inhibit convective transport into the culture chamber. In order for the gradient to remain stable, the characteristic diffusion time across the culture chamber must be less than the characteristic convection time through the source and sink channels. A flow rate of 8 nL/min was found to be suitable for a soluble gradient of VEGF diffusing through liquid culture medium. The distribution of a fluorescently labeled marker (e.g., 20 kDa FITC-dextran) can be quantified to validate the stability and linearity of the gradient (Fig. 4d, e) [30]. This model molecule formed a quantitatively predictable gradient at steady-state after 40 min. At this flow rate, convective forces in the culture chamber are predicted to generate a maximal shear stress on the cell-surface of $10^{-5}$ dyn/cm$^2$ (using a Stoke's drag approximation), which is orders of magnitude less than the amount of shear known to induce cellular alignment of ECs [65]. In contrast, devices that use flows within the cell culture chamber to generate steady-state gradients expose cells to shear on the order of $10^{-2}$ dyn/cm$^2$ [66].

Use of this microfluidic device has demonstrated that (1) the magnitude of the VEGF gradient must meet a minimum threshold to induce EC chemotaxis, and (2) chemotaxis is independent of concentration across the VEGF range of 18–32 ng/mL [30]. Chemotaxis in response to identical gradient steepness but different average concentrations was quantified by counting net accumulation or depletion of cells after 6 h in each quadrant of a device, (Fig. 5a, b). By this measure, human umbilical vein endothelial cell (HUVEC) chemotaxis was induced by a VEGF gradient of magnitude 14 ng/mL/mm (Fig. 5c), whereas a magnitude of 2 ng/mL/mm was insufficient to induce chemotaxis (Fig. 5d, e). This threshold is a consequence of stochasticity in factor diffusion and receptor binding. Although the probability that a gradient imparts its direction to a cell via spatially biased receptor potentiation increases linearly with the magnitude of the gradient, cells have intrinsic randomness in motility that must be overcome before chemotaxis can happen [67]. In addition to reductively assessing gradient magnitude, this experiment decoupled the effects of the average VEGF concentration from the VEGF gradient steepness. During analysis, the cell culture chamber was divided into four

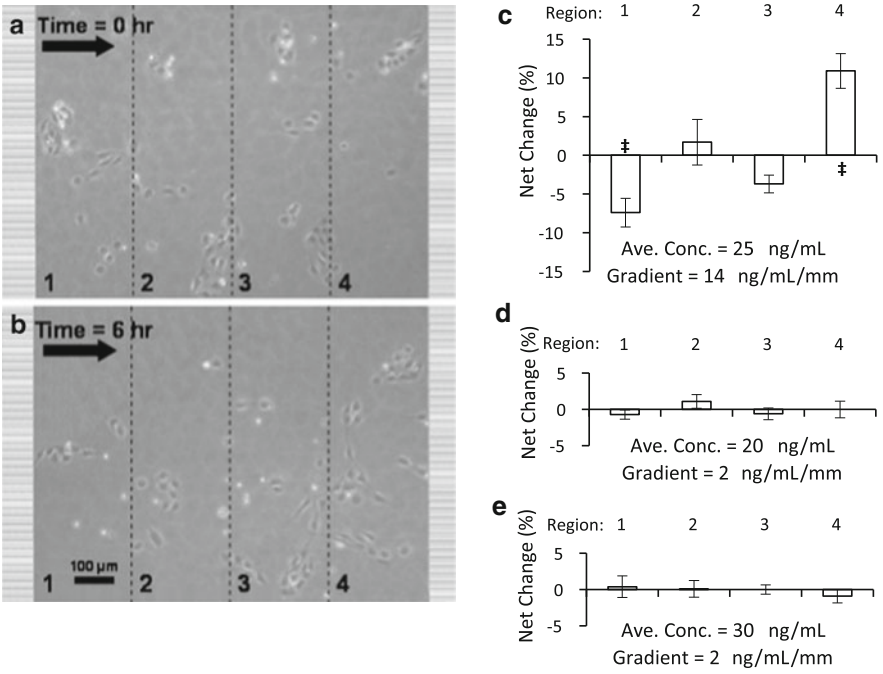


**Fig. 5** Chemotaxis of human umbilical vein endothelial cells. **a**, **b** Representative phase contrast images of devices at time = 0 and 6 h under a gradient of 14 ng/ml/mm and average concentration of 25 ng/mL. The chamber is divided into four equal regions of increasing VEGF concentration. The *arrow points* in the direction of the VEGF gradient. **c–e** The average net accumulation in the number of cells within each region under three linear concentration profiles, ±standard deviation (n = 3–5 devices). ‡Statistically significant accumulation/depletion of cells, $p < 0.05$. Originally published in [30]. *Reproduced by permission of The Royal Society of Chemistry,* http://dx.doi.org/10.1039/B719788H

regions that ranged in average concentration from 19 to 31 ng/mL and, had identical gradient steepness (Fig. 5a, b). Over this concentration range, similar numbers of cells were observed to chemotaxis in each region. In regions 2 and 3, accumulation of cells from the region of lower concentration is equal to depletion of cells to the region of higher concentration; therefore, there is no net change in cell number. Similarly, depletion of cells from region 1 (with the lowest VEGF concentration) was equal to accumulation in region 4 (with the greatest VEGF concentration). Greater average concentration has been theoretically predicted and experimentally shown to decrease chemotaxis, with sensitivity to the gradient scaling with one over the square root of concentration [67]. These results suggest that a higher average concentration would be required to observe a loss of directed migration in this system. In addition to the VEGF concentration profile, the density or type of substrate is known to impact 2D migration [68]. While these experiments utilized a fibronectin-coated surface, future studies could evaluate chemotaxis on a variety of

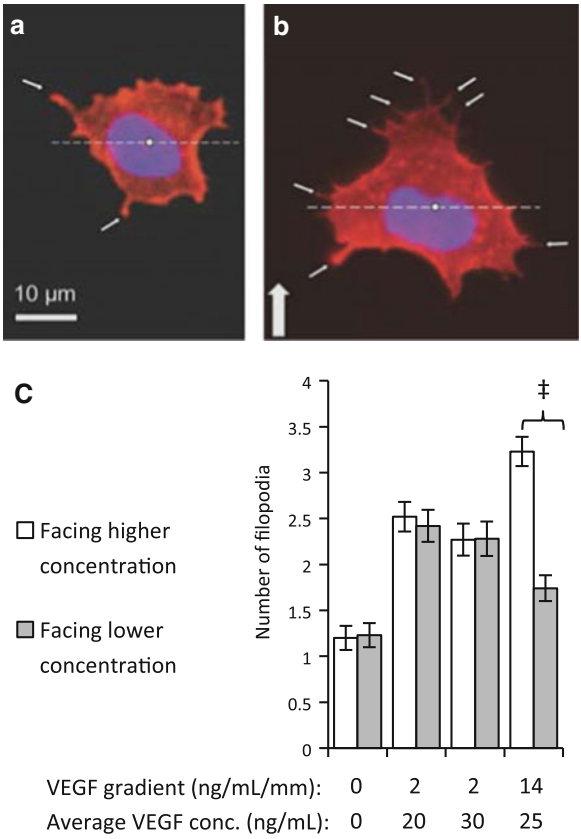


**Fig. 6** Human umbilical vein endothelial cells (*HUVECs*) extend filopodia with a directional bias if cultured under a gradient above a threshold magnitude. Representative fluorescent micrographs, nulclei = *blue*, actin cytoskeleton = *red*, of HUVECs in the **a** absence or **b** presence of a VEGF gradient of magnitude 14 ng/mL/mm and of direction indicated by the *thick white arrow*. We define filopodia, indicated by fine *white arrows*, as protrusions from the cell membrane of length $> 3$ μm and width $< 1$ μm. To assess possible directional bias in extensions, each cell is divided through its centroid into sections facing higher and lower concentrations of VEGF. **c** Number of filopodia per cell facing each direction under different linear concentration profiles of VEGF. Means are of $n > 70$ cells ±standard deviation. ‡Statistically significant, $p < 0.001$. Originally published in [30]. *Reproduced by permission of The Royal Society of Chemistry,* http://dx.doi.org/10.1039/B719788H

substrates to enable a multifactorial study of VEGF gradients and cell-surface interactions.

The importance of analyzing gradient steepness and average concentration as separate inputs is further emphasized by studies of filopodia extension in 2D [30]. Cytoskeletal remodeling is a prerequisite for chemotaxis, so cytoskeletal asymmetry is expected under conditions that induce chemotaxis. Similar to the results discussed above, a gradient steepness of 14 ng/mL/mm was sufficient to induce asymmetry in

filopodia extensions (Fig. 6a), while shallower gradients of 2 ng/mL/mm were ineffective (Fig. 6b). As predicted, a higher number of filopodia were observed on the cell edge facing higher VEGF concentrations. While the filopodia distribution was found to depend on the gradient steepness, the total number of filopodia was found to be a function of average VEGF concentration (Fig. 6c). VEGF concentrations of 20 ng/mL and greater were observed to stimulate significant filopodia extension (Fig. 6). These data suggest a minimum VEGF activation threshold is required to induce a migratory phenotype ($\sim$20 ng/mL) while a minimum VEGF gradient steepness is required to induce directionality ($\sim$14 ng/mL/mm).

Although cell polarization and chemotaxis can be conveniently studied in 2D, other important angiogenic processes such as sprouting morphogenesis and lumen formation are inherently 3D. To reductively study how soluble concentration profiles affect these processes, microfluidic devices that produce stable, quantitatively determined gradients within 3D matrices are required. In addition, the ideal experimental platform would enable tuning of the soluble gradient independently from choice of 3D matrix in order to probe the effects of matrix properties, such as matrix biochemistry, density, or stiffness in a reductionist manner. Devices that impose transient gradients or rely on flow through the cell culture chamber do not meet this criterion, because choice of matrix will impact diffusivity of the soluble cue, thereby altering the time-course of a transient gradient and/or the flow profile through the matrix, making comparisons across matrices impossible.

To achieve stable, matrix-independent gradients, the microfluidic device described previously can be slightly altered. To enable injection of matrix components, the height of the culture chamber can be increased. To decrease the time required to reach steady-state, the flow rates in the reagent channels is increased to 40 nL/min and the size of micro-capillaries is increased. In a 3D experimental study, these device changes are not observed to increase convective flow within the culture chamber because the matrix itself serves as a barrier to flow. To initiate 3D sprouting morphogenesis, endothelial cells are cultured as a 2D monolayer on collagen-coated beads. These cell-coated beads are then suspended within a fibronectin/collagen matrix. Sprouting morphogenesis was quantified in matrices of identical fibronectin concentration and varying collagen density from 0.3 to 2.7 mg/mL, resulting in materials with storage moduli of 7–700 Pa [69]. While increasing the matrix density increased the time required to reach a stable VEGF gradient, all matrices resulted in quantitatively consistent gradients of 50 ng/mL/mm VEGF within 2 h, a time scale that is much shorter than the experimental time scale of sprouting morphogenesis (1–4 days). As matrix density increased, cellular behavior changed from being mainly migratory to mainly proliferative (Fig. 7). At a low density of 0.3 mg/mL, isolated cells migrated into the matrix and underwent single cell chemotaxis. In intermediate densities, cells coordinated their chemotaxis; cells migrated as multiples at 0.7 mg/mL and as stable sprouts at 1.2 and 1.9 mg/mL. At a high density of 2.7 mg/mL, cell proliferated on the bead surface to form stumps that did not migrate into the surrounding matrix. Together, these data demonstrate that matrix density influences cell migration and proliferation, and stable sprouting morphogenesis occurs only when these two cellular behaviors are in balance.

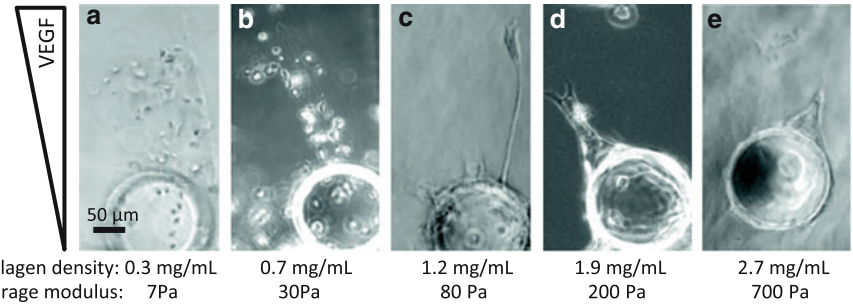


**Fig. 7** Representative phase contrast images of sprouting morphologies within collagen/fibronectin matrices of different collagen densities and identical VEGF gradients. **a**, **b** At low collagen densities (0.3 and 0.7 mg/mL), cells primarily migrate into the matrix as isolated cells. **c**, **d** At intermediate collagen densities (1.2 and 1.9 mg/mL), cells balance proliferation and migration to form stable sprouts. **e** At a high collagen density (2.7 mg/mL), cells form thick protrusions with minimal migration into the matrix. Originally published in [69]. *Reproduced by permission of The Royal Society of Chemistry,* http://dx.doi.org/10.1039/C005069E

In addition to affecting sprout morphology, matrix density was observed to affect the capacity of a sprout to align with a soluble gradient. Time-lapse microscopy was utilized to image sprout elongation over 4 days in the microfluidic devices. From these images, the initial angle (i.e., base of the sprout) final angle (i.e., tip of the sprout) of each sprout's growth could be measured with respect to the direction of the VEGF gradient (Fig. 8a, b) [70]. An angle of zero means that that sprout is perfectly aligned and growing towards the higher VEGF concentration. An angle of 180° signifies that growth is completely opposed to the gradient (Fig. 8c). For matrices of lower density (1.2 mg/mL), sprouts aligned to the gradient over time, with greater fidelity to gradients of steeper magnitude (46 vs. 23 ng/mL/mm) (Fig. 8d, f). In contrast, sprouts in matrices of higher density (1.9 mg/mL) did not align over time (Fig. 8e, g). Instead, these sprouts were much more likely to align with the gradient as soon as the sprout left the bead surface, with increased alignment observed in steeper gradients. From these data, we conclude that identical VEGF concentration profiles can result in dramatically different sprout pathfinding behaviors depending on the matrix properties. Lower density matrices are more permissive of sprout turning, while higher density matrices prevent misalignment of initial sprouting. These results demonstrate the critical importance of decoupling cell-matrix, cell-soluble signal, and soluble signal-matrix interactions. Furthermore, these results suggest that studies of soluble factor potency (e.g., determination of activation and saturation thresholds) will be dependent on the choice of 3D matrix.

Matrix density was also observed to mediate lumen formation, a morphological process that is required for perfusion and sprout stabilization to occur. Because microfluidic devices can be fabricated from optically clear materials (glass and polydimethylsiloxande) fluorescence staining and confocal microscopy can be performed directly on the samples at the end of the culture period (Fig. 9a, b). The

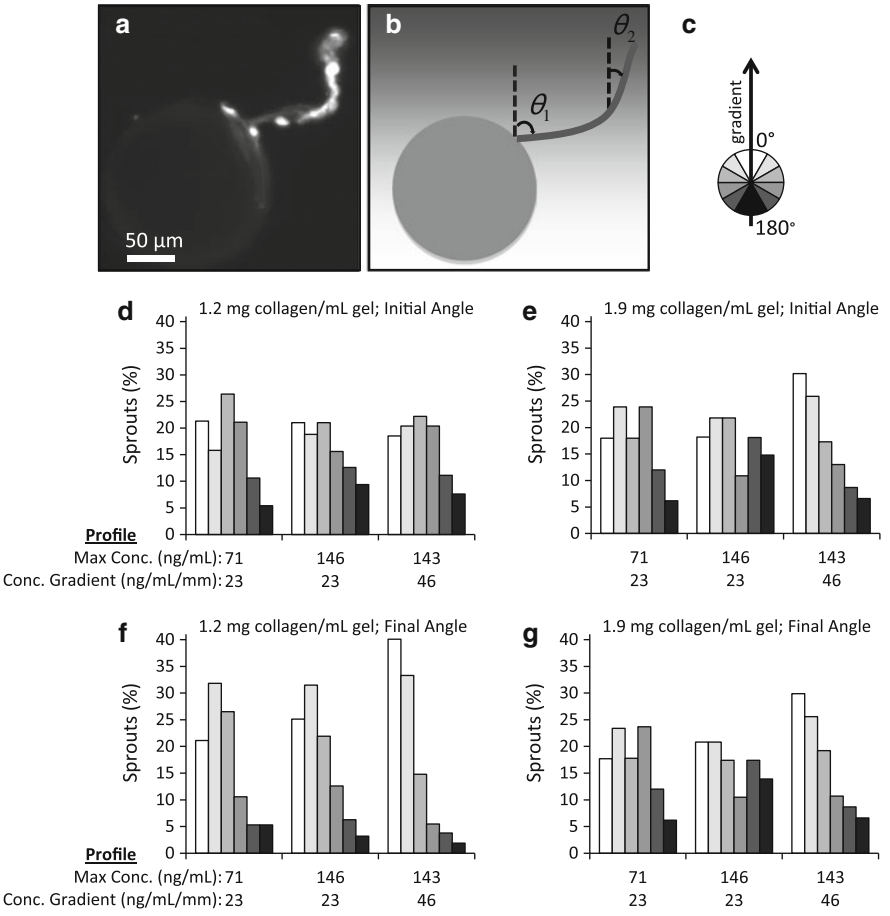


**Fig. 8** Sprout pathfinding in different linear VEGF gradients. **a** Fluorescent images of sprout pathfinding are analyzed by quantifying **b** the initial ($\theta_1$) and final ($\theta_2$) angles of sprout growth with respect to the gradient. **c** The growth angle is binned into six angular regions, colored as described by the pinwheel. An angle of 0° is completely aligned with the gradient, and an angle of 180° means the sprout is growing anti-parallel to the gradient. Distributions are shown for **d**, **e** initial and **f**, **g** final angles of sprouts grown in matrices of **d**, **f** 1.2 mg/mL and **e**, **g** 1.9 mg/mL collagen. Three VEGF concentration profiles are shown. Increased alignment with the gradient between initial and final angles indicates sprout pathfinding. For each condition, n = 4 microdevices, with an average of 80 sprouts. Originally published in [70]

extent of lumen formation was quantified by determining the fraction of the sprout length with a hollow core (Fig. 9c) [69]. As an additional measure of sprout maturation, cell density within the sprout was quantified as the number of cell nuclei found within a 10 µm length of the sprout. It is hypothesized that a higher cellular density is more likely to support lumen formation. Both markers of maturity are significantly increased in the 1.9 mg/mL collagen matrix compared to

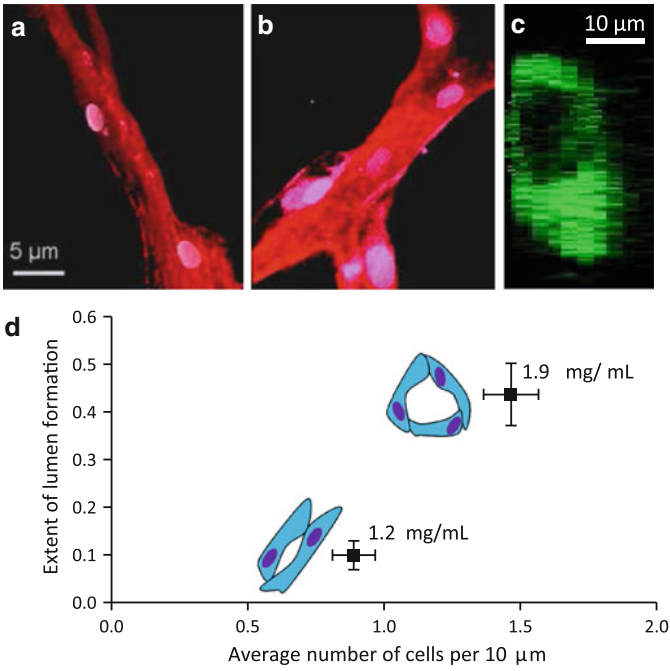


**Fig. 9** Morphological markers of maturity. Representative confocal images of sprouts formed within **a** 1.2 and **b** 1.9 mg/mL collagen matrices. Nuclei are stained white, actin cytoskeleton *red*. **c** Confocal microscopy cross-sections of sprouts grown in 1.9 mg/mL collagen have a clearly defined lumen. **d** Maturity is quantified in terms of lumen formation and cellular density. Extent of lumen formation is the fraction of sprout length containing a lumen. Cellular density is quantified as the number of cells per 10 microns of sprout length. Schematics of cross-sections from each condition are shown. Error bars are standard deviation, n = 20. The average number of cells per cross-section is two and three within 1.2 and 1.9 mg/ml matrices, respectively. Originally published in [69]. *Reproduced by permission of The Royal Society of Chemistry,* http://dx.doi.org/10.1039/C005069E

the 1.2 mg/mL counterpart (Fig. 9d). This faster rate of sprout maturation in the higher density matrix could be due to increased mechanical resistance to elongation, decreased proteolysis of the matrix, increased density of pro-proliferation signaling from the matrix, decreased diffusivity of autocrine/paracrine secretions, or some combination of these processes. Future studies utilizing well-defined, tunable biomaterial matrices (as opposed to naturally harvested collagen), are expected to shed light onto these mechanistic processes.

An additional micro-environmental cue that is thought to mediate sprout maturation is interstitial flow. Recently, Kamm et al. have reported the development of a microfluidic device that exposes the matrix directly to flowing reagent channels in order to mimic the interstitial flow profile experienced in vivo [71]. Micropillars inside the culture chamber stabilize the matrix to prevent degradation in response to shear stress from the reagent streams. This device may be particularly beneficial

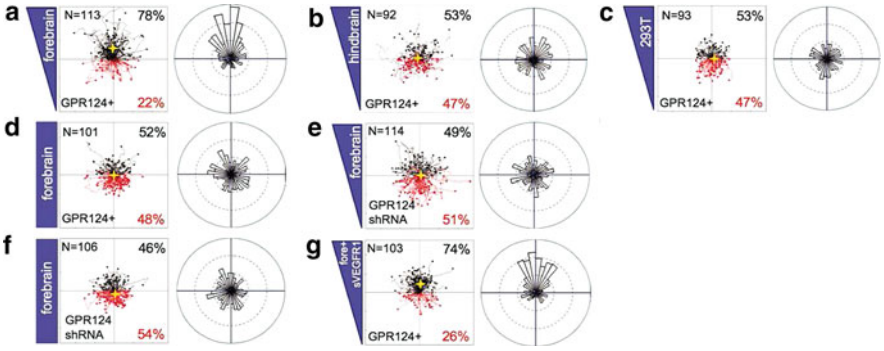


**Fig. 10** GPR124 is a receptor that mediates endothelial cell chemotaxis in response to paracine factors secreted by the embryonic forebrain. **a** GPR124-overexpressing bEND3 cells (GPR124+) migrate within a gradient of conditioned medium from cultured E12.5 forebrain cortical neurons. Migration is random under **b** an equivalent hindbrain gradient, **c** a gradient from HEK293T conditioned medium, **d** a uniform concentration of forebrain-conditioned medium. **e**, **f** Directional bias is ablated by treatment with shRNA against GPR124. **g** Chemotaxis is preserved under sequestration of VEGF by a recombinant soluble VEGFR1 ectodomain (400 ng/mL). Originally published in [72]

in studies of matrix remodeling during sprouting morphogenesis, as the proteolytic degradation of existing matrix and the deposition of new matrix are both expected to be intimately connected to interstitial flow.

Although VEGF is the most commonly studied angiogenic cue, gradients of other factors also play a role in endothelial cell chemotaxis and sprouting morphogenesis. Recent microfluidic experiments identified the G-protein coupled receptor 124 (GPR124) as a critical regulator of organ-specific angiogenesis in the central nervous system [72]. GPR124 is an endothelial receptor of previously unknown function that induces chemotaxis of brain-derived endothelial cells through a non-VEGF pathway. Expression of GPR124 caused cells from the brain vascular endothelial cell line bEND3 to chemotax along a gradient of paracrine secretions from cortical neurons of the murine embryonic forebrain (Fig. 10a). In contrast, a gradient of hindbrain-derived secretions did not elicit chemotaxis (Fig. 10b). As controls for specificity, a gradient of conditioned medium from the 293T cell line (Fig. 10c) and a uniform concentration profile of forebrain-conditioned medium (Fig. 10d) did not induce chemotaxis. Similarly, knocking down GPR124 through use of small hairpin RNA (shRNA) prevented chemotaxis (Fig. 10e, f). Blocking the VEGF activity through inhibition with a soluble receptor did not inhibit forebrain-specific chemotactic signaling (Fig. 10g). These results corroborate data from GPR124-knockout mice, which are deficient in the embryonic development of forebrain vasculature [72]. Together, these data support the hypothesis that GPR124 is a critical mediator of brain-specific endothelial cell chemotaxis and that the GPR124 ligand is secreted by forebrain cortical neurons.

To further demonstrate that the GPR124 ligand promotes stable sprout formation, sprouting morphogenesis assays were performed in a microfluidic device

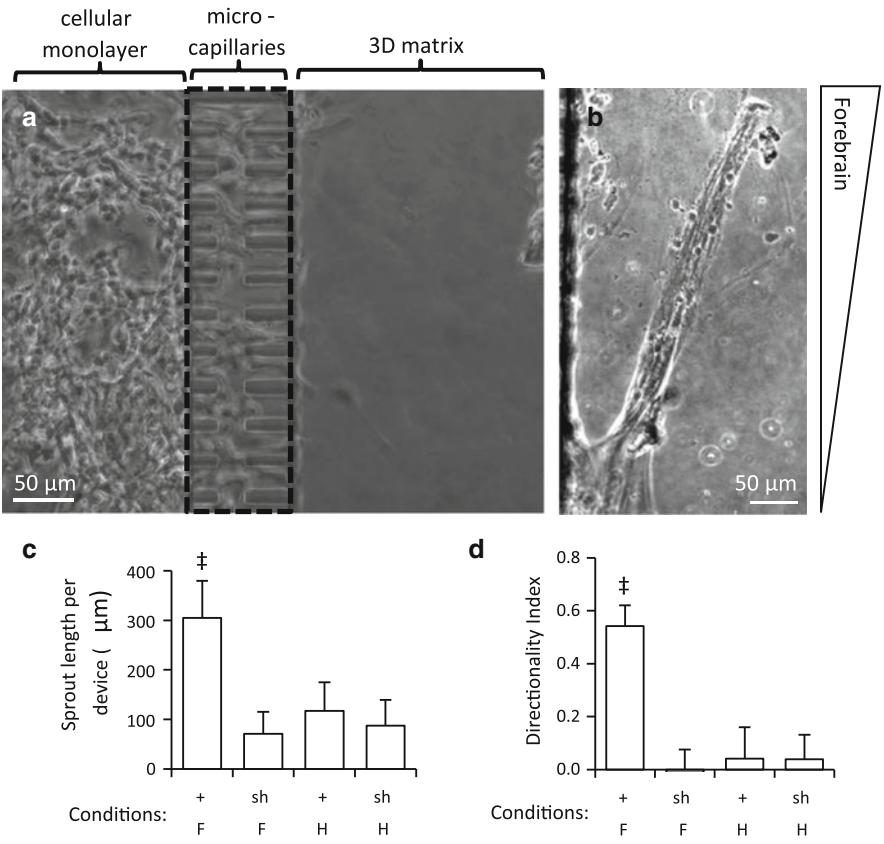


**Fig. 11** Brain endothelial cells (bEND3) expressing GPR124 were induced to form sprouts by gradients of forebrain-conditioned medium. **a** Schematic of microfluidic device to induce sprouting morphogenesis within a gradient of conditioned medium (*CM*). **b** A representative sprout cultured within a gradient of CM from E12.5 forebrain cortical neurons. **c**, **d** Directional sprouting morphogenesis is enhanced in cultures expressing GPR124 (+) and exposed to gradients of forebrain-conditioned medium (*F*). Cells treated with GPR124 shRNA (sh) or exposed to gradients of hindbrain-conditioned medium (*H*) resulted in significantly less sprouting morphogenesis and did not display pathfinding behavior (‡ $p < 0.05$). Originally published in [72]

modified to enable both 2D and 3D endothelial cell culture (Fig. 11a). In this assay, bEnd3 cells were initially cultured as a monolayer within a side chamber that was connected to the main culture chamber though 30 µm micro-capillaries. The main culture chamber was filled with a 3D matrix blend of collagen I (1.9 mg/mL) with fibronectin (50 µg/mL). The cells undergo 2D migration through the micro-capillaries followed by sprouting morphogenesis into the 3D matrix, where they are exposed to a stable gradient of paracrine secretions from cultured cortical cells of murine forebrain origin (Fig. 11b). This gradient is oriented perpendicular to the initial sprouting direction. Total sprout length was specifically increased by

signaling from GPR124/forebrain paracrine factors (Fig. 11c). In agreement with the chemotaxis data, significant sprout alignment with the gradient only occurred when cells expressed GPR124 and did not occur for cells treated with GPR124 shRNA (Fig. 11d). As a control, sprouts exposed to gradients of hindbrain-conditioned medium displayed significantly less directionality (Fig. 11d). These data demonstrate the powerful use of microfluidic devices as reductive screening assays to discover new regulators of organ-specific vascularization, which have been hypothesized to be critical during embryonic development and may also prove to be beneficial in regenerative medicine therapies [73].

In summary, the case studies presented here illustrate how this type of "restricted-flow" device can be utilized in two different angiogenic models. First, these devices enable 2D chemotactic studies of shear-sensitive endothelial cells in a reductionist manner. Second, these devices enable the reductionist study of sprouting morphogenesis, sprout pathfinding, and sprout maturation within 3D matrices. These devices have been used both in quantitative studies to evaluate the concentration profile requirements to induce endothelial cell polarization of known chemotactic cues (i.e., VEGF) as well as in discovery-driven experiments to identify novel regulators of organ-specific endothelial cell chemotaxis. Our results highlight the fact that endothelial responses to a specific gradient of soluble factors is context-dependent. Identical gradients can result in different cellular responses depending on the matrix microenvironment and potentially other biophysical and biochemical cues. Given the inherent complexity in the processes that regulate angiogenesis, these restricted-flow microfluidic devices are especially well suited for systematic experimental studies due to their quantitative control, substrate independence, and stability of the generated gradients.

## 5 Potential Impact and Future Opportunities

Building on the successful use of these devices to study 2D endothelial cell chemotaxis and 3D sprouting morphogenesis, future opportunities exist to further utilize this experimental platform in the development of clinically translatable therapies.

The controlled delivery of soluble factors from hydrogels [74], nanoparticles [75], or coacervates [76], to treat pathological angiogenesis such as chronic ischemia and cancer is a highly active area of research. A variety of innovative encapsulation and release strategies have been developed to enable delivery of specific concentration profiles; however, often the optimal profile required to elicit the desired cellular response is unknown. Due to the small volume of reagents required, microfluidic devices are ideal platforms to quantitatively screen multiple concentration profiles to identify conditions that promote or inhibit processes such as sprouting morphogenesis. These studies are expected to enable more efficient, goal-driven design of the delivery system. Moreover, the timed delivery of multiple factors is also known to affect therapeutic outcome. For example, a

promising pair of soluble factors is VEGF and PDGF-bb, which have been shown to stimulate sprouting and maturation when delivered sequentially [77, 78]. The time-dependent delivery of multiple soluble factors can be easily simulated in vitro using microfluidic devices programmed to deliver known amounts of several different source reagents. In addition, these microfluidic devices can be used in controlled delivery studies to optimize the soluble factors themselves. For example, protein-engineered variants of chemotactic factors, which have been shown to enhance chemotaxis and wound healing [79], could be screened quickly using multiple devices operated in parallel. The use of microfluidic devices to independently optimize soluble factor concentration profiles, temporal profiles, and potency can potentially save time and resources compared to traditional approaches to drug delivery optimization.

In addition to manipulating angiogenesis in native tissue, the creation of vascular networks within tissue-engineered constructs is another area of great potential. Without a perfusable network to mediate nutrient exchange, tissue-engineered constructs cannot support cell viability throughout the entire bulk structure. This challenge involves the use of a biomaterial scaffold, through which endothelial cells must penetrate and develop into functional blood vessels. Microfluidic sprouting morphogenesis assays can be used in the high-throughput screening of candidate biomaterials exposed to specific soluble concentration profiles. Although natural materials such as collagen and alginate have some ability to be tailored, novel synthetics, which are multivariately tunable for cell adhesion, mechanical strength [80], immobilized ligand density [81], and cell-sensitive degradation [82], are especially suited to this systematic approach. For example, crosstalk between cell adhesion and growth factor signaling is known to impact cellular proliferation and migration [83] and is likely to affect sprouting morphogenesis. Engineered matrices can also provide additional anisotropic signals such as lithographic nanopatterns [84], nanofibrous structures [85], and gradients of bound growth factors [86], adhesion ligands [87], or stiffness [88]. All of these anisotropic microenvironments are expected to interact with and modify directional signaling from soluble gradients.

Another potential application for these devices is the study of pathological angiogenesis such as chronic ischemia and cancer angiogenesis. By mimicking specific characteristics of the diseased tissue within the quantitatively controlled environment of the microfluidic device, these studies may help to provide systematic understanding of disease processes by teasing apart interdependent variables. Ischemia in peripheral arterial disease results from a failure of angiogenesis, leading to loss of mobility and independence [89]. Angiogenesis in cancer, i.e., tumor-elicited vascularization, is a critical mechanism of malignancy [90]. Mechanistic insight into these complex processes may be gleaned through reductive analysis of individual components. For example, the microfluidic device could be utilized to quantitatively examine the effects of paracrine factors secreted under hypoxic conditions, phenotypic changes displayed by vascular endothelial cells, or aberrant matrix remodeling, all of which are known to change over the course of disease progression. The device microenvironment could also serve as an

in vitro mimic of diseased tissue for the development of novel interventions, such as engineered growth factors [91] and gene delivery strategies [92]. These mechanistic studies are expected to help elucidate the complex etiology and possible treatments of vascular disease.

## 6 Conclusion

Restricted-flow microfluidic devices are ideal for performing quantitative, reductive, and systematic studies on the role of soluble gradients in sprouting morphogenesis. By allowing a diffusion gradient to form across a cell culture chamber that is shielded from convective flow, these devices create stable concentration profiles that both avoid shear-activation of endothelial cells and are independent of culture substrate and dimensionality. In addition, these devices enable gradient-mediated anisotropic signaling to influence cultured cells over indefinite time periods, thereby allowing time-lapse observation of cellular behavior over multiple days. Current use of the devices has focused on (1) the quantitative, reductionist evaluation of VEGF concentration requirements for 2D chemotaxis, (2) the interplay between 3D matrix microenvironment and VEGF gradient signaling, and (3) the identification of novel regulators of organ-specific angiogenesis. Future applications of the device may include optimization of multiple soluble gradients to enhance their angiogenic potential in regenerative medicine therapies, high-throughput screening of pro-angiogenic biomaterials for tissue engineering applications, and mechanistic studies of chronic ischemia and tumor vascularization.

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# Reactive Oxygen Species in Physiologic and Pathologic Angiogenesis

**Alisa Morss Clyne**

**Abstract** Reactive oxygen species (ROS), including superoxide and hydrogen peroxide, play a major role in angiogenesis. High ROS doses induce oxidative stress and subsequent cell death in a variety of cardiovascular diseases, including hypertension and atherosclerosis. However, low doses of externally applied ROS directly promote angiogenesis by causing sub-lethal cell membrane damage and subsequent fibroblast growth factor-2 release, by increasing growth factor production, or by enhancing growth factor binding to their receptors. Once angiogenic growth factor signaling is initiated, ROS are produced intracellularly through NAD(P)H oxidases and manganese superoxide dismutase as messengers in downstream growth factor signaling for proliferation, migration, and tube formation. This chapter discusses our current understanding of the vascular ROS balance in both physiologic and pathologic angiogenesis, as well as innovative approaches to applying ROS to induce angiogenesis.

## 1 Overview

Angiogenesis, the growth of new blood vessels from existing vessels, is tightly controlled by a balance of pro- and anti-angiogenic factors. Pro-angiogenic growth factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2), promote extracellular matrix degradation by cell secreted

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proteases followed by endothelial cell proliferation, migration, and tube formation [1, 2]. As the extracellular matrix breaks down, it releases additional growth factors that were bound to heparan sulfate proteoglycans. Concurrently, matrix protein breakdown products such as the plasminogen fragment angiostatin and the collagen XVIII fragment endostatin, inhibit angiogenesis [1]. This balance prevents angiogenesis from proceeding unchecked.

Reactive oxygen species (ROS) also help maintain this angiogenic balance. Whereas low ROS levels stimulate angiogenesis and are in fact required for many angiogenic signaling pathways, high ROS levels inhibit angiogenesis and promote death of many cell types critical to the angiogenic process [2]. In addition to dose, timing and application mode may also influence whether ROS are pro- or anti-angiogenic. Antioxidant therapies to inhibit ROS and shift the angiogenic balance have met with limited success, perhaps due to the complexity of ROS effects on angiogenesis [3].

In cells, most ROS are formed as by-products of mitochondrial electron transport. ROS can also be formed by NADPH oxidase, xanthine oxidase, and nitric oxide synthase, among others [2]. Along with promoting angiogenesis, ROS increase endothelial cell permeability, enhance cell adhesion molecule surface expression, inhibit endothelial cell dependent vasodilation, and at large doses induce apoptosis [4]. While ROS are important in a variety of physiological processes, basal ROS levels are altered by many diseases including atherosclerosis, hypertension, diabetes and cancer [5–7]. Interestingly, angiogenesis also plays a critical role in many of these diseases.

In this chapter, we review biologically relevant reactive oxygen species and their role in initiating angiogenic processes and acting as messengers for intracellular angiogenic signaling. We then discuss how altered ROS levels in disease affect angiogenesis. Finally, we describe new methods to apply ROS to promote angiogenesis.

## 2 Reactive Oxygen Species

ROS are highly reactive molecules or free radicals derived from molecular oxygen ($O_2$) (Fig. 1). Superoxide ($O_2^-$) is formed by one electron reduction of $O_2$ by a variety of enzymes including NADPH oxidase. Singlet oxygen ($O_2(^1\Delta_g)$) is the electronically excited state of $O_2$ ($(^3\Sigma_g^-)$ $^3O_2$), in which the two unpaired electrons adopt antiparallel spins in the same orbital [8]. Oxygen reduction by two electrons forms hydrogen peroxide ($H_2O_2$), which is catalyzed by superoxide dismutase. The hydroxyl radical (OH·) is produced by tri-electron reduction of $O_2$ in the presence of metal ions through the Fenton reaction. ROS, especially hydroxyl radicals, interact with amino acids and proteins to form longer lived organic hydroperoxides (RO·, ROO·)

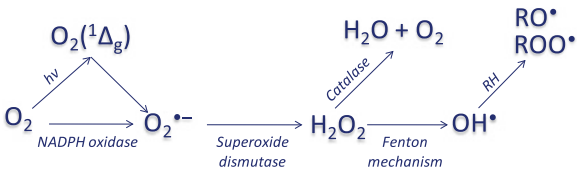


**Fig. 1** Biologically relevant ROS production

## 2.1 Superoxide ($O_2^-$)

In cells, the mitochondrial oxidation–reduction system generates superoxide ($O_2^-$). Specifically, enzymes such as NADH/NADPH oxidase, xanthine oxidase, lipooxygenase, mitochondrial oxidase and cyclooxygenase reduce molecular $O_2$ during oxidative phosphorylation for ATP generation (Eq. 1) [9]. Approximately 2 % of the $O_2$ reduced by mitochondria is converted into $O_2^-$. $O_2^-$ is also generated in vivo by phagocytic cells such as macrophages, monocytes, neutrophils, and eosinophils in response to foreign bodies such as bacteria [10].

$$O_2 + e^- \rightarrow O_2^- \tag{1}$$

Due to its negative charge, $O_2^-$ cannot readily cross biological membranes. However, aqueous $O_2^-$ at low pH forms hydroperoxyl radical ($HO_2\cdot$), which can easily enter the cell. Superoxide is removed from cells by a spontaneous dismutation reaction (Eq. 2), during which two $O_2^-$ are converted to molecular oxygen and hydrogen peroxide. The reaction rate constant is $8 \times 10^4\ M^{-1}\ s^{-1}$, and the reaction proceeds four orders of magnitude faster in the presence of the enzyme superoxide dismutase [11, 12]. Most of the biological effects of superoxide are mediated through the dismutation to hydrogen peroxide.

$$O_2^- \cdot + O_2^- \cdot + 2H^+ \rightarrow H_2O_2 + O_2 \tag{2}$$

## 2.2 Singlet Oxygen ($O_2(^1\Delta_g)$)

Molecular oxygen exists in the triplet state ($(^3\Sigma_g^-)^3O_2$), with two unpaired electrons of parallel spin in separate orbitals. Singlet oxygen is the electronically excited state of oxygen when these electrons adopt antiparallel spins and occupy the same orbital, thus opening an opportunity to accept electrons of either spin [10]. Singlet oxygen is formed in cells by energy transfer from photochemically excited photosensitizers to triplet state molecular oxygen or during an inflammatory reaction. For example, $O_2^-$ produced by NADPH oxidase (Eq. 3) undergoes dismutation to form $H_2O_2$ and $O_2$ (Eq. 4). This $H_2O_2$ is in the singlet state if dismutation is spontaneous rather than catalyzed by superoxide dismutase. $H_2O_2$ produces hypochlorite in the presence of myeloperoxidase (Eq. 5) which in turn reacts with $H_2O_2$ to yield $O_2(^1\Delta_g)$ (Eq. 6).

$$2\,O_2 + NADPH \rightarrow 2\,O_2^- \cdot + NADP^+ + H^+ \quad (3)$$

$$2\,O_2^- \cdot + 2\,H^+ \rightarrow H_2O_2 + O_2 \quad (4)$$

$$H_2O_2 + Cl^- \rightarrow OCl^- + H_2O \quad (5)$$

$$H_2O_2 + OCl^- \rightarrow Cl^- + H_2O + O_2\left(^1\Delta_g\right) \quad (6)$$

Singlet oxygen both impairs and activates several signaling processes. Singlet oxygen generation during inflammation helps fight infection [10]. It also interacts with DNA to produce 8-oxo-7, 8-dihydro(deoxy)guanosine (8-oxo-(d)G) moieties which trigger DNA repair [10]. At very high $O_2(^1\Delta_g)$ concentration, single strand DNA as well as double strand breaks occur. Some of the amino acids most sensitive to singlet oxygen oxidation are tryptophan, cysteine, methionine and histidine. Cysteine oxidation by singlet oxygen inactivates several enzymes including tyrosine phosphatases, resulting in activation of signaling pathways such as p38/JNK and NF-$\kappa$B [10, 13, 14]. Tryptophan and tyrosine oxidation produces peptides that inhibit caspases, thus preventing apoptosis [15].

## 2.3 Hydrogen Peroxide ($H_2O_2$)

$H_2O_2$ is mainly produced by $O_2^-$ dismutation by superoxide dismutase. It is also directly produced by several enzymes such as monoamine oxidases and glycolate [16]. $H_2O_2$ is a stable and long living species since there are no unpaired electrons present. Due to their small size and neutral charge, $H_2O_2$ molecules freely dissolve in solution and readily diffuse into cells through cell membrane aquaporins [17, 18].

At high concentrations, $H_2O_2$ is associated with DNA, lipid and protein damage [7, 16]. However, hydrogen peroxide concentrations below 20–50 μM are less toxic to human cells [16]. The deleterious effect of $H_2O_2$ is attributed to its conversion into OH· radical. In the presence of iron or copper ions, $H_2O_2$ reacts with $O_2^-$ to produce highly reactive OH· (Eq. 7). Ultraviolet radiation also converts $H_2O_2$ into OH· radicals by homolytic fission of the O–O bond (Eq. 8). At low concentrations, $H_2O_2$ takes part in intracellular signaling and gene expression, including tyrosine phosphorylation of growth factor receptors, activation of MAP kinases, and increased affinity of DNA transcription factors for their binding sites [19–23].

$$O_2^- \cdot + H_2O_2 \xrightarrow{\text{Fe or Cu ions}} OH \cdot + OH^- + O_2 \quad (7)$$

$$H_2O_2 \xrightarrow{UV} OH\cdot \quad (8)$$

### 2.4 Hydroxyl Radical (OH·)

Hydroxyl radicals are the most reactive of all ROS even though they have a short half-life [24]. OH· is formed in biological systems from $H_2O_2$, as described previously. Due to its high reactivity, OH· reacts non-specifically with any cell component (DNA, lipids, proteins, amino acids and sugars) causing loss of cell integrity and function [25, 26]. α-tocopherol effectively converts OH· by forming stable tocopheroxyl radicals, which are then reduced by ascorbate and NADH/NADPH-dependent reductase enzymes into phenol [27].

### 2.5 ROS Scavengers

To protect against ROS damage, cells produce antioxidant enzymes. Major intracellular ROS scavengers include superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase [9]. SOD catalyzes the dismutation of superoxide into hydrogen peroxide, and GSH-Px removes $H_2O_2$ by oxidizing reduced glutathione (GSH) into glutathione disulfide (GS–SG, Eq. 9). Catalase decomposes $H_2O_2$ into $H_2O$ and $O_2$ (Eq. 10). Antioxidant enzyme expression is altered by a variety of factors, including angiotensin II, tumor necrosis factor-α (TNF-α), interleukin-1$\beta$, and hydrogen peroxide [28–31].

$$2GSH + H_2O_2 \rightarrow GS - SG + 2H_2O \tag{9}$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \tag{10}$$

## 3 Reactive Species in Angiogenesis

A variety of ROS play critical roles in angiogenesis. Low dose ROS initiate angiogenesis, often through physiologic ROS production in response to an applied stimulus. However, externally applied ROS can also initiate angiogenesis. Once the angiogenic process is underway, ROS act as second messengers in angiogenic signal transduction pathways. While conflicting reports describe pro-and anti-angiogenic effects of ROS in wound healing [32–35], ROS dose and timing as well as cell redox state are critical to determining their cellular response [36].

### 3.1 ROS-Induced Initiation of Angiogenesis

ROS that initiate angiogenesis can come either from biological production by adjacent cells or from sources external to the tissue. In wounds, leukocytes digest

damaged tissue and destroy microorganisms via hydrogen peroxide and superoxide release, which is referred to as "respiratory burst" [37]. Reoxygenation following hypoxia leads to ROS formation [38]. Changes in the extracellular tissue environment, including elevated glucose, ethanol, UV light, and ionizing radiation can also stimulate high levels of cell-produced ROS [39–42]. For in vitro and in vivo studies of ROS effects on angiogenesis, hydrogen peroxide or the ROS of interest is often applied directly.

While early, high ROS doses (e.g., 250–1000 μM $H_2O_2$) damage cells and tissue, after the initial injury lower ROS concentrations initiate angiogenesis and tissue repair [43, 44]. Endothelial cell exposure to hydrogen peroxide induced actin stress fiber formation and focal adhesion kinase (FAK) activation, likely through hydroxyl radical formation [45]. Hydrogen peroxide and ROS donors further stimulated cell proliferation, migration, and tube formation when added to 2- and 3-dimensional endothelial cell culture [46–48]. When ROS came from hypoxia, ischemic preconditioning, or ethanol instead of being exogenously added, actin reorganization, cell migration, and capillary tube still occurred [38, 40, 49]. Similarly, inflammatory cell-mediated angiogenic activity, including that from activated polymorphonuclear leukocytes, also appeared to be related to ROS [50–55].

Exogenous ROS affect angiogenic pathways in a variety of ways. Independent of ligand binding, UV light clustered growth factor receptors via ROS, and exogenous $H_2O_2$ activated PDGF and EGF receptors [41, 56–58]. Exogenous ROS stimulated VEGF mRNA and VEGF secretion, perhaps via enhanced NF-$\kappa$B binding [59]. Additional evidence suggests the important of NF-$\kappa$B in ROS-mediated angiogenesis, since NF-$\kappa$B antisense nucleotides inhibited tube formation in response to hydrogen peroxide [48]. In a 3D collagen gel model, hydrogen peroxide stimulated tube formation, which was blocked by an ets-1 antisense oligonucleotide. Ets-1 regulates genes involved in matrix degradation, such as urokinase plasminogen activator and matrix metalloproteinase-1, therefore ROS may also promote endothelial cell invasion by controlling matrix degradation [47]. Exogenous ROS may also signal via secondary ROS production. Hydrogen peroxide activated NADPH oxidase superoxide production in vascular smooth muscle cells, fibroblasts, and mouse pulmonary arteries [60, 61]. These secondary ROS may contribute to vascular cell injury due to ROS, however they have also been shown to induce cell proliferation [46]. Exogenous $H_2O_2$ has even been shown to mimic growth factors by directly inducing protein tyrosine phosphorylation and MAPK activation [62–64].

Exogenous ROS also contribute to angiogenesis through FGF-2, also known as basic fibroblast growth factor (bFGF) [65]. FGF-2 is associated with cell survival, proliferation, migration and differentiation [66–68], however it does not have a recognized signal sequence for secretion. FGF-2 is therefore released following cell injury often caused by ROS, for example by ionizing radiation, pulsed electromagnetic field, or elevated glucose [39, 69–71]. Released FGF-2 promotes cell survival and also increases FGF-2 and VEGF expression in endothelial cells, smooth muscle cells, and cardiac myocytes [72–77]. FGF-2 signaling in turn acts through ROS. Exogenous FGF-2 increased FGF-2 expression in pulmonary arterial

smooth muscle cells by increasing superoxide levels via NADPH oxidase activation. FGF-2 expression could also be stimulated by other factors known to increase ROS, including endothelin-1 and transforming growth factor-$\beta$1 [78].

## 3.2 ROS in Angiogenic Signaling

ROS not only initiate angiogenesis but they additionally play a critical role in intracellular signaling during angiogenesis. In endothelial cells, NADPH oxidase basally produces low superoxide levels, but it is stimulated acutely by growth factors [79]. VEGF specifically stimulates superoxide production via Rac-1 dependent activation of NADPH oxidases [80]. When VEGF binds to its receptor (primarily VEGFR2), the small GTPase Rac1 is activated. Rac1 moves to the plasma membrane to stimulate NADPH oxidase. ROS produced by the NADPH oxidase then inactivate the protein tyrosine phosphatases which negatively regulate VEGFR2 (Fig. 2). This enhances VEGFR2 autophosphorylation and VEGF signaling for endothelial cell proliferation and migration [80–82]. The role of ROS in VEGF signaling is supported by studies showing that antioxidants attenuated VEGF receptor tyrosine phosphorylation and subsequent endothelial cell proliferation and migration [80]. In addition to communicating downstream VEGF signaling, NADPH oxidase-produced ROS also support future growth factor signals by inducing VEGF and FGF-2 expression [78, 83].

Growth factor-stimulated primary superoxide production from NADPH oxidase may then stimulate secondary hydrogen peroxide formation via superoxide dismutases. VEGF induced manganese superoxide dismutase (MnSOD) expression via the NAPDH oxidase pathway [81]. MnSOD overexpression led to increased mitochondrial $H_2O_2$, which enhanced phosphosphoinositol-3-kinase (PI3K) signaling and activated its downstream targets, including Akt. The MnSOD-derived ROS then upregulated VEGF expression and promoted both endothelial cell sprouting in a collagen gel as well as blood vessel formation in the chick chorioallantoic membrane assay [84]. Similary to VEGF, FGF-2 induced angiogenesis was 3-fold higher in transgenic mice with elevated Cu/Zn superoxide dismutase. In vitro, the SOD inhibitor disulfiram inhibited hydrogen peroxide formation and DNA synthesis in capillary endothelial cells [85]. These results suggest that primary ROS may then induce secondary ROS production, which is critical for angiogenesis.

While ROS affect a wide variety of cell components, ROS may influence angiogenic signaling pathways by oxidizing redox-sensitive proteins. Hydrogen peroxide reversibly oxidizes low-pKa cysteine residues in proteins such as NF-$\kappa$B [86], hypoxia inducible factor (HIF) [87], and protein tyrosine phosphatases (PTPs) [14]. Hydrogen peroxide increased NF-$\kappa$B binding activity, and antisense NF- $\kappa$B inhibited tube formation by $H_2O_2$ [48, 59]. HIF-1 activates many angiogenic genes and pathways in response to hypoxia, in particular VEGF expression. ROS produced by NADPH oxidases induced HIF-1 in vascular cells [88]. PTPs associate with

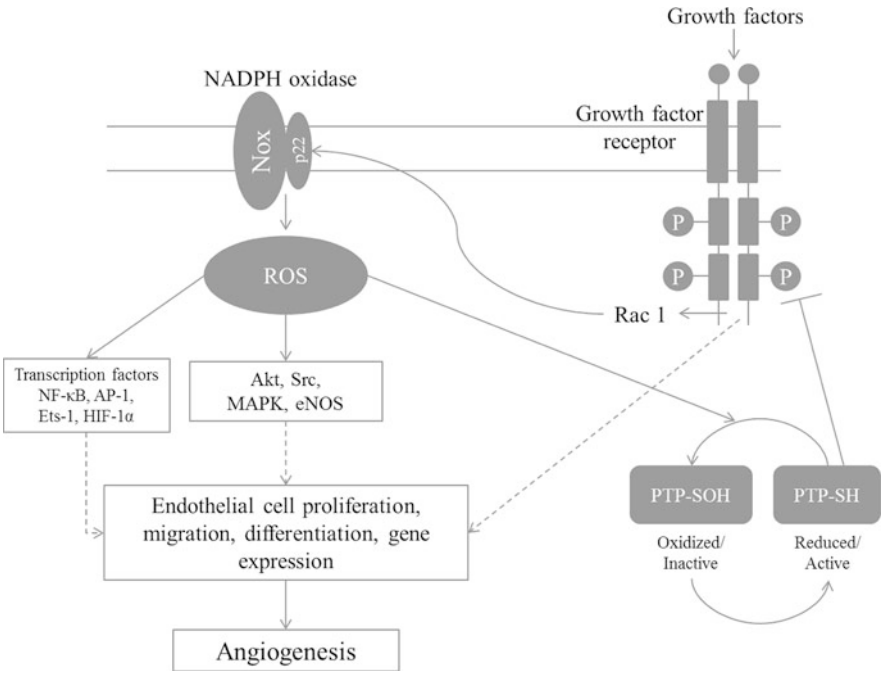


**Fig. 2** Signaling pathways regulated by NADPH oxidase-derived ROS leading to angiogenesis. Growth factor binds to its receptor leading to translocation of GTPase Rac 1 into plasma membrane, stimulating ROS production by NADPH oxidases. These ROS inactivate negatively regulating protein tyrosine phosphatases (PTP-SH → PTP-SOH) enhancing receptor autophosphorylation. Multiple signaling pathways leading to angiogenesis are activated

VEGFR2 after VEGF stimulation to inhibit VEGF-induced VEGFR2 autophosphorylation and subsequent endothelial cell migration and proliferation [89–91]. Thus PTP inactivation by ROS allows increased VEGF signaling.

ROS also activate downstream signaling pathways. Mitogen activated protein kinases (MAP kinases), which are important to cell proliferation and migration, are redox sensitive. Hydrogen peroxide activated p38 MAPK in endothelial cells, leading to actin reorganization, stress fiber formation, and vinculin recruitment to focal adhesions [92]. Akt plays a key role in cell survival. Hydrogen peroxide stimulated Akt in smooth muscle cells, and angiopoietin-1 enhanced Akt phosphorylation and angiogenesis via NADPH oxidase ROS production in endothelial cells [93, 94]. Akt activation could occur either through enhanced receptor tyrosine kinase signaling through PTP inactivation, or ROS may inactivate the phosphatase PTEN which is a negative regulator of PI3K that is upstream of Akt [95]. Additionally, ROS may act more directly through Rac1 to decrease cell–cell adhesion and promote cytoskeletal reorganization required for endothelial cell migration [96, 97].

## 4 Reactive Oxygen Species in Disease—Effects on Angiogenesis

ROS play a significant role in many vascular diseases, including atherosclerosis, hypertension, diabetes and cancer among others [98]. Elevated ROS in these conditions negatively affect a multitude of vascular functions. Endothelial cells directly exposed to high ROS undergo apoptosis, and chemically-induced apoptosis through oxidized LDL, angiotensin II, high glucose, and tumor necrosis factor-α is inhibited by antioxidants such as superoxide dismutase and N-acetyl cysteine [99]. ROS enhance inflammatory adhesion molecule expression [100, 101]. ROS, especially superoxide, inactivate nitric oxide to inhibit endothelium-dependent vasodilation [102]. In addition, individuals who suffer from these diseases may have altered angiogenesis.

ROS are important in all stages of atherosclerosis, from atherosclerotic plaque formation to plaque rupture to the intervention response. Angiogenesis related to elevated ROS may also play a role, since atherosclerotic vessels have intimal microvasculature whereas in normal vessels the microvasculature is confined to the adventitia [103]. These vessels may assist in plaque growth by recruiting inflammatory cells or providing essential nutrients [104]. Both mechanical and chemical factors may increase ROS to promote this angiogenesis. While laminar shear stress produced a transient prooxidant signal that is quickly decreased by antioxidant enzyme upregulation, the oscillatory shear stress present in atherosclerotic regions activated NADPH without compensatory SOD upregulation [105]. Hypercholesterolemia [106] and oxidized lipids [107] activate PKC and increase vascular ROS formation via NADPH oxidase [108, 109]. The importance of angiogenesis in atherosclerotic lesion progression is supported by reduced plaque growth and intimal neovascularization with anti-angiogenic therapy [110]. Plaque rupture correlates with angiogenesis, with a higher prevalence of plaque neovascularization measured clinically in lesions with plaque rupture [111]. Since angiogenesis requires proteases, plaque angiogenesis could directly contribute to plaque disruption [112, 113]. In fact, nonatherosclerotic explanted human coronary arteries showed homogenous low superoxide levels, whereas atherosclerotic arteries had increased superoxide in the vulnerable plaque shoulder [114]. Finally, baboon balloon-injured arteries demonstrated increased VEGF expression in the neointima, which correlated with the lipid peroxidation product 4-hydroxynonenal, an endogenous ROS present in atherosclerotic lesions [115].

All types of hypertension exhibit oxidative stress, which is important in hypertensive pathogenesis [116]. In the most direct ROS effect, NADPH oxidase-produced superoxide inactivates nitric oxide, thus inhibiting endothelium-dependent vasodilation and increasing peripheral resistance [117]. A number of hypertensive factors upregulate NADPH oxidases. Angiotensin II, an important prohypertensive agent, induces and activates NADPH oxidases [118]. Knockout of specific NADPH oxidase components prevented angiotensin II-induced stimulation of superoxide, which in turn enhanced nitric oxide availability and attenuated

hypertension [119, 120]. Hypertensive oxidative stress inhibits angiogenesis and in fact leads to microvascular rarefaction, the disappearance of capillaries and pre-capillary arterioles. Rarefaction, a hallmark of hypertension, can occur when elevated blood pressure causes capillary endothelium dysfunction, followed by microvessel constriction and disappearance [121]. Microvascular rarefaction increases peripheral resistance, thereby reducing blood flow and further elevating blood pressure. Furthermore, impaired angiogenesis in development may predispose to hypertension in later life [122, 123]. Some new angiogenesis inhibitor drugs used to treat cancer lead to hypertension through endothelial dysfunction and capillary rarefaction [124, 125], and vasodilator antihypertensive treatments (particularly angiotensin converting enzyme inhibitors) can improve capillary density [126]. Thus hypertension may systematically elevate vascular oxidative stress to inhibit angiogenesis.

A wide variety of cancers have been linked to oxidative stress, including breast, liver, lung, and skin cancer [127–130]. Human cancers produced hydrogen peroxide at levels comparable to activated neutrophils, and $H_2O_2$ was involved in lymphocyte-mediate angiogenesis in tumors [51, 131]. Oxidative stress may initially induce DNA damage leading to tumor formation. Subsequently, ROS may promote cancer cell survival by activating Akt, or increase cell proliferation via MAPK and NF-$\kappa$B [132–135]. Angiogenesis is critical to tumor growth and metastasis, and a number of cellular stress factors, including ROS, are important angiogenic stimuli in cancer [136]. ROS stabilized HIF-1$\alpha$ and induced angiogenic factor production by tumor cells [137]. The importance of ROS in tumor angiogenesis is supported by the fact that antioxidants attenuated angiogenesis due to tumor secreted products [138].

In diabetes, vascular ROS are increased due to both high glucose and advanced glycation end products (AGE). High glucose leads to superoxide leakage from mitochondrial respiration, increases NADPH oxidase activity in endothelial cells through the PKC pathway, and upregulates NAPDH oxidase expression [139–141]. AGE increased NADPH oxidase expression in cardiac myocytes [142], and downregulation of NADPH oxidase attenuated endothelial cell activation in response to AGE [143]. Blood vessels from diabetic subjects showed increased ROS due to enhanced NADPH oxidase activity and subsequent eNOS uncoupling [144–146]. Interestingly, angiogenesis in diabetes is vascular-bed dependent. Excess angiogenesis in renal glomeruli leads to loss of renal filtration function and diabetic nephropathy. Fragile new capillaries formed due excessive angiogenesis in the eye create a unique retinopathy [147]. In contrast, reduced angiogenesis in the extremities contributes to poor wound healing [148]. While no direct link has been shown, there is strong correlation between ROS production, neovascularization and VEGF expression in eyes of diabetics [149, 150]. This correlation is supported by the fact that antioxidants inhibited neovascularization in the mouse model [151, 152]. ROS dose and timing are particularly important in wound healing [32]. High ROS levels are initially produced at the wound site to prevent wound infection. As the wound progresses out of the inflammatory phase and into the proliferative phase, lower ROS levels initiate wound healing processes, especially angiogenesis [32]. However,

excessive ROS production or their impaired detoxification causes extended oxidative stress, which leads to chronic, non-healing wounds with decreased angiogenesis [153].

## 5 Novel Methods for Applying Reactive Species to Stimulate Angiogenesis

Current therapeutic angiogenesis techniques lack the required clinical efficacy. While angiogenic growth factors including VEGF [154, 155] and FGF-2 [156, 157] are critical to the angiogenic process, their success depends on precise timing, dose, and gradients [158–161]. Efforts to apply growth factors either in ischemic tissue, wounds, or tissue engineered constructs to induce angiogenesis have met with limited success [161–163]. ROS are known to promote angiogenesis, however precise dosing of these species is difficult to achieve. We recently showed that non-thermal dielectric barrier discharge (DBD) plasma could apply ROS and stimulate angiogenesis in endothelial cells in 2D and 3D culture.

### *5.1 Dielectric Barrier Discharge Plasma*

Plasma is an ionized gas composed of charged particles (electrons, ions), excited atoms, molecules, radicals, and UV photons [2]. Naturally occurring plasmas include the solar corona and lightning, and man-made plasmas are used in television, fluorescent light bulbs, and microelectronics processing. Man-made plasma is created with an electrical discharge and a large electric field. Electrons, being much lighter than molecules, atoms, and ions, absorb electrical energy first. Given sufficient time, electrons transfer a substantial part of their energy to ions, molecules, and atoms through collisions. When the temperature of electrons and the heavier plasma components equilibrates, the entire plasma becomes hot. Such plasma is called thermal or equilibrium plasma. In non-thermal plasma, which is far from thermal equilibrium, the electron temperature is much higher than the heavy particle temperature. Gas in a non-thermal plasma remains close to room temperature.

Thermal plasmas such as the argon plasma coagulator have been widely used for blood coagulation as well as for pathological tissue removal [164–168]. In recent years, non-thermal plasma emerged as a novel technology for medical applications. Since gas temperature remains close to room temperature, these plasmas can be applied directly to cells and tissue without measurable damage [169]. Non-thermal plasma has primarily been used to coagulate blood, sterilize tissue and equipment, or kill bacteria and cancerous cells [170–176].

DBD plasma is generated at atmospheric pressure in air when short duration, high voltage pulses are applied between two electrodes, with one electrode being insulated to prevent an increase in current [177, 178]. Depending on the applied voltage, dielectric material and interelectrode distance, the DBD plasma characteristics in air vary, including electron density, reduced electric field, gas temperature, and active species concentration [178]. For living tissue treatment, a floating electrode DBD (FE-DBD) is employed where an insulated high voltage electrode acts as one electrode while the live tissue acts as the second electrode [170]. Non-thermal DBD plasma produces a variety of biologically active reactive species, in particular ROS [178]. Non-thermal DBD plasma has a g-factor (ROS generated per electron volt) between 0.3 and 0.5 [179]. Thus, for every plasma dose of 1 J/cm$^2$, 1.88−3.13 × 10$^{16}$ ROS are produced in gas phase. Non-thermal plasma devices, specifically DBD plasma, have great potential in medicine due to their selectivity, portability, scalability, ease of operation, and low manufacturing and maintenance costs.

## 5.2 DBD Plasma Induced Endothelial Cell Proliferation, Migration, and Tube Formation

DBD plasma had previously been shown to kill bacteria and cancer cells using high ROS doses [175, 180]. However, we hypothesized that lower ROS doses from the DBD plasma could induce angiogenesis via FGF-2 release. We had previously studied altered FGF-2 release, storage, and cellular effect induced by ROS production due to high glucose [39, 181]. Others had also demonstrated that mechanical forces, ionizing radiation, and pulsed electromagnetic fields lead to angiogenesis via FGF-2 [69–71, 182–184]. However, non-thermal plasma has advantages over irradiation and electromagnetic fields in that the latter are penetrating and injure surrounding tissue, or they need an expensive setup to be safely generated.

We initially demonstrated that while longer term plasma treatment induced endothelial cell death via apoptosis, lower plasma doses (4.2 J/cm$^2$) led to FGF-2 release peaking 3 h after plasma treatment. This FGF-2 release then enhanced cell proliferation, which was abrogated by an FGF-2 neutralizing antibody or ROS inhibitors *N*-acetyl cysteine and sodium pyruvate [179]. We further showed that plasma treatment enhanced endothelial cell 2- and 3-dimensional migration, again through FGF-2 release via plasma-produced ROS. Using a collagen gel assay, we demonstrated that plasma-produced ROS could induce endothelial cell tube formation [185]. To ensure that plasma-produced ROS caused these effects, we measured an increase in intracellular ROS about 1 h after plasma treatment, with oxidative stress returning to baseline levels by 3 h. By analyzing each plasma-produced ROS individually, hydroxyl radicals and hydrogen peroxide were identified as the key plasma-produced ROS responsible for the angiogenic effect [186].

### *5.3 DBD Plasma Induced Secondary ROS*

When growth factors such as FGF-2 bind to their receptors, ROS are produced by NADPH oxidases [187]. This ROS signaling following growth factor stimulation may enhance growth factor binding to receptors, induce receptor tyrosine kinase phosphorylation, or signal along growth factor pathways [58, 82, 188]. In human coronary endothelial cells, FGF-2 induced cell proliferation and migration through ROS production by NADPH oxidase [189]. The NADPH oxidase inhibitor DPI prevented secondary ROS formation and decreased cell proliferation. FGF-2 stimulation also increases FGF-2 expression in endothelial cells, smooth muscle cells and cardiac myocytes. Using an in vitro scrape cell injury model, Ku et al. showed that scrape-released FGF-2 induced a 4-10 fold increase in FGF-2 mRNA levels [72]. This process may also be mediated by FGF-2 induced intracellular ROS production [74–76, 190]. In pulmonary arterial smooth muscle cells, FGF-2 expression following exogenous FGF-2 addition was associated with increased intracellular ROS via NADPH oxidase activation. FGF-2 expression was attenuated by the intracellular ROS scavenger *N*-acetyl cysteine [78]. FGF-2 release caused by injury has also been shown to enhance FGF-2 expression in intestinal epithelial stem cells and human lens cells [68, 191].

We hypothesized that FGF-2 released by DBD plasma causes cell proliferation through intracellular secondary ROS production. We measured longer term plasma effects on intracellular ROS (up to 24 h after plasma). ROS levels increased rapidly following plasma and returned to untreated control levels by 6 h. However, at 24 h intracellular ROS peaked a second time. When cells were incubated with an FGF-2 neutralizing antibody, the initial ROS peak was higher (40 % increase), occurred later (3 h), and declined more slowly over time. No secondary ROS peak was observed at 24 h with the FGF-2 antibody. When the secondary ROS peak was blocked with sodium pyruvate, plasma no longer increased endothelial cell proliferation. Finally, we were interested in observing the effect of multiple plasma treatments, or essentially repeated oxidative stress. When endothelial cells were plasma treated a second time either 48 or 72 h after the initial plasma treatment, plasma-induced FGF-2 release increased significantly. This effect was also blocked by an FGF-2 neutralizing antibody. These data show that initial oxidative stress induces FGF-2 release, which then signals for cell proliferation via intracellular secondary ROS production. The initial FGF-2 release induces FGF-2 production, so that any subsequent oxidative stress causes a larger FGF-2 release.

## 6 Conclusions

ROS modulation is an important therapeutic option for a wide variety of angiogenesis dependent and independent diseases. However, current oxidant and anti-oxidant therapies fail to incorporate the complexities of ROS type, dose, and timing.

By further understanding the role of ROS in physiology and pathology, we will enlarge our biochemical toolbox for both enhancing and inhibiting angiogenesis.

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# Microfluidic Devices for Angiogenesis

**Vernella Vickerman, Choong Kim and Roger D. Kamm**

**Abstract** Cell culture has played a central role in developing our understanding of angiogenesis, and a wide variety of culture systems have been adapted for this purpose. Despite the value of this approach, many of the systems employed have suffered from a lack of precise control over culture conditions, an inability to visualize the process of angiogenesis in real time, and limitations in the ability to replicate the in vivo situation in which multiple cell types interact over distances of 100s of microns. With the advent of microfluidics, many of these obstacles can be overcome, and in vitro experiments can be produced with closer relevance to the in vivo situation. In this chapter, we describe the evolution of these microfluidic devices in the context of angiogenesis and describe current capabilities.

## 1 Introduction

Angiogenesis is important in health and disease, as has been abundantly demonstrated in other chapters of this text. During embryonic development, for example, angiogenesis facilitates the expansion of the primitive vascular plexus, thus meeting the demands for oxygen and nutrient requirements during embryogenesis. In healthy adults physiologic angiogenesis occurs during endometrial and placental formation, wound granulation after injury and hair follicle vascularization [1]. Furthermore, angiogenesis has been implicated in and directly linked to the pathogenesis of a growing list of diseases [2]. Given the far-reaching impact, it is not surprising that the radical theory of angiogenesis proposed by Folkman during

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the 1970s has spawned an entirely new field of both basic and translational research. Moreover, there is considerable interest in understanding how to modulate vascularization and insights from angiogenesis are used to design new therapies for cardiovascular diseases [3] or more boldly, the in vitro creation of vascularized tissues or organs by implementing tissue engineering approaches for replacement therapies.

## *1.1 In Vitro Versus In Vivo Models of Angiogenesis*

Over time, investigators have implemented different types of in vivo and in vitro models in an effort to recapitulate natural angiogenesis. The requirement for more affordable, reliable, reproducible and well-characterized model systems has contributed to vast progress over the years. Despite these advances, there still remain challenges with either approach, evident by the limited success in the translation of basic research to the clinic or bedside. Nevertheless, the research community is aware of these shortcomings, and has identified the extensive criteria that must be met [4] for successfully recreating angiogenesis in the lab. Thus, the field continues to evolve, as newer models are developed and older ones are refined.

In vivo *systems:* Three main types of in vivo angiogenesis assay have been described; (1) microcirculatory preparations in chick embryo and rodents; (2) recruitment of vessels by biocompatible polymer matrix implants; and (3) excision of vascularized tissues (see [4] for an extensive review). One of the earliest in vivo angiogenesis models dates back to the late 1930s, where Ide et al. [5] demonstrated the vascularization of an implant of Brown-Pearce epitheliaoma using the transparent rabbit ear window developed by Sandison [6]. Since then, several other in vivo assays have been developed including cranial windows, chick chorioallantoic membrane (CAM), corneal micropocket assays among others [4].

In vitro *systems:* In vitro assays traditionally take what could be viewed as a minimalistic approach; the angiogenesis cascade is decomposed and investigated as the sum of its individual steps, namely migration, proliferation and tube formation. Two research groups, Jaffe et al. and Gimbrone et al. were among the first to report of the successful long-term culture of endothelial cells in vitro [7, 8]. However, it was not until methods for the successful culture and clonal expansion of endothelial cells (ECs) in vitro [9] were developed that the first in vitro angiogenesis assay was established. In 1980, Folkman and Haudenschild demonstrated that cloned capillary ECs cultured on a gelatinized Cuprak dish in tumour-conditioned medium could initiate angiogenesis [10]. Since then a variety of in vitro models have been developed in an attempt to simulate and analyze the process of neovascularization. These in vitro assays can be broadly classified as two-dimensional (2D) or three-dimensional (3D). In 2D models, cells are plated on culture surfaces that are coated with thin layers of adhesion proteins whereas in 3D models cells are cultured on or in 3D matrices. In 3D cultures, cells are able to invade or migrate within the matrix, which better recapitulates the 3D nature of the

in vivo microenvironment. A common theme involves the culture of ECs in the presence of different extracellular matrix components and angiogenic factors. Although these models have been extremely valuable for understanding several aspects of the cellular and molecular mechanisms operative in angiogenesis, they lack the capability of including other important factors such as chemical gradients, surface shear stress and interstitial flows, as discussed in more detail later.

Physiologically relevant and well-controlled models that better mimic normal and pathological angiogenesis would narrow the gap between bench-top discoveries and clinical applications. In vivo models have physiological relevance yet inherently lack a high level of control. On the other hand, in vitro models have the potential for greater degree of control, yet lack critical elements of the in vivo microenvironment. There remains much room for improvement, and this has motivated many to explore microfluidic methods in the search for greater in vivo relevance in an in vitro model.

## *1.2 Traditional Methods of Studying Endothelial Chemotaxis*

Directional migration toward a gradient of soluble chemoattractant, or chemotaxis, is an essential feature common to many cell types that is vital for organ development, wound repair, inflammation, neurite outgrowth, angiogenesis, tumor invasion and metastasis. During angiogenesis, activated endothelial cells degrade their underlying basement membrane, interpret directional cues and migrate toward a chemotactic gradient of angiogenic factors (e.g. VEGF, bFGF [11]). A modified Boyden chamber assay, also known as the Transwell® assay is widely used [12–15] to study endothelial chemotaxis. The Boyden chamber consists of upper and lower compartments separated by a porous filter barrier. Filters are typically available in different membrane materials (e.g. polycarbonate, polyester, and cellulose nitrate), surface treatments and pore sizes which are chosen based on cells of interest. In this method, cells are seeded in the upper compartment and chemoattractant placed in the lower. After a predetermined incubation period, typically 4–6 h, the number of cells that have migrated across the entire width of the filter is quantified. Two major weaknesses with this method include (1) uncertainty in the nature of gradient generated and (2) the inability to visualize and continuously monitor cells during the course of the experiment. Other traditional methods for studying endothelial migration include the wound assay [16], Teflon fence assay [17] and phagokinetic track assay [18]. In each of these, there is an inherent challenge in distinguishing between chemokinesis and chemotaxis. Consequently, many in the research community have recently looked to microfluidic approaches that facilitate the establishment of stable gradients and permit real-time monitoring.

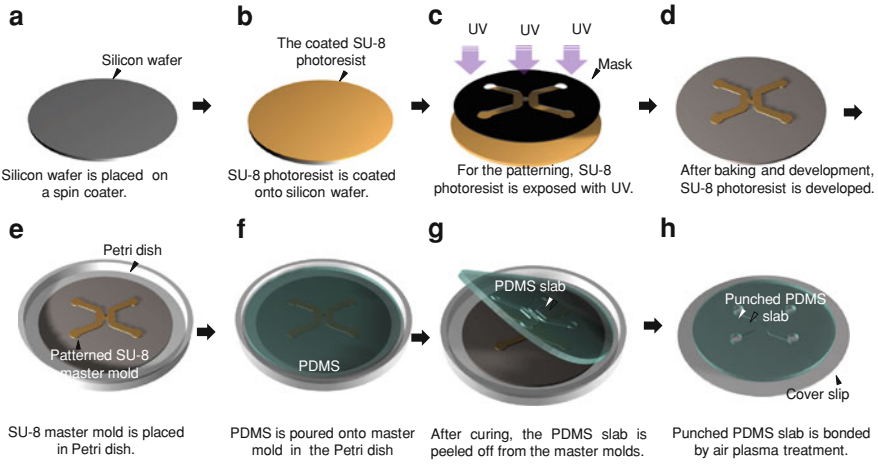


**Fig. 1** Schematic of Microfluidic Device Fabrication: The microfluidic chip is fabricated via conventional soft lithography (**a**–**d**) and PDMS replica molding (**e**–**h**). **a** A silicon wafer is placed on a spin-coater to achieve the desired SU-8 resist film thickness. **b** SU-8 photoresist is coated onto the silicon wafer and baked on a hot plate. **c** For patterning, the SU 8 photoresist on the silicon wafer is exposed to conventional UV (350–400 nm) radiation through a photomask. **d** After baking, the SU-8 resist is developed with SU-8 developer, and an SU-8 master mold is made. **e** The SU-8 master mold is placed in a large Petri dish for PDMS preparation. **f** PDMS is poured onto the master mold in the Petri dish and degassed in the vacuum chamber. **g** After curing (70 °C), the PDMS replica is peeled away from the master mold. **h** For bonding, the PDMS slabs are bonded with a cover slip after oxygen plasma treatment

## *1.3 The Advent of Microfluidics*

Early fluidic devices were developed in the 1980s and used in studies of the effects of shear stress on endothelial monolayers [19–21]. While not "micro" in scale, they typically involved channels on the order of $10^{-3}$ m; large enough to enable easy fabrication by standard machining methods, yet small enough to limit the need for large numbers of cells or large volumes of reagents. In parallel, microfluidics emerged as a multidisciplinary research field since its inception at Stanford University where the technology was first applied to the fabrication of gas chromatographic air analyzers [22] and for designing the nozzle component for the first inkjet printers by IBM [23]. The foundation for microfluidic fabrication was laid by the microelectronics industry that developed fabrication techniques for creating high-resolution features in microelectronic components. Early microfluidic systems were fabricated in silicon or glass using standard photolithography (other lithographic techniques include electron lithography, X-ray lithography, ion lithography) and chemical etching methods [24]. In recent years, there has been a shift towards elastomeric materials that permits rapid prototyping of microfluidic systems, discussed further in Sect. 3 and illustrated in Fig. 1 [25, 26].

Current applications for microfluidic devices span multiple disciplines, with applications in biotechnology and biochemical processing [27]; clinical and forensic analysis [28]; combinatorial chemistry [29]; systems biology [30]; tissue engineering [31], cell-based biosensors [32]; diagnostics and personalized medicine [33]; and embryo production [34]. The biology community has greatly benefited from these advances, and progress in microfabrication technologies has paved the way for new approaches to manipulate and observe cells in microenvironments that more closely mimic in vivo conditions. Microfluidic-based cell culture systems provide new capabilities for continuous monitoring of dynamic processes, such as angiogenesis, at high spatial and temporal resolution in a controlled microenvironment [35].

## 2 The Biology of Angiogenesis and Endothelial Cell Chemotaxis

### *2.1 Angiogenesis in Development*

Although the processes by which microvascular networks form are described in detail in other chapters, a brief background is useful as motivation for in vitro studies in microfluidics to follow. The embryonic vasculature is formed by two distinct processes, vasculogenesis—the de novo vessel formation from endothelial progenitors, angioblasts—and angiogenesis—the expansion of a pre-existing vascular network which occurs during the later stages of development. Interestingly, these two processes of vascularization occur in distinct embryonic regions which are defined by the three germ layers: ectoderm, mesoderm and endoderm. Vasculogenesis, which gives rise to the primitive vascular plexus, occurs in tissue of a splanchnopleural origin (includes endoderm and splanchnopleural mesoderm), while angiogenesis predominantly occurs in tissues of a somatopleural origin (ectoderm and somatopleural mesoderm) [36]. Here, in keeping with most of the existing microfluidic literature, we mainly focus on angiogenesis.

### *2.2 The Pathways of Angiogenic Sprouting and Network Formation*

Angiogenesis is a complex, multi-step process involving a series of well delineated steps. Once endothelial cells have acquired an angiogenic phenotype, the following processes occur: (a) protease production increases facilitating degradation of basement membrane; (b) directional cues initiate migration towards the angiogenic stimulus; (c) proliferation; (d) tube formation; and (e) maturation. The regulatory chemical signals produced at the onset of and during angiogenesis

originate from many cell types including tumor cells, fibroblast, keratinocytes and macrophages [37]. Up-regulation of pro-angiogenic factors and the simultaneous down-regulation of endogenous angiogenesis inhibitors are normally triggered when the tissue environment becomes hypoxic [38, 39] or inflammatory [40].

Oxygen sensing is important for many biological processes including development, pH homeostasis and angiogenesis [41]. Critical molecular mediators of hypoxia and cellular oxygen-signaling pathways, hypoxia-inducible factor (HIFs), are known to directly activate the expression of such pro-angiogenic factors as VEGF, PDGF-B, Ang-1, Ang-2 and receptors VEGFR-1, VEGFR-2 and Tie-2 [39, 42], (see also Table 1). In particular, hypoxia induces a dramatic increase in VEGF messenger RNA levels [43]. VEGF is essential for embryonic vasculogenesis and angiogenesis, as demonstrated by the observation that VEGF gene inactivation is lethal [44, 45]. In vitro, VEGF promotes EC proliferation, migration, differentiation and capillary formation. It is also widely appreciated that tumor cells secrete angiogenesis-related proteins under hypoxic conditions [46, 47]. Recently, a linear correlation was established between hypoxic growth conditions and the expression levels of eight angiogenesis-related proteins including VEGF, IL-8, PDGF-AA, PDGF-AA/BB, TGF-$\beta$1, TGF-$\beta$2, EGF, and IP-10 [48]. In addition, stromal cells promote angiogenesis [49, 50] and also enhance vascular maturation [51].

Inflammatory cytokines and chemokines represent another group of signaling molecules with critical roles in angiogenesis regulation during tumor growth, wound healing and ischemia [40]. Furthermore, chronic inflammatory conditions such as rheumatoid arthritis (RA) are considered as angiogenic diseases, as the excessive neovascularization contributes to their pathogenesis. Pro-inflammatory factors such as TNF-$\alpha$, IL-8/CXCL8 and SDF-1/CXCL12 are known angiogenic mediators while IL-4, LIF and PF4/CXCL4 exert an inhibitory role [52, 53]. For extensive reviews on additional topics related to angiogenesis in inflammation see [54].

## *2.3 Angiogenesis in Disease Processes*

In healthy adults, endothelial cells remain virtually quiescent—the result of a strict balance between pro- and anti-angiogenic factors. The transition from a quiescent to an angiogenic phenotype occurs when this balance is disrupted and pro-angiogenic factors dominate. While physiologic angiogenesis occurs in a tightly regulated fashion, deregulation of this process contributes to the progression of a variety of diseases (Table 2). The pathogenesis of the diseases may be characterized by either an excessive or insufficient growth of blood vessels. Interestingly, while the cellular and molecular regulators of normal and pathological angiogenesis have much in common, the latter is characterized by uncontrolled, aberrant vascular growth [55]. For example, VEGF signaling plays a pivotal role in normal and tumor angiogenesis, however tumor vasculature is chaotic and structurally abnormal [55].

**Table 1** Pro-and anti-angiogenesis factors

| Positive/negative regulators | Soluble ligands/receptors | Impact on endothelial cells | Notes |
|---|---|---|---|
| Pro-angiogenic Factors | *VEGF*<br>VEGFR-1 (Flt-1)<br>VEGFR-2 (Flk-1/KDR) | Chemotactic [11]<br>EC migration, proliferation, anti-apoptosis, differentiation | *Targets* EC specific mitogen<br>*Sources* Smooth muscle cells, keratinocytes, macrophages, tumor cells Gene<br>*Regulation* Oxygen tension, growth factor (TFG-alpah,PDGF), inflammatory cytokines (IL-1-alpha,IL-6); Hormones (TSH, ACTH); oncogenes (ras) [111] |
| | *Angiopoietin-1*<br>Tie-2 | *in vitro* EC migration, tube formation, survival<br>*in vivo* Maturation, signal recruitment of mural cells | [112] |
| | *FGF*<br>bFGF binds FGFR-1 aFGF | *Chemokinetic*<br>↑ EC proliferation | Synergistically with VEGF. Broad spectrum, multiple cell types [11] |
| | *PDGF-BB*<br>PDGFR-αα<br>PDGFR-ββ<br>PDGFR-αβ | ↑ EC migration, require bFGF pretreatment | *Targets* VSMCs/pericytes mitogen and chemoattractant. bFGF sensitizes EC [113] |
| | *IL-8/CXCL8*<br>CXCR1<br>CXCR2 | *Chemotactic*<br>↑ EC proliferation<br>↑ EC migration<br>↑ MMP production | ELR-positive chemokine [114] |
| | Stromal cell-derived factor 1<br>*SDF-1/CXCL12*<br>CXCR4 | *Chemotactic*<br>*in vivo*; dermal angiogenesis recruitment of EPCs | [115] |
| | TGF-β | Induces EC apoptosis ↑ bFGF expression, ↑ VEGF synthesis | [116] |
| | Hepatocyte growth factor/ scatter factor(HGF/SF)<br>Met | ↑ EC proliferation<br>↑ EC migration | Down-regulate TSP-1 in tumor cells [117] |

(continued)

**Table 1** (continued)

| Positive/negative regulators | Soluble ligands/receptors | Impact on endothelial cells | Notes |
|---|---|---|---|
| Anti-angiogenic factors | Angiostatin | Inhibit EC proliferation | 38 kDa plasminogen fragment [118] |
| | Endostatin | Inhibit EC proliferation | 20 kDa C-terminal collagen XVIII fragment [119] |
| | Interleukins (IL-4, IL-13) | Inhibit EC migration Inhibit tube formation | Cytokine [120], [121] |
| | *Platelet factor-4 (PF4)* | Inhibit EC proliferation | ELR-negative chemokine [122] |
| | CXCR3-B | Inhibit EC migration | |
| | Thrombospondin-1(TSP-1) | Inhibit EC migration | [123] |
| | Vasostatin | Inhibit EC proliferation | Fragment of calreticulin [124] |

**Table 2** Angiogenesis in disease process

| Angiogenesis | Diseases | Notes/references |
|---|---|---|
| Excessive/ abnormal angiogenesis | Cancer/solid tumors hemangiomas | [125] |
| | *Retinopathies* Proliferative ischemic retinopathies: proliferative diabetic retinopathy (PDR), retinopathy of prematurity (ROP). Age-related macular degeneration (AMD). Retinoblastoma | Blindness, excessive pre-retinal blood vessel growth leading to retinal detachment [126] |
| | Atherosclerosis | Inflammatory disease [127–129] |
| | Inflammatory rheumatic diseases: rheumatoid arthritis (RA) | [130] |
| | Psoriasis | Chronic Inflammatory disease of skin and small joints [131] |
| | Asthma | Chronic Inflammatory disease: [132] |
| | Endometriosis | Gynecological disorder, proliferation of endometrial glands and stroma outside uterine cavity [133] |
| | Chronic liver disease: portal hypertension (PH), fibrosis, cirrhosis, non-alcoholic steatohepatitis (NASH), hepatocellular carcinoma | [134] |
| Insufficient angiogenesis | Coronary artery disease | [135] |
| | Ischemic stroke | [136] |
| | Chronic wounds | [137] |
| | Inflammatory rheumatic diseases: systemic sclerosis or systemic scleroderma (SSc) | [130] |

## *2.4 Angiogenesis in Tissue Repair*

During tissue repair, for example in wound healing, angiogenesis is necessary for generating a functional granulated tissue. In healthy individuals, this phase of wound repair progresses naturally. However, in certain situations, for example in diabetes, growth factor deficiencies, impaired keratinocyte and fibroblast migration and proliferation, and accumulation of anti-angiogenic glycation end-products in their tissues impair the angiogenic response and subsequent tissue repair [56]. Furthermore, tissue damage is inevitable in diseases characterized by inadequate vascular perfusion and interventions to correct vascular insufficiency are required. One approach, therapeutic angiogenesis, has been extensively explored. Therapeutic angiogenesis is the delivery of exogenous factors (e.g. small molecules, genes or cells) or the use of mechanical devices to stimulate neovascularization, restore form and function to tissues [1]. Growth factor therapy with recombinant proteins, gene therapy using non-viral (e.g. plasmid DNA, liposomes, nanoparticles) or viral (e.g. recombinant retrovirus, adenovirus, adeno-associated virus,

Herpes simplex virus type-1) techniques to deliver pro-angiogenic factors, tissue-engineered products, hyperbaric oxygen and negative pressure wound therapy are current avenues for stimulating angiogenesis [1, 57]. Recombinant growth factors including VEGF, bFGF and PDGF-BB have been used in animal models of chronic limb ischemia [58, 59]. More recently, using plasmid-based gene delivery systems, local intramuscular administration of FGF-1 was shown to be safe and improved amputation-free survival in patients with critical limb ischemia [60]. Hyperbaric oxygen therapy (HBOT), the intermittent exposure of patients to 100 % oxygen at pressures above 1 atmosphere, has been known to promote angiogenesis and collagen synthesis but the underlying mechanisms remain unclear [61]. Another strategy, negative pressure therapy has been used extensively in clinical management of wounds [62]. The underlying mechanism by which topical negative pressure stimulates angiogenesis has been investigated using in vitro methods and linked to increased endothelial cell migration and proliferation [63, 64].

Angiogenesis is clearly a complex process requiring the coordination of multiple cell types and integration of a host of chemical and mechanical microenvironmental signals. Despite these enormous challenges small successes provide a map for future directions and motivation for further pursuits in the application of angiogenesis principles for tissue repair and regeneration.

## 3 Endothelial Cell Culture in Microfluidics

### *3.1 Pre-formed Microvascular Systems*

Microfluidic cell culture began approximately 30 years ago with studies in which the cells were plated on channels on a 2D rigid surface [65]. This was a natural extension of studies in traditional culture dishes, adding some advantages in terms of improved imaging capabilities and a more natural means of introducing fluid shear stress, a factor that had been recognized to play a significant role in numerous endothelial cell processes since the early experiments by Gimbrone, Davies and Dewey [66, 67] beginning in the 1980s. These early flow devices were not "micro" in scale, however, and were typically machined out of hard plastic, limiting their use and their ability to mimic the actual in vivo microenvironment. In particular, the plastic surfaces were orders of magnitude higher in stiffness than the basement membrane these cells adhere to in blood vessels, and were incapable of being stretched by 5–10 % as would normally occur in vivo as a consequence of normal blood pressure pulsatility. Despite these limitations, these studies greatly advanced our understanding of endothelial biology, for example, leading to a progressively deeper appreciation of the importance of fluid shear stress and of the wide range of biological processes influenced by it (see e.g., reviews by Davies [68]).

Although the surfaces of these devices had always been treated with some form of adhesion factors (e.g., collagen, fibronectin, etc.), an important step toward greater realism occurred when these devices began to incorporate compliant substrates, either fabricated from elastomeric materials or incorporating regions of hydrogel that the cells could be plated onto, thereby relaxing the constraint of using an unrealistic substrate stiffness. Hydroglels such as collagen, fibrin, Matrigel$^{TM}$, and others (gelatin, laminin, alginate, and chitosan) came into common usage [69]. These became widely referred to as three-dimensional (3D) substrates, even though the cells generally remained on the 2D surface of the gel. Most of these hydrogels naturally contained or were functionalized with motifs that could bind to integrin receptors (e.g., the RGD sequence), giving rise to cell adhesion, but also to the signaling associated with integrin activation. Results from these compliant systems differed from those on rigid substrates in numerous ways, but most noticeably in terms of the nature of the focal adhesions formed, being more focal in nature on rigid substrates, but more diffuse and ill-defined on 3D gels [70].

Studies were also conducted on or in rigid substrates in 1D, 2D, and even 3D [71, 72] with the aim of developing microvascular networks for applications such as tissue engineering. While these could sustain a flow, they lacked endothelial cells, and were often not biocompatible. They did, however, demonstrate the capability of fabricating complex vascular-like structures using micro- and nano-fabrication methods.

A second major advance occurred with the introduction of soft lithographic methods as a means of producing microfluidic channels and systems, typically from poly(dimethylsiloxane) (PDMS), a biocompatible elastomer [26]. Numerous advantages accrued from this new approach. One was that, since the systems were fabricated by means of casting the PDMS onto silicon wafers, the wealth of knowledge gained from the use of silicon technology in the semiconductor industry could immediately be applied to the fabrication of these miniaturized devices for cell culture. Ultimately, the inherent low Young's modulus of PDMS, although far in excess of biological tissues, could be used to allow substrate stretch, simulating the deformations of the vascular wall, or to measure contractile forces of cells using systems of micropillars upon which the cells could be cultured [73]. Finally, the high permeability of PDMS to gas and liquid [74] proved a mixed blessing. On one hand, gas exchange in an incubator helped to maintain desired levels of $O_2$ and $CO_2$. Given the reduced volumes of these miniaturized systems (larger surface-to-volume ratio), this no doubt helps limit the need for frequent media changes. On the other hand, it also led to higher rates of evaporation, a limitation in some experimental situations. Finally, the tendency for PDMS to adsorb small hydrophobic molecules also has a negative influence in some experiments, especially in the context of drug screening [75].

Despite the obvious advantages of microfluidics in studying vascular biology, its use for investigations of angiogenesis is a relatively recent development. Early attempts to produce microvascular networks focused not on angiogenic responses, but rather on the fabrication of preformed vascular network patterns in PDMS or stiff gels, and subsequent seeding with endothelial cells. This work was motivated

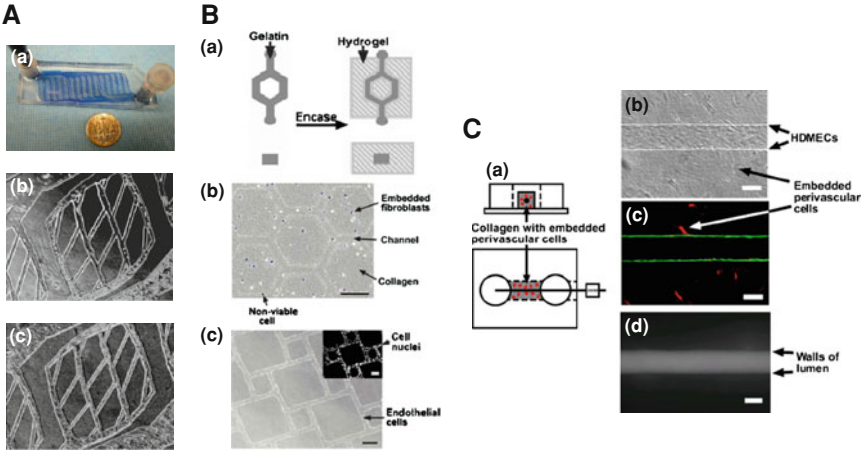


**Fig. 2** Preformed microvascular networks: **A** (a) Macroscopic view of the microfabricated PDMS network. (b) Endothelial cells immediately after seeding onto the pre-fabricated networks. (c) Endothelial cells lining the networks after monolayer formation [77]—*Reprinted with permission from Springer*. **B** Formation of tubes that incorporate endothelial cells and fibroblasts. (a) Schematic diagram showing the fabrication of microfluidic gels. Sequential introduction of Pluronic and liquid gelatin into the channels, and gelation, yielding a gelatin mesh that could be separated from the channels. (b) Collagen gel with embedded fibroblasts. (c) Collagen gel with a monolayer of endothelial cells lining internal channels. Inset, Hoechst-stained microvascular network [78]—*Reproduced by permission of The Royal Society of Chemistry*. **C** (a) Schematic of gel substrate with embedded perivascular cells (PCs) and a channel for endothelial cell seeding. (b) Phase-contrast image and (c) corresponding fluorescence image of the center plane of the tube with PCs embedded within the collagen matrix. (d) Fluorescence image after 20 min of perfusion with fluorescently labeled BSA. Scale bars = 100 μm [80]—*Reprinted with permission from Elsevier Ltd*

by the need for vascular networks in organs being developed for engineered tissues. One of the first demonstrations of microvessel growth in PDMS channels was published in 2002 [76, 77] (Fig. 2A), demonstrating that the vessels could be maintained for up to 2 weeks under flow conditions in channels as small as 35 μ. Of course, the use of these "engineered" vascular networks would require that either the channels be cast in a hydrogel that could be seeded with cells, as has recently demonstrated [78, 79] (Fig. 2B), or that the networks be removable from the mold without causing irreparable damage.

An alternative "engineered" approach was developed by Tien and colleagues [80, 81] (Fig. 2C). In their approach, a cylindrical channel was cast around a rigid needle in a hydrogel such as type I collagen. Once gelled, the needle was removed, leaving an open cylinder into which endothelial cells could be seeded, forming confluent EC-lined lumens after about 2 days in culture. These vessels could subsequently be perfused, and in doing so, became stabilized through a combination of physiological levels of fluid shear stress and hydrostatic pressure [81]. Functional assays were performed, demonstrating levels of permeability for albumin comparable to those observed in vivo ($\sim 2 \times 10^{-7}$ cm/s) and well-defined, continuous

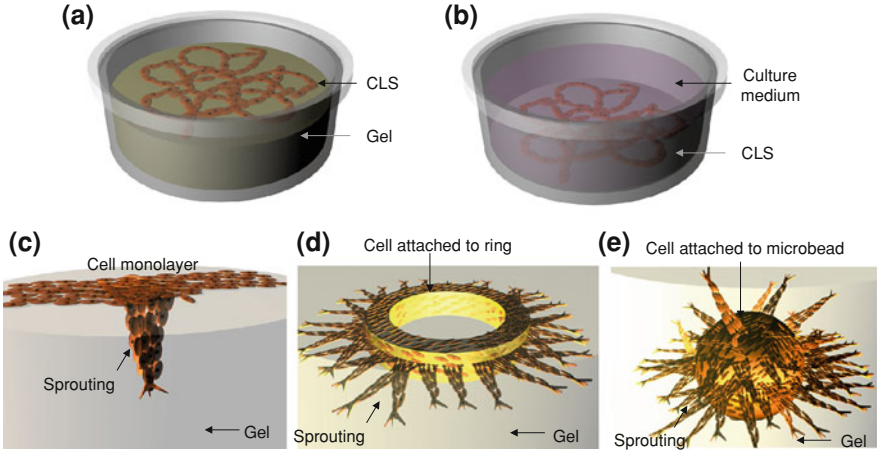


**Fig. 3** Conventional in vitro methods to study angiogenesis: Schematic representation of 2D and 3D assays. **a** 2D assay with capillary-like structures (CLS) developed on the surface of a gel adopting a planar configuration. **b** 3D sandwich assay in which ECs are seeded between two gel layers and form networks. **c** Monolayer angiogenesis assay. **d** Aortic ring assay embedded in Matrigel and incubated. **e** Cell attached to microbeads seeded in gels [138]

staining for VE-cadherin, indicative of tight junctional complexes. These vessels appeared stable, and could be maintained for over 2 weeks in culture. And while these vessels were not branched, several could be placed in parallel in order to produce uniform perfusion of a block of tissue.

Three dimensional structures could also be formed, in this case through a method employing two gels, one (gelatin) that was used as a sacrificial material in the formation of 3D microvascular networks with channels as small as 6 $\mu$ in diameter, and that could be seeded with endothelial cells and maintained in culture for several days [78] (Fig. 2B). Another advantage of this two-gel system is that a second cell type, fibroblasts in this case, could be seeded into the remaining gel, presumably to allow interactions with the endothelial monolayer.

## *3.2 Angiogenesis and Vasculogenesis Assays*

None of the methods described above allowed, however, for natural angiogenesis, the objective being instead, the designed formation of a microvascular network by seeding cells into a cast mold. In a different set of studies, the objective has been to induce an endothelial monolayer to form sprouts and networks into a 3D hydrogel (Fig. 3). This requires the ability to seed cells either on or in a matrix material that is receptive to sprouting, and the addition of angiogenic factors to the medium in order to promote angiogenesis, either in a spatially-uniform manner, or as a gradient to induce sprouting in the direction of higher growth factor concentration.

Assays for angiogenesis have varied in their design, ranging from the more conventional gel systems, where endothelial cells are plated onto the surface of a gel [82] (Fig. 3a) or sandwiched between two layers of hydrogel [83] (Fig. 3b) with growth factors added to stimulate vascular network formation or sprouting, to systems in which the cells are plated as a monolayer on a gel surface (Fig. 3C), or vessel rings [84] (Fig. 3d) or endothelial-coated microbeads are suspended in matrix [85] (Fig. 3e).

Two different types of approach have been used, one to study vasculogenesis and another for angiogenesis. While we focus most of the discussion here on angiogenesis, a short summary of some of the important microfluidic work on vasculogenesis is first presented.

Vasculogenesis occurs by the transformation of angioblasts into endothelial cells that assemble to form a microvascular network [86]. This process usually occurs during development (although there is some evidence that it can also occur in adults), whereas angiogenesis can occur both in the embryo and the adult organism [87]. Vasculogenesis, or at least one model for it, is accomplished by sandwiching endothelial precursor cells or endothelial cells themselves, between two layers of gel [83]. In this case, vascular networks form as a consequence of the seeded cells stretching out toward their neighbors, making contact, forming a 3D network and in many cases, ultimately creating an interconnected array of tubes with diameters comparable to those of a capillary. Generally, these networks have been non-functional in the sense that it is not possible to perfuse them due to the manner in which they are connected to the channels of the device, but systems having the capability to link with these formed networks now exist and it is just a matter of time before these systems can be perfused and presumably used to provide nutrients and gas exchange to other resident cells.

Several microfluidic assays for angiogenesis have been developed consisting of medial channels that run parallel to a gel region. One such system consists of a hydrogel (e.g., collagen) seeded with embryoid bodies or embryonic kidneys and flanked on both sides by medium-filled channels [88] (Fig. 4C). Molded from PDMS and sealed by vacuum against a petri dish following the placement of tissue-seeded gels, gradients were produced in this system by the flow of growth factor-containing medium (VEGFA, VEGFC, FGF2) on one side and control medium on the other. Vascular sprouts were observed to form preferentially in the direction of higher growth factor concentrations. The authors noted the potential for real-time imaging and the retrieval of tissue specimens at the end of the experiment for subsequent biochemical analysis.

A major challenge in using this system was the containment of gel solution, which these authors accomplished by means of PDMS plugs inserted into the channels during gel polymerization. Another approach is to use posts cast in the PDMS replica to define the gel region, and to rely on surface tension along these posts to contain the gel and also to stabilize it after gelation (Fig. 4A, B) [35, 89]. Initial systems accomplished this by direct microinjection of the gel solution, followed immediately (before polymerization) by attachment of a coverslip to the plasma-treated PDMS surface. In these studies, medium and endothelial cells were

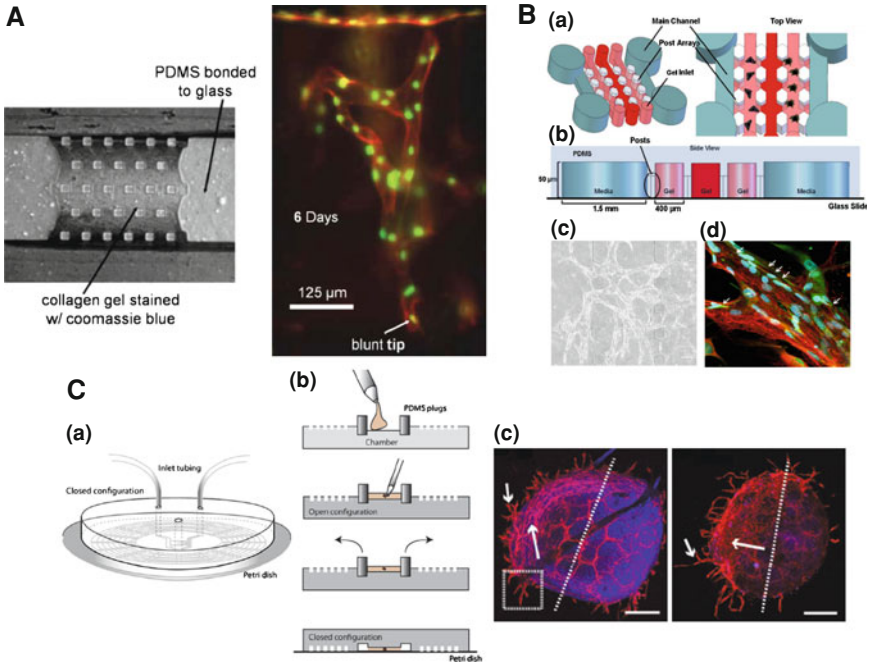


**Fig. 4** Microfluidic assays for angiogenesis: **A** Developed microfluidic system. *Left* A photograph of a 2-channel system (*top* and *bottom*) with a gel region in between. *Right* Fluorescent image showing the sprouting of new vessels into the hydrogel from the endothelial monolayer seen at the top of the image [35]—*Reproduced by permission of The Royal Society of Chemistry*. **B** A system in which ECs and fibroblasts are co-seeded in the gel region and form a microvascular plexus. (a) Schematic representation of the microfluidic system with gel regions (*red*) and media channels (*cyan*). Gel channels are each 400 μm wide and are supported by hexagonal posts, 100 μm in diameter. (b) Side view of the device showing the relative positions of the polymerized hydrogels and the media channels (1.5 mm wide). (c) Phase-contrast image (day 14) of a primitive capillary plexus. Scale bar = 100 μm. (d) Collapsed z-stack of confocal images showing a 3D projection of the structures [89]—*Reprinted with permission of John Wiley and Sons.* **C** (a) Schematic of the microfluidic chip shown assembled by connecting a vacuum to the outer network of channels. (b) Assembly of the system. A liquid gel solution is introduced to the chamber. Then the biological sample is added. Finally, the flow channel plugs are removed and the device is assembled. (c) Embryonic kidney stimulated with a gradient of VEGFA, FGF2, and VEGFC for 48 h showing the asymmetric sprouting of microvessels. [88]—*Reproduced by permission of The Royal Society of Chemistry*

introduced to the side channels after attaching the coverslip, lining the channel walls including the lateral face of the collagen. Seeded in this way, growth factors introduced either uniformly or as a gradient were shown to control vascular sprouting into the gel [35]. A major advantage of this system was the capability to image sprout formation in real-time, with sprouts growing approximately in the imaging plane. This enabled high resolution imaging of vascular sprouts that could grow several hundred microns in length over just a 24 h period [35]. In other

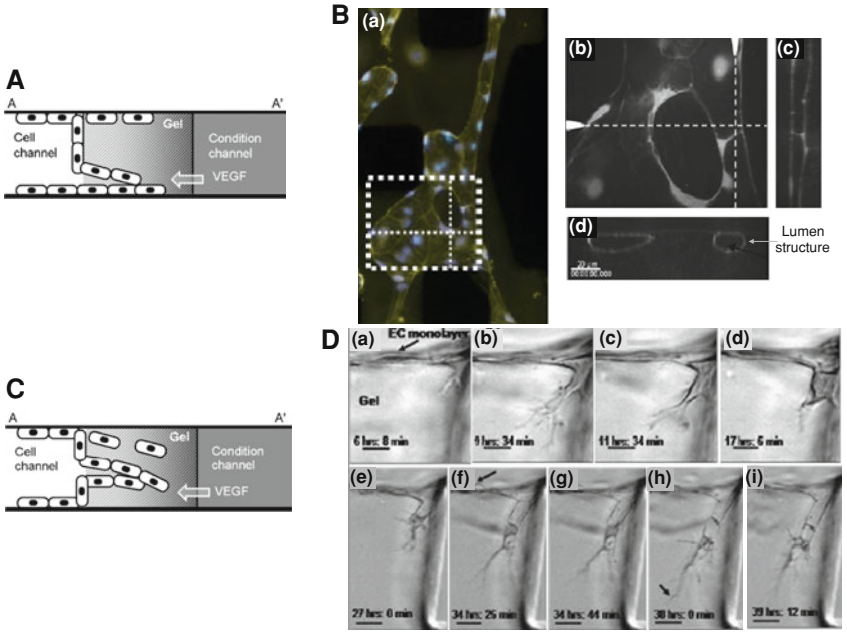


**Fig. 5** Types of invasion into the gel: **A** 2D capillary morphogenesis in the collagen treated channel and **C** 3D sprouting into the gel. **B** (a)–(d) confocal micrographs showing branched structures with lumens formed by migration of cells along the bottom of the gel region that eventually close to form patent lumens across the gel. Scale bar = 250 μm [35]—*Reproduced by permission of The Royal Society of Chemistry*, [139]—*Reprinted with permission of John Wiley and Sons*. **D** Microvascular endothelial cells sprout to form capillary-like structures. (a)–(i) Sequence of images demonstrating capillary formation and the initiation of lumen formation [35]—*Reproduced by permission of The Royal Society of Chemistry*

experiments, the cells were seeded either individually in the gel or along the 2D surface of the coverslip. New capabilities demonstrated in these studies were the generation of flow either in the media channels or across the gel itself, creating an interstitial flow. Two types of vascular structures were observed; in one, the endothelial cells invaded into the gel along the surface of the coverslip, but eventually folded around to form closed and empty lumens which were demonstrated to be patent by both confocal microscopy and the flow of microbeads.

An alternative method is to add gel-filling channels to the system that permit the introduction of gel after bonding the coverslip by plasma treatment [90]. These systems were used in multiple investigations. In one, the effect of device coating was studied [91]. It had been observed that the endothelial monolayer would often adhere to and migrate along the walls of the microfluidic device, or that contraction of cell-seeded gels would cause gaps to appear between the gel and the device walls, especially if the gel concentration was low (Fig. 5A, B) [35]. Two methods were found to mitigate this tendency, either to increase the gel stiffness or to increase the adhesion of the gel to the device surface by an initial coating of

poly D lysine (PDL), both of which led to more sprouts with a more natural appearance [90] (Fig. 5C, D). Gel stiffness could be increased in type I collagen either by using a higher collagen concentration or by gelation at low pH [92].

## 4 Factors that Influence the Properties of Vascular Sprouts

### *4.1 Biochemical Factors*

In recent experiments, the combined effects of VEGF and ANG-1 were studied [93]. Using a microfluidic system, an endothelial monolayer was induced to grow into a type I collagen gel. When under the influence of a VEGF gradient alone, the sprouts were observed to often become unstable, and the tip cell would frequently break loose from the stalk and migrate separately. This tendency could be greatly reduced by the additional of ANG-1, a known vascular stabilizing factor. While this represents but one of many possible combinations, it demonstrates the enormous potential of microfluidics to study the combined effects of multiple factors that can either accelerate or slow and stabilize the growth of microvascular sprouts.

While it is well established that biochemical factors influence angiogenesis, the effects of physical factors on the sprouting of endothelial monolayers into gel have been much less studied. Of studies conducted in microfluidic systems, two primary matrix-related factors have been studied. One is the stiffness of the gel [92], which can be varied either by the use of different gels (e.g., type I collagen, fibrin, matrigel), by varying the concentration of gel, or by regulating the extent of cross-links [92]. While the gel type has been extensively studied in macro-culture assays, this has been relatively less studied in microfluidic systems.

### *4.2 Effects of Hemodynamic Shear*

In one recently published set of experiments, Kang and co-workers [94], examined the effect of shear stress on the apical (luminal) side of the monolayer on endothelial sprouting. They found that, in the presence of sphingozine-1-phosphate (S1P), one of the factors found to promote angiogenesis although it also has stabilizing effects, shear stress on the apical surface of cells adherent to a gel surface, more aggressively invaded into the gel, forming sprouts at a higher frequency than monolayers lacking shear stress. In their experiments, the system could be visualized only after fixation, so their data all examine a single time point (24 h after the initiation of shear). Interestingly, in a different study, using VEGF rather than S1P to induce sprouting, shear stress was found to exert a stabilizing effect; that is, the monolayer was less likely to extend sprouts into the gel when

subjected to fluid shear stress of 1.2 Pa over the apical cell surface. It remains to resolve this apparently contradictory behavior.

In these studies the effects of hemodynamic shear stress parallel to the monolayer surface was investigated. But in the vascular system, flows also occur *across* the vascular endothelium (transendothelial flow) due to differences in hydrostatic plus oncotic pressure between the blood and the interstitial space. These flows across the arterial wall have been estimated to be on the order of 0.01 μm/s in normal arteries, but could easily rise much higher either when endothelial cell turnover occurs [95], as a result of vessel wall trauma, or in vascular beds that that more permeable than normal, such as in the vascular networks that form in the vicinity of malignant tumors [96].

### *4.3 Effects of Transendothelial Flow*

Two groups have examined the effects of interstitial flow across an endothelial monolayer on angiogenesis in microfluidic systems. One used a system in which a gel was formed in a PDMS chamber, on one side of which an endothelial monolayer was grown, across which flow could be directed from the apical surface into the gel [97]. These experiments showed that flow enhances the rate of vascular ingrowth by a Src-mediated process at mean flow velocities ranging up to 50 μm/min. Vascular sprouts appeared to emerge from localized islands of activated Src. More recent studies, however, raise questions about this observation. Using a microfluidic system containing a gel positioned between two media channels so that flow could be directed either in the apical-to-basal or basal-to-apical direction, Song and Munn observed a different response [98]. Whether or not shear stress was applied to the apical surface, and with or without a VEGF gradient, they found that apical-to-basal flow tended to reduce the tendency for angiogenesis as compared to the case with no flow, or with flow from the basal-to-apical side. The mechanisms or signaling pathways activated by this process were not investigated, and this remains an interesting area for further investigation. It is worth nothing, however, that in other studies interstitial flow has been shown to elicit a biological response from smooth muscle cells and fibroblasts [95], and that this effect could be inhibited by the introduction of heparanase, presumably removing the cell-secreted glycocalyx in these collagen I gels. In addition, transendothelial flow has been shown to cause a reduction in monolayer hydraulic conductivity and protein permeability [99]. The link to angiogenesis is that sprouting is often accompanied by a loosening of the cell-cell junctions, which would also cause an increase in permeability.

In a separate study [35] it was observed that interstitial flows of ~90 μm/min past endothelial cells seeded *inside* the gel helped to promote the formation of an interconnected network, and also induced the cells to form a monolayer on the upstream face of the gel region. These results, taken together, suggest that flows of a magnitude that would be expected in vivo, either parallel to or perpendicular to an endothelial monolayer, can have a dramatic influence on angiogenesis. Studies are just now appearing in

the literature, in large part due to the newly developed capabilities of microfluidic systems, and many questions regarding mechanisms of action remain to be answered.

## 5 Heterotypic Cell Culture in Microfluidics

### *5.1 The Importance of Co-Culture in Angiogenesis*

A number of recent studies have begun to use microfluidics to examine interactions between multiple cell types, and some have specifically examined interactions in the context of angiogenesis. There exists a strong motivation for studies of this type. Clearly, in an attempt to replicate true physiology, many types of cell–cell interactions need to be replicated, and the addition of other, non-endothelial cell types helps in that regard. Whether the objective is to create an engineered organ or to screen for new drugs, the more closely one can mimic in vivo conditions, the better. At the same time, however, the addition of other cell types greatly complicates the process of understanding all of the relevant communication pathways. In order to provide some added realism while avoiding the overwhelming complexity of the in vivo situation, investigators have typically introduced only one or two accessory cell types; cells that communicate with ECs in a way that influences the particular behavior of interest.

### *5.2 Experiments with Multiple Cell Types*

One example of this has been the co-culture of endothelial cells with tumor cells, either as isolated cells [90] or as tumor spheroids. Experiments of this type have demonstrated that, depending upon the tumor cell type, the nature of the interaction can be very different, giving rise to different forms of invasion, or producing various levels of stimulation of the EC monolayer (Fig. 6a). In either case, one objective has been to study the role of endothelial-secreted factors that might act as chemoattractants to cancer cells, as a stimulus drawing the cells to the vascular or lymphatic system where they might intravasate and be transported to another location where they could initiate a metastatic tumor. Cells have been introduced both suspended in matrix material [100] (Fig. 6c) or plated onto the surface of a separate channel [101], and induced to migrate preferentially toward the endothelial cells.

Of greater relevance to this chapter, factors secreted by tumor cells have induced sprouting angiogenesis. In a series of preliminary studies using a microfluidic platform consisting of 2 or 3 media channels and 1 or 2 gel regions, the role of various cell types on endothelial invasion into gel has been investigated (e.g., [102]). Interestingly, the effects of different cancer cell lines have shown

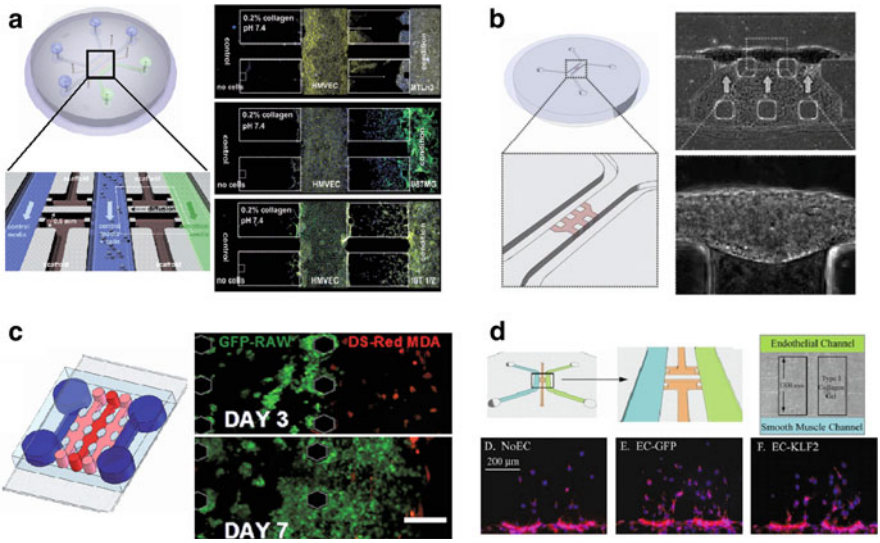


**Fig. 6** Some of the various types of experiments conducted in microfluidic systems: **a** Heterotypic cell culture investigating the interactions between GFP-expressing rat mammary adenocarcinoma cells (MTLn3) and GFP-expressing human glioblastoma cell line (U87MG), human microvascular endothelial cells (HMVEC) and a smooth muscle precursor cell line (10 T1/2) [90]—*Reproduced by permission of The Royal Society of Chemistry*. **b** Endothelial cells seeded in one channel and hepatocyles seeded in the other. Hepatocytes form bile cannaliculi and ECs sprout into the gel [102]—*Reprinted with permission from FASEB*. **c** Tumor cells (MDA) and macrophage (RAW) co-seeded in hydrogel and interacting through intercellular signaling [100]—*Reproduced by permission of The Royal Society of Chemistry*. **d** Investigating SMC (Human aortic vascular SMC CRL-1999) migration in response to endothelium-derived factors [103]—*Reprinted with permission from The American Society for Biochemistry and Molecular Biology*

sometimes contradictory behavior. When endothelial cells are allowed to interact with a particular breast cancer cell line [90], the endothelial cells were observed to actively sprout into the collagen type I gel, while the rate of tumor cell invasion was relatively subdued. In contrast, when interacting with a glioblastoma cell line (U87MG), the tumor cells became highly activated, migrating at a high speed toward the endothelium, while the endothelial cells remained relatively quiescent [89]. Clearly, multiple signals are being exchanged between tumor and endothelial cells, and one cannot readily generalize from one cell line to another as their behavior can be markedly different.

In another set of experiments using the same microfluidic device [103], the interactions between an endothelial monolayer and the 10 T1/2 cell line were considered (Fig. 6d). Since the 10 T1/2 cells are known to be smooth muscle precursor cells, the hypothesis was that when placed in close proximity, they would both differentiate into mature SMCs and also migrate to the endothelial monolayer, stabilizing it and forming an adjacent layer of cells. Results were

somewhat disappointing, however, in that there was little evidence to support the hypothesis.

It is well known that the hypoxic environment found in and around tumors is a major factor influencing the transition of a quiescent endothelium to an activated, angiogenic phenotype. These results from prolonged lifetime of HIF-1α caused by intermittent hypoxia [104]. This, in turn, increases the synthesis and secretion of angiogenic factors such as VEGF, bFGF, and the initiation of sprouting. This has spawned an interest in developing microfluidic systems that can produce a controlled hypoxic environment. In one of these [105], small alginate gel disks seeded with U87 tumor cells were formed in PDMS microwells, then a layer of endothelial cells were plated onto the surface. Since the rate of oxygen consumption was known from separate experiments, computational methods could be used to estimate the degree of hypoxia and the spatial distribution of oxygen tension.

When vascular networks grow in vivo, they are acted upon by numerous factors, both pro- and anti-angiogenic, as described above. Most of both types of factors are synthesized and secreted by resident cells, such as in response to hypoxic conditions. In addition, as the network matures, it undergoes a process of pruning and stabilization, mediated largely by pericytes and smooth muscle cells [106]. For this reason, some have begun to introduce other, accessory cells into their microfluidic cultures in order to promote maturation and stabilization. In one study [89], fibroblasts or mesenchymal stem cells were added to endothelial cells to produce structures having many of the characteristics of a primitive vascular plexus. In another study, a co-culture of endothelial cells and hepatocytes gave rise to vascular sprouts with well-defined lumens and good long-term stability [102] (Fig. 6b). More studies of this type are certain to appear since the interactions between multiple cell types have clear advantages in the formation and stabilization of vascular structures.

### *5.3 Angiogenesis in Tissue Engineering*

Angiogenesis, as mentioned earlier, is highly desired in engineered organs, although the structure of the vascular network can be drastically different between organ systems. This is nowhere more evident than in tissues such as the liver that are both highly vascularized and possess a unique microvascular structure that is central to organ function. For this reason, much work has been directed toward inducing microvessles to grow and develop the desired structure in in vitro systems, either to function ex vivo or ultimately to be implanted. Several microfluidic systems have been developed for this purpose, but only a few contain endothelial cells and offer the hope of natural vascularization [102, 107, 108].

## 6 Quantification Methods and the Role of Modeling

An issue that often arises in studies of angiogenesis in microfluidic systems is how best to quantify the nature of the networks. Various metrics have been developed such as the projected area of the sprouts [89, 90], the total length of sprouts or the average length [90], the average number of cells contained in a sprout, and the number of branches. Recently, a Matlab-based tool, Rapid Analysis of Vessel Elements (RAVE) has been made available, that should prove useful in quantitative analysis [109].

A new approach that has just recently been developed is to use a computational model as a means of controlling in real time an in vitro experiment in angiogenesis, with the objective of regulating the growing microvascular bed [110]. This approach would have obvious advantages in the creation of a vascular system to meet the needs of a specific engineered tissue. In this approach, network growth is observed over time, in this instance, simply in terms of network morphology, and a simulation is used to predict future behaviors under a variety of stimulatory patterns, such as the spatiotemporal distributions of angiogenic factors. Microfluidic systems are particularly well suited to this method in that they allow both for real-time imaging of vascular growth, and the ability to control the delivery of growth factors over time and space in a precise manner. First attempts at this employ a simple model that relates vessel diameter to the speed of tip cell migration; by controlling the latter via changes in the VEGF gradient, the diameter of the vessels as a function of position can, in theory, be controlled. Many hurdles yet need to be overcome, however, if this method is to succeed, not least of which is the fact that vessel grow at a particular instant in time is a consequence of numerous signaling events that have occurred in the past. In order to effectively control the process, it will be necessary to identify read-outs of earlier signaling such as through the use of fluorescent reporters.

## 7 Future Directions

New microfluidic methods are being developed at a rapid pace, and it is clear that future methods will enable further improvements in realism, control of the critical biochemical and biomechanical factors, and imaging capabilities. From the current studies of angiogenesis, assays are likely to emerge that facilitate the growth of entire microvascular networks in co-culture with perivascular cells that are capable of being perfused and stabilized for extended periods of time. These “engineered” networks will contribute an essential element in the quest for vascularized organs for implantation and enable new, more detailed studies of angiogenesis. At the same time, as our understanding of the underlying biological processes evolves, new approaches to modeling can be developed that will eventually facilitate the active regulation of in vitro angiogenesis.

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# Vascular Cell Physiology Under Shear Flow: Role of Cell Mechanics and Mechanotransduction

**Devon Scott, Wei Tan, Jerry S. H. Lee, Owen J. T. McCarty and Monica T. Hinds**

**Abstract** Whether examined at the micro- or macroscale, biological phenomenona are not exempt from physical laws and principles. The vasculature is frequently utilized as a model system to better understand and analyze the consequences of biophysical forces on biochemical processes and ultimate biological phenotypes. Given the complexities of biological systems, there is an inherent need to focus in order to properly elucidate mechanisms. Mechanotransduction and cell mechanics in various stages of angiogenesis have long been examined at distinct length-scales ranging from subcellular, cellular, multi-cellular, tissue, and beyond. This chapter will highlight research over the past decades that have contributed to revealing the importance and interplay between biophysical forces (compressive and shear flow) and biological behavior (motility, regulation of smooth muscle cells, polarity). Abnormal biophysical forces, such as hypertension, contribute significantly to vascular diseases, including atherosclerosis and aneurysm formation. Understanding the relationship between biophysical forces and biological behavior is required to understand the mechanisms of vascular disease.

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## 1 Introduction

Blood vessels are multi-branched elastic vessels that transport blood throughout the entire body. There are three types of blood vessels: arteries that carry blood away from the heart, capillaries that enable the exchange of nutrients, $CO_2$, and $O_2$ between tissue and blood, and veins that carry blood back to the heart. Blood vessels are composed of three layers, the intimal, media and adventitia. The innermost layer, the intima, consists of a single layer of endothelial cells held together by an intercellular matrix surrounded by connective tissue with elastic lamina woven throughout. The middle layer, the media, is the thickest layer; it consists of smooth muscle cells, elastin fibers, and bundles of collagen fibrils, which acts as a mechanically homogeneous material. The adventitia or outer layer consists of fibroblasts, collagen, and elastin with connective tissue.

Blood vessels, including arteries, arterioles, capillaries, venules and veins, provide pathways for blood to travel. The heart consists of two pumps: one, the pulmonary circulation, drives blood to the lungs for oxygen and carbon dioxide exchange; while the second pump, the systemic circulation, brings blood to all other tissues in the body. Cardiac output is intermittent due to the periodic stimulation of heart muscles, however the distensibility of the large arteries and its branches during ventricular contraction (systole) and the elastic recoil of the arterial wall during ventricular relaxation (diastole) enables continuous flow through the arteries to the periphery [1].

As arteries branch the vessels become narrower and the walls become thinner, the arteries change from an elastic structure to a semi-muscular structure to a muscular structure at the arterioles (Table 1). Large elastic arteries serve two main functions, a conduit function and a compliance buffering function. The conduit function is the driving force for blood flow to the lungs and other organs in the pulmonary and systemic circulations. Due to low vascular resistance and a forward pressure gradient, the conduit function provides a pathway for the blood supplied from the heart through the arteries. The compliance function allows the artery to act as a pressure reservoir to smooth flow pulsations, from the cyclic action of the heart to a nearly steady flow across the capillaries, which reduces the ventricular workload during systole and conserves heart energy expenditure, alleviating pulsatile stress in the peripheral arteries [2]. The compliance function buffers flow pulsations to steady flow in the systemic circulation and semi-steady flow in the pulmonary circulation (Fig. 1). Arterial compliance reflects the total amount of blood that can be stored in an artery with a given pressure increase, enabling the conduit arteries to expand to store large volumes of blood ejected from the heart. As the heart relaxes, the arterial walls recoil and push blood into downstream vessels. The Windkessel model is the concept that blood vessels act as elastic storage vessels, relating the pressure waveform to the interaction between the stroke volume (i.e. the output of blood with each heart cycle) and the compliance of the large elastic pulmonary arteries [3]. The compliance or Windkessel effect prevents excess rise of pressure during systole and

**Table 1** Size and function of the arteries found in the pulmonary circulation [11]

| Artery | Diameter | Artery Type |
|---|---|---|
| Elastic artery | >2 mm | Elastic |
| Semi muscular artery | 400 μm–2 mm | Semi-Elastic |
| Muscular/microcirculation | <400 μm | Resistance |

maintains flow during diastole. The storage capacity of the elastic vessels is given by the distensibility, $D = dV/dp$, or the change in volume over the change in pressure.

According to the distensibility equation, the greater stroke volume, the greater amount of blood to be stored in the elastic arteries, and thus greater rise in pulse pressure. If the vessels were not compliant, pressure and flow pulsatility in downstream arteries would become exceedingly high, exerting a detrimental hemodynamic load on downstream arteries. In addition, if large arteries were not distensible, the blood flow through tissue would only occur during systole, while blood flow would cease during diastole [5]. Blood flow, $Q$, from the ventricle into the arteries, can be calculated by taking into account the blood pressure in the elastic artery, $p$, and the peripheral resistance of the small arteries, $R$. For an elastic artery, the change in volume flow is proportional to the pressure, $Q = p/R$. When the ventricle ejects blood from the heart the proximal elastic arteries are first distended until the pressure in these arteries rises higher than the pressure in the arterioles, once a pressure gradient is established blood flows to other parts of the arterial tree. The more compliant the elastic vessels, the longer the blood flow wave takes to reach the periphery, while the less compliant or stiffer the elastic arteries, the faster the velocity of the pressure pulse and blood flow propagation to the microcirculation. Therefore, propagation of blood flow to the microcirculation is driven by arterial compliance.

As the arterial lumen diameter decreases through the arterial tree, a muscular smooth muscle cell layer replaces the elastic lamina, and elasticity decreases. In the semi-muscular and muscular vessels of the microcirculation, pressure pulsations become smaller until the pulsations dampen to a semi-steady flow in the capillaries. The muscular arterioles located at the periphery, are the major resistance vessels and are the main sites for arterial wave reflection [6]. As an artery reaches an organ, it branches into many smaller-radius vessels; resistance in these smaller diameter vessels will be relatively high due to friction from the vessel wall. The greater the surface area of the vessel, the more friction, and thus more resistance, between the blood and the vessel wall. Vessel resistance dampens the pulsatile flow by limiting the amount of blood through the vessel, the greater the resistance the harder it is to advance blood to the next arterial segment. Therefore, small changes in arterial resistance have a large effect on the upstream arteries. For a small increase in resistance, the pressure in the large arteries must increase to advance the flow to the arterioles [7]. The arterioles contain very little elastin, yet have a thick layer of smooth muscle cells (SMCs) that will contract the vessel, increasing the resistance and decreasing the flow (vasoconstriction). Vasoconstriction and vasodilatation (arterial expansion) occurs in the proximal as well as the peripheral arteries.

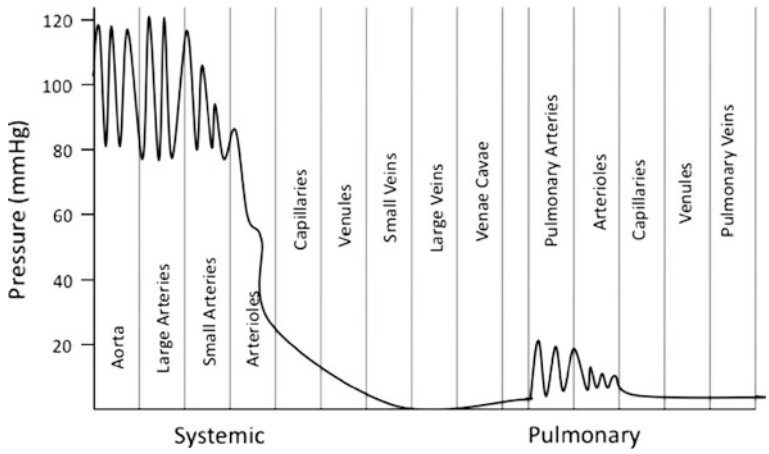


**Fig. 1** Normal blood pressure in the circulatory system. The pulmonary circulation has much lower pressures and pulsations that extend into the capillaries (Redrawn from Sherwood [4])

Vasoconstriction is regulated by factors including, paracrine factors, hormones and neurotransmitters, while vasodilatation is regulated by paracrine factors and activation of the nervous system. The SMCs also play a role in increasing the diameter of the vessel during vasodilatation allowing for the decrease of vascular resistance enabling blood flow and pressure to decrease [4].

The cyclic pumping function of the heart creates a pulsatile component in the arteries, thus blood flow and pressure are also pulsatile. The heart cycle includes periods of systole, heart contraction, and diastole, heart relaxation. During systole, blood is pumped out of the heart into the arteries. Pulse pressure is a measure of the change in blood pressure during heart contraction, or systole. When the heart is at rest (diastole) no new blood is pumped into the arteries, however, blood will advance to peripheral arterial segments. Large arteries that carry blood away from the heart provide a reservoir for high pressure allowing pulse pressure to decrease in the downstream arteries and cause the flow to become more steady or uniform. There are two major factors that affect pulse pressure, the stroke volume (blood output of the heart) and the compliance of the arterial trees [8]. The higher the stroke volume, the higher the volume of blood that must be accommodated in the arterial tree which results in either a higher pressure during systole or a higher pulse pressure. The less compliant the arterial tree the greater the pulse pressure to pump blood into the arterioles.

## 2 Vascular Stiffness

Many vascular diseases, such as pulmonary hypertension and coronary heart disease, are characterized by increased blood flow and pressure in the arteries [9]. Arteries maintain a homeostatic stress/strain range, stress or strain outside of this

range leads to adaptation and changes in the tissue composition, which result alterations in arterial compliance. When the vessel walls of large arteries harden due to hypertension, aging or diabetes, their compliance and thus capabilities of modulating flow pulsatility diminish. Mitchell and colleagues showed that decreases in large artery distensibility due to ageing lead to increased flow pulse pressure in downstream arteries [10]. Decreases in large artery distensibity are caused by vascular remodeling induced by oxygen deficiency, changes in external load, disease altering stress–strain relationship within the vascular wall, changes in arterial pressure, or alterations in smooth muscle tone [11]. Arterial stiffness is increasingly recognized as an important component of cardiovascular risk [11] because stiffness alters the way that the cardiovascular system responds to stress and pressure changes. The efficiency of the arterial buffering depends on the viscoelastic properties of the arterial wall and the vascular diameter.

Modifications in the composition of the arterial wall, known as vascular remodeling, as well as chronic increases in pulse pressure can lead to reductions in the arterial compliance or vascular stiffening; therefore, a stiffer artery will require a higher distending pulse pressure for a given diameter increase [12]. Characteristic vascular changes, including intimal hyperplasia/fibrosis, medial hypertrophy, and extensive extracellular matrix modulation, lead to decreased compliance of the vasculature and changes in vasoactivity. Vascular stiffening due to increased smooth muscle hyperplasia and hypertrophy may produce an increase in collagen fibers and a thinning of elastin fibers, creating less compliant arteries [13]. In compliant arteries, elastin bears the mechanical load caused by arterial pressure and circumferential stretch. In stiffer arteries, the elastin in the walls of the conduit vessels breaks down, resulting in arterial dilation, which further increases the stress on the vessel wall. Arterial stiffening occurs as the mechanical load is transferred from elastin to the stiffer collagen fibers. The increase in mechanical load on the collagen fibers causes an increase in collagen crosslinking, which results in a stiffer vessel [14]. Increases in stiffness also increase the downstream pulsatile tensile and shear stresses. Shear stress is increased as a result of decreased flow dampening during systole. Thus, more blood is ejected downstream during systole producing higher peak flows in the systole upstroke of the flow wave. The tensile stress on the arterial wall increases in proportion to the increase in pulse pressure. SMCs contribute to arterial stiffness, by transmitting contraction through the arteries, smooth muscle contraction can effect even the large arteries [12]. In the small arteries, contraction of the SMCs can cause large changes in the lumen which correspond to changes in the peripheral resistance [11].

Arterial stiffening alters the way that the vascular system can respond to stress and pressure changes. When the compliance of the vasculature decreases, pulsations from the heart are not efficiently dampened; this alters the smooth continuous flow that normally dominates in downstream arteries [7]. Therefore, arterial stiffening can increase pulsatile flow in the downstream arteries, which may lead to further microvascular damage in some vital organs, such as brain and kidney. In these organs, extensive arterial branching does not exist to dampen the pulsatile flow before it enters the microvasculature [15], and thus studies have shown that

microvascular changes are closely related to proximal arterial behavior [16]. An example was shown in hemodialysis patients, where larger amplitude pressure waves, caused by stiffer arteries, led to other cardiovascular diseases such as peripheral artery disease, ischemic heart disease, and heart failure [17]. Pulmonary hypertensive patients will also experience increased pulsatile flow into the small arteries and capillaries due to large artery stiffness, eliciting changes in the vessel wall which in turn cause an increase in arterial resistance. Pressure and flow induce wall distensibility by influencing: pulse patterns which are also distorted by branching of the arterial tree, the resistance to forward motion of flow, and the configuration and velocity of flow through the arterial tree [18]. However, the cellular mechanism underlying the role of vascular stiffening and pulsatile flow in the vascular remodeling process is still poorly understood, possibly due to the lack of a model system that can be used to examine the relationship between pulse flow waves and vascular cells. In addition, this is further complicated by the lack of understanding of the crosstalk and integration required between length-scales as one moves from sub-cellular genotype to cellular behavior to disease phenotype. An excellent example of this the set of diseases described by mutations in the LMNA gene, collectively known as laminopathies [19]. The most understood of these is progeria, where patients exhibit accelerated aging and have notable similarities in cardiovascular disease incidence [20] but surprising differences in disease ultrastructure [21].

A biomechanical study comparing the pulmonary arteries of a normo-tensive and a hypertensive (stiff artery) rat suggested that increased crosslinking of the extracellular matrix structural proteins may be a mechanism for the pulmonary artery stiffening [22]. The elastic modulus ($E$) determines the stiffness of the vessel wall; the modulus varies both longitudinally and circumferentially throughout the arteries. These changes reflect changes in material composition and configuration of the collagen, elastin and smooth muscle fibers. The elastic modulus can be calculated from the stress and strain of the artery:

$$E = \frac{d\sigma}{d\varepsilon},$$

where $\sigma$ is the stress and $\varepsilon$ is the strain. Changes in arterial stiffness alter the arterial pressure; Lame's equation for stress in thick walled tubes can relate strains to pressures as a result of changes in stiffness:

$$\sigma_1 = \frac{P_i[r^2 + (r+T)^2]}{(r+T)^2 - r^2}, r = r_0(1+\varepsilon)$$

where $\sigma_1$ is the stress in a thick walled artery, $P_i$ is the mean pressure, $T$ is the vessel thickness, $r_o$ is the internal radius of the artery in the initial state and $r$ is the internal radius of the artery in a strained state. Lame's equation can be used to calculate the systolic and diastolic arterial moduli [23]. Thus, from Lame's equation, the stress due to a change in pressure can be calculated and the elastic modulus can be determined to assess the impact of change in pressure on arterial

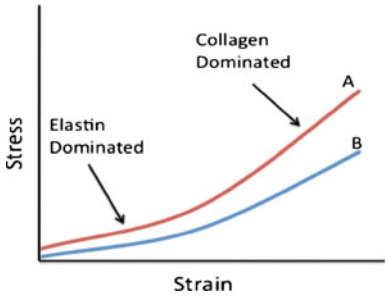


**Fig. 2** Stress strain curve of arteries illustrating the effects of elastin and collagen. Curve A represents a stiffer artery then Curve B

stiffness. The mechanical behavior of the blood vessel can be described by the strain energy function, which quantifies changes in blood vessel function due to changes in structure with a hyper elastic nonlinear arterial model (Fig. 2). Experimental stress strain data can be used to plot this curve using the pseudo-elastic strain energy function:

$$\rho_o W = \frac{1}{2}(a_1 E_{xx}^2 + a_2 E_{yy}^2 + 2a_4 E_{xx} E_{yy})$$

where $a_1$, $a_2$, and $a_4$ are material constants with units of stress, $E_{xx}$ and $E_{yy}$ are the circumferential and longitudinal moduli of the artery [24]. The medial portion of the artery is responsible for most of the physical properties. Tension at low distending pressures or stresses is born by the elastin fibers while collagen fibers remain folded. At higher pressures or stresses, the less extensible collagen fibers bare the stress, thus invoking a higher stress on the vessel due to an increase in strain, resulting in increased vessel stiffness.

The interactions between the large and small arteries have significant implications on the transmission of the pulsatile pressure and flow through the arterial circulation. Large pulmonary arteries dampen flow pulsations resulting from the intermittent ventricular ejection; consequently, small arteries deliver semi-steady optimal blood flow to the lungs. Interactions between the macro- and micro-circulations are based on pulse pressure and flow wave transmissions [25]. Alterations in one part of the system can affect the other. The microcirculation can influence the pulse pressures in the macro circulation through increased vascular resistance [26]. In turn, the compliance of the macro circulation regulates pulse pressures waves and influences extension of pulsations into the microcirculation [27].

## 3 Vascular Resistance

The resistance arteries consist of small arteries which are less than 400 μm in diameter [28]. An increase in pulmonary vascular resistance (PVR) is primarily caused by a decrease in the lumen diameter of small arteries and arterioles. As described by Poiseuille's law, resistance is inversely proportional to the fourth

power of the vessel radius. Thus, a small decrease in radius will greatly increase the resistance. The arterial resistance, a response to changes that mainly reside in the distal arteries, can be caused by a number of factors associated with structural, mechanical, and functional properties of the arterial wall. PVR, blood pressure, and flow are all related; an increase in blood flow or a decrease in pressure in the small arteries will result in a decrease in resistance, whereas a decrease in blood flow or an increase in pressure results in an increase in resistance. Increased PVR could be caused by pruning, vasoconstriction, or medial hypertrophy.

Pruning is a process in which there is a reduction in the total number of small peripheral arteries. Pruning of the peripheral arteries may be paired with dilation of the proximal arteries, resulting from constriction of the muscular arteries in response to increased pressure [29]. Pruning is a structural change that may increase vascular resistance in vascular diseases such as pulmonary hypertension [30]. The increase in resistance is due to the loss of pathways through which the blood can flow.

Vasoconstriction, or the decrease in blood vessel diameter, also contributes to increases in PVR. Vasoconstriction involves the contraction of SMC due to changes in environmental factors. For example, the elevation of calcium in pulmonary SMCs leads to pulmonary vasoconstriction [31]. As the SMCs constrict, the diameter of the small artery lumen will decrease, thus increasing the resistance in the arteries. Hypoxic vasoconstriction develops to maintain the local ventilation-perfusion relationship; this allows blood to be diverted from hypoxic regions to regions with proper ventilation [32]. However, chronic hypoxia will lead to narrowing of the arteries through muscularization and SMC proliferation, resulting in an increase in PVR related to vasoconstriction. Vasoconstriction is controlled through the production and release of mediators of vascular tone.

Medial hypertrophy involves the thickening of the arterial medial layer and leads to the narrowing of the vascular lumen, which causes a greater resistance to flow. This is the main contributor to increased PVR in pulmonary hypertension [28]. Medial hypertrophy can be caused by a number of different factors including, recruitment of other cells, SMC hypertrophy, SMC proliferation and fibroblast cell migration (Fig. 3). Endothelial cell dysfunction can cause the release of paracine factors, such as growth factors, to induce SMC proliferation, or chemokines to recruit circulating inflammatory cells [33]. The circulating inflammatory cells that infiltrate the media are primarily composed of monocytes and macrophages. These circulating cells attach to the endothelium and are able to penetrate into the vascular wall increasing the medial thickness thus increasing PVR [34]. Fibroblasts migrating to the medial layer may also contribute to medial hypertrophy. The transdifferentiation, induced by environmental factors, of fibroblasts to myofibroblasts is a key aspect in vascular remodeling. Once transformed, myofibroblasts are able to migrate from the adventitia to the media. Growth of the media can also result from an increase in SMC size, or number, or both. Increased pressure in the small arteries can cause SMC to undergo morphological and functional changes. Medial thickening can change SMC from a contractile to a proliferative phenotype, where

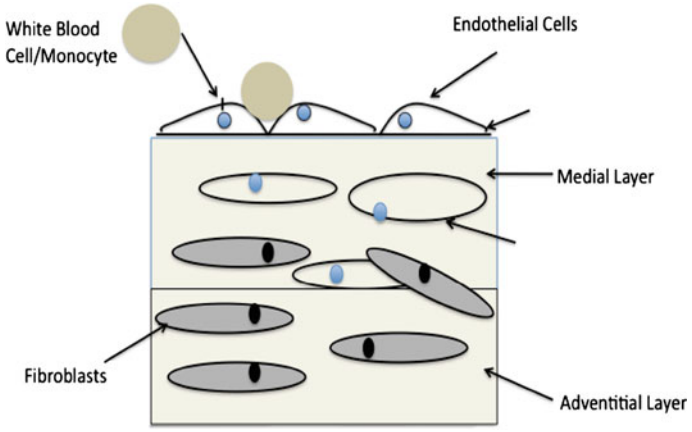


**Fig. 3** Cell recruitment, fibroblast migration, and SMC proliferation and hypertrophy all contribute medial hypertrophy

abnormal cell growth dominates [35]. SMC hypertrophy accounts for the increase in SMC mass thickening of the medial layer in hypertension [36].

## 4 Arterial Wall Mechanical Stresses

Endothelial cells live in mechanically active environments, and experience stress and strain in both physiological and pathological conditions. The biomechanical stress imposed on cells in vivo is manifested as a complex multi-axial stress, often including various anisotropic biaxial stress conditions. The artery wall continually adapts to the changing mechanical environment, due to growth, remodeling, repair, and disease. Therefore, the structure undergoes irreversible changes due to blood flow. Blood vessels are subjected to mechanical forces of hydraulic pressure, circumferential stretch, and shear stress due to the pulsatile nature of blood flow. Blood pressure (BP) determines the amount of mechanical stretch on a vessel; BP creates radial, normal, and tangential, or shear, forces on the vessel wall. Normal stresses on the vessel wall affect all layers, the intima, media, and adventitia, while shear stress only directly affect the layer that it is in contact with the blood flow, the endothelial cells of the intima (Fig. 4).

Blood pressure is a major determinant of vessel stretch; it creates radial and tangential forces on the blood vessel wall that affect all cells types (Fig. 4). Arterial stretch or strain is the radial distension and longitudinal elongation of the vessel which are caused by changes in pressure and flow of the blood. Strain is defined as $\varepsilon = {}^{\Delta r}/_{r}$ or the ratio of the change in radius of the vessel to the original radius of vessel before pressure application. Circumferential strains on the wall are very complex, but can be estimated from measurement of blood pressure,

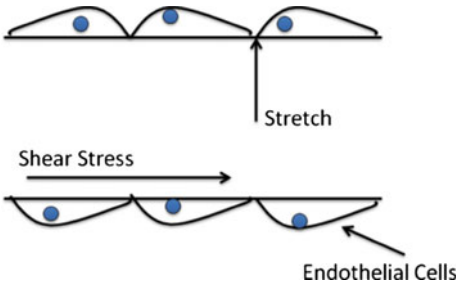


**Fig. 4** Fluid shear stress acts longitudinally on the endothelial cells; cyclic stretch acts perpendicular to the flow direction caused by the pulse pressure of the flow

mechanical constraints, wall thickness, and geometry. Pressure extends the artery like a balloon, the best way to measure the circumferential stretch caused by this distension is the mean circumferential hoop stress [17]. The circumferential hoop stress is given by, $\sigma = Pr/h$, where $P$ is the blood pressure, $r$ is the vessel inner radius and $h$ is the vessel wall thickness. Circumferential strains usually affect the smooth muscle cells and the tunica media. The pulsatile nature of blood flow forces the artery to stretch with every heart beat, and therefore stretch is affected by the compliance of the arteries through the level of pulsation [37].

Shear stress directly affects endothelial cells that make up the intima layer [38]. Fluid shear stress is the result of friction created by blood flow and is also affected by arterial stiffness. Shear stress is a product of blood viscosity and the velocity gradient at the vessel wall. Although blood is a non-Netonian fluid, an assumption can be made that the non-Newtonian behavior of blood in the circulation does not affect the dynamics of the circulation as a whole. While this assumption may not hold true at specific points, such as in a vessel junction, in general, fluid shear stress can be calculated from the shear rate and the viscosity of the blood, through the change in velocity in the vessel over the change in radius, $\tau = \mu \frac{dv}{dr}$. For laminar steady flow of a Newtonian fluid, the calculation of shear stress is given by: $\tau = \frac{4\mu Q}{\pi r^2}$, where $\mu$ is the viscosity, $Q$ is the flow rate and $r$ is the lumen radius. Shear stress depends on the diameter of the vessel, therefore an increase or decrease in the shear stress is associated with the increase and decrease of vessel diameter. Thus, when compared to stiff vessels, elastic vessels with an increased diameter could reduce the shear rate up to 30 % [39]. In a physiological artery, the magnitude of shear stress is in the range of 10–40 dynes/cm$^2$, whereas in a diseased state, the shear stress can dramatically increase relative to normal levels [40]. The shear stress influences many vascular functions, such as the permeability of the vessel, the activity of the endothelial cells, the integrity of formed elements in the blood, and coagulation of blood [41].

Stiffness of the proximal arteries alters the flow stress. Changes in the upstream flow pattern are transferred to the downstream arteries affecting the mechanical stresses on the microcirculation. Increases in pulsations caused by a stiffer artery will result in increased shear stress and increased circumferential stress in the microcirculation. These mechanical changes not only affect the blood flow, but they also affect the cells and fibers embedded in the tissue walls.

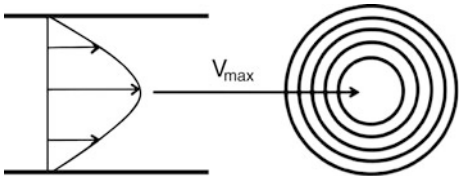


**Fig. 5** Laminar steady parabolic flow is present in most of the cardiovascular system. The arterial walls slow the flow down and the maximum velocity is found in the center of the arteries. Flow is distributed in concentric circles in the diameter of the vessel

## 5 Fluid Mechanics of the Vasculature

High blood flow as a result of increased large arterial stiffness may induce abnormal vasoreactivity of the artery, which could lead to the development of diseases such as pulmonary hypertension [42]. Proximal arterial characteristics, such as stiffness, influence blood spatial and temporal flow patterns in downstream vessels [43]. Elasticity of the upstream arteries determines the flow pattern on downstream small arteries. The less elastic the upstream pulmonary arteries, the higher flow pulsations in the small arteries, which normally experience low flow pulsations [44]. Decreased elasticity in the artery could lead to enhanced energy transmission to the smaller arteries, which could lead to vascular damage. The major determinants of blood flow are pressure, the viscosity of the blood, and the elasticity of the vessel.

Normal flow in the pulmonary artery is laminar, which is characterized by concentric layers of fluid moving parallel through the length of a vessel (Fig. 5). The velocity of the fluid is affected by the spatial geometry of the vessel. Fluid closest to the blood vessel wall is slowest, while it reaches a peak velocity at the center of the vessel. Thus, steady laminar flow exhibits a parabolic profile across the width. Pulsatile flow is also laminar, but it is more complicated than steady flow as its spatial flow profile is a flattened parabola. Figure 6 demonstrates that near the wall the velocity of pulsations is dampened to near zero flow, while toward the mid axis of the vessel, the maximum pulse flow velocity is reached. Therefore, there is a spatial flow gradient across the width of the blood vessel for both steady and pulsatile flow in the arteries.

### *5.1 Steady Flow Behavior*

Steady flow is described by the Poiseuille equation, which states that the pressure gradient is directly proportional to the length of a vessel, and to the rate of flow through the tube, while the viscosity of the liquid is inversely proportional to the width of the vessel (Fig. 7). Flow increases exponentially with increases in the radius.

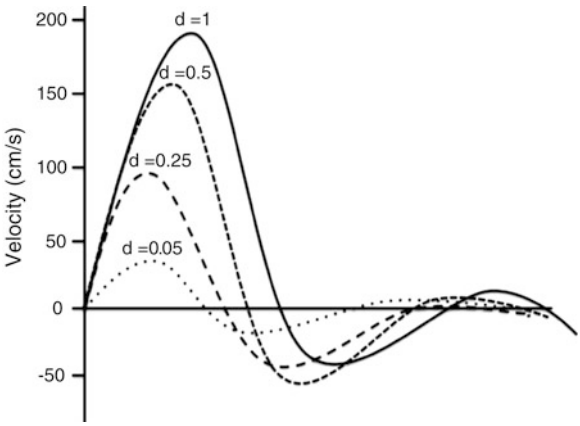


**Fig. 6** The pulsatile velocity profile across the width of the blood vessel to the middle axis. The velocity at the wall is zero, d = 0.05 represents the effect of the fluid by friction next to the wall, d = 1 represents the flow at the midline of the vessel (Redrawn from [11])

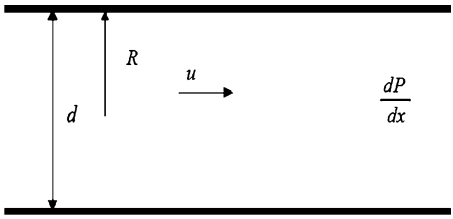


**Fig. 7** The relationship between steady flow and pressure in a tube

Poiseuille's equation is derived from the Navier–Stokes momentum equation:

$$\rho\left(u\frac{\partial u}{\partial x}+v\frac{\partial u}{\partial y}\right)=-\frac{\partial P}{\partial x}+\mu\left(\frac{\partial^2 u}{\partial x^2}+\frac{\partial^2 u}{\partial y^2}\right) \tag{1}$$

where $\rho$ is the density, $u$ is the velocity in the $x$ direction, $v$ is the velocity in the y direction, $P$ is the pressure, and $\mu$ is the viscosity. In order to determine the flow rate, the volume of a parabaloid with a parabola profile must be solved.

$$Q=2\pi\int_0^R Vrdr. \tag{2}$$

Simplification of the Navier–Stokes equation by assuming fully developed incompressible flow and $u$ the only velocity results in:

$$\mu\frac{\partial^2 u}{\partial y^2}=\frac{\partial P}{\partial x}. \tag{3}$$

This equation is solved with double integration using the condition of no slip at the walls and is then be incorporated into Eq. 2 to get the solution for steady laminar flow in a tube:

$$Q = 2\pi R^4 \frac{(P_1 - P_2)}{8\mu l} \tag{4}$$

where Q is the flow rate, R is the radius of the chamber, $\mu$ is the viscosity and $l$ is the length of the vessel. Or for a rectangular chamber such as the parallel plate flow chamber:

$$Q = \frac{h^3 w}{12\mu} \frac{(P_1 - P_2)}{l} \tag{5}$$

where $h$ is the height of the channel and $w$ is the width. Shear stress is then calculated for a rectangular channel using the equation $\tau = \mu(dV/dx)$ producing the equation for steady shear stress:

$$\tau = \frac{6Q\mu}{wh^2}. \tag{6}$$

These flow equations are the most basic measurement for flow velocity, with the assumptions that the flow is laminar and does not vary with time. Poisuille's equation predicts fully developed viscous flow with a parabolic profile (Fig. 5). If flow in arteries is not steady, it will display a pulsatile behavior. Steady flow can only be applied to extremely small vessels where steady flow dominates. Vessels beyond the size of arterioles will still experience pulsatile flow. Pulsatile behavior is a function of the pumping of the heart, the pulse flow behavior changes in the arterial system due to extensive branching and vessel diameter.

## 5.2 Pulsatile Flow Behavior

The Womersley parameter is a measure of the degree of departure from normal parabolic flow characteristic of pulsatile flow. The Womersley number is given by: $\alpha = R\sqrt{\omega\rho/\mu}$. Where $R$ is the radius of the vessel, $\omega$ is the frequency, $\rho$ is the density, and $\mu$ is the viscosity. This equation exemplifies that the degree of departure from parabolic form increases with the Womersley number and frequency.

The equation to describe the flow of a pulsatile fluid was first derived by Womersley in 1955. The equation of motion of a liquid is:

$$\frac{\partial^2 w}{\partial r^2} + \frac{1}{r}\frac{\partial w}{r} - \frac{1}{\upsilon}\frac{\partial w}{\partial t} = -\frac{1}{\mu}\frac{\partial P}{\partial z}. \tag{7}$$

The pressure gradient $\frac{\partial P}{\partial z} = \frac{P_1 - P_2}{L}$ can be represented by a simple harmonic function:

$$\frac{\partial w^2}{\partial r^2} + \frac{1}{r}\frac{\partial w}{\partial r} + \frac{i^3 n}{\upsilon} = -\frac{A}{\mu} e^{i\omega t}. \tag{8}$$

In order to solve this equation, a substitution can be made: so that Eq. 7 becomes:

$$\frac{d^2u}{dr^2} + \frac{1}{r}\frac{du}{dr} + \frac{i^3 n}{\upsilon}u = \frac{A}{\mu}. \tag{9}$$

This equation can now be solved with a Bessel function of order zero:

$$u = \frac{A}{i\omega\rho}\left(1 - \frac{J_1\left(\alpha i^{3/2}\right)}{\alpha i^{3/2} J_o\left(\alpha i^{3/2}\right)}\right) \tag{10}$$

where $J_0$ and $J_1$ are Bessel functions of the order zero and one, and $\alpha = R\sqrt{\omega/\upsilon}$ or the Womersley number. The function in the brackets was termed $[1 - F_{10}]$ and the values were tabulated by Womersley. Then, allowing the real portion of the pressure gradient to be $M\cos(\omega t - \phi)$, we get a flow rate of:

$$Q = \frac{\pi R^2}{\omega\rho} M[1 - F_{10}]\sin(\omega t - \phi). \tag{11}$$

Equation 8 can be put in a more physical form by putting it in terms of the modulus $\left(M'_{10}\right)$ and the phase $(\varepsilon_{10})$, these values were tabulated by Womersley based on the Womersley Number.

$$Q = \frac{\pi R^4 M}{\mu}\frac{M_{10}}{\alpha^2}\sin(\omega t - \phi - \varepsilon_{10}). \tag{12}$$

For a parallel plate flow chamber this equation becomes:

$$Q = \frac{h^3 wM}{\mu}\frac{M_{10}}{\alpha^2}\sin(\omega t - \phi - \varepsilon_{10}). \tag{13}$$

## 6 Endothelial Cell Physiology

Endothelial cells (ECs) line the inner surface of blood vessels throughout the entire cardiac circulation; they form a layer between the circulating blood lumen and the rest of the arterial wall. ECs are continuously exposed to shear stress, pressure and strain. Shear stress is the most influential factor on ECs; the application of shear orients the cells in the direction of flow and affects their functions. The endothelium is essential for vasodilatation and vasoconstriction in response to blood flow related shear stress [45]. The endothelium normally releases vasoconstrictors and growth promoting factors or vasodilators and growth-inhibiting factors to regulate vascular homeostasis. The release of these factors enables the ECs to regulate underlying vascular SMC behavior and contraction. These mediators are thought to control pulmonary vascular tone, homeostasis and vascular injury repair and growth.

Endothelial dysfunction can lead to impaired production of these factors [35]. Once ECs are activated in a diseased state, they may start to express factors that can increase SMC proliferation and vasoconstriction. In pulmonary hypertension, EC dysfunction results in the reduction of pro-vasodilator and antiproliferative molecules such as nitric oxide, while pro-vasoconstrictive and pro-proliferative molecules such as ET-1 are increased.

Nitric oxide (NO) is an essential signaling molecule in many physiological processes; it is responsible for resting pulmonary vasorelaxation and vascular tone. Endothelial NO synthase (eNOS) catalyzes the conversion to produce NO; this enzyme is expressed in ECs and SMCs and can react with a variety of molecules. eNOS can be modified by biomechanical stimuli such as shear stress and increased pulmonary blood flow [46]. eNOS is a constitutive enzyme controlled by the intracellular calcium concentration, when shear stress increases the calcium concentration an increased production of NO results in the inhibition of the underlying SMCs. If there is a sudden decrease in NO, SMCs will contract due to the withdrawal of the inhibitory effect of NO production [47]. Along with the vasconstrictive effect, NO is also antiproliferative, thus a reduction in its production could contribute to proliferation of SMCs. Decreased levels of eNOS have been observed in patients with pulmonary hypertension [48].

Endothelin-1 (ET-1) is an amino acid polypeptide produced by vascular ECs, and binds to SMC through the $ET_A$ receptor. ET-1 is a vasoconstrictive and pro-proliferative molecule, which induces dysfunction of ECs, SMCs, and fibroblasts. ET-1 levels are increased in patients with pulmonary hypertension. In hypertensive animals, ET-1 was abundant in the ECs of pulmonary arteries with medial thickening and intimal fibrosis and showed a strong correlation with increased pulmonary vascular resistance. ET-1 receptors are highly expressed in the media of arteries and on SMCs, where they mediate vasoconstriction and proliferation. The level of ET-1 is inversely proportional to the magnitude of blood flow and cardiac output.

Angiotensin-1 converting enzyme (ACE) is a circulating enzyme that contributes to vasoconstriction and increased blood pressure; it is secreted by endothelial cells. ACE converts angiotensin-I to angiotensin-II, a vasoconstrictor, and it degrades bradykinin, a vasodilator. ACE is highly expressed in the lungs. Studies have shown that Angiotensin-II induces pulmonary vasoconstrcition and ACE inhibitor has prevented the development of pulmonary hypertension in animal models [49].

Platelet derived growth factor (PDGF) regulates cell growth and division, and is synthesized by many different cell types including SMCs and ECs. PDGF is a heterodimer that consists of two chains, A and B, which are 40 % homologous to each other. PDGF is an important mitogen for mediating SMC hyperplasia, hypertrophy, migration, and for vascular remodeling. Over expression of this growth factor can lead to a diseased state. High levels of PDGF have been found in patients with pulmonary hypertension, indicating that this growth factor is critical molecule contributing to elevated resistance and pressure in the pulmonary vessels.

Transforming growth factor beta (TGF-$\beta$) is a protein that controls proliferation, and cellular differentiation. TGF-$\beta$ is a modulator of smooth muscle morphology, phenotype, and stimulates extracellular matrix production, it is secreted in latent form by ECs. TGF-$\beta$ induces the production of collagen and other extracellular matrix proteins, such as fibronectin, and has been shown to remodel tissues. The different isoforms of this growth factor are encoded by distinct genes and have specific functions for tissue and development. Transforming growth factor beta 1 (TGF-$\beta$1) mRNA is expressed in ECs. TGF-$\beta$1 exerts its effects on SMCs including, inhibiting proliferation, altering extracellular matrix synthesis and cell differentiation. However, in the pulmonary artery SMCs from patients with pulmonary hypertension, TGF-$\beta$1 caused enhanced cell proliferation compared to an inhibitory effect in normal cells [50, 51].

## 7 Mechanotransduction

ECs respond to flow through mechanotransducers, which convert the mechanical signal of fluid shear stress to biochemical signals. Many mechanotranducers have been identified, including ion channels, integrins, adhesion proteins, glycocalyx, primary cilia, tyrosine kinase receptors, G-protein coupled receptors, and cytoskeleton. However, the interplay and coordination between these mechanotransducers remains ill-defined.

Initial responses to fluid shear stress include the activation of mechanosensitive ion channels and oscillations in intracellular calcium [52]. These changes occur in a matter of seconds. Several minutes of fluid shear stress induces changes in signaling cascades including mitogen-activated kinases (MAPK) and activation of transcription factors including nuclear factor kappa B (NF$\kappa$B) [52]. It has been suggested that cells can distinguish between athero-protective and athero-prone fluid shear stress through mechanosensory proteins at the cell surface that transmit the signal throughout the cell via the cytoskeleton to nucleus, focal adhesions, and cell–cell contacts [53, 54]. This cytoskeleton-mediated distribution of signaling throughout the cell is known as “decentralization theory” [54].

Evidence suggests that many parts of a cell respond to the mechanical stimulation imposed by fluid shear stress including the cell surface, cell–cell junctions, focal adhesions, and the nucleus [53–55]. These cellular locations are connected by the cytoskeleton [53]. The cytoskeleton is critical in the transmission of the apical shear stress signal to the basal integrins or lateral adhesion proteins, which is where mechanotransduction events occur. The decentralization model suggests that mechanotransduction is transmitted by the cytoskeleton throughout the cell, thus initiating the myriad of fluid shear stress-induced cell responses. An active area of research in the past decade has aimed to better understand the complicated orchestration by small Rho GTPases on such responses, such as the potentially separate, but intimately linked, microtubule regulation of cell polarity [56, 57] and

actin mediation of cellular stiffness [58, 59]. Therefore, the highly dynamic cytoskeleton not only provides cell structure, but is also integral in cell signaling.

Anchor points, including focal adhesions and cell–cell junctions, are the major sites for fluid shear stress-induced tension on the cytoskeleton [53, 54]. Focal adhesions are locations where a cell interacts with the ECM through integrin clusters. Focal adhesions are also linked to the cytoskeleton through linker proteins, such as vinculin. Under high, unidirectional fluid shear stress, ECs remodel and align their focal adhesions in the direction of fluid shear stress, demonstrating that focal adhesions are responsive to mechanical stimuli [60]. ECs elongated on micropatterned lanes have also demonstrated alignment of focal adhesions [61]. The alignment of focal adhesions and the cytoskeleton may promote mechanotransduction due to internal cellular tension imposed by the elongated EC, independent of fluid shear stress. Furthermore, the type of ECM associated with the focal adhesions contributes to the type of signal cascade triggered by tension due to fluid shear stress [62]. Interestingly, the focal adhesions comprised of integrin $\alpha_v\beta_3$, predominantly formed on fibronectin, are responsible for tension-induced upregulation of I$\kappa$B kinase, which phosphorylates and degrades I$\kappa$B [62].

Cell–cell junctions act as anchor points between neighboring cells through molecules such as cadherins and cell adhesion molecules. Cell–cell junctions also are connected to the cytoskeleton through linker molecules. High, unidirectional fluid shear stress modulates expression and phosphorylation of cell–cell junctions including VECAD, catenins, and tight junctions [55]. While the signaling cascades induced by tension on cell–cell junctions are largely unknown, MAP kinase pathways including MEK and ERK1/2 have been implicated [63]. Functionally, the upregulation of cell–cell junctions, e.g. VECAD, is often associated with decreased EC permeability [64]. Recent data also suggests that the formation of cell–cell junctions regulates EC proliferation through tension-induced signaling via the actin cytoskeleton [65].

ECs elongated on micropatterned lanes have limited surface area that can facilitate the formation of cell–cell contacts (Fig. 8) [66]. This is due to the nature of micropatterned surface, such that an entire side of the cell is freely exposed to a passivated BSA-coated region. Cell–cell junctions can form end-to-end, with a maximum contact area of 25 μm (the width of the micropatterned lanes). Multiple cells can adhere and spread across the 25 μm wide lane, providing a neighboring cell for the formation of cell–cell contacts, but these cells usually lack cell junctional anchor points at the opposing cell surface. Thus, internal mechanical tension derived from cell–cell junctions of MPECs is likely to be minimal compared to confluent ECs. The large number of cell–cell contacts formed under high, unidirectional fluid shear stress may offer additional tension-induced signaling mechanism that act to either: (1) enhance cytoskeletal-alignment dependent signaling or (2) possibly generate additional cascades that stimulate gene expression solely dependent on fluid shear stress.

Fluid shear stress is a mechanical stimuli, and mechanotransduction is mediated by the cytoskeleton [53, 67]. Thus, fluid shear stress mechanical stimulation, and not cytoskeletal alignment, may be required to trigger cytoskeletal-dependent signaling

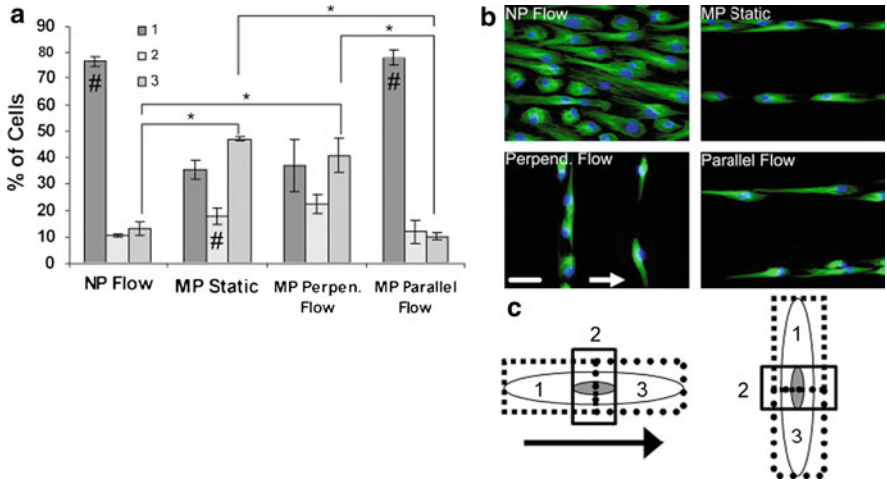


**Fig. 8** Polarization of MTOC in MPECs and non-patterned (*NP*) ECs [66]

for some molecules, such as thrombomodulin. This suggests that certain signaling cascades are only activated after surpassing a specific mechanical threshold. Furthermore, the additive effects on cytoskeletal alignment and fluid shear stress on expression of VCAM-1, vWF, and TFPI implies that aligned cytoskeletal conformation is required for optimal mechanical force-induced cytoskeletal signaling. These complex signaling cascades would then affect other larger cellular structures, such as the nucleus, where recent evidence [68] has demonstrated that failure in such crosstalk at the sub-cellular and cellular levels may help explain why only certain tissues and organs, including the cardiovascular system, mimic natural aging degradation in progeria patients but not others [69]. Indeed, with the advancement in high-content and high-throughput screening platforms, and increased awareness of biophysical contributions, additional studies are needed to begin deciphering this multi-scale dialogue in order to better understand the mechanisms of disease.

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# Matrix Mechanics and Cell Contractility in Angiogenesis

**Joseph P. Califano and Cynthia A. Reinhart-King**

**Abstract** Angiogenesis is a complex process that relies on the interplay of chemical and mechanical signaling events that ultimately result in the formation of new blood vessels. While much work has uncovered the chemical signaling events that mediate angiogenesis, the role of the mechanical environment is less understood. In this chapter, we will discuss how the mechanical microenvironment regulates angiogenesis by examining how matrix stiffness and cellular contractility mediate endothelial cell behaviors that are necessary for the progression of angiogenesis. Specifically, we will describe the roles of matrix stiffness and cell contractility as regulators of endothelial cell adhesion and shape, migration, growth, cell–cell interactions, and cell–matrix remodeling. Collectively, these findings implicate endogenous cellular forces and matrix stiffness as critical components of the angiogenic microenvironment, and suggest that both are important parameters for tissue engineering applications and a greater understanding of angiogenesis during disease progression.

## 1 Introduction

Angiogenesis is controlled by numerous chemical and mechanical signaling events that result in the formation of new capillaries. It requires normally quiescent endothelial cells to undergo changes in shape, proliferation, migration, and extracellular matrix (ECM) remodeling to form a new capillary network [1]. While

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significant attention has been paid to investigating the role of chemical events that initiate and affect angiogenesis, e.g. paracrine and autocrine growth factor signaling, the role of the mechanical environment in mediating angiogenesis is not as well described.

Endothelial cells (ECs) reside in a complex mechanical environment that consists of exogenous forces applied to cells, endogenous forces generated by cells, and the local stiffness of the extracellular matrix (ECM) [2]. Exogenous forces include the effects of hemodynamic forces due to blood flow (shear stress [3, 4], cyclic strain [5, 6] and pulsatile pressure [7]), and forces applied by other cell types (e.g. transmigrating leukocytes [8]) or tissues [9] as described in other chapters within this book. In addition to exogenous forces, the mechanical environment of angiogenesis also includes the endogenous forces generated by ECs against their ECM, more commonly referred to as cellular contractility and traction stresses.

In this chapter we will describe how the mechanical environment influences angiogenesis by exploring how matrix stiffness and cellular contractility mediates EC behaviors that enable the progression of angiogenesis. Specifically, we will discuss how matrix stiffness alters EC shape, contractility, proliferation, cell–cell interactions, and cell–matrix remodeling.

## 2 Endothelial Cell Shape, Contractility, and Growth

Cell-ECM interactions control EC behaviors such as adhesion, spreading, and growth that are crucial for the process of angiogenesis [10]. These responses are facilitated by actomyosin interactions that generate cellular contractility regulated in part by the Rho family of GTPases. In general, Rho-Kinase (ROCK) is activated by GTP-bound Rho and alters the activity of myosin light chain kinase, an effector of cell contractility. These interactions are sensitive to the stiffness of the extracellular environment and govern changes in EC spreading and growth.

### *2.1 Matrix Stiffness Alters Endothelial Cell Spreading and Contractility*

Endothelial cell spreading and shape is directed by the mechanical microenvironment. Endothelial cell spread area increases with increasing substrate stiffness [11, 12]. On compliant substrates, ECs adopt a spindle shaped bipolar morphology, while EC shape on stiffer substrates is more multipolar and isotropic [13]. Changes in EC shape are accompanied by alterations in the density of focal adhesions, clusters of integrins and adaptor proteins that anchor actin stress fibers to the

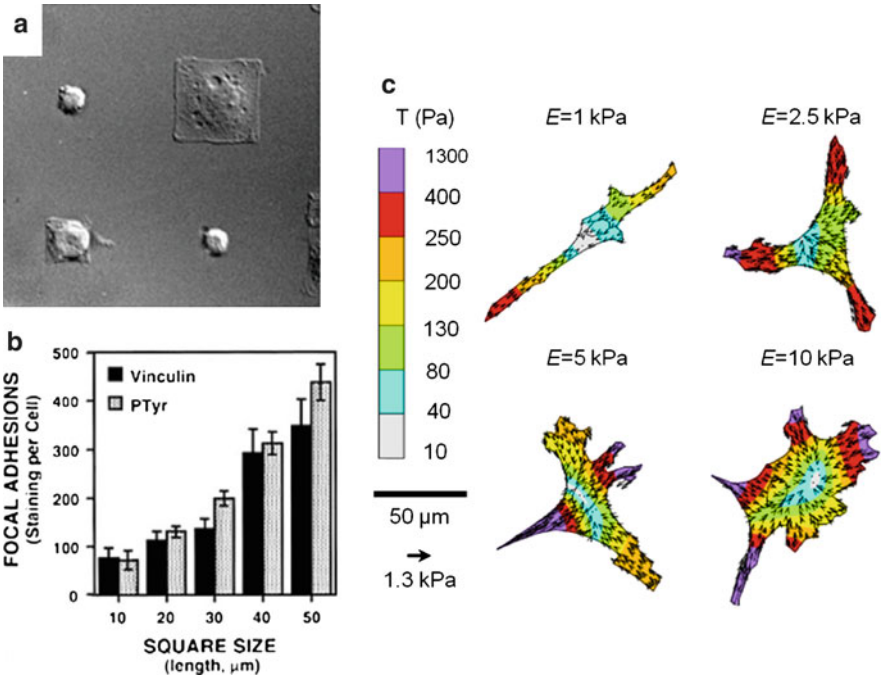


**Fig. 1** Changes in cell shape accompany alterations in focal adhesion formation and cell contractility. **a** Endothelial cells cultured on square micropatterned adhesive islands were used to determine the role of shape on focal adhesion formation (clockwise from top left, the square side lengths are 10, 40, 5, and 20 μm, respectively. **b** The number of vinculin- (a focal adhesion protein required for normal cell spreading [30]), and phosphotyrosine- (PTyr, a marker for tyrosine kinase signaling) positive focal adhesions increases with increasing spread size (from [15] with permission). **c** EC spreading and traction force generation increase with increasing substrate stiffness. Cells exhibit bipolar spreading on compliant ($E = 1$ kPa) matrices and increase spreading as stiffness is increased. The total magnitude of the traction stress $T$ also increases with increasing substrate stiffness (from [13] with permission)

plasma membrane [14]. When ECs spread, there is an increase in focal adhesion density that increases with the degree of cell spreading [15] (Fig. 1a, b).

Focal adhesion assembly is also sensitive to cell contractility and cell–cell interactions [15, 16]. Increased endothelial cell–cell contact decreases cell spreading and the size and number of focal adhesions [17]. However, Nelson et al. showed that when cells are patterned to be in contact, but changes in spreading are prevented, focal adhesion formation increases. These changes result from VE-cadherin-mediated increases in cytoskeletal tension (increasing RhoA activity) that increases focal adhesion formation. Similar to integrin-mediated contractility, cadherin-mediated increases in contractility are actin-dependent. Interestingly, cadherins have been shown to transmit traction forces [18, 19] that increase with increasing substrate stiffness, and require actin cytoskeletal assembly and myosin activity [20]. Furthermore, changes in Rho-mediated contractility influence

cell–cell interactions. Increasing RhoA-mediated myosin-generated contraction (e.g. treatment with thrombin) disrupts cell–cell contacts and adherens junction assembly [21]. This response is sensitive to the stiffness of the mechanical microenvironment. Increased matrix stiffness promotes disrupted VE-cadherin mediated cell–cell junctions and results in an increase in EC monolayer permeability [22]. These data are indicative of crosstalk and feedback mechanisms that exist between integrins and cadherins [23] that mediate cellular force balance, and suggest that matrix stiffness alters endothelial cell–cell and cell–matrix connectivity.

In addition to mediating changes in cell spreading, matrix stiffness may directly impact traction force generation. As substrate stiffness is increased, there is an increase in endothelial cell traction force generation [13] (Fig. 1c). Measurements of force generation and cell area coupled with regression modeling indicate that both area and matrix stiffness are significant predictors of EC traction forces in single cells and cells in contact [13]. While the total magnitude of the force exerted by ECs is linearly related to cell area during spreading events, cells are capable of exerting significant traction forces in the absence of notable focal adhesions or stress fiber formation [12], typical attributes of well-spread cells. These results indicate that matrix stiffness alters EC traction force generation, and suggest that ECs may utilize small nascent focal complexes to exert traction forces, a mechanism first described in fibroblasts [24].

Changes in matrix stiffness-mediated EC contractility associated with spreading or focal adhesion organization may be due to cell shape changes that physically alter the localization and activity of contractile adaptor proteins. During cell spreading after plating, the actin-binding protein filamin binds the Rho GTPase activating protein (GAP) p190 (p190RhoGAP) thus preventing its accumulation in membrane lipid rafts and allowing high Rho activity [25]. When cells are spread the protease calpain cleaves filamin allowing the accumulation of p190RhoGAP in lipid rafts that inhibit Rho activity. These data indicate that cell spreading alters the localization and activity of mediators of cellular contractility. They suggest that matrix stiffness alters cell shape and contractility via changes in protein localization; however, more work is needed to understand the crosstalk of intracellular proteins that participate in these mechanisms.

Notably, p190RhoGAP has been shown to regulate capillary formation in vitro and retinal angiogenesis in vivo by altering the transcription factors TFII-I and GATA2, regulators of VEGFR2 expression [26]. Moreover, GATA2 and VEGFR2 expression levels increase with increasing substrate stiffness. Interestingly, VEGF stimulates stress fiber and focal adhesion organization [27] through Rho and ROCK signaling [28]. More recent work indicates that VEGF induces a two-fold contractile response mediated by VEGFR2 and ROCK [29]. These data suggest that angiogenesis is mediated by EC sensitivity to growth factor signaling that is intimately associated with matrix stiffness and the contractile machinery of the cell.

## 2.2 Endothelial Cell Shape and Cytoskeletal Tension Regulate Proliferation

Endothelial cell growth is critical to angiogenesis, and shape has emerged as an important regulator of proliferation. Early experiments with ECs determined that increases in spread area due to increasing density of substrate-bound fibronectin are accompanied by an increase in cell proliferation [31]. The investigation of cell shape on the control of cell cycle progression indicated that ECs must remain spread for most of $G_1$ to enter S phase [32]. As ECs spread during $G_1$, increasing nuclear volume and DNA synthesis predict the proliferation response [33].

The use of micropatterned substrates that control cell spreading has identified cell shape as a switch between EC life and death [34, 35]. ECs grown on microadhesive islands less than 500 $\mu m^2$ undergo apoptosis, but on substrates greater than 1,500 $\mu m^2$ cells spread and grow [36]. When grown on small adhesive islands that prevent spreading, cells fail to progress from $G_1$ to S phase despite normal activation of the Erk1/2 MAPK pathway [37]. Failure to progress into S phase is attributed to an inability to increase cyclin D, reduce the cyclin-dependent kinase inhibitor $p27^{Kip1}$, and phosphorylate Rb in late $G_1$ (Fig. 2). Changes in EC shape and spreading that predict growth are tied to tension in the cytoskeleton, and cell cycle progression can be blocked by cytoskeletal disruption. Disruption of the actin filament network with cytochalasin D or microtubules with nocodazole leads to apoptosis similar to that seen in cells on small adhesive islands that restrict spreading [38]. Simultaneous disruption of actin and microtubules leads to increased rounding, dephosphorylation of the serine/threonine kinase Akt (also known as Protein Kinase B), decreased expression of Bcl-2, and increased caspase activity, hallmarks of apoptosis (Fig. 2).

Cytoskeletal tension and cell spreading have been identified as a late $G_1$ restriction point that acts independently of MAPK/Erk signaling to promote the $G_1$-S transition during growth [39]. When actin polymerization is disrupted with cytochalasin D, cyclin D expression is decreased, ECs accumulate $p27^{Kip1}$, and cell cycle progression is arrested. Separate work determined that when EC spreading is inhibited by using substrates with reduced ligand density, cells also accumulate $p27^{Kip1}$ and arrest in mid $G_1$ [40]. This response is rescued with constitutively active Rho that increases Skp2 expression (required for degradation of $p27^{Kip1}$ and progression through $G_1$) and promotes $G_1$ progression by altering the balance of mDia1 and ROCK, effectors of Rho that mediate cell contractility and actin cytoskeletal organization (Fig. 2). These findings indicate that EC cytoskeletal tension and shape are important cell cycle checkpoints that mediate cell cycle progression.

Cell–cell interactions also contribute to EC growth decisions by modulating cytoskeletal tension. VE-cadherin-mediated cell–cell contacts decrease cell spreading and proliferation, but if spreading is prevented by growing cells on micropatterned substrates, proliferation increases via Rho signaling and cytoskeletal tension [41]. Use of dominant negative RhoA can block cell–cell contact-mediated

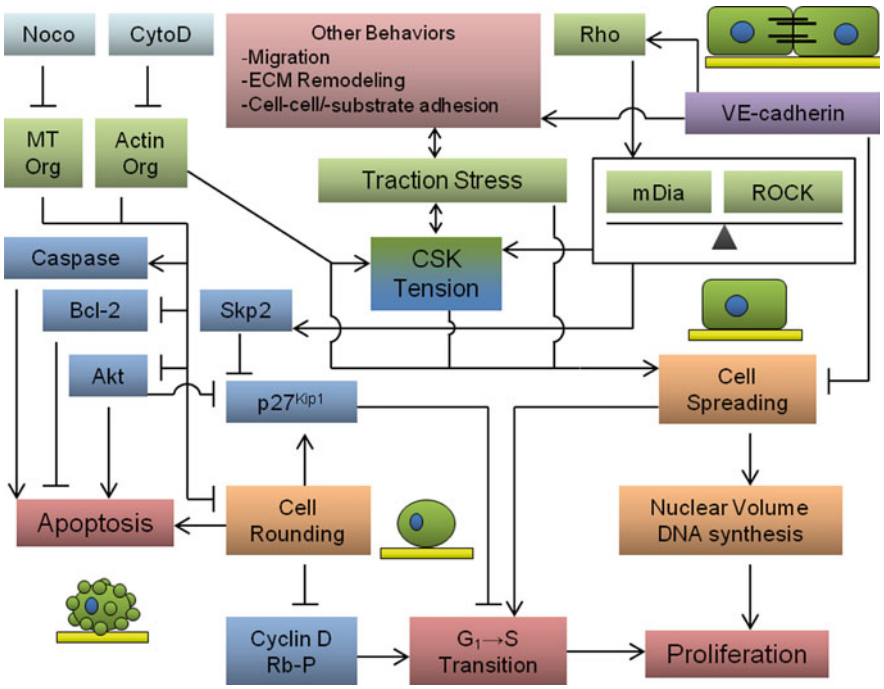


**Fig. 2** Signaling diagram depicting the interplay of cell shape and cytoskeletal tension on EC proliferation. Mediators of cell contractility (classical Rho-mediated elements) are shown in *green*, mediators of cell-cycle progression are shown in *blue*, and cell-cycle decisions are shown in *red*. *Noco* nocodazole; *CytoD* cytochalasin D; *MT Org* microtubule organization; *CSK* cytoskeletal; *Rb-P*, phosphorylated-Rb. See text for citations

increases in actin stress fiber formation indicating that RhoA is required for contact-mediated increases in proliferation [42]. Additionally, regions of EC proliferation have been shown to be correlated with high traction stresses [43]. Inhibiting acto-myosin tension or cadherin-mediated cell–cell interactions can alter the pattern of proliferation. These data indicate that cell–cell interactions influence cytoskeletal interactions that mediate EC proliferation.

## 3 Endothelial Cell Mechanosensing

In addition to mediating changes in cell spreading and growth, phenotypic changes important for the progression of angiogenesis, the mechanical environment also influences the mechanical properties of ECs. Sensitivity to the external mechanical environment is made possible by cellular machinery that converts mechanical signals into changes in cell stiffness [44] or gene expression [22].

### 3.1 Integrins and the Actin Cytoskeleton Mediate EC Mechanosensitivity

Early experiments uncovered a mechanical linkage between the nucleus and cell-surface receptors, whereby exerting exogenous force on integrins reoriented cytoskeletal filaments, distorted the nucleus, and redistributed nucleoli within ECs [45]. This work suggested that integrin engagement was related to processes governed by the cellular nuclear machinery, and in fact integrin binding directly activates the expression of the immediate-early genes c-fos, c-myc, and c-jun that regulate cell growth [46]. In addition, integrins ligated by RGD-coated magnetic beads and twisted with a magnetic stimulator increase endothelin-1 (a vasoconstrictor) gene expression, a response blocked by disrupting the actin cytoskeleton [47]. These findings suggest that mechanical forces, as sensed by integrins, may play an active role in mediating EC transcription-regulated responses governing angiogenic processes.

Force transmission throughout the cell is largely facilitated by the actin cytoskeleton. Experiments disrupting cell-substrate adhesion and inducing cell retraction indicate that the cytoskeleton is prestressed, and that the actin cytoskeleton is the primary stress-bearing member in ECs [48]. Cell stiffness increases when cells are rapidly strained suggesting time-dependent mechanical properties. Indeed actin stress fibers behave as viscoelastic cables tensed by myosin motors where actin cytoskeletal prestress is largely determined by myosin light chain kinase [49] (Fig. 3a). Furthermore, a separate study using AFM nanoindentation characterized the mechanical properties of individual stress fibers in living cells. It was found that actomyosin contractility regulates the mechanical properties of stress fibers [50]. Stress fiber stiffness can be altered by pharmacological perturbation of contractility (e.g. blebbistatin decreased stiffness while calyculin A increased stiffness). While the baseline mechanical properties of stress fibers are approximately linear, they become heterogeneous with increasing cellular contractility. These data suggest that the mechanical microenvironment of ECs alters the mechanical properties of stress fibers within the cell via actomyosin contractility.

ECs exhibit viscoelastic mechanical properties that are sensitive to changes in cell spreading. When grown on substrates of increasing fibronectin ligand density, ECs exhibit increases in spreading, cytoskeletal stiffness, and apparent viscosity, [51]. Separate studies using magnetic pulling cytometry determined how cells adapt to changes in mechanical forces applied at integrins [52]. Cells exhibit a four-phase response: immediate viscoelastic; early strengthening response to short pulse forces (both prevented by inhibiting Rho); robust strengthening after prolonged force; large-scale repositioning after prolonged force application. These responses are prevented by inhibiting Rho, blocking mechanosensitive ion channels, or inhibiting Src tyrosine kinase. Separate work indicates that treatment of ECs with VEGF increase reduces the elasticity of the cytoplasm, a responses that is dependent on ROCK [53]. These findings indicate that ECs have robust

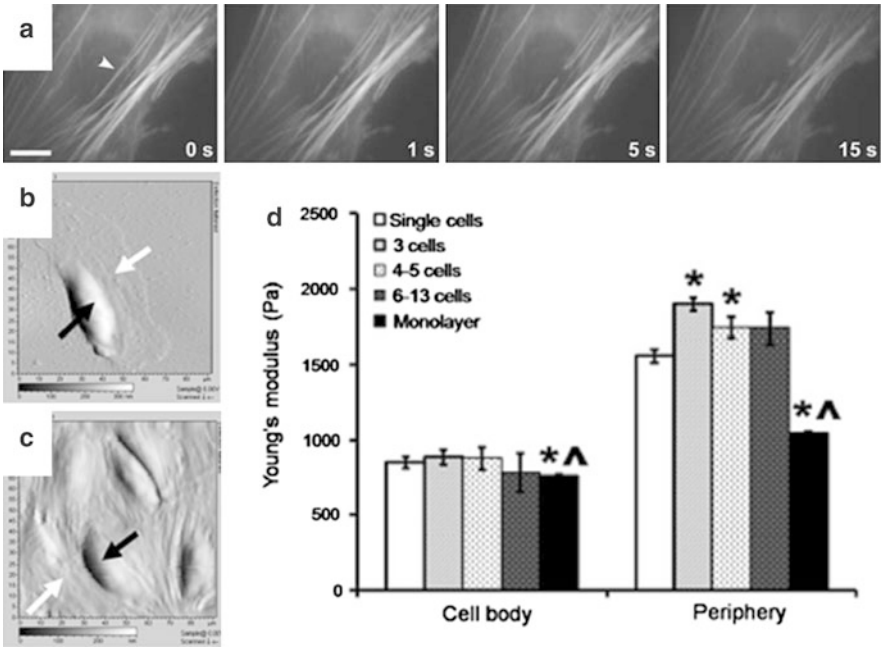


**Fig. 3** The mechanical properties of endothelial cells are regulated largely by the actin cytoskeleton. **a** After laser ablation, actin stress fiber bundles undergo viscoelastic retraction (arrowhead represents laser position, scale bar is 10 μm) (from [49] with permission). Cell stiffness is sensitive to the extent of cell–cell interaction. **b–d** Atomic force microscopy of single cells **b** to cells in a monolayer **c** indicated that peripheral forces are greater than cell body forces for all degrees of contact, and that the stiffness of a single cells is greater than that of cells in monolayer. Black arrows indicate cell body; white arrows indicate cell periphery; images **b–c** are 90 × 90 μm (from [54] with permission)

mechanisms that alter their intrinsic mechanical properties in response to the external mechanical environment.

Similar to other EC responses (e.g. proliferation), the mechanical properties of ECs are affected by cell–cell interactions. ECs in groups (3–5 cells) are larger and stiffer than single isolated cells [54] (Fig. 3b–d). Treatments that weaken cell–cell junctions (cytochalasin B, VE-cadherin blocking antibody) result in an increase in cell-substrate adhesion (increased focal adhesion size and density) and stiffness of cells in monolayers that is similar to single cells. These results indicate that cell–cell interactions play an important role in mediating the mechanics of ECs.

Changes in the mechanical properties and force balance in ECs may ultimately be required for normal cell mechanosensing and function. Importantly, aberrant Rho sensitivity is implicated in tumor vessel formation, where tumor-derived ECs lose the ability to respond to physical cues and form abnormal vessels [55]. These cells exhibit an increase in baseline Rho and ROCK activity, exert increased traction forces, exhibit an enhanced ability to form capillary networks in vitro, and do not reorient their cytoskeleton under external cyclic strain. Interestingly, ROCK

inhibition recovers the sensitivity of cytoskeletal organization to strain application and normalizes capillary network formation. These data indicate that the mediators of cell contractility involved in mechanosensing are an important switch between normal and abnormal function during angiogenesis.

Taken together, these data indicate that changes in the external environment (matrix stiffness or exogenous forces) may ultimately alter the intrinsic mechanical properties of ECs, and suggest that these changes in turn affect mechanosensing and cell function. This is of fundamental importance in understanding the relationship between the mechanical environment and angiogenesis, in both the design of suitable scaffolds for vascular tissue engineering constructs, and in disease states with changing mechanical environments (e.g. tumor angiogenesis).

## 4 Endothelial Cell Network Assembly

An important event during angiogenesis is the spatial reorganization of ECs into a capillary morphology. In 1980, Dr. Judah Folkman published seminal work describing the intrinsic ability of ECs to reorganize into a capillary-like phenotype in vitro [56]. Since then, the mechanical properties of the ECM have emerged as a critical regulator of the development of such capillary-like morphologies [57].

### *4.1 Matrix Stiffness Regulates Endothelial Cell–Cell Assembly and Sprouting*

In general, compliant ECMs support the formation of networks of ECs, a morphology reminiscent of the capillary beds seen in vivo [58] (Fig. 4a). Early experiments indicated that capillary tube formation occurs on compliant ECM or when cells retract and elevate above stiff culture dishes [59]. In 2D studies, EC network formation decreases as substrate stiffness is increased on fibrin gel [60] (Fig. 4b), collagen gel [58], or matrigel substrates [61, 62]. Similarly, capillary-like network formation in 3D decreases with increasing stiffness of fibrin [63] and collagen gels [64] (Fig. 4c). These studies indicate that ECs have a propensity to self-assembly in sufficiently compliant mechanical environments.

Work in our lab with variably compliant polyacrylamide gels has determined that compliant substrates ($E < 1$kPa) promote the spontaneous assembly of EC networks, and the propensity of endothelial cells to assemble into networks decreases with increasing matrix stiffness ($E = 2.5$–$10$ kPa) [11] (Fig. 4d). In contrast to previous work utilizing collagen or fibrin gels, experimental systems where gel stiffness is coupled to changes in protein concentration, the stiffness of polyacrylamide substrates is tuned independently of the protein ligand concentration available to adherent cells. This system allowed us to determine that

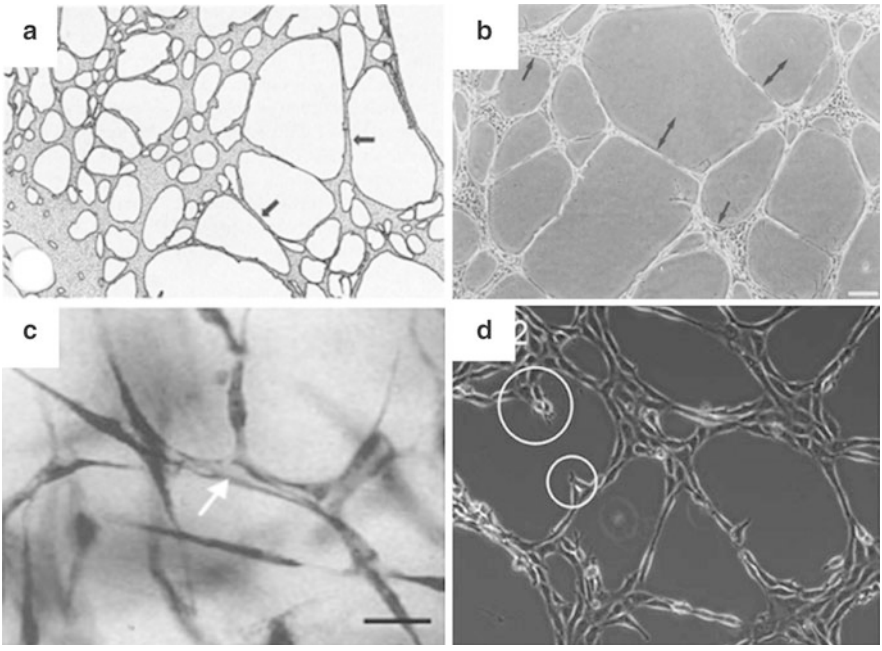


**Fig. 4** Examples of EC network assembly. **a** ECs in culture are capable of forming networks that mimic the morphology of capillaries in vivo (sketch from [58] with permission). ECs form networks on compliant fibrin gels **b** (from [60] with permission), within collagen gels **c** (from [64] with permission), or on polyacrylamide substrates **d** (from [11] with permission). Note the typical ring-like morphologies of ECs despite substrate material. *Double-headed arrows* in **b** denote tubular structures, scale bar is 250 μm; *arrow* in **c** points to a lumen-like region, scale bar is 25 μm; *circles* in **d** outline sprouting cells that will make an additional cell–cell connections

network assembly is modulated by substrate stiffness alone [11], independent from changes in ligand density. Interestingly, EC network assembly is induced on stiff matrices by reducing the concentration of the surface-bound ligand [11]. These findings suggest that EC network formation results from a balance of cell–cell and cell-matrix interactions in order to optimize mechanical input to the cell [65]. On compliant substrates where cell-substrate resistivity is reduced, ECs may seek out additional mechanical input from interactions with adjacent cells that result in the formation of networks. In contrast, stiff substrates may provide adequate mechanical input to cells thus reducing a propensity to seek cell–cell interaction.

As networks form, ECs sprout to form additional cell–cell connections. This response is also sensitive to matrix stiffness. Vascular sprouting of ECs embedded in 3D fibrin gels decreases as matrix density increases, a response that can be recovered in stiffer matrices by the addition of mesenchymal stem cells that promote matrix metalloproteinase (MMP) upregulation [66]. VEGF-induced sprouting in 3D is further regulated by matrix density and stiffness according to a Goldilocks principle: intermediate matrix is ideal for sprouting while low density

promotes uncoordinated migration and high density promotes lack of sprout elongation [67]. In addition, EC protrusions (lamelipodia, filopodia, microspikes) extend preferentially from regions of greatest traction forces [68]. These data indicate that matrix stiffness mediates a sprouting phenotype and suggest that cytoskeletal tension contributes to sprouting decisions in ECs.

## *4.2 Supporting Cell Types Enable EC Network Assembly in Stiff Matrices*

Angiogenesis in vivo is dependent on supporting cells [69], and co-culture models have begun to elucidate the mechanical role of support cells, such as fibroblasts or stem cells, in EC capillary formation.

ECs invade 3D fibrin gels but do not form capillaries unless they are co-cultured with mesenchymal stem cells (MSC) or fibroblasts [70]. Specifically, adipose-derived stem cells (ASC) promote angiogenesis via the plasmin axis of serine proteases, while bone marrow-derived stem cells utilize MMPs [70]. Promotion of EC angiogenesis by association with ASCs is similar to results found with fibroblasts that promote angiogenesis by exhibiting the angiogenic cytokines urokinase plasminogen activator, hepatocyte growth factor, and tumor necrosis factor alpha [71]. When ECs are cocultured with mesenchymal cells, the formation of impermeable vessels is greatly enhanced in vitro, [72]. Importantly, such prevascularized tissue constructs have been shown to accelerate anastomoses and promote tissue remodeling after implantation in vivo [73].

While these results suggest that EC angiogenesis in coculture is dependent on chemical signaling from supporting cells, evidence implicating the mechanical stiffness of the 3D construct as a mediator of angiogenesis is emerging. For example, when ECs are embedded in 3D fibrin gels with a monolayer of fibroblasts seeded on top of the gel, changes in ECM density alter matrix mechanics and EC traction force generation that influence capillary morphogenesis [74]. Increasing the density of fibrin gels inhibits capillary morphogenesis without the addition of such supporting cells [66]. This response is also established in vivo in SCID mice, where increasing fibrin matrix density inhibits vascular formation that can be partially recovered with the addition of MSCs [75]. While the role of the mechanical properties of 3D constructs on angiogenesis is still emerging, these findings suggest that the mechanical interplay between ECs and supporting cells is a critical regulator of angiogenesis in 3D.

## *4.3 Matrix Stiffness and Cell Contractility Alter EC Migration*

Inherent to cell–cell interactions during angiogenesis is the ability of ECs and vascular cells to migrate. It is well established that endogenous traction forces

drive cell motility [76]. While early experiments implicated roles for ligand density, integrin expression level, and ligand binding affinity as mediators of cell migration [77], substrate stiffness [78, 79], and strains created in compliant substrates [80, 81] also guide motility. Cell migration in response to a stiffness gradient has been termed durotaxis and has been demonstrated in ECs as well as vascular smooth muscle cells, fibroblasts, and stem cells [80, 82–84]. ECs are sensitive to strains created in compliant matrices by the traction stresses of neighboring cells, and this response is dependent on matrix stiffness [81]. On sufficiently compliant matrices, pairs of ECs exhibit hindered migration compared to single cells indicating that compliant matrices promote cell–cell interactions. On compliant substrates, the traction forces exerted by one cell may create a perceived stiffening in the substrate that allows cells to sense each other at a distance [81], and organize into multicellular networks [85]. Such network formation on compliant polyacrylamide substrates may be disrupted by growth factor (bFGF, VEGF)-induced motility [86]. These observations suggest that matrix stiffness-mediated EC network assembly results from tightly-regulated migratory behavior.

While more research is needed to fully understand the role of substrate mechanics in mediating EC migration during angiogenesis, the study of collective cell migration in epithelial cell sheets has uncovered some relationships that guide the interplay between cell contractility and global motility. Collective cell migration and traction force generation in Madin-Darby canine kidney cell (MDCK) epithelial sheets indicates that cell scatter correlates with adhesion strength and actomyosin-dependent contractility that transmits tension to the cell periphery [87]. Traction forces generated by MDCK monolayers are greatest at the edge of the monolayer and greater than forces measured in isolated cells [88], and may be generated in the bulk of the cell sheet away from the leading edge [89]. Furthermore, MDCK assembly is anisotropic along the stiffest substrate direction of anisotropic substrates and correlates with traction force and actin cytoskeletal orientation [90]. These data indicate that collective cell migration in epithelial cells is sensitive to matrix stiffness that alters cell contractility. Future work should identify the role of matrix stiffness in mediating collective cell migration in ECs.

## 5 Matrix Remodeling

During angiogenesis, new vessel formation is facilitated by the disruption of the basement membrane and the development of a provisional matrix containing proteins such as collagen and fibronectin [1]. While disruption of the basement membrane is usually attributed to the action of MMPs [91] (as discussed by Grainger and Putnam in this book), the contractile machinery of ECs is implicated in ECM remodeling [92].

### *5.1 Traction Forces Remodel Collagen and Fibronectin ECM*

Early experiments indicate that ECs in confluent monolayers generate tension that elevates webs of ECM above the cell monolayer that support spontaneous capillary network formation [59]. Similarly, ECs and smooth muscle cells form networks only after the appearance of organized basement membrane matrix [93]. Specifically, ECs synthesize and organize collagen into cables that promote spindle-shaped morphologies and network formation, a response that requires traction forces [58]. These data suggest that matrix remodeling is requisite for network assembly.

The fibronectin ECM may be particularly important for mediating angiogenesis. In 3D fibrin matrices, EC tubulogenesis requires the fibrillogenesis of a fibronectin matrix that promotes cytoskeletal organization and tension [94]. Fibronectin assembly increases intracellular stiffness that helps ECs match the stiffness of the surrounding matrix during vessel formation. Separate studies indicate that ECs form capillary-like structures with a central lumen atop tendrils of fibronectin [36]. In 2D, fibronectin polymerization is requisite for the maintenance of stable cell–cell interactions during network assembly [11]. Interestingly, fibronectin is assembled into fibrils by cells [95] through endogenous Rho-mediated contractility, and fibronectin fiber orientation is subsequently guided by traction forces [96]. These interactions are sensitive to cell–cell interactions. Cadherin-mediated cell–cell adhesions mediate cellular contractility that directs fibronectin fibril formation [97]. During *Xenopus* morphogenesis, cell–cell adhesions transfer tension to integrins that direct fibronectin fibril formation in the blastocoel roof. These data suggest that EC contractility, traction forces, and cell–cell interactions enable fibronectin ECM assembly that is requisite for network assembly during angiogenesis.

## 6 Conclusion

Matrix stiffness and cell contractility are important regulators of cell shape and growth, network assembly, EC mechanics, and ECM remodeling. While it is clear that the mechanical microenvironment mediates angiogenesis, much work remains to understand its ramifications. Future work should focus on 3D constructs that recapitulate the endothelial microenvironment. In addition to the role of matrix stiffness, the role of other mechanical cues (e.g. traction forces generated by supporting cells; exogenous forces such as shear stress) will better elucidate the complex interplay between the mechanical microenvironment and angiogenesis. The clarification of the role of the mechanical microenvironment on angiogenesis in vivo will better our understanding, and enable our control, of blood vessel formation.

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# Computational Modeling of Angiogenesis: Towards a Multi-Scale Understanding of Cell–Cell and Cell–Matrix Interactions

**Sonja E. M. Boas, Margriet M. Palm, Pieter Koolwijk and Roeland M. H. Merks**

**Abstract** Combined with in vitro and in vivo experiments, mathematical and computational modeling are key to unraveling how mechanical and chemical signaling by endothelial cells coordinates their organization into capillary-like tubes. While in vitro and in vivo experiments can unveil the effects of, for example, environmental changes or gene knockouts, computational models provide a way to formalize and understand the mechanisms underlying these observations. This chapter reviews recent computational approaches to model angiogenesis, and discusses the insights they provide into the mechanisms of angiogenesis. We introduce a new cell-based computational model of an in vitro assay of angiogenic sprouting from endothelial monolayers in fibrin matrices. Endothelial cells are modeled by the Cellular Potts Model, combined with continuum descriptions to model haptotaxis and proteolysis of the extracellular matrix. The computational model demonstrates how a variety of cellular structural

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properties and behaviors determine the dynamics of tube formation. We aim to extend this model to a multi-scale model in the sense that cells, extracellular matrix and cell-regulation are described at different levels of detail and feedback on each other. Finally we discuss how computational modeling, combined with in vitro and in vivo modeling steers experiments, and how it generates new experimental hypotheses and insights on the mechanics of angiogenesis.

## 1 Introduction

Blood vessel growth is essential during embryogenesis, but is also a prominent aspect of diseases such as cancer, rheumatoid arthritis and retinopathy. Angiogenesis research can benefit from computational models in three ways. Firstly, computational models help to gain an overview in this complex system by testing which components and interactions are minimally required. These components and interactions can then be examined to understand their function and predict their effects. Computational models are therefore not only useful to gain mechanistic understanding of angiogenesis, but also to find new therapeutic targets. Secondly, computational models can discriminate between and select from alternative hypotheses. Often, more than one hypothesis explains a biological observation, such as network formation from dispersed endothelial cells. Computational models can test the sufficiency of each hypotheses to reproduce the biological observations. Predictions that result from these models can be validated experimentally to support or reject the tested hypotheses. Thirdly, computational models can connect and combine knowledge on single proteins and mechanisms to examine angiogenesis as a system. Experimental research is often limited to a specific step or protein in angiogenesis and does not grasp how this part is integrated in the whole. Ultimately, computational models include processes at multiple scales, like extracellular matrix, cells, and cell-regulation simultaneously. Such multi-scale models are the next step in computational modeling to make the transition to angiogenesis in the body.

In the first section, computational models of network formation and sprouting are reviewed. These models address questions that have been raised by experimental observations and thereby give new insights in angiogenesis. It concludes by discussing the current state of multi-scale modeling. The next section gives a practical example of how computational models can be used in angiogenesis research and shows how systems biology, a continuous cooperation between computational and experimental biologists, drives development of computational models. To do so, we introduce a new computational model of sprouting, based on an experimental model of capillary-like tube formation by Koolwijk et al. [1]. Finally we will discuss which steps should be taken in angiogenesis research to further evolve computational modeling.

## 2 Computational Models of Angiogenesis

The first models of angiogenesis were continuum models that describe angiogenesis in terms of the spatial density of cells [2–5]. The main advantage of these models is that they can often be solved analytically, but they are often too abstract to mimic angiogenesis realistically. More complex techniques allow for a more detailed description of angiogenesis, which yields more realistic models. Such techniques include discrete methods such as particle based modeling that describe cells as point-like particles [6, 7] and cell-based models [8–10] that also explicitly model the cell shape and membrane. These discrete methods are often combined with continuum models, creating a hybrid model [11–14] in order to utilize the strength of both methods.

This section reviews computational models of angiogenic network formation and sprouting. Network formation involves the collective behavior of cells and the interaction of cells with their environment. Models of sprouting angiogenesis are used to describe angiogenesis induced by cells in hypoxic tissues, e.g., a tumor.

### *2.1 Network Formation*

During early vascular development endothelial cells join into a primitive vascular network. Vascular network formation can be mimicked in vitro by seeding endothelial cells on a suitable matrix containing nutrients and angiogenic factors; for example Fig. 1a shows endothelial cells seeded on Matrigel matrix forming a network-like pattern. The conditions in in vitro network formation experiments differ greatly from in vivo angiogenesis. Yet, specific cases of angiogenesis result in similar vascular networks such as angiogenesis in the yolk sac and retinal angiogenesis. In both cases the vasculature arises from a vascular plexus containing endothelial cells.

In vitro experiments showed that, after the network is formed, almost all matrix is located beneath the cells [15]. This led to the hypothesis that cells pull on the matrix, resulting in matrix accumulation below cell clusters. The pulling forces of the cells also cause the formation of tension lines, radiating from the clusters, in the surrounding matrix, along which cells migrate [3]. This model assumes that cells can exert traction on the matrix, which results in matrix deformation and heterogeneity of strain in the matrix. Cells preferentially move along the orientation of high stress. The model suggests that matrix remodeling suffices for network formation.

Namy and coworkers combine the effects of cell traction with haptotactic cell migration along matrix gradients [4] (Fig. 1b). They found an optimal cell density at which networks can be created, corresponding with experimental observations [16]. Similarly, a range of matrix stiffness, which is linked to the fibrin density of the experimental matrix, was tested. This model suggested that active cell migration may

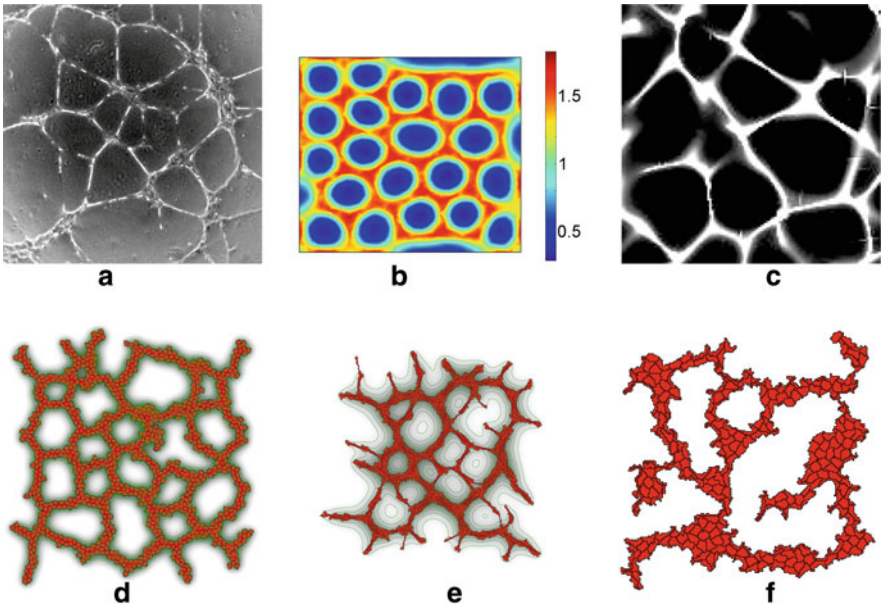


**Fig. 1** Overview of vascular network formation. **a** Shows a vasculature grown in vitro with HUVEC on Matrigel. **b** Illustrates the networks formed with the mechanical continuum model [4]. **c** Shows the outcome of the chemical continuum model [5]. **d** and **e** Show the networks formed with the chemical cell-based model, respectively with contact inhibition (**d**) [14] or cell elongation (**e**) [13]. **f** Illustrates the networks formed with the cell-based model with preferential attraction to elongated structures [9]. All images were reproduced with the publishers permission

be required for network formation which contradicts the observations by Manoussaki et al. [3].

Both previous models consider mechanical interactions between cells and the matrix to be the driving forces for network formation. Serini et al. [5, 17] proposed that chemotaxis is the driving force of network formation [5]. In the in vitro models cells move predominantly towards regions of high cell density suggesting that the cells are attracted by a chemoattractant secreted by the cells. Therefore, the computational model assumes that cells secrete a chemoattractant to which cells move preferentially. This model produces network-like patterns as shown in Fig. 1c. Two important predictions are made based on this model. First, the model predicts an optimal cell density for the formation of stable vascular networks and second, the size of the meshes in the network depend on the diffusivity and decay rate of the chemoattractant.

The mechanical and chemical hypotheses for vascular network formation have also been combined in one mechanochemical model [18]. This continuum model hypothesizes that network formation consists of two stages. First, cells move upwards chemical gradients. Second, at higher local cell density, the cells do not sense the gradient, but the high cell density signals them to start remodeling the matrix. This then attracts cells to the high density regions. The mechanochemical

model showed that the assumptions indeed lead to network formation and that chemotaxis drives the formation of networks while mechanical interaction stabilize the formed network. In this model matrix elasticity does not affect the properties of the networks. The mechanochemical model is not able to reproduce all observations from both chemical and mechanical angiogenesis models; a more detailed description of the matrix mechanics is required that also influences early cell migration.

Clearly, multiple hypotheses can be used to explain the experimentally observed network formation. Moreover, model observations and predictions for both the mechanical and the chemotaxis model could be reproduced in vitro [4, 5]. The mechanical models show that matrix thickness and stiffness may be determining factors in network formation, as has been show experimentally [15]. The chemical models reproduce the VEGF dependence that has been observed in vitro [5] as well as a characteristic length of the networks that depends on the diffusivity of the chemoattractant [19]. Both models only produce one similar prediction; there is an optimal cell density for network formation, below this density cells disconnect and above this density cells aggregate [16]. Therefore, it remains unclear whether the two mechanisms are involved in angiogenesis in different environments, or that the two mechanism act consecutive or simultaneously during angiogenesis.

**Cell-based models** The models discussed so far use a continuum description for both cells and mechanical or chemical fields, meaning that cells and fields are described as densities. This kind of description is appropriate for mechanical and chemical fields; for example, the concentration of a specific chemical can be measured at a specific position and can have any value. However, generalization of cells into cell densities ignores cell behavior, cell properties and cell–cell interactions, which are often key to morphogenic processes such as angiogenesis. Therefore, cells should be the basis of an angiogenesis model. Cell–based models incorporate detailed cell–cell interactions as well as cell properties such as cell shape and size, which can also be measured experimentally for quantification of the parameters and the predictions of the models [20]. Dynamic cell properties and behavior can be added by extending each cell with regulation networks, such as signaling or genetic pathways. Altogether, cell-based models are a solid basis for computational angiogenesis models that can be used to explain tissue effects at the cell level [21].

Different hypotheses have been implemented and compared using cell-based models. One of these models is a hybrid cell-based model, using the Cellular Potts Model (see also Sect. 3.1), which is based on the assumption that cells chemotact toward a chemoattractant that they themselves secrete [13, 14, 22, 23]. This assumption is similar to the assumption used for the continuum chemotaxis model [5]. In this cell-based model the cells' shape, size and membrane surface are described explicitly, and chemicals are described as continuous fields. One of the main advantages of this cell-based model is the more realistic chemotactic response of cells. This cell based model can be used to simulate network formation solely by defining cell behavior and properties. When only autocrine chemotaxis is

implemented, network formation only occurs for narrow parameter ranges: strongly adhering cells or steep chemical gradients [22, 23]. Therefore, Merks et al. proposed two hypotheses for which network formation occurred for a much wider range of parameters: contact inhibition [14] and cell elongation [13].

The contact inhibition hypothesis proposes that cells only respond to the autocrine chemoattractants where the cell membrane is not in contact with other cells. This exclusive sensing is thought to be mediated through the dual function of VE-cadherin; it acts as a homophilic trans-membrane cell-adhesion molecule and it plays an inhibitor role in the VEGF signaling pathway [24] which increases cell motility. Contact-inhibition locally reduces the cell motility. Therefore, cells within the cluster do not respond to the chemoattractant that all cells secrete. This process appears to contribute to both network formation (Fig. 1d) and sprouting angiogenesis. The reasons for this are best understood in the context of sprouting angiogenesis and will therefore be discussed in Sect 2.1.

The cell elongation hypothesis is based on the biological observation that cells elongate during network formation. In this model, the combination of elongated cells with autocrine chemotaxis results in network formation [13]. The final network, which can be observed in Fig. 1e, is similar to in vitro networks. When cell elongation is omitted, the cells aggregate instead of forming network, indicating that cell elongation drives network formation in this model. The evolution of network properties over time, such as the number of nodes and meshes, correspond with data from in vitro experiments with HUVECs on Matrigel. This suggests that cell elongation may play an important rule during network formation. In this model network formation occurs at two time-scales. First, cell elongation induces a persistent movement along the long axis of the cell. This causes the formation of thin branches of connected cells. Second, the network coarsens by fusion of branches and mesh collapse. This is driven by the chemotaxis that enables slow migration of cells along their short axis.

An alternative hypothesis was proposed by Szabó et al. [7, 9, 10]. Their experiments suggested that neither mechanical interactions nor chemotaxis are required for network formation [7] and that cells move preferential towards elongated cells. From these observation they propose that network formation is driven by the *preferential attraction to elongated structures*. This hypothesis has been used as a basis for both a particle based model [7] and a cell-based model [9, 10]. In the particle based model cells are represented by point particles that diffuse and adhere to their neighbors. While this model lacks some key cell properties, including cell shape, it suffices as a *proof-of-concept* model for preferential attraction to elongated structures. The models are used both to investigate network formation from dispersed cells [9] and sprouting from a blob of cells [10]. This model suggests that cells can indeed form network only due to cell-cell interactions, as is shown in Fig. 1f. Sprouts formed in these networks only become stable when they connect to other sprouts, suggesting that anastomosis stabilizes the formed network.

Because they all produce similar morphological patterns, none of the modeled hypotheses can be ruled out as a driving force for network formation. Cell-based

models [13, 14, 22, 23] suggest that autocrine chemotaxis, combined with cell properties such as contact inhibition of cell elongation, may drive angiogenesis. Other cell-based angiogenesis model [7, 9, 10] have suggested that autocrine chemotaxis may not be necessary at all. Moreover, mechanical interactions between cells and the matrix have not yet been modeled with a cell-based model. Adding this mechanical interaction to cell-based angiogenesis models will help to gain a true understanding of the mechanisms involved in angiogenesis

## *2.2 Sprouting*

Sprouting angiogenesis is the formation of new vessels by creating a sprout in the wall of the existing vessel. This form of angiogenesis is often observed in the vicinity of hypoxic tissue that secretes angiogenic factors, e.g., a growing tumor, which activate and attract endothelial cells from the existing vessels [25].

By stimulating the formation of a new vasculature, a tumor is able to grow and proliferate. The mechanisms underlying the dynamics of sprouting angiogenesis are still poorly understood. What mechanisms guide the growing sprout? How do biochemical and biomechanical interactions of the ECM with cells effect sprouting? Is proliferation required and where is proliferation located in the sprout? How are tip cells selected in the vessel and what causes sprouts to branch? Computational models have contributed to a better understanding of these issues.

In corneal angiogenesis sprouting is restricted in absence of proliferation; sprouts will not reach a tumor when cells are not able to divide [26]. A continuum model [2] describes the change in cell density over time due to cell migration driven by cell diffusion, chemotaxis and haptotaxis. The initial configuration of the simulation consists of a blood vessel at one side and a tumor at the other side of the simulation domain. This tumor secretes a chemoattractant, resulting in a gradient of chemoattractant that attracts cells towards the tumor. Haptotaxis is induced by fibronectin that the cells secrete themselves. The highest levels of fibronectin are present where the cell density is maximum. Therefore, haptotaxis and chemotaxis work in opposite directions. The continuum model suggests that, in absence of proliferation, the sprouting is restricted. The authors propose that this is caused because haptotaxis outweighs chemotaxis and increasing the number of cells would increase the chemotactic response.

A problem with this model is that it describes cells as a density field, hence it cannot describe how the sprout breaks up due to lack of proliferation. Therefore, a discrete modeling approach has been introduced to study cell proliferation in the sprout [8]. As illustrated in Fig. 2a the model mimics a cornea with a lesion in the center from which VEGF is secreted. A sprout grows from the periphery and consist of multiple cell types; one leading tip and multiple following stalk cells. The tip cell migrates towards the center induced by the VEGF gradient. Tip cell migration is limited by the elasticity of the tip cell and the strength of the adhesion between stalk cells. Adding proliferation enables unlimited sprout extension.

This model suggests that basic cell properties can explain the need for proliferation in sprouting.

The model by Szabó et al. [10] describes cell shape, cell membrane and cell migration in much more detail. The model does not consider chemotaxis or cell-matrix interactions. The cell properties and behavior that are specific for this model are preferential attraction to elongated structures, cell polarity and self-propulsion (i.e. persistence of motion). The model also differentiates between tip and stalk cells. The tip cell is polarized, causing directed movement in the direction of the polarization vector. The results shown in Fig. 2c suggest that both preferential attraction and self-propulsion are necessary to reproduce realistic sprouting behavior. Cell polarization may be regulated by cell–cell contacts and VE-cadherin may be a key player for this. Moreover, the model suggest that differential behavior at the tip of the sprout may drive sprout formation. Therefore, this model suggests that proliferation may not be required, as long as the supply of cells from the main vessel is sufficient.

**Cell-matrix interactions** The previous two cell-based sprouting models have only considered cell properties and cell behavior, ignoring all ECM and stromal tissue. Anderson and coworkers [6] created a particle based, hybrid model describing sprouting angiogenesis. In this model cells are represented as point particles on a grid while the chemotactic and haptotactic fields are still described as continuum equations. This model was used to investigate how the balance of haptotaxis and chemotaxis influences branching and anastomosis. As shown in Fig. 2b branching and anastomosis occur in the model, but these behaviors only occur when cells are able to move perpendicular to the chemotactic field, which is enabled by haptotaxis. When the haptotactic forces are strong enough branches can split and reconnect in order to form a functional vasculature.

Anderson et al. model [6] suggests that haptotaxis is key to branching, but it did not show how cells interact with their heterogeneous environment. A more recent, cell-based model, represents the ECM as a static, heterogeneous configuration of matrix fiber bundles, interstitial fluid and immobile tissue-specific cells [11]. The endothelial cells in the model are motile and adhere stronger to matrix fibers than to the surrounding matrix. Immobile cells act as obstacles that hinder the migration of endothelial cells. The tip cell is influenced by a chemoattractant field and it degrades ECM components. Degradation of the extracellular matrix during sprouting enables cells to migrate and branch off the main sprout as shown in Fig. 2d. The model suggests that a heterogeneous composition of the matrix is necessary for the formation of branches; the inhomogeneities in the matrix enable cells to split from the main branch. Furthermore, the model suggests that the proliferation region determines sprouting dynamics but does not affect the final sprout morphology.

A follow-up model was used to investigate cell-ECM interaction in more detail [27]. In this model all cells respond to the chemoattractant and that the immobile tissue cells are removed, ie, only fibers cause matrix heterogeneity. The model suggests that sprouting only occurs in a specific range of matrix densities, which corresponds with experimental observations. Moreover, simulation results suggest

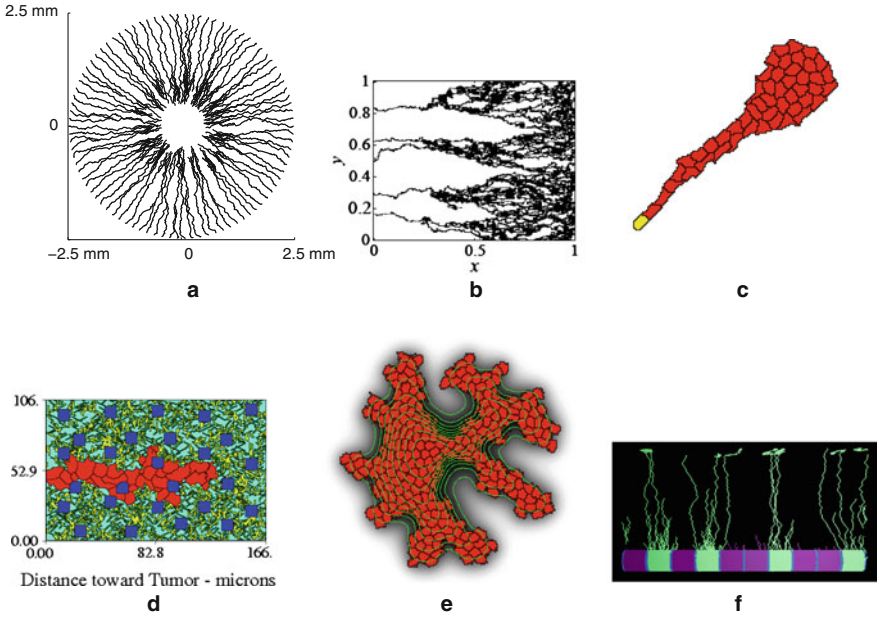


**Fig. 2** Overview of the computational models of angiogenic sprouting. **a** Shows corneal angiogenesis as modeled in the discrete model based on tip cell elasticity and stalk cell adhesion [8]. **b** Shows the networks formed with the discrete model with chemotaxis and fibrinectin induced haptotaxis [6]. **c** Shows sprouting induced by preferential attraction to elongated structures in a cell-based model [10]. **d** Shows the outcome of the cell-based model of sprouting angiogenesis in a heterogeneous ECM [11]. **e** Illustrates how contact inhibition induces sprouting in the cell-based chemotaxis model [14]. **f** Shows how tip cells form sprouts in the agent-based tip cell selection and sprouting model [12]. All images were reproduced with the publishers permission

that low fiber density results in cell elongation. Similar changes were observed when the random fibers were replaced by a specific fiber pattern, for example long fibers cause cells to elongate in the same direction as the fibers. The authors propose that contact guidance, due to cell-matrix interactions, is key to role in vascular sprouting because it enables sprout branching in an inhomogeneous matrix.

Another model suggests branching can occur in the absence of matrix heterogeneity [14]. The model assumes cells are attracted towards an autocrine chemoattractant, using similar rules as in chemotaxis-based network model (Sect. 2.1). Contact inhibition mediated by VE-cadherin causes cells to be only sensitive to the chemoattractant at positions of the cell membrane adjacent to the ECM. Sprouting occurs in two ways. First, when cells are arranged in an aggregate, only the outer layer of cells sense the chemoattractant. These cells tend to migrate towards the center of the aggregate causing a buckling instability that induces sprouting. This effect enables cells, even those with a low motility, to move against the chemotactic gradient. Second, another mechanism may explain sprouting for

highly motile cells. To move away from the mother vessel and form a sprout, cells must migrate *against* a steep gradient of self-secreted chemoattractant. Once a small sprout is created by a motile cell, the gradient around this outgrowth is less steep than the rest of the gradient, so cells within the sprout have higher motility than elsewhere, causing an instability.

Although most of the previous models simplified angiogenesis by assuming endothelial cells all have identical properties, in fact differentiation between leading "tip" cells and following "stalk" cells is key to sprouting angiogenesis. Bentley et al. [12] investigate the molecular and biophysical mechanisms driving tip and stalk cell differentiation using an agent-based, computational model [12]. The model represents a row of cylindrical endothelial tip and stalk cells made up of multiple agents. Only the tip cells can extend filopodia outwards, representing an new sprout as shown in Fig. 2f. The model is used to study the interaction between Dll4-Notch1-signaling with VEGF-induces tip cell activation [28]. The robustness of tip cell selection is investigated by applying a VEGF gradient perpendicular to the vessel [29]; each cell senses the same level of VEGF, which combined with a Dll4-Notch1-based lateral inhibition mechanism produces a pattern of alternating tip and stalk cells. The tip cells grow long filopodia that may meet up to form a connected vessel; anastomosis. When the common surface area of the connecting tip cells has increased sufficiently, one of the two cells becomes a stalk cells and the vessel stabilizes. Thus, the model suggests that the common surface area is a determining factor for tip cells selection; if the common surface area is too small lateral inhibition does not work. A second application of the model involved cellular competition for the tip cell position. Time-lapse microscopy has shown how stalk cells migrate along the sprout, take up the role of tip cell, and inhibit the original tip cell become a stalk cell [30]. Cell variants with higher levels of VEGFR2-expression have a competitive advantage over the wild-types: they end up more often at the tip of the sprout, but only if Notch1 can inhibit Dll4 expression. The advantage of the variants diminishes when all cells have low levels of Notch1. These observations suggest that Notch limits the levels of VEGFR2 in wild-type cells. Bentley tests this hypothesis in her agent-based angiogenesis model by applying a VEGF gradient along the sprout and by allowing cells to switch places. Switching is regulated the level of VEGFR2 and Notch expression; VEGFR2 promotes switches towards VEGF while Notch1 inhibits the same switches. With these assumptions the experimental observation could be reproduced, suggesting that this mechanism may explain tip cell shuffling.

## *2.3 The Future of Angiogenesis Modeling*

The models discussed so far, all isolated specific aspects of angiogenesis to predict the outcome of proposed in vitro experiments. To study angiogenesis in vivo, we must incorporate the interaction with the rest of the body in a multi-scale model.

Angiogenesis is induced by hypoxic tissue which, for example, can be a tumor or an active muscle. The change in oxygen and nutrient supply due to the new vasculature changes the signals coming from the tissue, resulting in a dynamic feedback loop between angiogenesis and the needs of the tissue. Also blood flow may be key to this feedback. Disfunctional vessels are not able to support blood flow and do not contribute to the perfusion of the tissue. Endothelial cells change their behavior due to the shear stresses induced by blood flow [31]. The inclusion of these processes in a multi-scale angiogenesis model would be a great tool to study whether pathological processes either involve excessive or insufficient blood vessel growth. Such multi-scale models can not only be used to formalize and validate hypotheses, they can also be used to predict the effects of pro- or anti-angiogenic therapies on the vasculature and the other tissues involved.

In order to build these multi-scale models, researchers often extend existing models. For example, the particle-based sprouting model by Anderson et al. [6] has been extended with blood flow [32]. This model suggested that most vessels are not perfused due to the lack of anastomosis, and thus drugs can not reach the target. More complex approaches have been used to combine more detailed angiogenesis models with blood flow and the kinetics of oxygen and VEGF [33–35]. This model has show to produce vascularization similar to experimental observation in a heterogeneous extracellular matrix [34] and in the skeletal muscle [36].

In the previous models the surroundings of the vasculature are static and are not being changed by the growing vessels and the increasing supply of oxygen and nutrients. This means that a part of the feedback is missing, for example a tumor can grow when the blood supply increases and a larger tumor needs a bigger supply of blood. Shirinifard et al. [37] combined cell-based Cellular Potts models (see Sect. 3.1) of blood vessel formation and tumor growth to investigate how tumor growth and vascular remodeling interact. This high level of detail gives insight in how specific cell properties influence tumor growth and angiogenesis.

Cell-based modeling would be a suitable approach to create predictive multi-scale models. Cell behavior in such a model must be linked to biological or physical cell properties. The extracellular matrix as well as blood flow could be added to the model. Then, the cell properties could be linked to matrix interactions and local levels of oxygen, nutrients and other chemicals. A cell-based model could simulate emergent angiogenesis and blood vessel remodeling and could be used to predict the effects of therapeutic agents.

## 3 Cell-Based Model of In Vitro Sprouting

The previous section discussed how multi-level computational models aim to fuse models to incorporate different aspects of angiogenesis, such as cell behavior, matrix interactions and blood flow. Processes like chemotaxis and haptotaxis can be described with continuum models, while we argued that the representation of cells requires a cell-based approach. Cell-based models explicitly model cell

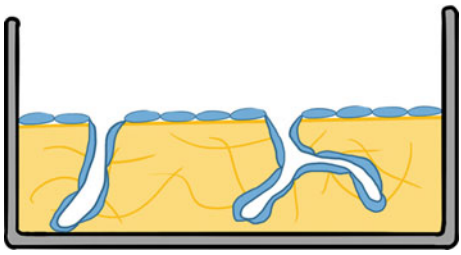

**Fig. 3** A schematic cross-section of the experimental model by Koolwijk et al. [1]. Endothelial cells are seeded on a three-dimensional fibrin matrix and form capillary-like tubular structures upon stimulation

structures (e.g. cell membranes) and cell behaviors (e.g. cell-cell adhesion and pseudopod extensions). These models are intuitive and relate well with biological observations. To illustrate the approach, we created a computational cell-based model of an in vitro model of capillary-like tube formation, introduced by Koolwijk et al. [1].

A schematic representation of a cross-section of the experimental model is given in Fig. 3. A monolayer of isolated human microvascular endothelial cells (hMVEC) is seeded on a three-dimensional fibrin matrix. The composition of this matrix, consisting of only fibrin (Fig. 7), is completely controlled and reproducible. The endothelial cells grow into the matrix and form capillary-like tubular structures upon stimulation with an angiogenic factor, VEGF and/or bFGF (basic fibroblast growth factor), in combination with the inflammatory mediator TNFα (tumor-necrosis factor alpha). TNFα is suggested to induce receptor-bound u-PA (urokinase-type plasminogen activator) activity to enable the cells to degrade the fibrin matrix [38]. Although VEGF and bFGF are both growth factors, TNFα completely inhibited the growth factor-induced proliferation in this experimental model.

The computational model of sprouting is designed to be easy to relate to the experimental observations. The model can be used to explain and predict angiogenic patterning on tissue level, based on quantitative descriptions of cell behavior. The most important cell behaviors, such as cell shape, cell adhesion and haptotaxis are therefore included, and their effects and relative importance in sprouting can be examined. Endothelial cells have a wide range of interactions with the extracellular matrix, of which the function as well the effects are often still unclear. Endothelial cells for instance secrete proteolytic enzymes to degrade the matrix. The proteolytic activity is regulated by a complex system and the computational model can help to gain insight in the relative importance of the components in this system, in the regulation of the system and in the effect of this regulation on sprouting.

## *3.1 Computational Sprouting Model*

To mimic the experimental set-up of Koolwijk et al., the computational model starts with a monolayer of cells, of which one is a tip cell and the rest are stalk cells, on a fibrin matrix and a basement membrane (BM) in between. The Cellular

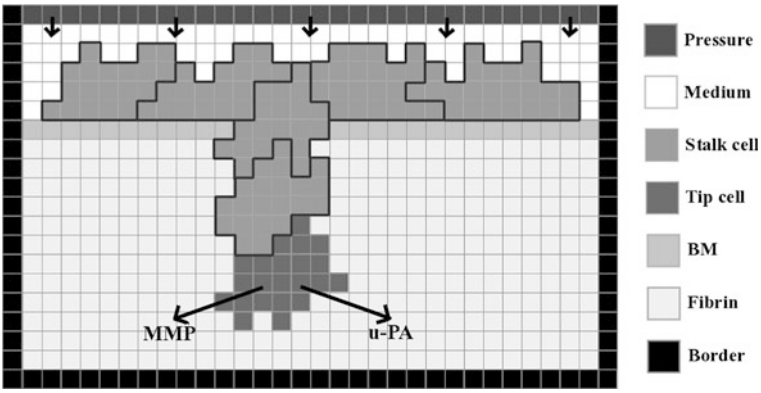


**Fig. 4** Schematic representation of the cellular potts model. The different colors depict the types defined in the sprouting model. The tip cell secretes MMP and u-PA (*large arrows*) to degrade the extracellular matrix and the small arrows on top represent a the small chemorepulsive pressure on the cells

Potts model [39, 40] describes the shape, behavior and movement of the cells. The tip cell will secrete proteolytic enzymes, matrix metalloproteinases (MMPs) and u-PA, to degrade the BM and fibrin respectively. The endothelial cells are attracted to higher concentrations of fibrin, which is also modeled as a concentration field, and migrate into the created space to form a sprout. On top, a local chemorepulsive field exerts a small pressure on the endothelial cells in the monolayer to keep them flattened and well connected. A schematic overview of the model is given in Fig. 4.

The Cellular Potts model represents cells, BM and fibrin as patches of grid sites (Fig. 4), which are differentiated by types $(\tau)$ with specific properties and behaviors. Cells can migrate by the addition and removal of grid sites at their cell membranes, which can be seen as extensions or retractions of pseudopodia. A grid site $(\mathbf{x})$ is added or removed by copying the contents of a neighboring grid site $(\mathbf{x}')$ (Fig. 5). Whether such a copy is allowed depends on an energy function, which summarizes the balance of forces resulting from cell behavior: $\mathrm{E} = \mathrm{E}_{\mathrm{contact}} + \mathrm{E}_{\mathrm{shape}} + \mathrm{E}_{\mathrm{connectivity}}$. The change in this local energy ($\Delta$E) for a certain copy is a measure for its favorability. Energy decreasing copies will always be accepted; while a copy that increases energy will be accepted according to a Boltzmann probability function: $\mathrm{P}_{\mathrm{accept}}(\Delta\mathrm{E}) = e^{\frac{-\Delta E}{\mu}}$. The ability to accept copies that cost energy resembles active cell motility, with the parameter $\mu$ a cell motility parameter.

Adhesion or repulsion by cells is modeled by contact energy between types $(J_{\tau 1,\tau 2})$: lower energies resemble stronger adhesions between types. The model is surrounded by a border (Fig. 4) which has high contact energy with cells to prevent them from sticking to the edges. The overall contact energy sums the contact energies between cells and between cells and their surrounding:

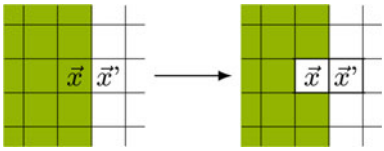


**Fig. 5** Graphical representation of a copy attempt. A random grid site ($\mathbf{x}$) is chosen to copy the state of a neighboring grid site ($\mathbf{x}'$) to simulate pseudopod extensions and retractions

$$\mathrm{E}_{\mathrm{contact}} = \sum_{(\mathrm{x},\mathrm{x}')} J_{\tau(x),\tau(\mathbf{x}')},$$

with $(\mathbf{x}, \mathbf{x}')$ a pair of adjacent grid sites at the cell membrane. Migrating cells have a typical shape, depending on cell size (area) and membrane surface (perimeter). Therefore, the energy increases with the deviation of a target value. $\lambda_{\mathrm{area}}$ and $\lambda_{\mathrm{perimeter}}$ indicate the weights of the constraints.

$$\mathrm{E}_{\mathrm{shape}} = \sum_{c\in\mathrm{cells}} \lambda_{\mathrm{area}}(\tau_c)\big(A(c) - A_{\mathrm{target}}(\tau_c)\big)^2 + \lambda_{\mathrm{perimeter}}(\tau_c)\big(P(c) - P_{\mathrm{target}}(\tau_c)\big)^2$$

Biological cells do not break up, thus all pixels of one cell must be connected. Therefore, a large penalty energy ($\mathrm{E}_{\mathrm{connectivity}}$; see Table 1) is added to the energy function when a tip or stalk cell has lost its connectivity [41].

The tip and stalk cell are surrounded by extracellular matrix, that is fibrin or BM. Unlike the migrating cells, fibrin and BM are immobile types. Endothelial cells can preferentially migrate towards higher concentrations of adhesion sites in the extracellular matrix, this process is called haptotaxis. In order to model this, fibrin and BM are also modeled as static homogeneous concentration fields that attract tip and stalk cells. The tip cell secretes u-PA and MMP to locally degrade the concentration fields. The probability that the types 'fibrin' and 'BM' are degraded depends on their local field concentrations by a Hill equation: $\mathrm{P}_{\mathrm{degradation\ of\ X}} = \frac{[X]^n}{[k]^n+[X]^n}$. The concentrations of the proteolytic enzymes are described by the following partial differential equation: $\frac{\delta c}{\delta t} = D\nabla^2 c + kc + s_\tau$, where $D$ refers to the diffusion constant, $k$ to the decay constant and $s$ to the secretion of the enzyme $c$ by type $\tau$. The local degradation of the haptotactic fields by the secreted proteolytic enzymes results in concentration gradients, a cell will preferentially extend up the gradient [42]. The effect of haptotaxis is calculated for every copy attempt, implemented as:

$$\Delta\mathrm{E} = \mathrm{E}_{\mathrm{new}} - \mathrm{E}_{\mathrm{old}} - \Delta\mathrm{E}_{\mathrm{haptotaxis}} \quad \text{where } \Delta\mathrm{E}_{\mathrm{haptotaxis}} = \lambda_{\mathrm{haptotaxis}}\big(c(\mathbf{x}') - \mathbf{c}(\mathbf{x})\big),$$

with $c$ the concentration of the attracting component and $\lambda_{\mathrm{haptotaxis}}$ describes the weight of the constraint.

**Table 1** Default parameters for the sprouting model

| Property | Value |
|---|---|
| Contact energy | $J_{\text{stalk,medium}} = 1, J_{\text{stalk,tip}} = 1, J_{\text{tip,BM}} = 100$ |
| | $J_{\text{stalk,border}} = 10^7$, $J_{\text{stalk,pressure}} = 10^7, J_{\text{tip,border}} = 10^7$, $J_{\text{tip,pressure}} = 10^7$ |
| Area | $A_{\text{target}}$, (tip, stalk) = 50, $\lambda$(tip,stalk) = 70 |
| Perimeter | $P_{\text{target}}$, (tip, stalk) = 30, $\lambda$(tip) = 25, $\lambda$(stalk) = 5 |
| Haptotaxis | $\lambda$(tip, stalk) = 1000 |
| Chemotactic pressure | $\lambda$(tip, stalk) = −0.1 |
| Pressure field | $D = 10^{-2}$, $k = 0.01$, $s = 1,000$ |
| MMP and u-PA field | $D = 10^{-6}$, $k = 0.01$, $s = 500$ |
| Fibrin degradation | Type: $n$ = 4, $k$ = 1.9, initial concentration = 2 |
| | Field: $-10^{-7} \cdot$ [u-PA][fibrin] |
| BM degradation | type: $n$ = 3, $k$ = 1, initial concentration = 2 |
| | field: $-5 * 10^{-8} \cdot$ [MMP][BM] |

## 3.2 Integration of the Experimental and Computational Model

In Sect. 3.1, we showed how a conceptual model of tube formation translates to a computational model. Basic cell behaviors and properties are included in the computational and the default parameters can be found in Table 1. First, the model is used to test a hypothesis concerning the relation of proteolytic enzyme secretion and sprout morphology. Subsequently, the model will be extended, mainly focusing on matrix interactions, to differentiate between forces that drive migration. With these examples, we emphasize how computational and experimental biologists can benefit from each other's models in their quest to understand angiogenesis.

### 3.2.1 Matrix Degradation and Sprout Morphology

To induce sprouting experimentally, endothelial cells are stimulated with an angiogenic factor, VEGF and/or bFGF, in combination with the inflammatory mediator TNFα [1, 43]. Both the angiogenic factors as well as TNFα induce proteolytic enzyme activity. The growth factors are suggested to stimulate secretion of a soluble form of plasminogen activators, t-PA (tissue plasminogen activator), which becomes active upon contact with fibrin and degrades fibrin in a diffuse manner. TNFα is suggested to induce u-PA (urokinase-type plasminogen activator) production and thereby stimulate receptor-bound u-PA activity [24]. u-PA is inactive until it is bound to its membrane-bound receptor, which localizes proteolytic activity to the membranes of the receptor expressing cells. Besides u-PA, TNFα can also induce the plasminogen activator inhibitor, PAI-1, which inhibits u-PA and t-PA. Proteolytic activity during sprouting is closely regulated by endothelial cells and results from a balance between the proteolytic enzymes and their inhibitors. Endothelial cells secrete MMPs (matrix metalloproteinases) to

degrade the basement membrane. The production and activity of u-PA and MMPs is interlinked; u-PA activity is suggested to induce MMP activity indirectly and at least one membrane-bound MMP (MT1-MMP) is known to be capable of fibrin degradation as well [44].

Proteolytic activity can be manipulated by stimulation of the cells with angiogenic factors or inflammatory factors [43]. To induce sprout formation, low amounts of TNFα are added to the monolayer of endothelial cells in combination with a growth factor. Stimulation with angiogenic factors alone induces a uniform degradation of fibrin and the monolayer of endothelial cells does not sprout but as a result lowers as a whole. This uniform degradation of fibrin can result from a combination of diffuse proteolysis induced by the angiogenic factors and the absence of inflammatory factor induced inhibitors of proteolysis. Excessive plasminogen activation, in endothelial cells seeded in suspension into a three-dimensional fibrin matrix, results in the formation of round cyst-like structures [45]. Cyst-like structures are also observed in the endothelial monolayer model [1] after stimulation with a higher dose of TNFα in combination with angiogenic growth factors by Koolwijk et al. (data not shown).

We aim to find the conditions that suffice to explain the experimental observations on sprout morphology. This hypothesis will then be tested with the computational model. We hypothesize that the intensity of proteolytic enzyme secretion (u-PA and MMP) as well as the distribution of secretion over different cell types is responsible for the observed phenotypes in angiogenesis. After low stimulation with TNFα, tip cells might already secrete proteolytic enzymes at maximal rate and are therefore insensitive to stimulation. In contrast, stalk cells normally do not secrete many proteolytic enzymes and are therefore more sensitive for stimulation than tip cells. Indeed, the tip cell was seen to have the most u-PA receptors during sprouting, which facilitate proteolysis to degrade fibrin [46]. When both tip and stalk cells secrete high levels of proteolytic enzymes, the fibrin will be degraded uniformly.

To test this hypothesis in the computational model, we assumed that both tip and stalk cells can secrete u-PA and MMP. As discussed above, proteolysis is a complex system that involves membrane-bound and soluble proteolytic enzymes as well as inhibitors. For simplicity, only membrane associated proteolytic activity and no inhibitors are considered in this first attempt. Since inhibitors are not modeled explicitly, proteolytic activity and secretion of proteolytic enzymes are directly coupled. The presence of inhibitor is modeled by lower secretion rates of the proteolytic enzyme. To model proteolytic activity at the membrane, the diffusion constants for the proteolytic enzymes are set to a very low value (Table 1). Secretion of the proteolytic enzymes could also be thought of as expression of the receptors that facilitate the activity of the enzymes at the membrane. Soluble proteolytic enzymes are not modeled, although high secretion of proteolytic enzymes does result in proteolytic activity at a larger distance. MMPs are assumed to exclusively degrade the basement membrane, while u-PA degrades the fibrin matrix. Mitosis of stalk cells is also included in the model to supply the monolayer with new cells, simply by dividing a cell over its short axis when it has increased

by an arbitrary factor of 1.28. The secretion of the proteolytic enzymes by the tip cell is kept constant at a maximal rate.

We study how u-PA and MMP secretion by stalk cells affects sprout morphology. Figure 6 gives an overview of the simulation results as a function of the secretion rates of u-PA and MMP. Because the simulations are stochastic, variation is seen between simulations with the same parameter settings; representative simulations were selected for the morphospace in Fig. 6. Sprout morphology can be grouped in four categories: sprouts, solid round cysts, hollow cysts and monolayers. Sprouts have a cord-like orientation of cells, while cysts are more round and multi-cellular. A high secretion of both u-PA and MMP (Fig. 6d) by stalk cells results in lowering of the monolayer. Sprouts are formed for low secretion of u-PA (Fig. 6a), while solid cyst-like structures are formed for medium levels of u-PA secretion (Fig. 6b) for all MMP secretion levels by stalk cells. High secretion of u-PA and low secretion of MMP (Fig. 6c) results in hollow-cyst like structures. Occasionally (6 out of 128 simulations), no sprouting occurs for low levels of MMP secretion by stalk cells because stalk cells position themselves between the tip cell and the BM and thereby prevent degradation of the basement membrane. An interesting transition is seen between a hollow cyst-like structure and monolayer lowering for a secretion of MMP between 8 and 12 %. The hollow cyst-like structures can be formed since the BM remains intact for attachment of endothelial cells before this transition. These structures are likely to collapse if gravity was included in the model. Experimentally, tubular structures can also disappear due to excessive fibrinolysis [43].

u-PA can activate MMP, thus the activity of both enzymes are likely to increase simultaneously. The sprout morphologies that are seen along diagonals of the morphospace in Fig. 6 are therefore biologically most probable. Along the diagonal, we see sprout formation for low secretion of both enzymes by stalk cells, cyst formation for medium secretion and monolayer lowering for high secretion. This is consistent with the experimental results by Collen et al. [43] and our own hypothesis as discussed above. The intensity and the distribution of proteolytic enzyme secretion over different cell types (tip and stalk cells) thus seems to be a sufficient explanation for the tissue behavior observed in the laboratory.

The computational model is oversimplified, because it only describes proteolytic activity at the membrane and no diffuse matrix degradation or inhibitors. The notion that TNFα induces secretion of proteolytic enzymes as well as their inhibitors implicates a more complex regulation of matrix degradation during sprouting. Lowering of the endothelial monolayer, when exclusively stimulated with angiogenic factors, is likely to depend on soluble rather than membrane-bound proteolytic enzymes. Inhibitors and soluble proteolytic enzymes should be included to understand the basic principles of angiogenesis.

Beside proteolytic degradation of the extracellular matrix, it is likely that the structure of the matrix influences tube formation. Matrix structure for example effects the sensitivity of fibrin matrices to proteolytic degradation [43]. The composition of fibrin matrices can be controlled experimentally [47]. High molecular weight (HMW) and low molecular weight (LMW) fibrinogen can be

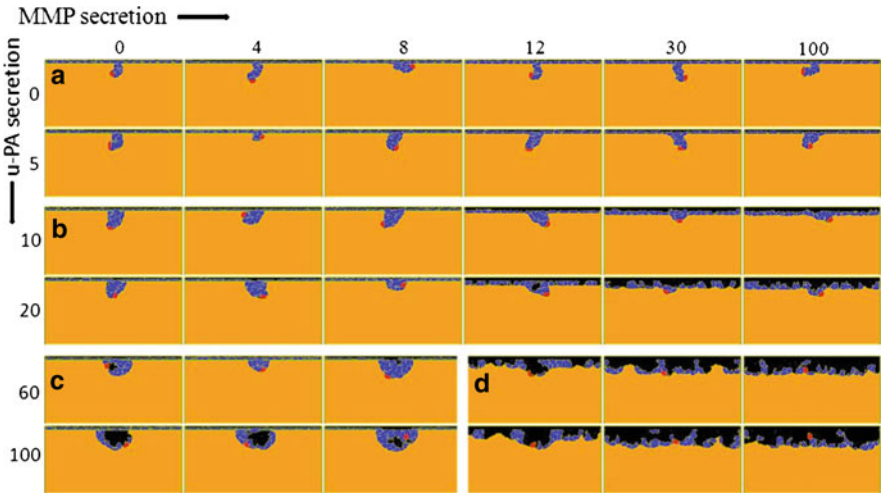


**Fig. 6** Morphospace of proteolytic enzyme secretion by stalk cells. Tip and stalk cells secrete u-PA and MMP to degrade fibrin and the basement membrane respectively. The secretion of u-PA and MMP by stalk cells is expressed in percentage of the maximal secretion rate as secreted by the tip cell. This results in different sprout morphologies: sprouts (**a**), solid cyst-like structures (**b**), hollow cyst-like structures (**c**) and monolayers (**d**)

isolated from unfractionated fibrin and used to create different densities of the matrix. A matrix composed of only LMW fibrinogen has thinner fibers and has a denser structure than a matrix made from only HMW fibrinogen (Fig. 7). Small differences in fibrin matrix lead to different gene expression patterns in endothelial cells [44] and influence tube formation [47]. Tube formation was more extensive in HMW than in LMW matrices. We plan to use the computational model to examine how these differences in matrix structure influence sprouting.

### *3.3 Modeling Perspectives*

Koolwijk et al. developed an in vitro model to examine tube formation in angiogenesis [1]. To gain knowledge in the mechanism and key players involved, we created a computational model that resembles the experimental in vitro model. The computational model is used to validate conceptual models for basic mechanisms of sprouting and to predict how alterations in cell behavior will affect sprouting. The model explains experimental observations concerning the effects of proteolytic enzyme activity on sprout morphology by supporting the hypothesis that not only the level, but also the distribution of proteolytic enzyme secretion over tip and stalk cells is responsible for different sprout morphologies. Several experimental observations and techniques can help to validate conclusions drawn from the model. Contradictions between experimental data and the computational

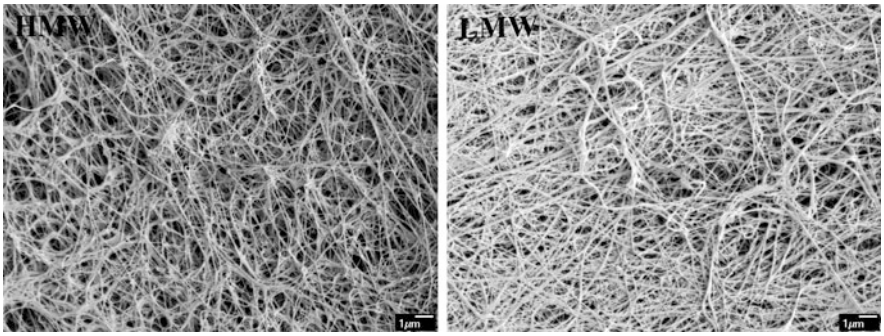


**Fig. 7** Fibrin matrices. Scanning electron microscopic analysis of fibrin networks. High molecular weight (*HMW*) fibrinogen is thicker and forms more open network structures (*left figure*) than low molecular weight (*LMW*) fibrinogen (*right figure*). *Bars* represent 1 μm

predictions point out gaps in our understanding. A clear focus point for improvement is the structure of the extracellular matrix and the interactions of cells with the matrix.

We could model the extracellular matrix in detail. Endothelial cells strongly interact with the matrix, mechanically as well as chemically. Cells can adhere to the fibers in the matrix and rearrange the fibers. By modeling the actual fibers and their alignment, physical obstruction of the matrix, haptotaxis and directional guidance of migration can be considered. Endothelial cells can also degrade the matrix with a sensitive and complicated system of proteolytic enzymes. Proteolysis of the matrix locally releases chemotactic components that stimulate sprouting. Each of these mechanical, haptotactical and chemotactical forces resulting from the surrounding tissue influence angiogenesis and are therefore important to understand.

Besides modeling the extracellular matrix more intensively, the interactions within the cell should be focused on. Endothelial cells adhere to the extracellular matrix with integrins, which are also linked to the cytoskeleton of the cell. By modeling cytoskeleton remodeling and integrin-mediated binding at a molecular level, we can study mechanical matrix interaction. Additionally, tip cell selection is an interesting molecular interaction within and between cells to include in the model. Tip cell selection depends on Delta-Notch signaling [28] and is required for experimentally observed branching of tubes.

In conclusion, we aim to create a multi-level model of angiogenesis that includes the molecular, cellular and tissue level. Each level should be modeled simple and intuitively and the interaction between the levels must be taken in close consideration. To understand which components should be modeled and how they interact, a continuous feedback between experimental and computational modelers is needed.

## 4 Conclusion

In this chapter, we have reviewed how computational models shed new light on questions involving network formation and sprouting. Firstly, we discussed how computational models are used to test alternative hypotheses on the mechanisms that drive network formation. Cellular and environmental factors in these models are studied to predict their effects on angiogenesis. A comparison of experimental results and these computational predictions can show which mechanisms are most likely the driving forces of network formation. Secondly, we focused on computational models that provide new insights in the mechanisms of sprouting. These models address questions about the regulation of sprouting, such as the necessity and location of proliferation, dynamics of tip cell selection, and the influence of angiogenic factors and the extracellular matrix. The discussed models use different techniques to model cells: continuum models describe cells as densities, while discrete models represent them as particles. The functioning of endothelial cells depends on thousands of interacting proteins and genes. Cell-based models are discrete models that represent the results of these gene and protein interactions by a set of cell properties (e.g. cell and membrane size) and behaviors (e.g. adhesion and chemotaxis), suggesting that a few cell behaviors sometimes suffice to explain complex collective cell behaviors like angiogenesis [21].

To illustrate this approach in more detail, we discuss a cell-based model to study angiogenic sprouting. This computational model is based on an in vitro model of sprouting in a fibrin matrix by Koolwijk et al. [1]. The model is used to formalize the mechanisms that are minimally required for sprouting and it predicts the effects on the dynamics of tube formation of varying cell properties, such as matrix degradation. Predictions from the model can lead to new insights and drive experimental research. The observations and results from the experimental research are crucial for the validation and further development of the computational model. A focus point for further study is the interaction of endothelial cells with the extracellular matrix. Various interactions with the matrix strongly influences sprouting, but it is difficult to separately study them experimentally. We plan to model the extracellular matrix itself and its interactions with endothelial cells in more detail and extend our model to a multi-scale model, including molecular, cellular and tissue levels, to gain insight in these interactions. In a close cooperation between experimental and computational biologists, we can reach a thorough understanding of how the interactions between multiple levels of organization lead to counterintuitive effects, which experiments alone would not unveil.

Multi-scale modeling is thought to be the next step in computational modeling. If different scales and their interactions are modeled simultaneously, we can identify the global and local (side) effects of a therapeutic drug. Some multi-scale models of angiogenesis have already been developed [32–37], as discussed in Sect. 2. So far, many of these models are based on phenomenological rules and the results are direct results of the implemented rules. In order to make the step to explanatory rather than descriptive multi-scale models, a thorough understanding of the mechanisms at the

separate levels is needed. A few international projects recognize this need for explanatory multi-scale models, like the Physiome Project and the Virtual Physiological Human Project [36]. We argue that cells and their behavior should still have a central role in these models, since cell behaviors and properties can also be observed and measured experimentally, which allows validation and quantification of the computational model. A constant feedback loop between computational and experimental models is thus needed to reach a functional and multi-level understanding of angiogenesis, a strategy called systems biology.

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# ECM Remodeling in Angiogenesis

**Stephanie J. Grainger and Andrew J. Putnam**

**Abstract** Remodeling of the extracellular matrix (ECM) is an essential component of the complex vascular biology that drives each step within the angiogenic cascade. The process of angiogenesis involves a series of events that depend heavily on proteinases and their ability to remodel the ECM, originating with degradation of the basement membrane to allow for endothelial cell (EC) breakthrough, migration, and proliferation. This is followed by organization into nascent blood vessel sprouts, vessel maturation and stabilization, deposition of basement membrane around the new vessels, and finally pruning or remodeling of the new vasculature for physiological needs. There is evidence that ECs cooperate with supporting stromal cells to orchestrate these remodeling events and ultimately to create pericyte-stabilized functional networks of vessels. During angiogenesis, proteinases not only directly breakdown the ECM to create a physical path for new EC sprouts, they also indirectly expose cryptic sites hidden within the ECM to alter the adhesive microenvironment for pericytes and endothelial cells during sprouting. Physiological control of angiogenesis is achieved in part by the angiogenic switch, in which a balance of pro- and anti-angiogenic factors serves to maintain vessel homeostasis under normal conditions. Proteinases, and certain matrix metalloproteinases (MMPs) in particular, function on both sides of the angiogenic switch. They degrade the basement membrane and nearby ECM surrounding established blood vessels at the onset of angiogenesis, and release pro-angiogenic growth factors that would remain otherwise bound to the ECM. However, they also negatively control angiogenesis, as some proteolytic fragments of the ECM possess anti-angiogenic properties. In addition to the chemical specificity of proteinases, emerging evidence suggests that their ability to proteolytically remodel the ECM during angiogenesis

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may also depend on the physical properties of the ECM. In this chapter, we will discuss the important factors that govern ECM remodeling during angiogenesis, focusing on the links between proteinases, stromal cells, and matrix physical properties. The impact of these possible links on therapeutic and pathologic angiogenesis will also be discussed.

## 1 Introduction

During angiogenesis, the ECM dynamically evolves, changing and adapting to cellular processes that are taking place within its structure. As ECs differentiate into tubular structures containing lumens and associated pericytes, significant matrix remodeling occurs. Proteinases carve out areas to allow for invasion of the nascent tubular structures into the surrounding stroma, creating new vasculature in response to hypoxia. A new basement membrane is laid down, and supporting interstitial matrix is layered beneath the newly formed structures. This chapter will discuss angiogenesis as it relates to the surrounding ECM, the various proteinases that are able to modulate key steps of this process, and finally, the link between ECM mechanical properties and cellular remodeling during angiogenesis.

Vasculogenesis and angiogenesis are two distinctly different processes by which blood vessels form (Fig. 1). In embryonic development, angioblastic cells assemble into a primary capillary plexus to create nascent vasculature de novo via vasculogenesis. By contrast, angiogenesis refers to the formation of capillaries via branching from existing vasculature after initial embryonic development. This requires a complex series of events starting with basement membrane degradation of the existing vasculature, followed by endothelial cell activation, migration, and proliferation, organization into immature vessel sprouts with leading tip cells, maturation and vessel stabilization via mural cell association, and finally, basement membrane deposition and pruning of the new vessels in response to the physiologic demands of the tissue [1]. Each step in this process requires interaction between cells and their surrounding ECM.

A balance between pro- and anti-angiogenic proteins, known as the ‘angiogenic switch’, is crucial to the control of angiogenesis, with soluble factors and insoluble factors actin regulating this switch. When the scale is tipped in favor of molecules that inhibit angiogenesis, the switch is ‘off’ until the levels of activating (pro-angiogenic) molecules are increased and able to overcome the inhibiting molecules. In healthy adults, the switch is typically maintained in the ‘off’ position, unless a pathological state which requires the formation of new vasculature occurs, such as cancer, wound healing, or ischemic disease. (In cancer, tumor growth beyond a threshold is achieved in part by recruiting host vasculature; however, because a detailed discussion of tumor angiogenesis is beyond the scope of this chapter, readers should refer to other reviews on the topic instead [2].) Many signals can tip the switch in favor of angiogenesis, such as hypoxia, low

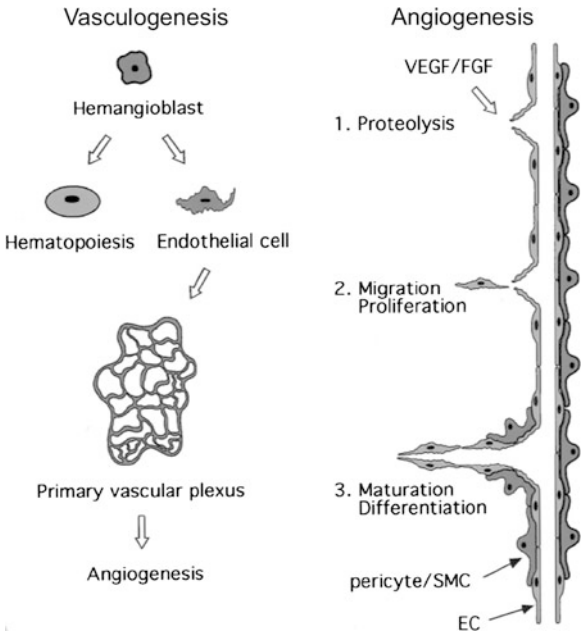


**Fig. 1** Schematic depiction of the two different processes by which new blood vessels form in the body. Vasculogenesis typically occurs in development, with angiogenesis occurring throughout life. This chapter focuses on how the ECM is remodeled during the latter process. (Figure reproduced from [139] with permission from Elsevier.)

environmental pH, mechanical stresses, tumor growth, or the presence of immune or inflammatory cells. Soluble growth factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (FGF), and hepatocyte growth factor (HGF), are potent pro-angiogenic factors. Stabilizing immature vessels requires molecules such as angiopoietins-1 and -2, TGF-$\beta$, and sphyngosine-1 phosphate (S1-P), which are also considered pro-angiogenic. On the other side of the balance, suppressive signals from angiogenesis inhibitors include $\alpha$-interferon, platelet factor-4, and thrombospondin-1, as well as other cryptic protein fragments [3, 4]. Matrix metalloproteinases (MMPs) and their endogenous inhibitors can also be considered in the context of the angiogenic switch, as they function as both pro- and anti-angiogenic molecules and are essential in each step of capillary formation and remodeling [5].

## 2 Changes in the ECM Accompany Each Stage of Angiogenesis

During the initial stages of angiogenesis, contact between ECs and the ECM is a key controller of angiogenic signaling. ECs must adhere to the ECM in order to properly migrate, a necessary requirement for initiating angiogenic sprouting [6]. The ECM immobilizes angiogenic cytokines, and thus coordinates signals transduced to ECs via both growth factor receptors and integrin cell adhesion

receptors [6–8]. Integrins, in turn, can regulate EC proliferation, survival, and the formation of functional vessel lumens [8–11]. If the ECs fail to adhere to the ECM, proliferation ceases and angiogenesis thus also stops [7, 12–15].

At the earliest stages of angiogenesis, the basement membrane, consisting primarily of laminin-1 and type IV collagen, gets degraded to expose the ECs to the surrounding interstitial matrix [16]. In a quiescent state, the basement membrane helps insulate the ECs from this interstitial matrix and inhibits EC invasion and migration [7]. Following its degradation, a gradient of ECM components and the cytokines attached to them provide a set of cues to direct EC motility. In wound healing, for example, the interstitial matrix consists primarily of fibrin and type I collagen, and supports subsequent EC migration and sprouting [17]. VEGF plays a particularly critical role at this stage, as it is known to induce $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrin expression, both of which bind type I collagen. Type I collagen also helps to transform the leading ECs into a tip cell phenotype [18]. One mechanism known to disrupt angiogenesis for therapeutic or alternative purposes is to disrupt the formation of the collagen triple helix via alteration of the prolines. This results in a cessation of collagen recognition and binding by the ECs, effectively halting angiogenic invasion and tubule formation [19].

Once a nascent tubule escapes the basement membrane and begins to invade the interstitial matrix, extension of the capillary sprout begins. Type I collagen induces nascent cord formation and a migratory EC phenotype in part by suppressing cyclic AMP, which causes increased actin polymerization and stress fiber formation within the EC cytoskeleton [7]. This enables the ECs to generate substantial contractile forces and apply tension to the matrix over relatively large distances, which in turn supports capillary cord formation along the matrix fibers. Other cell types do not migrate and produce cords when implanted in fibrin or collagen gels in the same way [20]. Disruption of vascular endothelial cadherin (VE-cadherin) intercellular junctions via signaling mechanisms induced by collagen I binding also help to induce initial sprouting of ECs from a base vessel [16]. Disrupted of their quiescent cell–cell contacts, ECs begin to migrate and develop into nascent cords [19, 21].

The next steps in the angiogenic process include the formation of hollow lumens, followed by maturation of the nascent vessels. As mentioned previously mentioned, integrin-mediated interactions between ECs and collagen, fibrin, and fibronectin provide key instructive signals [22–24]. $\alpha_2\beta_1$ and $\alpha_1\beta_1$ are known collagen receptors, while $\alpha_V\beta_3$ and $\alpha_5\beta_1$ are known fibronectin receptors that also permit EC interactions with fibrin. Lumen formation is dependent on the formation of these integrin-dependent intracellular vacuoles that are initially formed by the process of pinocytosis, or in which small vesicles formation form to create pockets within the cell. These vacuoles fuse together by exocytosis between adjacent ECs and start to direct an apical-basal organization and polarization [25, 26]. This polarization requires membrane type-MMP (MT-MMP) to interact with the ECM at the exterior of the newly formed lumens. The roles of these MT-MMPs will be discussed in greater detail later in this chapter.

The final step in the angiogenic process, tube stabilization, coincides with the production of laminin to form a new basement membrane along the basal surface of the nascent tubules. Integrins $\alpha_6\beta_1$ and $\alpha_3\beta_1$, which bind ECs to specific laminin isoforms, are known indicators of capillary maturation [27, 28]. The expression of these integrins suppresses several signaling pathways, and trigger EC quiescence [29, 30]. The laminin-rich basement membrane also provides an interface with which both ECs and stabilizing pericytes can interact [7].

## 3 Proteinases Involved in Angiogenesis

The ECM must be broken down and reformed for many processes throughout life, including embryonic development, various morphogenic processes, cellular reproduction, and tissue remodeling. The matrix itself serves as a platform for cell growth and support, but is also capable of controlling cellular attachment, proliferation, migration, and differentiation of cells via cell-ECM interactions. Many cytokines and growth factors can also be sequestered in the matrix and stored for later use. Several different types of angiogenic proteinases modulate the ECM in varying ways to influence angiogenesis. Some proteases indirectly promote EC proliferation, while others degrade the ECM to allow for tunneling ECs to invade and form tubules. Others control growth factor release from the matrix, altering cues that can direct or inhibit the angiogenic process. A final group of proteinases also controls cell adhesion to the matrix, inducing polarity within the blood vessels. These adhesions may direct cells to migrate, proliferate, or remain quiescent, based on the levels of varying integrins expressed, and the contents of the matrix to which they bind. MMPs are the main degradative enzymes responsible for modulating the ECM in a tissue. They are always contributing to the evolving matrix as it changes in different ways to support and encourage various cellular processes. In addition to both membrane-bound and soluble MMPs, ADAMs are another important group of proteins that influence ECM remodeling. A final grouping of players is the tissue inhibitors of metalloproteinases (TIMPs), which can control angiogenesis and subsequent matrix remodeling by maintaining vascular quiescence and halting angiogenic cues to maintain the angiogenic switch in the "off" position.

**A. Soluble Matrix Metalloproteinases**

Secreted matrix metalloproteinases are a family of zinc-containing endopeptidases that are able to degrade various ECM components. They are produced as pro-enzymes that are proteolytically processed to become activated. A sulfhydryl group in the pro-domain of all MMPs, known as a "cysteine switch," is able to work in sync with the zinc ion of the catalytic site to maintain cell quiescence [31]. By disrupting this cysteine-zinc binding, the MMP takes the first step toward activation [32].

In general, the naming scheme follows a simple numerical order, starting with the first to be discovered, MMP-1, which acts on collagens. It was originally

discovered when collagen gels were degraded by tadpole fin explants. The rest of the MMPs are divided into subgroups based on domain structure and substrate specificities: matrilysins (MMP-7 and MMP-26), collagenases (MMP-1, MMP-8, MMP-13, and MMP-18), stromelysins (MMP-3, MMP-10, and MMP-11), gelatinases (MMP-2 and MMP-9), enamelysins (MMP-20) and epilysins (MMP-28), and others that don't fit into one of these subgroups (MMP-19, MMP-21, MMP-22, MMP-23, and MMP-27) [33–35]. MMPs can also be classified based on the basic domain groups that are included in their structures. All MMPs have three common structural domains: the "pre" or signal sequence, the pro-peptide domain, and the catalytically active domain (Fig. 2). MMPs-7 and -26 contain only these 3 domains, have a broad range of substrate specificity, and are able to degrade many ECM proteins [36–38]. Addition of a hemopexin domain connected to the core region via a proline-rich hinge region at the catalytic domain allows for enhanced substrate specificity over those containing only the three basic regions. This hinge region is also responsible for binding the family of specific MMP inhibitors (TIMPs). Some examples of hemopexin-domain containing MMPs are collagenases, which degrade the native helix of fibrillar collagens such as types I, II, and III, as well as stromelysin-1 and -2, enamelysin, metalloelastase, and MMPs-19, -22, and -27, which again don't fit into a specific structural class of MMPs [33]. These stromelysins, with their hemopexin domains, still have a rather broad substrate specificity, degrading several groups of ECM proteins, including proteoglycans, fibronectin, and laminin [32]. Another similar grouping is the gelatinases (MMPs-2 and -9) containing three head-to-tail cysteine-rich fibronectin type II-like repeats within the catalytic domain [36, 39]. These MMPs also degrade types IV, V, VII, and X native collagens, as well as denatured collagen (gelatin), fibronectin, and laminin [39]. When the cysteine-rich repeats are instead furin-susceptible sites, stromelysin-3 and epilysin are classified as a grouping. Finally, there are two outlying MMPs, including MMP-21, which does not have a hinge region at all, and instead contains a vitronectin-like region within the propeptide region, and MMP-23, which also lacks the hemopexin domain, containing a cysteine and proline-rich region followed by an immunoglobulin-imitating region [40].

Soluble MMPs can have both pro- and anti-angiogenic roles. Their pro-angiogenic capacity is perhaps more obvious, given their ability to degrade ECM components and jumpstart the path toward angiogenesis. Many growth factors and cytokines are known to upregulate EC basement membrane degradation, as well as EC proliferation, migration, and differentiation into a pro-angiogenic phenotype. Examples of these molecules are VEGF, bFGF, and several interleukins, which increase the amounts of inactive [41] and active[42] MMP-2 and MMP-9 [43]. However, because certain ECM cleavage products have anti-angiogenic properties, MMPs may also be considered anti-angiogenic as well [44].

*1. Pro-angiogenic roles of soluble MMPs*

During basement membrane degradation, a subgroup of ECs, known as "tip cells", initiate sprouting. These cells possess high proteolytic activity, enabling them to successfully break down the matrix and tunnel through the interstitial

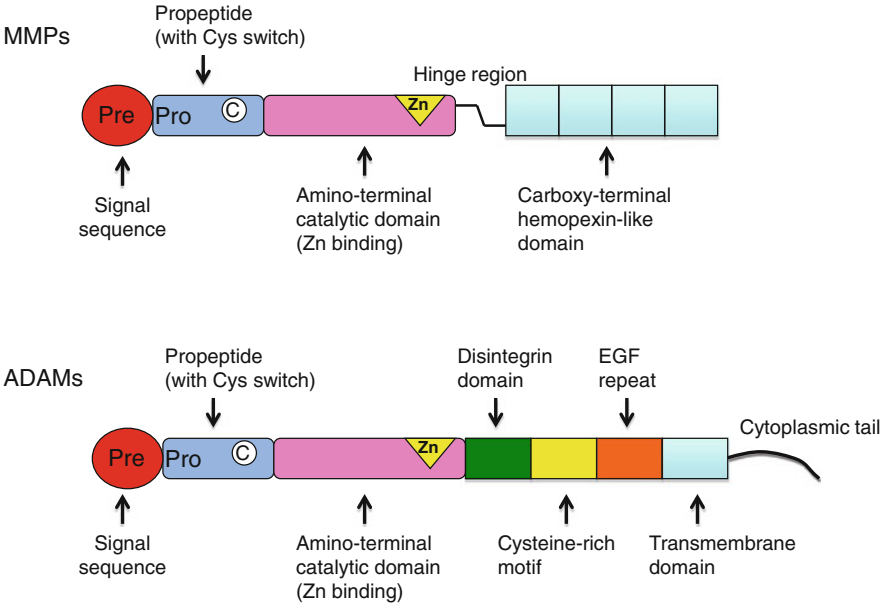


**Fig. 2** Schematic representation of the structures of MMPs and ADAMs. Both protease families contained conserved features, a "pre" or signal sequence, a propeptide domain (pro) (with either a cysteine switch or a furin-susceptible site), and a catalytic, Zn-binding domain. Additional sequences in some MMPs include a hinge region (H) and hemopexin domain, and other features not shown here. ADAMs contain a disintegrin domain, a cysteine-rich motif, an EGF repeat, a transmembrane domain, and a cytoplasmic tail

ECM and hypoxic tissue [45]. Upon signaling to initiate sprouting, the tip cells must first proteolyze the capillary basement membrane, which is primarily comprised of laminin, collagen IV, heparin-sulfate proteoglycans, and entactin [46]. Multiple MMPs can degrade these ECM components: MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, and MT1-MMP [33]. As mentioned previously, two important growth factors in the initiation of the angiogenic cascade, VEGF and bFGF, produce vesicles containing pro-MMP-2 and pro-MMP-9, as well as MT1-MMP. Upregulation of these MMPs is thus associated with increased basement membrane invasion abilities of ECs [47].

After the basement membrane has been broken down, ECs induce MMP expression from interstitial cells by secreting extracellular matrix metalloproteinase inducer (EMMPRIN) [48]. The majority of MMP production may be from these surrounding interstitial and inflammatory cells present in the matrix, rather than the ECs forming the actual new capillary sprouts. Interstitial flow from the vasculature to the lymphatics, which is enhanced following degradation of the basement membrane barrier, combined with this increased MMP production, creates chemotactic gradients that further encourage EC invasion into the ECM. This is perhaps achieved by the interaction of various ECM breakdown products

with the surface of the ECs [49]. Despite significant differences in pathologic and physiologic microenvironments, only small changes in soluble MMP expression are observed [50]. For example, switching from a physiologic ECM containing mostly collagen I to a provisional ECM comprised of fibrin, fibronectin, and vitronectin typically found during wound healing or prolonged ischemic diseases, results in slight modulation of the MMP expression profile [51].

Recent work suggests that there may be no single proteolytic mechanism utilized by ECs to degrade the ECM. Instead several MMPs are likely used in complement, and the specific combination and ratio of MMPs expressed and utilized depends on the identity of the matrix as well as the identity of stromal cell types that interact with the ECs. For example, when adipose-derived stem cells or a generic fibroblast source were included as interstitial cells to induce capillary sprouting in a fibrin matrix, the plasminogen activator-plasmin axis was the preferred proteolytic mechanism utilized for capillary invasion into the matrix and tubule lengthening, while MMPs appeared to play a distinct role regulating capillary diameter and stabilization only. In contrast, when mesenchymal stem cells from bone marrow were used in place of the adipose-derived cells, MT-MMPs were the sole proteases for ECM invasion and sprouting [52].

Further work illustrating knockdown of either of the gelatinases, MMP-2 and MMP-9, suggests that these two proteinases may work in concert to remodel the ECM during angiogenic processes. When one of the two is targeted for gene knockdown, sprouting is still able to occur. MMP-9 is unable to degrade type I collagen alone, so thus it does not serve to encourage tunneling and sprouting of ECs during angiogenesis via matrix proteolysis directly. Instead, its pro-angiogenic capacity may lie in its ability to release bound VEGF (secreted by stromal cells) from the matrix to induce sprouting. It is also capable of activating TGF-$\beta$, resulting in promotion of tissue remodeling [53, 54]. During *in vivo* wound healing and hind limb ischemia studies, the peak activity levels of these MMPs coincide with granulation tissue formation, fibroblast migration into the tissue, and vascularization of the wound [55, 56]. Several research groups have now fabricated synthetic hydrogels with linkages sensitive to MMP-2 and MMP-9 so that cellular invasion can occur in much the same way as in hydrogels of natural composition (e.g., collagen or fibrin). In vitro studies using RGD-functionalized version of these MMP-sensitive gels have demonstrated EC adhesion and capillary sprouting by mimicking key elements found in natural matrix proteins [57, 58]. Furthermore, tethering growth factors such as VEGF to the matrix via proteolytically sensitive linkages recapitulates the growth factor sequestration capacity of physiologic ECMs [59].

*3. Anti-angiogenic roles of soluble MMPs*

As mentioned previously, MMPs can be considered to be both pro- and anti-angiogenic. MMP expression and activity can impede blood vessel formation via one of two possible mechanisms. First, overactive MMPs can compromise ECM stability, which may result in vascular regression. Second, MMP activity can generate matrix fragments with anti-angiogenic capabilities.

With respect to the first possibility, the process of angiogenesis typically culminates with vascular pruning. During this process, vessels that have not been

stabilized regress as some of the interstitial collagen is broken down. Specifically, plasmin-mediated activation of MMPs-1, -10, and -13 has been shown to induce vascular regression as each of these MMPs (although predominantly MMP-1) can digest interstitial collagens [60]. This subset of MMPs, in conjunction with MMPs-2, -9, and MT1-MMP, may act together to digest multiple different ECM components based on these enzymes' specificities for different substrates [60, 61]. The first subset breaks down native type I collagen, while the gelatinases more efficiently degrade denatured collagens. A direct correlation between the levels of active MMPs in maturing capillary beds and the levels of vascular regression has been reported [62, 63].

With respect to the second possibility, the proteolytic degradation products of MMPs are anti-angiogenic. Proteolytic degradation of collagen IV, one of the primary components of basement membrane, generates anti-angiogenic fragments that include arrestin, canstatin, tumstatin, and metastatin [34]. MMP-9 predominantly produces free tumstatin, as well as smaller amounts of arresten and canstatin. Other MMPs, including MMP-2, -3, and -13, are also able to liberate tumstatin, although not as efficiently as MMP-9 [64]. Tumstatin targets the $\alpha_V\beta_3$ integrin, which is not expressed at measurable levels in physiologic angiogenesis, but is seen at much higher levels in tumor angiogenesis. Studies have explored the possibility of using tumstatin to reduce pathologic angiogenesis [64]. Arrestin, another collagen IV breakdown fragment, binds the $\alpha_1\beta_1$ integrin receptor for collagen I, and inhibits EC proliferation, as well as migration and further tube formation in vitro. Similarly, collagen XVIII is a component of the interstitial matrix beneath the basement membrane of the vasculature. Collagen XVIII breakdown products are endostatin and neostatins, which are small, varying molecular weight molecules that are the further breakdown products of endostatin. MMPs-3, -7, -9, and -13, as well as MT1-MMP, act on collagen XVIII to produce these fragments. Endostatin affects VEGF signaling, EC proliferation and migration as well, in part by acting on the $\alpha_5\beta_1$ integrin [65–68]. Another unique function of endostatin is its ability to inhibit MT1-MMP and MMP-2 activities [69].

**B. Membrane-Type Matrix Metalloproteinases (MT-MMPs)**

The membrane-type MMPs (MT-MMPs) represent another grouping of MMPs, so named because they are bound to the cell's plasma membrane via either a C-terminal transmembrane domain or a glycophosphatidyl inositol (GPI) anchor [39, 70]. Both classifications include a "pre" region, a propeptide region with a furin-susceptible site, a catalytic domain, a hinge region, and a hemopexin domain. After furin activation intracellularly, the proteinase gets processed and sent to the cell membrane, where the catalytic, hinge, and hemopexin domains lie extracellularly. These external regions are held at the cell surface by a transmembrane region attached to a short amino acid tail residing in the cell cytoplasm, or a GPI domain that is fixed in the cell membrane. These MT-MMPs provide spatial control of matrix breakdown directly at the cell membrane surface [33]. MT-MMPs degrade gelatin, fibronectin, and aggrecan, as well as several other ECM substrates [37, 38].

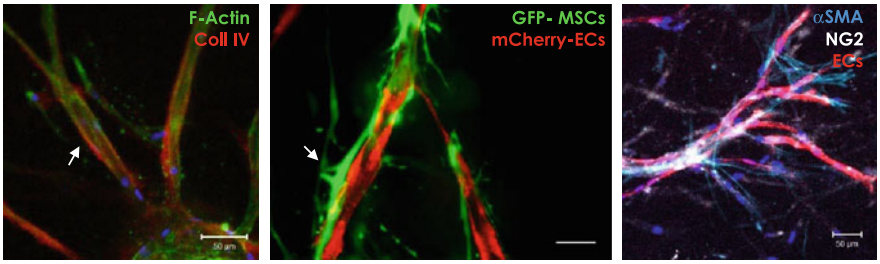


**Fig. 3** Three-dimensional co-cultures of ECs and stromal cells generate stable, pericyte-invested capillary networks in vitro. In these experiments, bone marrow-dereived mesenchymal stem cells (MSCs) MSCs were distributed throughout a fibrin-based 3-D ECM in the presence of microcarrier beads coated with ECs. **a** After 7 days, cultures were fixed and IF stained at day 7 for F-actin (*green*) and collagen IV (red) to visualize basement membrane deposition (white arrows). Scale = 50 μm. **b** MSCs expressing GFP were interspersed with mCherry-transduced ECs. Physical association of the ECs and MSCs were observed (white arrows). Scale = 25 μm. **c** Cultures containing mCherry-transduced ECs and MSCs were fixed and IF stained for pericyte markers αSMA (aqua) and NG2 (white). DAPI-stained nuclei are visible in the blue channel. Scale = 50 μm. (Figure reproduced from [74] with permission from Elsevier.)

Once nascent capillaries have formed, a basement membrane, a hallmark of a more mature vessel network, is deposited. Pericytes play a key role in this process. These cells are recruited from the host stroma in part via the secretion of PDGF-$\beta$ by ECs. PDGF receptor-$\beta$ signaling then initiates a cascade of events that control pericyte-EC binding, migration to the site, and proliferation. Interestingly, all of these processes are in some manner controlled by MT1-MMP [71–73]. Co-cultures of ECs and stromal cells of various origins in 3-D gels yield stable, pericyte-invested networks of capillaries characterized by the presence of basement membrane subjacent to the ECs along with the periendothelial location of the stromal cells (Fig. 3) [74]. In the absence of stromal cells, ECs express MT1-MMP, with very little basement membrane production, even at later time points. If stromal cells are included with ECs in culture, MT1-MMP expression is undetectable in the capillary stalks. The ECs in the stalk instead produce basement membrane proteins, and the expression of MT1-MMP is restricted to the tip cells. Similar expression profiles have also been see in vivo [45].

Further examination of the newly deposited basement membrane production yields some other interesting observations. The EC TIE-2 receptor is preferentially expressed in the stalk portion of a maturing capillary sprout, but are notably absent in the tip ECs. This expression is thought to be controlled by the pericytes, which produce Ang-1 to signal to the ECs via the TIE-2 receptor [45, 75]. This Ang-1/TIE-2 interaction is one mechanism by which pericytes and SMCs can regulate MMP activity of ECs. An alternative, and much more direct, means to achieve this control is via TIMP-1 secretion. TIMP-1 inhibits soluble MMPs, but also appears to stabilize vessels by inducing basement membrane protein production [76]. Other TIMPs also work in conjunction with both cell types to inhibit angiogenic sprouting and stabilize nascent vessels. TIMP-3 is secreted by perivascular cells,

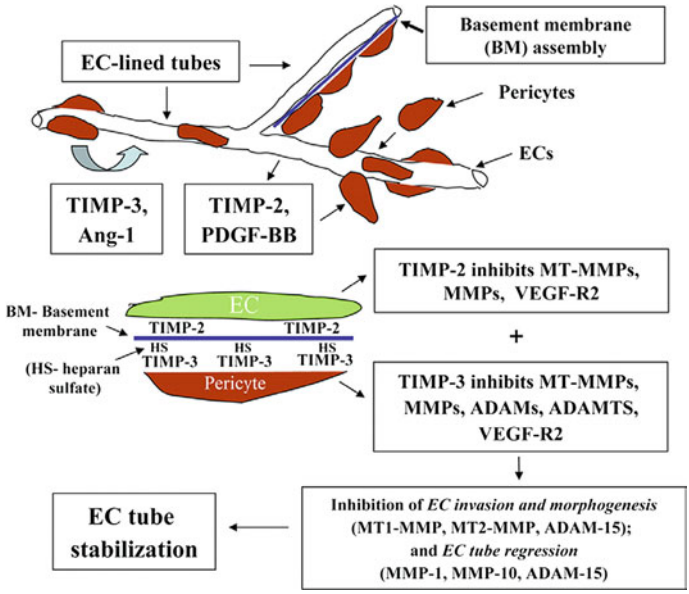


**Fig. 4** Schematic diagram illustrating the contribution of TIMP-2 and -3 to pericyte-induced vascular tube stabilization, as proposed in [77]. TIMP-2 is derived from ECs, whereas TIMP-3 is produced by pericytes. Together, they contribute to vascular stabilization by inhibiting a variety of MMPs, ADAMs, and VEGFR-2. The initiation of tube stabilization requires the blockade of both EC tube formation and EC tube regression, which further leads to the cessation of EC activation and the development of EC quiescence. Pericytes are required for ECs to assemble basement membrane matrices, which may locally capture and present TIMP-3 to ECs through heparan sulfate proteoglycans. (Figure reproduced from [77] with permission from The Rockefeller University Press.)

and is then presented to the ECs via heparin sulfate proteoglycans in the basement membrane, which suppress MT1-MMP activity and encourage sprouting of the ECs (Fig. 4) [77, 78]. Furthermore, all EC interactions with associating pericytes via Ang-1 or PDGF serve to inactivate and inhibit MT1-MMP after the basement membrane has been reproduced and to ensure vascular quiescence.

**C. A Disintegrin and metalloproteinase (ADAM)**

ADAMs are a family of secreted and transmembrane proteins that control cell adhesion, as well as proteolytic processing of the ectodomains of cell surface receptors and signaling molecules [79]. Like previously described MMPs, ADAMs have both pre- and propeptide domains, with the pro- domain acting as an intramolecular chaperone that controls protein folding [80] and enzyme latency via a cysteine-switch mechanism [81, 82]. At this point, the structures differ, with ADAMs having a disintegrin-like domain with a loop that is able to interact with neighboring cell integrins [83]. Following this region, a cysteine-rich domain, then an EGF-like domain, followed by a membrane-spanning region and a cytoplasmic tail. The cytoplasmic domain is able to interact with proteins of intracellular

signaling importance, as well as to control trafficking of proteins (Fig. 2). ADAMs are named such due to their original structural homology to the small proteins of hemorrhagic snake venoms that were able to bind platelet integrin $\alpha_{2b}\beta_{3a}$ to block platelet aggregation [84].

Because ADAMs are an active family of metalloproteinases, they are able to cleave ECM proteins and cause degradation of the bulk ECM in a locale. One example of this is ADAM-9, which is able to cleave laminin and promote invasion [85]. Several studies have shown a connection between ADAM-10 and cleavage of adhesion molecules such as VE-cadherin, where the ADAM is able to disassemble the junctional contacts that control permeability and assist with encouragement of EC migration and tubule sprouting [7, 86]. Other ADAMs that are known to control ECM degradation and release activators of ECs to a migratory phenotype that will start the angiogenic cascade, are ADAMS-15 and -17 [7]. ADAMs are also able to induce shedding of adhesion molecules such as PECAM-1, or their activity may mobilize growth factors, chemokines, or other soluble factors that can influence angiogenic processes [87].

**D. Inhibitors of Matrix Metalloproteinases**

Tissue inhibitors of metalloproteinases (TIMPs) are the main enodogenous inhibitors of MMPs. There are four mammalian TIMPs that have been identified and characterized within the literature. They are all known to regulate MMP activity during periods of tissue remodeling, with molecular weights between 20 and 29 kDa [88]. All TIMPs inhibit MMP substrates in a 1:1 stoichiometric ratio [89], with each TIMP binding the active site cleft in the catalytic domain of an MMP, in the same manner as an ECM substrate would bind the MMP [40]. Each TIMP has disulfide bonds of a three loop N-terminal domain, which is where interaction with the catalytic domains of MMPs occurs, and a complementary three loop C-subdomain [90].

All TIMPs are secreted proteins, but TIMPs-2, -3, and -4 can all be found near the surface of a cell, in association with different MMPs [91]. All four TIMPs are known to inhibit active forms of all MMPs; however, their inhibition abilities vary widely. The main exception to this rule is that TIMP-1 is a poor inhibitor of MMP-19, MT1-MMP, and MT3-MMP [92]. TIMP-3 easily inhibits many of the ADAMs [93]. Examples of preferential binding are the ability of TIMP-1 to preferentially bind with pro-MMP-9, and TIMP-2 preferentially binding with pro-MMP-2 to inhibit conversion to active forms of these MMPs [94, 95].

TIMP-2 has a special functional role in controlling the activation of pro-MMP-2, with MT1-MMP also acting as a modulator. This activation step takes place on the cell surface, thus the need for inclusion of MT1-MMP for activation [96]. According to Strongin et al., the increased activation of MMP-2 in the presence of TIMP-2 is the result of the N-terminal inhibitory domain of TIMP-2 binding to the active site of MT1-MMP, and the C-terminal domain of TIMP-2 interacting with the C-terminal hemopexin domain of pro-MMP-2 [97]. An additional unique feature of TIMP-2 is its ability to suppress angiogenesis by reducing EC proliferation cues from bFGF via its C-terminal region [98].

TIMP-3, as briefly mentioned earlier, can inhibit both MMPs and the ADAM family of proteinases [40]. TIMP-3 is also known to block VEGF to VEGFR-2 binding, which further contributes to the anti-angiogenic capabilities of TIMP-3 [99]. A third unique property of TIMP-3 is its restricted diffusion caused by its tight binding to heparin sulfate proteoglycans in the surrounding ECM. Because it does not readily diffuse, it is thought that TIMP-3 instead functions to regulate angiogenesis after the angiogenic switch has been flipped into an "on" position. As MMP degradation of the ECM liberates the matrix-bound TIMP-3, it can then inhibit any subsequent MMP activation at the cell-ECM level [100]. Normally, TIMP-3 functions to form complexes via its C-domain with MMPs-2 and -9, thus effectively slowing pro-angiogenic signaling [90].

TIMP-4 is found mainly in the human heart, with low levels of the inhibitor found in the kidney, colon, placenta, and testes [101]. Levels are dysregulated in various cardiovascular diseases. This TIMP functions mainly to reduce EC motility, as well as proliferation, and induce apoptosis as well. In in vivo models, addition of TIMP-2 results in suppression of angiogenesis, while addition of TIMP-4 is not able to have this same effect [102]. TIMP-4 deficient mice instead showed reduced cardiac function with aging, due to increased apoptosis of cells [103].

An additional inhibitor of MMPs, while not in the TIMP family, is the GPI-anchored glycoprotein, reversion-inducing cysteine-rich protein with kazal motifs (RECK). It is known to inhibit the release of pro-MMP-9 from the EC surface. It also effectively inhibits MT1-MMP, which will result in inhibition of MMP-2, as previously discussed [104, 105]. In RECK knockout mice, blood vessels cannot reach a mature stage, and mice die in utero. Overexpression of RECK in tumor models results in a reduction of new blood vessel sprouting to sufficiently nourish the tumor [88]. An additional proteinase inhibitor, $\alpha_2$-macroglobulin, is the primary MMP inhibitor found in blood plasma [106]. Finally, thrombospondin-1, a known inhibitor of angiogenesis, is also known to inhibit pro-MMP-2 and pro-MMP-9 from becoming activated. Thrombospondin-2 is also known to complex with MMP-2 to increase clearance via receptor-mediated endocytosis [106].

A schematic summarizing many of the effects of soluble and membrane-bound MMPs, as well as the TIMPs, is shown in Fig. 5.

## 4 Effects of Stromal Cells on the ECM

**A. Stromal cells influence ECM synthesis and degradation**

It has been widely established in the literature that the presence of pericytes covering EC tubules results in stabilization of the vessels, a decrease in vascular pruning, and decreased permeability of the nascent vessels [107]. Pericytes are a source of angiopoietin-1, which acts on EC TIE-2 to stabilize these heterogeneous cell–cell junctions [108]. Recent findings have shown that EC-pericyte interactions occur after ECs carve out "vascular guidance tunnels" within the ECM, which

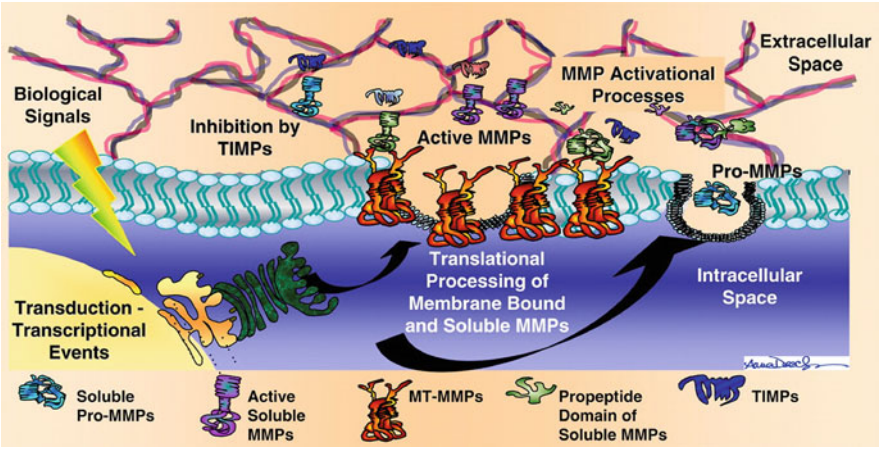


**Fig. 5** A simplified schematic of the current concepts depicting the roles and regulation of MMPs as they pertain to matrix remodeling. Figure reproduced from [140] with permission from The American Physiological Society.)

provide physical space for the EC-pericyte interactions to take place. Stratman et al. showed that the pericytes are recruited to the ablumenal surface and are able to move along the EC tubules through these pre-formed spaces to regulate tubule maturation and vascular basement membrane assembly [109, 110]. Basement membrane is deposited between ECs and pericytes within these tunnel voids. Further work by this group showed that PDGF-BB and HB-EGF are necessary for pericytes to accumulate within these tunnels and along EC tubules, and also for proper basement membrane deposition. Without these growth factors directing the behavior of the pericyte-EC interactions, their data suggest that no basement membrane will be laid down [111]. This growth factor-pericyte interplay is known to be regulated by MT1-MMP. Active MT1-MMP directly controls the binding to PDGF receptor-B of pericytes after PDGF- $\beta$ secretion by ECs. This cascade of events then controls pericyte migration to sites of need along the vasculature, as well as proliferation to induce greater vasculature stabilization and angiogenic quiescence [71, 73]. VEGF, an important initial cue for increased vessel permeability followed by basement membrane degradation and EC sprouting, also affects pericyte coverage. Treatment with VEGF antagonists results in increased pericyte coverage and improved microcirculatory function with lower permeability [112, 113].

The TIE-2 receptor of ECs on the stalks of sprouting capillaries is a signaling receptor that results in collagen IV expression throughout the stalk but not in the tip cells of capillary sprouts. It is thought to be regulated by Ang-1 in the presence of vascular smooth muscle cells (vSMCs) [45]. When vSMCs are absent, the distinction between stalk and tip cells is eliminated, and MT1-MMP is expressed throughout the structure [45]. Pericytes in co-cultures with ECs also express

TIMP-1, which inhibits the activity of soluble MMPs and promotes basement membrane deposition by ECs [76].

As mentioned earlier, pericyte contact with EC tubules reportedly plays a role in basement membrane deposition. Basement membrane proteins such as laminins and collagen IV are produced with the assistance of pericytes. In quiescent vessels, these basement membrane proteins inhibit tube morphogenesis by blocking access to the interstitial collagens that encourage EC migration and invasion. When pericytes are not present to encourage laminin deposition, ECs remain migratory, and vessel stabilization does not occur. Further work has also shown that EC-pericyte interactions induce production of fibronectin, nidogen-1, nidogen-2, and perlecan, as well as the laminins and collagen IV on the ablumenal surfaces of nascent EC tubules, all of which are constituents of basement membrane [109]. Additional work in this area showed that ECs produced the elevated levels of fibronectin found during these EC-pericyte interactions, while nidogen-1 was produced by the pericytes. These two proteins are both known to be binding molecules for the basement membrane specifically [7, 114]. Furthermore, fibronectin binds collagen IV and perlecan, and nidogen-1 binds collagen IV and laminin. Thus, fibronectin and nidogen-1 appear to be essential for assembly of the full basement membrane [114]. Collagen IV is mainly responsible for basement membrane structural support, and binds predominantly fibronectin and nidogen-1, and self-assembly of fibronectin that is induced by pericytes has a direct effect on collagen IV assembly as well [109].

Most mature capillary networks within the body have approximately 20–25 % of their area covered by pericytes [115]. Despite the fact that pericytes cover only about one fourth of the total EC tubule area, they appear to control basement membrane production and maintenance along the full length of each tubule (see Fig. 4). This may be due to their ability to move along the tube surface [115]. Collectively, these results suggest that heterotypic cell–cell contacts between ECs and pericytes, along with the constant pericyte motility along nascent tubules, are required events for basement membrane deposition and ultimate vessel stabilization.

While conventional wisdom suggests that stromal cells that differentiate into pericytes promote vessel stabilization, basement membrane production, and maturation of the interstitial ECM, there are several cases where stromal cells influence ECM protein degradation and subsequent remodeling. The recruitment of mural cells to the stalks of nascent EC tubules anatomically distinguishes tip cells from stalk cells, and leads to the further production of MMPs by the tip cells to facilitate their ability to break down and invade the matrix ahead of them. In aging adults, pericyte coverage can decrease, causing ultimate ECM breakdown and angiogenic activity increases [116]. Similar activities take place in various ischemic diseases and pathologies such as diabetes.

**B. Pericytes modulate integrin-mediated EC-to-ECM attachment**

Integrins mediate EC attachment to the ECM, and cross-talk with several growth factor receptors, and thus are of critical importance in the process of angiogenesis. ECs express a wide range of integrins depending on the location and

state of activation of the cells. The repertoire of integrins expressed by quiescent ECs enables adhesion to components of an intact basement membrane. On the other hand, angiogenic ECs upregulate the expression of a small subset of integrins, most notably the $\alpha_v\beta_3$ heterodimer, which has been reported to be required for angiogenesis [117]. It has been proposed that this integrin is upregulated in angiogenic ECs to permit the binding to a wide range of provisional matrix components, including fibrinogen, vitronectin, von Willebrand factor, and fibronectin. Consistent with a requirement for this integrin in angiogenesis, a landmark study showed that $\alpha_v\beta_3$ integrin antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels [118]. There is some evidence that integrin $\alpha_5\beta_1$ expression is also upregulated at the initiation of angiogenesis, perhaps because it allows the newly migrating ECs that are sprouting from a pre-existing vessel to bind the aforementioned components of the provisional matrix [119, 120]. A complete discussion of integrins and their roles in both normal and pathologic angiogenesis is beyond the scope of this chapter. (For an excellent review, the reader is referred to a paper by Stupack and Cheresh [121].)

However, two facts regarding integrin expression are particularly relevant in the context of this chapter on ECM remodeling. The first is that the integrin expression profiles of ECs undergoing angiogenesis are different than those of quiescent ECs, as noted in the preceding paragraph. This is due to the fact that angiogenic ECs are exposed to an interstitial matrix whose composition is significantly different than a basement membrane, as noted in the preceding paragraph. The second is that pericytes appear to also be involved in the regulation of EC integrin expression patterns. ECs that are in contact with pericytes express $\alpha_5\beta_1$ to attach to the basement membrane. When pericytes are not present, expression of this integrin is down-regulated, and the nascent vessels remain in an unstable immature state (perhaps more appropriately called an angiogenic state versus a quiescent state). Other integrin subunits with high affinities for basement membrane components (i.e. nidogens, laminins, and collagen IV), including the $\alpha_3$, $\alpha_6$, and $\alpha_1$ subunits, are also not expressed by the ECs in appreciable amounts when pericytes are absent. In contrast, $\alpha_2$ integrin, which recognizes collagen I (a component of the interstitial ECM, but not the basement membrane), is expressed at high levels in ECs cultured alone in 3-D collagen matrices, but is down-regulated in EC-pericyte co-cultures after pericytes have been recruited to initiate vessel maturation [109]. This study by Stratman et al. also showed that a strong induction of $\alpha_3$, $\alpha_5$, and $\alpha_6$ integrin mRNAs in ECs, combined with a dramatic increase in pericyte $\alpha_1$, $\alpha_3$, and $\alpha_6$ integrin mRNAs only when ECs and pericytes were cultured together and coincident with the deposition of basement membrane matrices [109]. Thus, there appears to be a complex and dynamic crosstalk between the ECs and pericytes that governs the integrin expression profiles of both cell types. These dynamic changes in integrin expression profiles permit EC adhesion to the interstitial matrix during the initial sprouting phases of angiogenesis, followed by the subsequent adhesion of both cell types to the EC-deposited basement membrane as the capillaries stabilize and mature.

## 5 Linking ECM Mechanical Properties and Remodeling

The ECM's ability to regulate angiogenesis is complex and multivariate. Not only does it impact capillary morphogenesis via biochemical regulatory mechanisms, including through growth factor sequestration, integrin-mediated adhesion, and protease susceptibility, it also acts as an instructive structural framework to support sprouting and nascent capillary functionality. An additional feature of the ECM that has been proposed as a regulator of angiogenesis is its mechanical resistance to cell-generated tractional forces [122]. Evidence from several studies corroborates the idea that mechanical cues directly impact tubulogenesis [123–130]. Vailhe et al. demonstrated that human umbilical vein ECs seeded on top of 2-D fibrin gels varying in concentration from 0.5 to 8 mg/ml only formed capillary-like structures on the softest of the gels. The authors concluded that the ECs do not form capillary-like structures on the more rigid gels because the cells are unable to generate the necessary contractile forces to remodel the more rigid substrate [125]. Another study conducted by Deroanne et al. showed that ECs seeded on collagen-functionalized polyacrylamide gels of different stiffness change morphologies from a monolayer to a tube-like phenotype as substrate rigidity decreases [123]. In 3-D culture, Urech et al. investigated angiogenic process extension in 3-D fibrin gels and manipulated their mechanical properties by adding exogenous factor XIII to form additional cross-links [127]. Sieminski et al. also studied the 3-D formation of capillary-like structures by two different types of ECs in freely-floating versus. mechanically-constrained (attached) collagen gels, and concluded that changing the collagen concentration modulates the formation of these structures by regulating the amount of traction force exerted by the cells [124]. Further evidence links EC-generated traction forces with branching [128], the formation of capillary-like structures [123, 124, 131], and the transcriptional control of soluble pro-angiogenic molecules [129]. A more detailed discussion of the role of ECM mechanics and EC-generated tractional forces is found elsewhere in this book.

In the scope of this chapter, it is important to recognize that the mechanical properties of the ECM are highly dynamic due to active remodeling induced both by the ECs and stromal cells. A study by Lee et al. used second harmonic generation and two-photon excited fluorescence to show that ECs induce quantifiable alterations in local collagen matrix density via a process that involved cell-generated forces [132]. Another study by Krishnan et al. tracked changes to the ECM during the process of angiogenesis using a 3-D culture model [133]. The authors reported an overall softening of the ECM as MMP activity increased during the initial sprouting phase, and then a slow stiffening as the MMP activity held steady and sprouts increased in length during tubulogenesis. Other studies have suggested that matrix stiffness may also indirectly modulate MMP activity in ECs [134–136]; however, the underlying mechanisms linking ECM mechanical properties and protease expression and/or activity remain to be elucidated.

In addition to EC-generated forces regulating angiogenesis, a recent study by Kilarski et al. reported that external forces generated by myofibroblasts pulling on the ECM during wound contraction mediated the formation of vascular loops by pulling on preexisting vascular beds [137]. This process, known as intussusceptive microvascular growth [138], demonstrates that stromal cells influence the process of angiogenesis in multiple ways. Not only do they secrete pro- and anti-angiogenic soluble factors, some of which influence the expression and activity of certain MMPs, and act as pericytes to stabilize the nascent vasculature, they also generate local forces that dynamically remodel the ECM and preexisting tissue structures. Understanding the complex interplay between ECs, stromal cells, and the ECM remains an ongoing challenge for the field.

## 6 Conclusions

The ECM continuously remodels in response to multiple instructive cues during the complex process of capillary morphogenesis. From early development of the capillary plexus in vasculogenesis, to the angiogenic sprouting of new vasculature in ischemic tissue in adults, many different proteinases work in concert with ECs and stromal cells to drive matrix breakdown, capillary sprouting, and subsequent maturation. Various soluble MMPs, membrane-bound MMPs, ADAMs, and inhibitors of each of these active players, play important roles to maintain the balance between pro- and anti-angiogenic cues in quiescent vessels, and to tip the scales to induce capillary morphogenesis and blood vessel development when needed. An increasing body of literature strongly suggests that pericytes are not only essential in promoting the stabilization and long-term functionality of capillary networks, but they can also exert their influence on vessel formation in a multitude of ways. As discussed here, pericytes also dynamically communicate with ECs to influence matrix proteolysis, synthesis, integrin expression profiles, and the mechanical properties of the interstitial matrix, all of which can influence angiogenesis. Further work is needed to dissect the exact roles of these various players on matrix remodeling and angiogenesis.

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# Barrier Maintenance in Neovessels

**Geerten P. van Nieuw Amerongen**

**Abstract** A hallmark of many pathologies is vascular leak. The extent and severity of vascular leakage is broadly mediated by the integrity of the endothelial cell (EC) monolayer, which is in turn governed by three major interactions: cell–cell and cell-substrate contacts, soluble mediators, and biomechanical forces. Despite its tremendous medical importance, no specific therapies are available directly targeting the endothelium to prevent or reduce vascular permeability. Endothelial cells constantly equilibrate contractile and adhesive forces to maintain vascular barrier integrity. Intracellular signalling, and in particular the involvement of small Rho GTPases in endothelial hyperpermeability responses to many inflammatory stimuli through actin/myosin-mediated cellular contractility, is well-understood. Surprisingly less is known about maintenance of the basal endothelial barrier integrity. Recent live cell imaging studies revealed that highly confluent endothelial monolayers actively maintain barrier integrity by a continuous remodeling of their cell–cell contacts, accompanied by a rapid opening and closure of small inter-endothelial gaps. Moreover, evidence is accumulating that mechanical cues determined by the local microenvironment of ECs are of eminent importance to the integrity of the endothelial monolayer. Here we will review chemical and mechanical signaling involved in maintenance of the integrity of the endothelial barrier.

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## 1 Introduction

The endothelium—the inner lining of all blood vessels—is the main barrier that actively regulates the exchange of water and solutes from the blood to the surrounding tissues and vice versa. All tissues require a continuing supply of nutrients and a means of clearing waste products. The vascular system is very well equipped to this task. Capillaries are the most important sites for physiological exchange of solutes and waste products, as they are sufficiently "open" (i.e., "permeable") to allow the ready exchange of small molecule nutrients and waste products between the blood and tissues [1], whereas pathological exchange (e.g under inflammatory conditions) mainly occurs at the level of the post-capillary venules. Important parameters that govern capillary permeability are: (1) vascular surface area available for molecular exchange; (2) thickness of the vessel wall; (3) hydraulic conductivity, a measure of capillary permeability to water; (4) reflection coefficient, a measure of solvent drag in relation to that of water; (5) diffusion; and (6) trans-vascular pressure gradients [1].

It is important to distinguish between the basal permeability levels of normal tissues and the greatly increased levels of plasma protein extravasation that occur in pathology [2]. These hyperpermeable states may be acute or chronic and differ from each other and from basal levels of permeability with respect to the vessels that leak, the composition of the extravasate, and the anatomic pathways that solutes follow in crossing vascular endothelium [1]. The contractility of the venular endothelium has long been recognized and this endothelial activity represents an important pharmacological target [2–4].

Breach of vascular integrity results in the accumulation of plasma, proteins and cells in the interstitial space, and is one of the cardinal signs of the inflammatory response [2]. Tight regulation of the vascular permeability barrier is required to hold both acute and chronic inflammatory disease in check, and failure to restore barrier function in a timely manner can result in a catastrophic loss of vascular volume, as in septic shock, or contribute to the development of chronic inflammatory diseases such as atherosclerosis [5].

Vascular permeability is regulated by the coordinated opening and closing of endothelial cell–cell junctions and relies on a complex interplay of junctional adhesion components, cytoskeletal rearrangements, and cellular adhesive and counter-adhesive forces [4].

The endothelium is exposed to a variety of chemical and mechanical factors. Mechanical forces are exerted by blood flow and blood pressure. Blood flow creates a frictional force on the endothelial surface known as shear stress, whereas blood pressure creates (cyclic) stretching forces on the vessel wall, known as wall stress. On top of these fluid forces, the vessel wall is locally subjected to stretch forces induced by rhythmic distension of organs, such as a beating myocardium or a breathing lung. In addition to these applied forces, other mechanical factors determined by the cellular microenvironment are perceived by the endothelium as well, such as geometry and rigidity of the extracellular matrix. Through mechanotransduction

systems, cells translate these mechanical stimuli into biochemical signals that control multiple aspects of cell behaviour, including proliferation, differentiation and cellular adhesion [6]. For many years, it has been recognized that changes in the magnitude and direction of shear stress have profound effects on the adhesion of endothelial cells to their sub-endothelial matrix as well as on the organization of inter-endothelial cell–cell interactions, as reviewed elsewhere [7]. Similarly, stretch also affects endothelial monolayer integrity, as is well-established based on the vascular leak-inducing effects of the ventilator, which contributes to severe lung injury in intensive care treatment, as reviewed elsewhere [8]. However, limited information is available on whether changes in rigidity of the extracellular matrix (ECM) might affect the integrity of the endothelial barrier. Knowledge about the relationship between ECM stiffness and barrier function is of importance as blood vessels progressively stiffen with aging [1, 2]. This stiffening is exacerbated by risk factors associated with common disorders such as diabetes, hypertension, cancer, atherosclerosis and renal disease [3–7, 9, 10]. All these disorders are associated to endothelial dysfunction and many of them with increased vascular permeability in particular, suggesting that stiffening of the vascular wall might destabilize the endothelial barrier.

Here we review recent findings that shed light on the relationship between chemical and mechanical factors that regulate the maintenance of the endothelial barrier. For information on the genetic interactions implicated in the maintenance of vessel integrity the reader is referred to a list of the genes involved [11]. Detailed information on regulation of barrier dysfunction [2], the signal transduction pathways involved [4] and its clinical implications [5, 12] exist to which the interested reader is referred.

## 2 Cell–Cell Junction Formation

VE-cadherin-based adherens junctions (AJs) and claudin-based tight junctions (TJs) form a semipermeable endothelial barrier between the vessel lumen and stroma. In contrast to blood–brain endothelial cells, in which the TJs form a sealing belt, TJs in most other endothelia have a mosaic structure leaving space for the passage of macromolecules. The AJs contribute largely to the barrier properties of these latter endothelia through their interaction with the actin cytoskeleton.

VE-cadherin, a calcium-dependent adhesion protein mediating transhomophilic interactions, localizes at cell–cell contacts, regulating the formation of adherens junctions, and linking the site of the junction to the actin cytoskeleton.

The procedure by which endothelial cells establish their adherens junctions has remained unclear for a long time. Recently, the dynamics of cell–cell junction formation and the corresponding architecture of the underlying cytoskeleton were nicely visualized by terrific imaging in cultured human umbilical vein endothelial cells [4]. It was shown that the initial interaction between cells is mediated by protruding lamellipodia. On their retraction, cells maintained contact through thin

bridges formed by filopodia-like protrusions connected by VE-cadherin–rich junctions. Subsequently, bridge bundles recruited nonmuscle myosin II and mature into stress fibers. Myosin II activity was important for bridge formation and accumulation of VE-cadherin in nascent adherens junctions. Thus, a novel lamellipodia-to-filopodia transition seems to be a mechanism of cell–cell junction formation in endothelial cells, where lamellipodia act as the initial protrusive contact, subsequently transforming into filopodia-like bridges connected through adherens junctions. The lamellipodia not only play a role in initial formation of endothelial junctions, but are also important in the maintenance of cell–cell interactions.

## 3 Endothelial Barrier Maintenance

Many studies have indicated the important contribution of the actin cytoskeleton to determining junctional integrity [4, 8, 13–15]. A cortical actin belt is thought to be important for the maintenance of stable junctions. In contrast, actin stress fibers are thought to generate centripetal tension within endothelial cells that weakens junctions. Many of these concepts have been based on studies in which endothelial cells are treated with inflammatory mediators known to increase endothelial permeability, and then fixing the cells and labeling F-actin for microscopic observation. These studies have missed essential information on the dynamic nature of the endothelial barrier under resting conditions. Recent live-cell imaging allows incorporation of the dynamic nature of the endothelial barrier into the studies of the mechanisms determining endothelial barrier integrity [13, 16]. Time-lapse images of fluorescently-labeled proteins in monolayers of confluent endothelial cells indicate that there exists time-dependent changes in endothelial barrier integrity.

These studies not only provide important information on processes involved both in endothelial barrier disruption and restoration under inflammatory conditions, but also highlight the dynamic nature of the maintenance of endothelial barrier integrity [13]. A remarkable feature of a quiescent endothelial monolayer is the rapid opening and closure of interendothelial gaps (see Fig. 1). Local lamellipodia all along the edges of endothelial cells are forming and regressing all the time in both nonconfluent and confluent cell monolayers.

In the quiescent endothelium, VE-cadherin-based junctions are subjected to continuous reorganization, which renders endothelial junctions highly dynamic and sensitive to extracellular stimuli. In fact, during the process of new vessel formation, endothelial cells undergo dynamic rearrangement upon angiogenic stimuli while continuously and simultaneously reorganizing cell–cell junctions and maintaining barrier function. This coordination is largely regulated by adhesion molecules at intercellular junctions and is particularly important for tube stabilization and maintenance or even restoration of the full barrier function. These subtle changes of the morphology at the cellular periphery are known as micromotion.

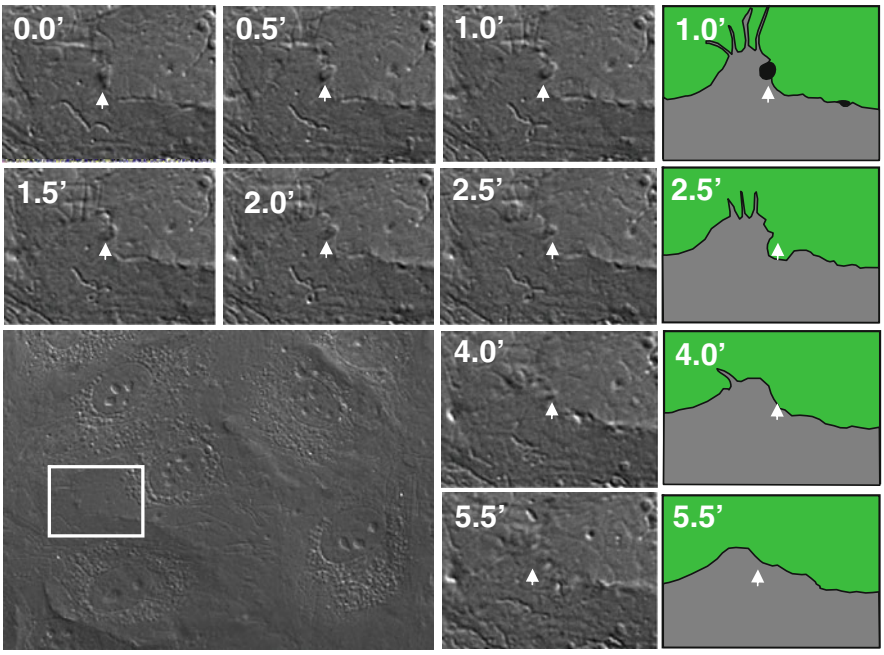


**Fig. 1** Spontaneous closure of inter-endothelial gaps. Differential interference contrast (*DIC*) time-lapse imaging shows the transient nature of spontaneous gaps in post-confluent endothelial cells. For identification, outlines of individual endothelial cells are shown in the images at the *right*

Micromotion can be detected by electrical cell impedance sensing or ECIS technology, and is reflected by the 'biological' noise or fluctuations in the impedance signal [17, 18]. As can be seen in Fig. 2, micromotion of resting endothelial monolayers (the noise) is largely dependent on activity of Rho kinase, a downstream target of RhoA signalling.

Thus, junction proteins play a critical role in controlling vascular integrity both in developing and existing vessels. Among these, VE-cadherin is crucial for this regulation for its capability to remodel the F-actin cytoskeleton via modifying the function of small GTPases of the Rho family (see below) [11].

## *3.1 Regulation by Rho GTPases*

Initial attention for Rho GTPases was drawn to RhoA activation and its role in cell contraction and endothelial hyperpermeability [9, 10, 19] Afterwards, the focus has moved to the role of Rac and Cdc42 in the assembly and stability of inter-endothelial junctions [14, 20]. More recently, we identified a role for RhoA and its effector Rho kinase in barrier maintenance [21].

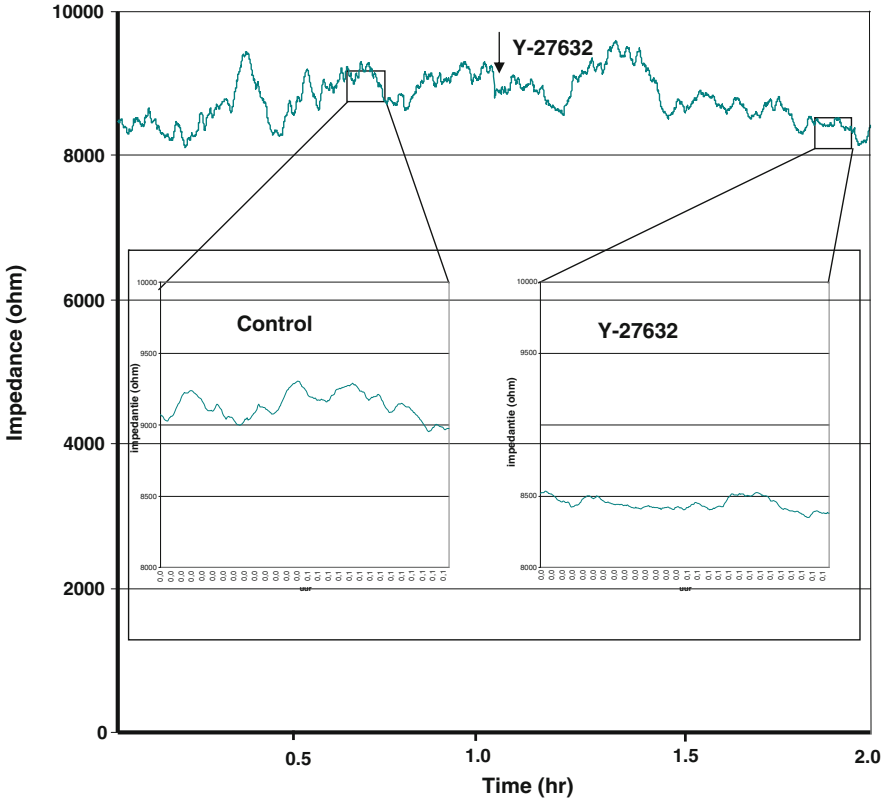


**Fig. 2** Endothelial micromotion is Rho kinase-dependent. Impedance analysis of confluent endothelial monolayers upon addition the Rho kinase inhibitor Y-27632 (10 μM). Cells were inoculated into electrode-containing wells and allowed to develop into confluent layers for 72 h. At the time indicated by the *arrow*, the Y-27632 was added, and the resultant changes in impedance were followed. Data were collected every second. Note that both the micromotion (biological 'noise') decreases upon addition Y-27632

Much attention has been given to the regulation of AJs and their pivotal protein VE-cadherin, a membrane spanning protein that forms homotypic interactions between adjacent endothelial cells. The intracellular part of VE-cadherin is connected to the F-actin cytoskeleton via both $\alpha$-and $\beta$-catenin. In addition, a third catenin, p120catenin (p120ctn), binds to VE-cadherin. P120ctn binding to VE-cadherin is an important step in the regulation of AJ stabilisation as well as in Rho GTPase regulation. Rac1 and Cdc42 are activated at sites where junctional complexes are formed, while RhoA activity is downregulated when the monolayer reaches confluence. Furthermore, many GTPase regulator activities are directly initiated by cadherin engagement [22, 23]. Thus, a precise spatial and temporal fine-tuning of the activity of Rho family GTPases is critically important in the establishment and maintenance of junctions.

Concerning basal endothelial barrier regulation, much attention has been given to the relationship between VE-cadherin, the catenins and Rho GTPases. After cadherin engagement p120ctn binding activates Vav2, a GEF for Cdc42 and Rac1. This GEF positively regulates Cdc42 and Rac1 activity. Both GTPases bind IQGAP (IQ motif containing GTPase-activating protein 1) [24, 25]. Unlike the name suggests IQGAP1 does not have GAP activity, meaning that it does not enhance the GTP hydrolytic activity of the GTPases. Instead its binding to Cdc42 and Rac1 inhibits their intrinsic GTPase activity, thus keeping them in their active, GTP-bound state. By recruiting IQGAP1, Rac1 and Cdc42 inhibit the interaction of IQGAP1 with $\beta$-catenin thereby initiating the binding of $\alpha$- and $\beta$-catenin. This cross-links the complex with the actin cytoskeleton and stabilises the AJ [25]. In contrast, inactivation of Rac1 and Cdc42 causes dissociation from IQGAP1 that then binds to $\beta$-catenin at the AJ complex. As a result $\alpha$- and $\beta$-catenin dissociate, leading to weaker adhesion and inter-endothelial gap formation [26].

Another AJ protein is the endothelial-specific HEG (heart of glass) transmembrane receptor, which interacts with a currently unknown extracellular ligand to activate a coordinated signalling response through inhibiting the activity of RhoA. This response requires the cerebral cavernous malformations proteins CCM1 (or KRIT1), CCM2 and CCM3 and leads to a cellular response that may include induction of endothelial vacuolisation and homotypic endothelial cell-to-cell junction formation. These cellular events are required for normal vascular development and maintenance of vascular integrity. Defects in these steps result in enhanced activation of RhoA and may lead to the pathological changes associated with hereditary cerebral cavernous malformations in humans [27, 28].

In conclusion, the maintenance of the endothelial barrier depends on RhoA, Cdc42 and Rac1 activation. RhoA activity decreases upon gap closure due to VE-cadherin and p120ctn engagement. The binding of the AJs to the actin cytoskeleton involves Cdc42, which is kept in its active state by binding to IQGAP1. Activation of Epac and RapGEF2 stimulate Rap1, which via Vav2 activates Rac1. Rac1 activation results in the inhibition of p115RhoGEF and thus a suppression of RhoA. Vav2 activity is further enhanced by p120ctn binding to the junctional complexes. The latter also directly activates p190RhoGAP thereby further inhibiting RhoA activity [26].

## *3.2 Regulation by Caveolae*

A close interrelationship between paracellular and transcellular pathways has been surmised for a long time, but was not well understood. A coupling between caveolae-mediated endocytosis to increased junctional permeability was indicated by the finding that loss of caveolin-1 results in not only the disappearance of caveolar structures but also destabilization of inter-endothelial junctions and formation of inter-endothelial gaps [29]. Recently more direct evidence has been obtained [30, 31]. Paracellular permeability of the endothelium is finely regulated by

constitutive caveolin-1–dependent inhibition of eNOS activity. Deficiency in caveolin-1 or activation of eNOS results in nitration of p190RhoGAP and the concomitant activation of RhoA, which induces adherens junction destabilization and increased endothelial permeability. Because of the causal relationship of eNOS-specific signaling to the impairment of p190RhoGAP activity, this mechanism induces RhoA activation in endothelial cells [30].

Taken together, these data indicate that caveolin-1 mediated signaling is critical in mediating the increased permeability of the vascular junctional endothelial barrier secondary to AJ disassembly; i.e., there is signaling cross-talk between caveolae-mediated endocytosis and junctional permeability.

## *3.3 Regulation by Soluble Mediators*

Many vaso-active agents are known to cause plasma leakage by increasing vascular permeability, but only a few soluble mediators are known to possess the opposite characteristics. Currently known endogenous barrier improving molecules include sphingosine 1-phosphate (S1P), adrenomedullin, Angiopoietin-1 (Ang1), Fibroblast Growth Factor (FGF), nitric oxide and adenosine [32]. Therapeutic efforts to enhance their function hold promise for the future because those compounds will likely be exploited in their ability to induce recovery of disturbed barrier functions and to act as prophylactic in stabilizing vascular barriers. The most prominent soluble mediators of endothelial barrier maintenance are discussed below.

### 3.3.1 Angiopoietins

The angiopoietin-Tie2 signaling system plays a crucial role in regulating endothelial barrier maintenance [33–35]. Ang-1 is a ligand of the endothelial cell receptor Tie2, and activation of Tie2 signaling enhances endothelial cell barrier integrity and endothelial-pericyte interaction, thereby promoting vascular stabilization. While expression of Tie2 is largely specific to the endothelium, Ang-1 production by mural and perivascular cells facilitates basal Tie2 signaling in quiescent endothelial cells that, in turn, is required for endothelial homeostasis. In contrast, Ang-2, produced and stored in Weibel-Palade bodies in endothelial cells, normally functions as an Ang-1 antagonist. Ang-2 destabilizes the quiescent vasculature and activates endothelial cells to respond to angiogenic stimuli [33]. Overexpression of Ang-2 in the mouse endothelium attenuates physiological Tie2 signaling and thus increases vascular permeability, suggesting that Ang-2 inhibits Tie2 signaling and counteracts the action of Ang-1 [34]. Consistent with these experimental data, circulating Ang-2 is related to vascular permeability and pulmonary dysfunction in critically ill patients [34].

### 3.3.2 Fibroblast Growth Factor

FGF signaling is directly required for maintenance of inter-endothelial adhesion. Suppression of FGF signalling leds to the dissociation of junctional adhesions in both arterial and venous vascular beds [36]. Murakami et al. [37] found that stimulation of endothelial monolayers with FGF1 increased p120- catenin-VE-cadherin interaction, whereas inhibition of FGF signaling induced uncoupling of p120-catenin from VE-cadherin, leading to VE-cadherin internalization [37]. The association of p120-catenin at the VE-cadherin juxtamembrane domain is known to inhibit VE-cadherin internalization by interfering with its binding to adaptor proteins of the clathrin-mediated endocytic pathway [38]. Therefore, the loss of endothelial barrier integrity in the absence of FGF signalling could be explained by destabilization of VE-cadherin homophilic adhesion and subsequent dissociation of AJs.

### 3.3.3 Sphingosine-1-Phosphate (S1P)

S1P, a sphingolipid metabolite found in high concentrations in platelets and blood, is a lipid mediator that interacts with GPCRs (S1P1–S1P5) to induce a variety of biological responses [39]. It is also able to enhance endothelial barrier integrity through S1P1 receptor (Edg1) signaling by promoting Rac1 activation and adherens junction assembly [40]. In confluent human umbilical vein endothelial cells, S1P significantly increases the abundance of VE-cadherin and ß-catenin at the cell–cell contact regions and enhances AJ assembly [41]. Administration of the S1P receptor agonist FTY720 in vivo potently blocks VEGF-induced vascular permeability, suggesting that S1P receptor on endothelial cells is able to regulate vascular permeability [42]. Furthermore, the S1P1 receptor is essential for normal vascular function since systemic antagonism of S1P1 receptor under basal physiological conditions causes S1P1 receptor internalization and degradation through receptor phosphorylation, leading to enhanced pulmonary vascular leakage.

## *3.4 Regulation by the Local Microenvironment*

There is growing recognition that—in addition to chemical factors such as vasoactive agents and adhesion molecules residing in the extracellular matrix (ECM)—mechanical factors, such as applied forces or the rigidity of the ECM, are able to crucially direct the form and function of cells in general and of vascular cells in particular [43, 44]. Consequently these mechanical factors modulate blood vessel morphology and function. Whereas the effects of shear and stretch forces have been studied in detail, remarkably less is known about the role of ECM rigidity in regulation of vascular barrier integrity. This is even more surprising, as the profound effects of stiffening of the vascular wall as a major cause of cardiovascular

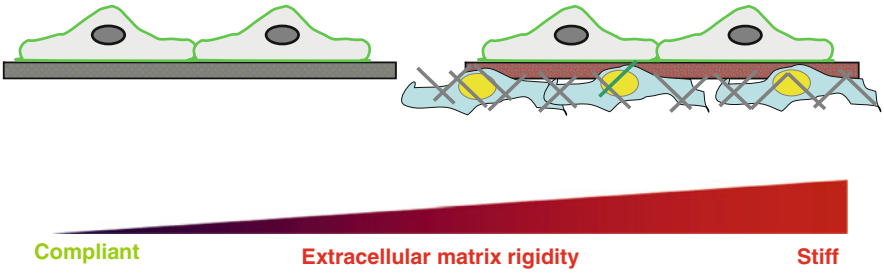


**Fig. 3** Blood vessels stiffen as a consequence of aging, diabetes, atherosclerosis, and ischemic heart diseases. A variety of mechanisms accounts for stiffening of the vessel wall, including cross-linking of matrix components, collagen deposition, increased pericyte coverage and atherosclerotic plaque formation. In addition, increased interstitial pressure as well as (periodically) stretching the vessel wall will result in an increased effective stiffness as sensed by the endothelial cells (*green outlining*)

degeneration have been appreciated for a long time [45]. Blood vessels stiffen as a consequence of aging, diabetes, atherosclerosis, and ischemic heart diseases. A variety of mechanisms accounts for stiffening of the vessel wall, including cross-linking of matrix components [46], collagen deposition, increased pericyte coverage [47] and atherosclerotic plaque formation (Fig. 3) [45]. In addition, increased interstitial pressure as well as (periodically) stretching the vessel wall will result in an increased effective stiffness as sensed by the endothelial cells. Recent data suggest a strong interaction between vascular wall stiffness and vascular permeability [3, 48]. The implications of vascular wall stiffening for other vascular functions such as angiogenesis as will be covered in detail by Califano and Reinhart-King elsewhere in this book.

In response to substrate stiffening, endothelial cells in culture modify their migration, morphology, spreading, and growth [49]. By virtue of these well known effects, substrate stiffness is recognized as a powerful determinant of endothelial form and function [44]. A key event in rigidity sensing is the modulation of cellular contractility. Cells on soft materials exert lower forces than cells on stiff materials, decreasing tension on force-bearing elements (Fig. 4) [44]. Through an as yet partially elucidated mechanism, stiffening results in elevated activity levels of the small GTPase RhoA, mediating enhanced contractile forces [3]. These responses are mediated by load-bearing subcellular structures, such as the cell-adhesion complexes and the cytoskeleton. Recent work has demonstrated that these structures are dynamic, undergoing assembly, disassembly [43]. Several studies have shown that cell–cell contacts bear considerable forces [50, 51] and, undergo dynamic, myosin-dependent elongation [52]. Applied forces [50] and stiffer substrata [53] enhance cell–cell contact assembly, indicating that these adhesions also undergo force-dependent adhesion strengthening. There is also evidence for actin flow along cell–cell contacts [54]. Vinculin is recruited to the junctions in a myosin-dependent manner, thereby contributing to adhesion strengthening [55]. Finally, a direct relationship between the total cellular traction

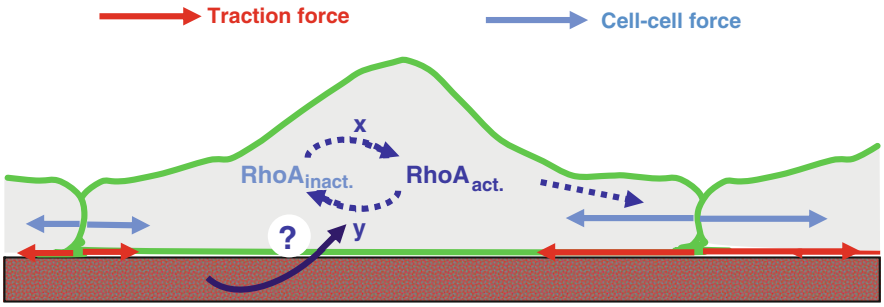


**Fig. 4** Stiffening of the extracellular matrix induces an increased tension in endothelial intercellular junctions. In endothelial monolayers forces are exerted at cell–matrix (traction forces, indicated in *red*) and at cell–cell interactions (cell–cell forces indicated in *blue*). The intercellular forces account for nearly one-half of the overall forces in the monolayer. These forces are enhanced substantially with substrate stiffening. Through an as yet partially elucidated mechanism[3, 48, 51], stiffening results in elevated activity levels of the small GTPase RhoA, mediating enhanced contractile forces. The nature of the guanine exchange factors (*GEFs*) and GTPase activating proteins (*GAPs*) remains to be identified

force on the ECM and the endogenous cell–cell force exists, such that the cell–cell tension is a constant fraction of the cell-ECM traction [3, 51].

Recent data indicate that stiffening of the endothelial microenvironment has a profound role in regulation of endothelial monolayer integrity. Stiffening of the extracellular matrix makes the endothelium more susceptible to hyperpermeability responses (Fig. 5). In response to thrombin on a compliant matrix, endothelial cells comprising the monolayer contract collectively, intercellular gaps do not form, and the monolayer stays intact. In response to thrombin on stiffer matrices, cells comprising the monolayer contract individually, large gaps arise between adjacent cells, and the monolayer becomes severely disrupted. These disruptive effects on stiffer substrates are promoted by larger physical force magnitudes [3]. Moreover, stiffening of extracellular matrix within the intima promotes endothelial monolayer permeability [48]. Similarly, the transendothelial transmigration of leukocytes also increased with increasing ECM stiffness suggesting similar underlying mechanisms [56]. A finding of particular interest is that these substrate stiffness-dependent effects are mediated by basal force differences in the endothelial monolayer.

In line with these findings, Ohayon et al. [57] demonstrated that, in addition to low endothelial shear stress and cyclic stretch, enhanced arterial wall stiffness may be a precondition for the initiation and development of atherosclerosis. These authors investigated the contribution of a contracting myocardium to local rigidity of the coronary arteries. It is well-known that elasticity of the coronary arterial wall is highly nonlinear, since a small variation in stretch induces a large increase in stress and wall stiffness. In this study very heterogeneous arterial wall stiffness was observed, with specific regions of distinct rigidity, originating from the axial distortion of the arterial wall caused by a contracting myocardium. Of particular

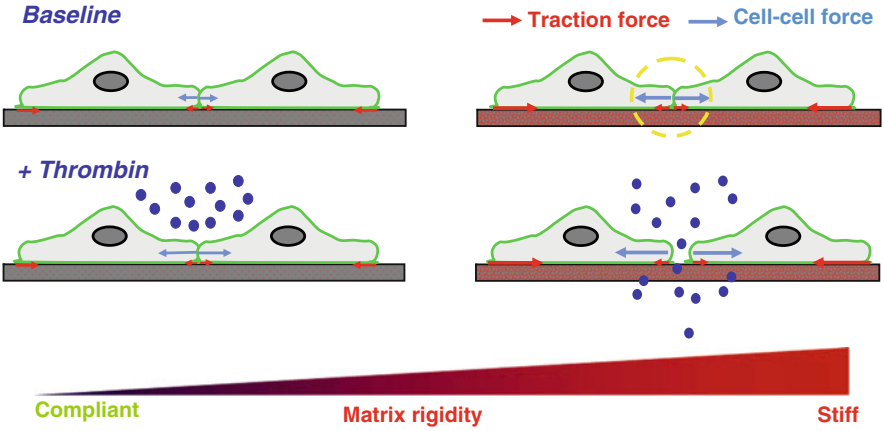


**Fig. 5** Stiffening of the extracellular matrix makes the endothelium more susceptible to hyperpermeability responses. In response to thrombin on a compliant matrix, endothelial cells comprising the monolayer contract collectively, intercellular gaps do not form, and the monolayer stays intact. In response to thrombin on stiffer matrices, cells comprising the monolayer contract individually, large gaps arise between adjacent cells, and the monolayer becomes severely disrupted. These disruptive effects on stiffer substrates are promoted by larger physical force magnitudes [3]

interest, these sites of high rigidity coincided with clinically identified atherosclerotic plaques. It is tempting to speculate that increased endothelial permeability induced by vascular wall stiffening at high-risk zones would play a central role in atherosclerotic lesion and plaque development, since leaky junctions within the endothelial monolayer contribute to LDL accumulation in the vessel wall [58].

Whether similar effects are applicable in diseases that involve progressive stiffening of the endothelial extracellular matrix, including acute lung injury, hypertension, and cancer, remains to be studied.

## 4 Conclusions and Perspectives

Originally thought to be a cellophane-like passive barrier lining all blood vessels, currently the endothelium is well-appreciated to act as an active barrier. A continuous remodelling of the junctions is ongoing, providing the endothelium with an enormous plasticity.

It has also become apparent that the maintenance of an existing barrier requires active cellular signaling which shares common features with signaling mechanisms involved in the barrier maturation process of new vessel formation [59]. The maintenance of the endothelial barrier depends on RhoA, Cdc42 and Rac1 activation. RhoA activity decreases upon gap closure due to VE-cadherin and p120ctn engagement. The binding of the AJs to the actin cytoskeleton involves Cdc42,

which is kept in its active state by binding to IQGAP1. In addition, cadherin engagement induces the activation of RapGEF2. This stimulates Rap1, which via Vav2 activates Rac1. Rac1 activation results in the inhibition of p115RhoGEF and thus a suppression of RhoA. Vav2 activity is further enhanced by p120ctn binding to the junctional complexes. The latter also directly activates p190RhoGAP thereby further inhibiting RhoA activity [26]. This signalling is controlled by circulating barrier promoting factors such as FGF, S1P and Ang1.

Recent data indicate that stiffening of the vascular wall might destabilize the endothelial barrier. Modulation of ECM properties bare impact on cell-ECM traction forces, consequently altering cell–cell tension. This relationship between ECM stiffness and endothelial permeability suggests that differences in local tissue compliance might contribute to differences in permeability observed in different parts of the vascular bed, such as seen in the lung (and brain) of which the tissue is soft, and where the barrier is tighter than at other places.

Moreover, it remains to be tested whether destiffening strategies would provide an effective means to combat diseases where vascular stiffening and vascular leak go hand in hand. Pharmacological treatment of stiffness of the vascular wall appears impossible. Attempts have been made with various drugs, including advanced glycation end-product crosslinking breakers, aimed to restore elasticity to the disorganized elastic fibers of the thoracic aorta [45, 46]. However, there is presently little evidence that these agents actually break pre-existing cross-links in vivo. Conversely, drugs currently used to treat arterial stiffness have effects on muscular arteries, and these dilate by $\geq$20 % with relatively small doses, but do not affect elastic arteries such as the proximal aorta and carotid artery. As therapies aimed at vasodilation do not directly target the structural composition of the vasculature, it is to be expected that they will have limited value in improving the integrity of the vascular barrier. Preventing the endothelial cells from responding to the stiffening through lowering of basal traction forces, and thus lowering of junctional tension, might provide a more promising approach to stabilize the vascular barrier in a stiffening microenvironment.

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# Computational Models of Vascularization and Therapy in Tumor Growth

**Benjamin Ribba, Floriane Lignet and Luigi Preziosi**

**Abstract** Computational and mathematical models are powerful tools to study the complexity in biological systems. The models, when validated with experimental evidence, can then be used to better understand the behavior of a complex system subjected to perturbations. In particular, a computational model can be used to test new hypotheses and, in the case of therapies for instance, to predict and optimize treatment outcomes in patients. Most models in biology rely on the description, using continuous or discrete mathematical tools, of the time-course of one or several biological entities. Its aim is to 'capture' the dynamics of a process, which by definition evolve in time. Almost all biological processes are characterized by a particular dynamic. Computational modeling relies on the premise that integrating the dynamics of a process can provide benefits in its understanding compared to a classical static analysis.

## 1 General Concepts

Computational and mathematical models are powerful tools to study the complexity in biological systems. The models, when validated with experimental evidence, can then be used to better understand the behavior of a complex system subjected to perturbations. In particular, a computational model can be used to test

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new hypotheses and, in the case of therapies for instance, to predict and optimize treatment outcomes in patients.

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An illustrative example is the successful use of changes in hematological variables on a continuous scale for assessing anticancer drug toxicity in early phase clinical trials. To appreciate the amplitude of myelosuppression following the administration of chemotherapy, clinicians used to consider the nadir (minimum) value of neutrophils or leukocytes. But in doing so, relevant information regarding the time course of hematological variables and duration of neutropenia was wasted. A predictive model of the time-course of leukocyte and neutrophil counts was developed and can be used for optimizing the design of clinical trials in oncology [1].

Often, however, the modeler faces some difficulties accessing data since experimentalists may sometimes omit the time factor when studying a biological process. As already stated, in the example of myelosuppression, the minimal value of neutrophil counts assessed at a given time was considered to evaluate the toxicity induced by the drug. To give a parallel example, the efficacy of anticancer drugs is still nowadays appreciated in early phase clinical trials by measuring the change in tumor size before and at a given time point after treatment. This is the base of the Response Evaluation Criteria in Solid Tumors (RECIST) [2, 3]. In preclinical experiments also, biologists often analyze the change of a biological marker at a given time point and not in a dynamic way. These limitations can be however, explained by the cost and time required for repeated measurements (using repeated MRI for instance to assess tumor size evolution during treatment).

More generally, in the drug development process, the development of computational models can facilitate the continuous integration of available information related to a drug or disease in order to describe and predict the behavior of studied systems and to address questions researchers, regulators and public health care bodies face when bringing drugs to patients.

The aim of the present chapter is to provide a partial and short overview of modeling efforts in the study of the process of angiogenesis and tumor growth. Models range from simple formalisms describing the time-course of a morphological variable, such as the tumor volume, to the description of molecular signaling pathways relevant to the process of angiogenesis.

## 2 Simple Computational Modeling in Oncology

The modeling of tumor growth started in 1964 when tumor cells in vitro were shown to grow following Gompertzian kinetics [4].

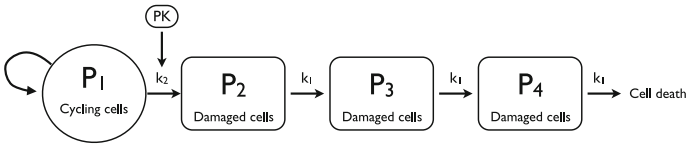


**Fig. 1** Illustration of the modified-Gompertz model that was used to describe untreated and treated xenograft tumors in animal models. Cycling cells become damaged as a function of the chemotherapy concentration. Damaged cells finally die with a time delay represented by a transit compartment. Adapted from [10]

The proposed Gompertz model was written as follows:

$$w(t) = w_0 e^{\frac{A}{\alpha}(1-e^{-\alpha t})}$$

where $w$ denotes the number of tumor cells, $w_0$ the initial number of tumor cells also called baseline tumor size, and $A$ and $\alpha$ two positive constants.

In 1970, the Gompertz model was successfully used to describe tumor growth in animal models [5]. The Gompertz model was successively applied to clinical data in 1972 with IgG multiple myeloma patients [6], and in 1976, Norton and Simon generalized the use of the Gompertz law for several ranges of solid tumors [7, 8]. The proposed model was at the basis of the so-called Norton-Simon hypothesis according to which increasing the dose intensity might lead to the optimization of treatment effects by reducing tumor regrowth between chemotherapy cycles. In 2003, this hypothesis reached validation in clinical trials [9].

In 2004, a model was proposed to integrate, with high accuracy, the effect of chemotherapy into the Gompertz modeling framework and to analyze the time-course of untreated and treated tumors in mice models [10]. In this study, a modification of the Gompertz model is proposed to remove the final plateau phase of the Gompertz equation. The equation for the so-called modified-Gompertz model:

$$\frac{dw(t)}{dt} = \frac{\lambda_0 w(t)}{\left[1 + \left(\frac{\lambda_0}{\lambda_1} w(t)\right)^{\psi}\right]^{\frac{1}{\psi}}}$$

where $w(t)$ denotes the tumor mass, and $\lambda_0$ and $\lambda_1$ are two positive constants regulating the growth in the initial exponential growth and in the linear phase respectively. $\psi$ is a constant parameter fixed to a high value (typically $\psi = 20$). In doing so, when $w$ is small, the model reproduces an exponential law, i.e. $\frac{dw}{dt} \approx \lambda_0 w$, while the growth tends to be linear, i.e. $\frac{dw}{dt} \approx \lambda_1$, while the tumor mass gets larger than the ratio $\frac{\lambda_1}{\lambda_0}$.

The previous tumor growth model was successfully integrated into a PK/PD framework based on transit effect compartments to reproduce both untreated and treated tumors with several therapeutic agents such as Irinotecan, 5FU and paclitaxel. Figure 1 shows an illustration of the model integrating the transit effect compartments.

Translation of those modeling attempts into clinical oncology have been limited so far mainly due to the lack of longitudinal data associated with clinical trials of new drugs in oncology. In particular, it is often not possible, due to ethical reasons, to find data for untreated patients. Nevertheless, very recently, models have been proposed to analyze tumor size dynamics in patients.

The analysis of longitudinal tumor size in non-small cell lung cancer (NSCLC) led to the development of a simple model combining linear growth and exponential decay as a function of drug concentration [11]. The model was successfully applied to four randomized clinical trials for NSCLC treatments that were submitted for registration: bevacizumab, docetaxel, elotinib and pemetrexed. One of the main results of this study was that the change in tumor size observed 7 weeks after treatment initiation was a better parameter to predict survival than the classical RECIST criteria. This study clearly demonstrates the interest of using a dynamic approach for evaluating the efficacy of anticancer drugs; and the potential benefit for forthcoming clinical trials is relatively similar to the results obtained with the model for myelosuppression [1]. To illustrate however, the issue of data availability previously mentioned, the analysis in [11] was carried out on more than 2,000 patients and still, to model tumor growth, a linear model (one parameter) was used. Other similar examples have been published. The reader can refer in particular to [12] and [13]. The last reference deals with tumor dynamic analysis in colorectal cancer patients.

Finally, it is worthwhile to mention a similar work where the objective was to analyze tumor growth kinetics in patients with metastatic renal cancer (mRCC) and gastrointestinal stromal tumors (GIST) treated by the anti-angiogenic compound Sunitinib [14].

So far, we have discussed models based on ordinary differential equations where the only considered variable of the system is time. It is worthwhile to mention here that several models have been proposed to integrate time together with space in the description of brain tumor growth and treatment. The proposed models describe the spatio-temporal evolution of tumor cells in the brain as "traveling waves" driven by two processes: uncontrolled proliferation and tissue invasion [15]. This proliferation-invasion description led to the suggestion that tumor diameter grows linearly over time with a velocity given by a combination of the two model parameters [16]. Swanson and colleagues showed the relevance of such a model for prediction of untreated glioma growth kinetics, specifically estimating net rates of proliferation and invasion for individual patients in vivo [17, 18]. These parameters were shown to be significant prognostic factors of therapy efficacy [19] and durations of survival [20, 21]. Mandonnet and colleagues studied the reliability of this model in determining low-grade gliomas dynamics [22–25] and showed it to be in agreement with the linear evolution of the mean tumor diameter observed in these tumors before transformation towards a higher grade of malignancy. The same model has been recently extended to integrate the process of angiogenesis for high grade gliomas [26].

In the following years, more and more models will continue to be developed addressing different cancer indications and different treatments such as targeted

therapy or radiotherapy. With the integration of more informative data (such as information for functional imaging in addition to morphological data), the newly developed models will also become more complex, integrating in processes that are important to tumor dynamics such as cell cycle regulation and angiogenesis.

## 3 Models of Tumor Growth and Angiogenesis

An extension of the Gompertz model that includes the process of angiogenesis was proposed in 1999 [27]. In this paper, the authors expanded the Gompertz model to incorporate the process of angiogenesis using a new variable, called "carrying capacity" to account for tumor vascularization. The proposed model is as follows:

$$\frac{dw}{dt} = -\lambda_1 w \, log\left(\frac{w}{k}\right)$$

$$\frac{dk}{dt} = -\lambda_2 k + bS(w,k) - dI(w,k) - ekg,$$

where $w$ denotes the tumor volume and $k$ denotes the carrying capacity. The first equation describes the Gompertz growth of the tumor volume. The growth saturates when $w$ reaches $k$. The evolution of $k$ depends on the process of angiogenesis. It is regulated by a positive 'pro-angiogenic' term denoted $S$, and a negative 'anti-angiogenic' term denoted by $I$. Two additional terms account for natural decay (at rate $\lambda_2$) and eventual degradation due to the effect of anti-angiogenic drugs, where exposure is denoted $g(t)$.

The authors showed that the model successfully predicts tumor growth inhibition by several anti-angiogenic compounds in mice models. Interestingly, this model has been subjected to mathematical analysis to study its main properties that highlighting the best strategies to optimize the delivery of anti-angiogenic drugs [28–32].

In 2011, a more complex model of tumor growth was proposed accounting for different types of tumor tissue, with a specific focus on hypoxic tissue that is known to play a crucial role in tumor angiogenesis [33]. The main innovation of this model is the integration, together with tumor size, of classical histological biomarkers such as those commonly retrieved in preclinical studies. This model integrates three types of tissue (proliferative non-hypoxic, hypoxic, necrotic) and is based on the following hypothesis: as the tumor grows, oxygen tends to lack within the spheroid and drives the formation of hypoxic tissue. Hypoxic tissues become in turn necrotic with a constant transfer rate. Depending on the whole tumor size, the carrying capacity increases as a result of the process of angiogenesis.

The model shows correct predictions of tumor size progression as well as the percentages of necrotic and hypoxic tissue in 30 mice that were xenografted with either HT29 or HCT116 colorectal cancer cell lines [34]. The diagram of the model is presented Fig. 2.

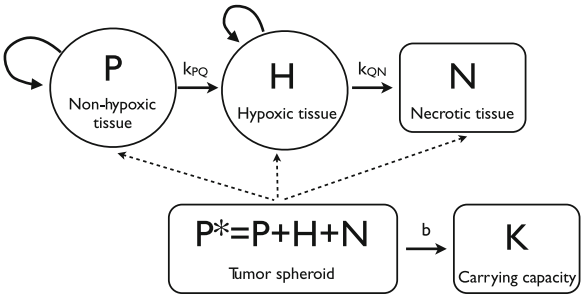


**Fig. 2** Illustration of the tumor growth angiogenesis model that was used to describe untreated xenograft tumors in animal models by combining tumor size measurements together with histological markers such as the percentage of hypoxic tissue. Adapted from [34]

The model is written as follows:

$$\frac{dP}{dt} = \lambda_P P(1 - s^\alpha) - k_{PH} P s^\alpha$$
$$\frac{dH}{dt} = k_{PH} P s^\alpha + \lambda_H H(1 - s^\alpha) - k_{HN} H$$
$$\frac{dN}{dt} = k_{HN} H$$
$$\frac{dK}{dt} = bP^* \text{ where } P^* = P + H + N \text{ and } s = \frac{P^*}{K}$$

$P^*$ denotes the tumor mean diameter as measured in mice and $s$ the hypoxic stress regulating the logistic growth of the non-hypoxic $(P)$ and hypoxic tissue $(H)$ and modulating the transfer between these two types of tissue. N denotes the necrotic tissue. The exponent $\alpha$ was fixed to a low value ($<1$) for which the logistic proliferation term tends to the Gompertz equation as used to model tumor growth in animal models [10, 27]. $K$ stands for the carrying capacity as in [27] and is here described by a simple growing function, as a function of the tumor size.

## 4 Multiscale Approach for Modeling Vascular Tumor Growth

To treat with model complexity, researchers have also put efforts on the development of 'multiscale' models. The aim of multiscale modeling is to account, in a single computational model, for several biological processes occurring at different space and time scales. Conceptually, this is a very promising approach but practically, many issues must be addressed. In particular, no real methodology has been proposed to assess validation and qualification aspects of those models. Nevertheless, it is believed that the development of multiscale models can provide the basis for an integrative holistic approach to predict drug response and effect [35].

Most of the mathematical multiscale models developed so far are theoretical attempts and none of them showed yet benefit for drug development. The reader

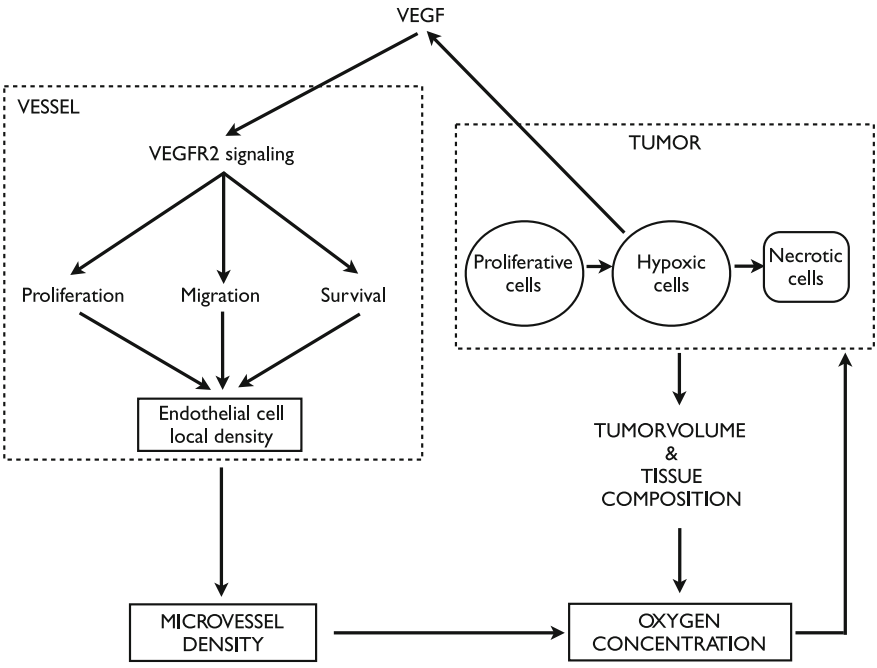


**Fig. 3** Illustration of the multiscale tumor growth and angiogenesis model integrating tumor regulation box and VEGF signaling-endothelial cells compartment. Adapted from [38]

can for instance refer to [36, 37]. These models incorporate a large number of parameters that do not have a biological meaning or are hardly measured experimentally, making model calibration a challenging issue. Finally, the models we are going to discuss in this paragraph are continuous model as they describe the evolution of continuous variable such as tumor size and density of blood vessels or endothelial cells. Many multiscale models are developed using discrete formalisms focusing on individual cell behaviors and integrating a population of cells to constitute a whole vascularized virtual tumor. Those aspects were previously discussed in the chapter by Boas et al.

We will shortly describe a model based on a set of partial differential equations that describe the behavior of endothelial cells, which constitute blood vessel walls, tumor cells, as well as of some major proangiogenic substances, such as the vascular endothelial growth factor (VEGF). This model can be viewed as an assembly of two major pieces that occur at different spatial scales. The first one is similar to the model presented in Fig. 2: it describes the regulation of tumor tissue as proliferative, quiescent and necrotic tissue. Hypoxic tumor cells are assumed to secrete VEGF, which in turns will bind the VEGFR2 receptors of endothelial cells constituting the vessels. The second one is the dynamics of the vascular network where the endothelial cell behavior, namely proliferation, migration and survival, is driven by intracellular signaling of VEGFR2 receptor. The proposed model consists of 11 equations and 45

parameters. Note that only a part of the parameters were found in the literature, the others were fixed arbitrarily. A schematic view of the model is shown Fig. 3.

Another example we would like to mention is the series of paper published by Agur and coworkers. The author proposed a complex model to describe the process of angiogenesis [39]. In this model, a particular attention is given to the modeling hypothesis based on experimental evidences provided by Suri and Maisonpierre, in particular, regarding the role of angiopoietin 1 and 2 [40, 41]. The authors have performed a validation study in comparing model predictions to observations in animal experiments [42]. Another validation study was performed in clinical data for mesenchymal patients [43].

The concept of multiscale modeling is indeed very appealing as it promises a framework to integrate models develop at different scales. However, the main problem relies in the excessive number of parameters they integrate for which it is not possible to perform one unique experiment. As an example, one cannot expect to measure molecular reaction rates and tissue dynamics in the same experimental set-up. So, the only possibility to cope with this problem is to couple pieces of models, carefully validated, together a modular structure. However, this modular framework does not go without important methodological problems and limitations that must be addressed. For instance, how to correctly balance a priori information (coming from literature for instance) and the data-driven knowledge? How to efficiently include or search for a priori information? This may require the development of sharing and re-use resources among the community. How to assess the validation of such piece-make models? And how to correctly 'jump' from one scale to another?

## 5 Specific Models for Intracellular and Intercellular Aspects of Angiogenesis

*a. Computational model of the dynamic of VEGFR2 signaling pathway*

Another important aspects for the development of relevant multiscale model of angiogenesis is the integration of intracellular mechanisms. The aim of this paragraph is to provide an example on a model targeting this level of description.

The Vascular Endothelial Growth Factor (VEGF) is a key molecule in angiogenesis, since it stimulates the migration, proliferation and survival of endothelial cells [44, 45]. The signaling pathways downstream its main receptor, the VEGFR-2 is well described in the literature [46, 47], and we can find mathematical models of the binding of VEGF to its receptor. For example, Alarcòn and coworkers have developed a stochastic model of the receptor dimerization and activation [48]. However, none of those models goes further than the binding of VEGF to its receptor.

A global view and comprehension of the intracellular pathways of VEGFR2 can be achieved based on information available in recent literature and specialized databases. Tools such as PubMed and Kyoto Encyclopedia for Genes and Genomes (KEGG1) are important to achieve this goal. Figure 4 shows a diagram of the VEGFR2 intracellular signaling pathways built based on the available information.

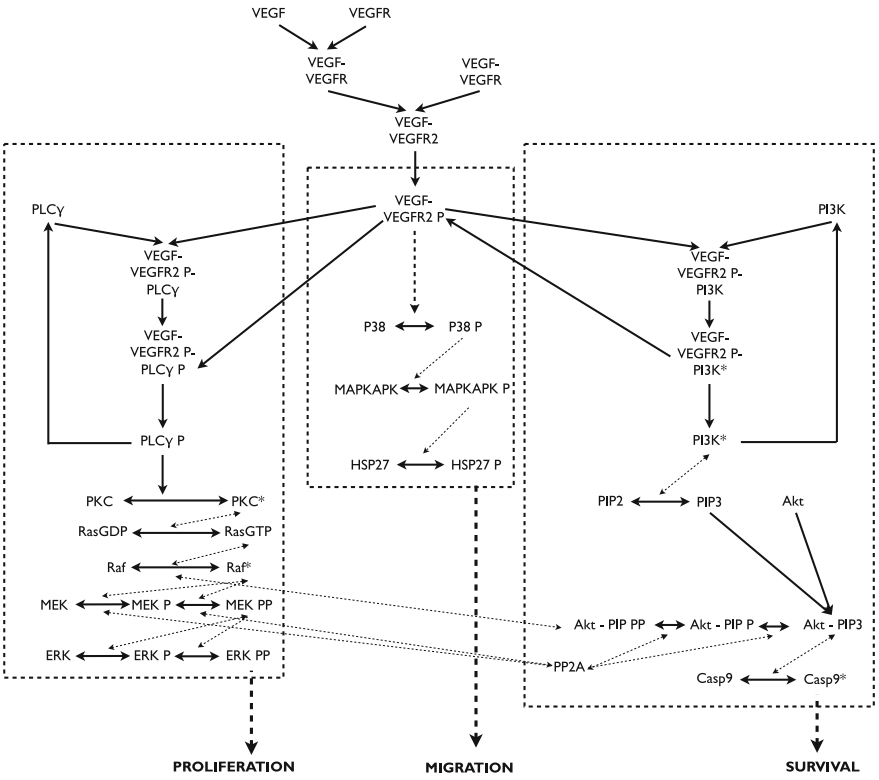


**Fig. 4** Diagram presenting a synthesis of knowledge and information extracted from recent literature (PubMed) and adequate databases (KEGG) on the VEGFR2 signaling pathway. *Dash lines* represent Michaelis–Menten reactions in which the catalyzer (e.g. RasGTP) is not consumed in the process. *Solid lines* represent mass-action kinetics

In this process, it is important to pay special attention to molecules for which the implication in the process of angiogenesis has been experimentally proven [45, 49] but also for which kinetic parameters of their reaction are already documented. Keeping the integrality of molecules that are known to interact on the pathway would lead to a system with an excessive complexity that might be of no help in the understanding of the overall pathway dynamic. Minimizing the number of components to fit with sparse data while keeping a good description of the molecular dynamics experimentally observed is however a challenging issue addressed in [50]. As far as the VEGFR2 signaling pathway is concerned, it seems reasonable, as a first attempt to restrict our description to pathways triggering proliferation, migration and survival of the endothelial cells; whereas VEGF is also known to drive variations of the vascular permeability, which is believed to have serious consequences on treatment efficacy in vascular tumors. As a first attempt, we can assume that permeability variation depends on modifications of the cells' shape and adhesion properties [46, 47].

We provide below a textual description of the main reactions occurring and illustrated in Fig. 4. VEGF binds to the VEGF receptor (VEGFR2) located at the surface of endothelial cells, which leads to the formation of homodimers of receptors and the autophosphorylation of their intracellular kinase domains [46] that then activates molecules at the top of signaling cascades. We can distinguish three main pathways:

- The phosphorylation of PLC$\gamma$ leads to the activation of PKC and then the Raf/MEK/ERK pathway [49] which stimulates cell proliferation [47].
- The MAPKAPK/hsp27 pathway is activated through the phophorylation of p38. This molecular network triggers actin reorganization and cell migration [51].
- The phosphorylation of PI3K permits the transformation of PIP2 into PIP3 which can bind to Akt and permit its bi-phosphorylation. Akt- PI-PP will then phosphorylate Casp9. Casp9 P is then unable to play its role in cellular apoptosis, which leads to an improvement of the cell survival [52, 53].

It is then possible to translate the molecular system into a system of ODEs. It is important to note, however, that this step implies the following hypothesis: the molecular concentrations are continuous, reactions happen in a homogeneous medium of a volume large enough, and reactions are deterministic.

The dynamics of reactions can then be modeled using mass action kinetics, which describes reactions rates as proportional to the concentrations. For example in the reaction $A + B \leftrightarrow AB$, where compounds $A$ and $B$ bind to each other to form a stable complex $AB$ at the rate $k_1$ and the product $AB$ dissociates itself into $A$ and $B$ at the rate $k_{-1}$. The speed of the reaction is then given by:

$$v = k_1[A][B] - k_{-1}[AB].$$

For some complex multi-step reactions, like phosphorylations, the Michaelis–Menten approximation can be used to reduce the complexity of the mathematical model and conserves the dynamic properties of the reaction. For instance, $A_P + B \rightarrow A_PB \rightarrow A_P + B_P$ where $A_P$ is a catalyzer of the activation of $B$ into $B_P$, can be simplified to $A_P + B \rightarrow B_P$ with the corresponding rate equation: $v = \frac{V[A_P][B]}{K[B]}$. When a unique catalyzer triggers several reactions, writing the rate equations is more complicated (the reader may refer to [50, 54] for further details).

In doing so, a differential equation can be associated with each reaction as follows:

$$\frac{dc_i}{dt} = \sum v_{pro,c_i} - \sum v_{con,c_i},$$

where $v_{pro,c_i}$ and $v_{con,c_i}$ are respectively the velocities of the reaction producing and consuming the concentration $c$ of the molecule $i$.

Writing all equations for the reaction depicted in Fig. 4 leads to a huge system composed by 39 equations with 78 parameters (reaction rates and Michaelis–Menten constants). Simulation of the model in principle reproduces the time evolution of the different elements composing the network following stimulation

by VEGF binding VEGFR2. It can be also used to theoretically investigate the effect of different therapeutic compounds acting at different level on the pathway. As a perspective, this model can be integrated into a multiscale framework describing tumor growth and angiogenesis such as in [38]. Indeed, in [38], three main processes govern the evolution of endothelial cell: proliferation, migration, and survival. In consequence, the model of VEGF intra-signaling, which also links to those three main behaviors, can be "plugged" into the endothelial cell equation. For this, and in order to homogenize the timescales between the two models, we could assume that at a given VEGF signal, we run the intracellular signaling model to the steady state and use those values as inputs for the endothelial cell equations. Alternatively, we could use the maximum values of concentrations downstream the VEGF signaling as they may better represent the force and amplitude of the signals towards proliferation, migration and survival. This approach was already used in a multiscale model for non-small cell lung cancer integrating the EGFR signaling pathway [55].

Scianna and coworkers have studied the link with the activation of calcium channels [56], a process also called non-store-operated calcium entry (NSOCE), because it is known that the absorption of calcium from the extracellular environment influences cell polarization, motility, and adhesion, all processes that are fundamental in vascular network formation. In fact, it has been recently demonstrated that mitogen-induced intracellular calcium signals and the relative pathways play a critical role in vascular progression both in vitro and in vivo (see for instance [57–61]). In this light they could also be in principle potential targets for anticancer drugs. In particular, different lines of evidence have shown that VEGF, after binding specific tyrosine kinase receptors with high affinity, triggers their dimerization and the auto-phosphorylation of critical cytoplasmic tyrosine residues, resulting in the indirect recruitment of PLA2 and eNOS enzymes. PLA2, in a single-step reaction, catalyzes the hydrolysis of esterified phospholipids (stored within the cell membrane), producing free arachidonic acid (AA), while eNOS releases its endothelium-derived nitric oxide (NO) [58, 61]. The complex interplay between these second messengers modulates the activity of probably more than one type of plasma-membrane calcium channels [62, 63]. The subsequent increase in intracellular calcium levels mediates cell motility, adhesion and cytoskeletal reorganization [57, 59, 60, 64], all cellular behaviors involved in tumor-derived blood vessel formation. A schematic view of the model is proposed Fig. 5. The model proposed by Scianna and coworkers [56, 65] introduces such subcellular mechanisms in a cellular Potts model, a discrete lattice Monte Carlo generalization of the Ising's model, based on an energy minimization principle. Typically, the CPM represents a collection of biological cells on a numerical grid, associating an integer index to each site to identify the space occupied at any instant by a cell or in a more detailed description by a subcellular compartment. Domains, i.e., collection of lattice sites with the same index, represent then subcellular compartments such as nucleus, cytosol, membrane which then form the considered individual cells and allow a better reproduction of cell motion and shape changes. The mathematical model is then a hybrid, because of the need to take care of the

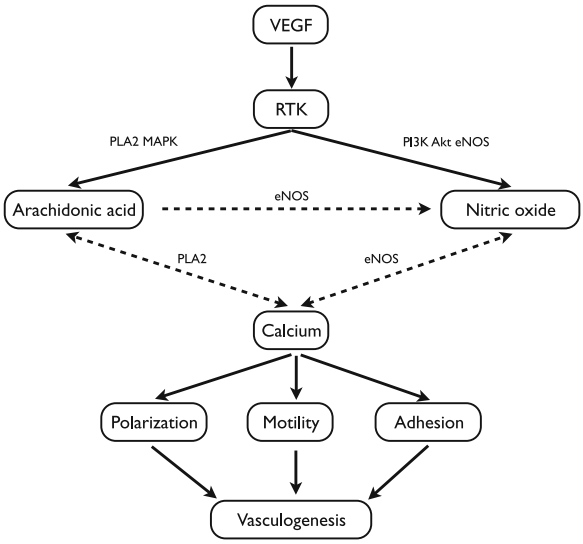


**Fig. 5** Schematic view of the model downstream the VEGFR receptor leading to the activation of the calcium channel. Adapted from [56]

diffusion of VEGF in the extracellular domain, the reception of signals via VEGF receptors located in the membrane subcompartment of the cell and their internalization and recycling, the activation of mechanotransduction pathways via the formation of cadherin-cadherin junctions, and the activation of the related signaling cascades within the cytosol. The expressions of specific molecules then feedback on the value of the modeling parameters of the cellular Potts model determining motility, adhesion and polarization. A schematic view of the multiscale structure of the model is proposed Fig. 6.

*b. Computational models of cell and vascular network organization.*

Another aspect that should be included in attempting a multiscale description of angiogenesis is the modeling of vascular network organization and structure.

It is known that in the embryo, the primitive vascular plexus forms through the process of vasculogenesis. In this process mesoderm-derived precursors of endothelial cells assemble by directed cell migration and cohesion. The resulting organization is a network characterized by a polygonal structure having a precise size that is found to be functional for oxygen transport into the tissues. In spite of the subsequent remodeling processes, this characteristic is maintained in the adult body where the capillary network embedded in the tissues and stemmed by the vascular tree has the same geometric shape of the minimal unit participating in the formation of the embryonic vascular network and is optimal for metabolic exchange.

The ability to form networking capillary tubes is a cell autonomous property of endothelial cells. At the site of vessel formation, soluble stimuli released by neighbouring cells modify the genetic program of endothelial cells allowing them to be responsive to permissive cues coming from the extracellular environment [66]. Several in vitro models support this concept. In particular, it is well known that culturing endothelial cells on a gel-scaffold markedly accelerates their morphological differentiation in to geometric tubular networks, which are almost

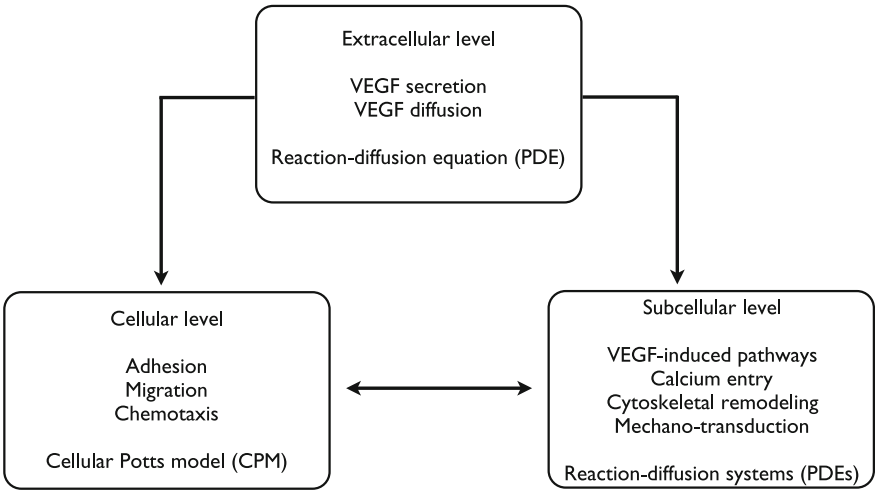


**Fig. 6** Schematic view of the multiscale model as proposed in [56]

identical to vascular beds formed in vivo by vasculogenesis. This phenomenon has been called in vitro angiogenesis or in vitro vasculogenesis [67].

In the experiments we discuss here, a Petri dish is coated with an amount of Matrigel, a natural basal membrane matrix which favours cell motility and has biochemical characteristics similar to living tissues. Human endothelial cells from large veins or adrenal cortex capillaries (HUVEC), or other similar types of cells, sediment onto the Matrigel surface from the physiological solution above. Cells then move on the horizontal surface, giving rise to a process of aggregation and pattern formation. The evolution of the process is the following: In the first 3–6 h endothelial cells migrate independently, keeping a round shape till they collide with closest neighbors. The cells eventually form a continuous multicellular network and adhere more strongly on the Matrigel multiplying the number of adhesion sites. Because of the increased number of activated adhesion sites, the network then slowly moves as a whole, undergoing a slow thinning process, while still leaving the network structure mainly unaltered. Finally, at the end of the process after nearly 20 h, individual cells fold up to form the lumen of the capillary, so that one has the formation of a capillary-like network along the lines of the previously formed two-dimensional structure.

Focusing on the trajectories of single cells, in most cases the direction of motion is well established and kept till the cells encounter other cells. Of course, a random component is present but is usually not predominant. The trajectories of individual cells then show what is usually called a degree of persistence in the direction of cell motion, i.e., a tendency to maintain its own direction of motion. In addition, in most cases the motion is apparently directed toward zones of higher concentration of cells, which suggests the presence of a mechanism of cross-talk among cells mediated by a protein secreted by the endothelial cells themselves.

A good candidate as soluble chemotactic mediator is VEGF-A, which as described in the previous section, is known to induce growth, survival, and motility in endothelial cells. Conversely, the addition of an anti-VEGF-A neutralizing antibody inhibits capillary network formation. In order to test the importance of chemotactic signaling mechanisms Serini and coworkers performed some experiments aimed at extinguishing VEGF-A165 gradients. Direct inhibition of VEGF-A caused an apoptotic effect [68]. To overcome this problem, they extinguished VEGF-A gradients spreading from individual endothelial cells plated of Matrigel by adding a saturating amount of exogenous VEGF-A165. Indeed, saturation of VEGF-A gradients resulted in strong inhibition of network formation. This observation is also confirmed in a set of experiments performed in Boyden chamber and evaluated by checkerboard analysis to study the chemotactic and chemokinetic activity of VEGF-A165.

It is found that in saturating conditions, cells maintain a certain degree of directional persistence, while the movement is completely decorrelated from the direction of simulated VEGF gradients.

On the basis of the phenomenological observations above and on the related experiments, Gamba and Serini proposed a mathematical model focusing on the early development of vascular network formation [68, 69]. Their basic assumption is that persistence and chemotaxis are the key features determining the size of the structure. Their mathematical model is a system of partial differential equations composed of:

- An equation describing the conservation of the number of endothelial cells, because no mitosis and no or very little apoptosis occur during the process;
- An equation describing how cells move that includes cell persistence and all the factors that influence a change in direction of cell motion, such as chemotaxis, drag-like dissipative interaction with the substrate, and response to compression to avoid overcrowding when the cells cluster. The chemotactic term may present a saturating term as suggested by Tosin and coworkers [70];
- An equation describing the diffusion of VEGF released by the endothelial cells and its degradation.

As shown in Fig. 7, the model proposed was able to successfully describe the early migration-dominated stages of network formation, yielding similar morphologies. It was also found that the size of the capillary structure is governed by the diffusion coefficient $D$ and the chemoattractant half-life $T$. In fact, the predicted average size of formed network structures is $L \sim DT^{1/2}$, in good agreement with phenomenological observations in vivo and measurements in vitro, confirming the fact that persistence and endogenous chemotaxis are essential for proper network formation.

There was another phenomenon that was also described by the same model, not previously foreseen. While it is thought that the chord length is nearly independent from the density of seeded cells in a certain range, it has been observed that outside this range one does not have a proper development of vascular networks, as observed in vivo by Fong and coworkers [71]. To enlighten this phenomenon,

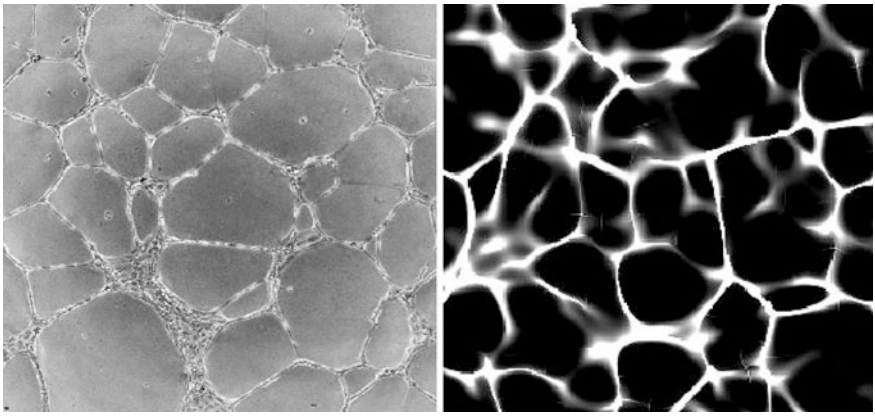

**Fig. 7** Results of the in vitro vasculogenesis experiment (*left*) and simulation (*right*) with 200 cells/mm$^3$

Serini and coworkers performed some experiments varying the density of seeded cells demonstrating the presence of a percolative-like transition at low densities and a smooth transition to a "Swiss-cheese" configuration at high density [68].

In fact, below a critical value around 100 cells/mm$^2$ the single connected network breaks down in groups of disconnected structures. On the other hand at higher cell densities, say above 200 cells/mm$^2$, the mean chord thickness grows to accommodate an increasing number of cells. For very high values of cell density (e.g., above 500 cells/mm$^2$), the network takes the configuration of a continuous carpet with holes named lacunae in the literature. In this case cells do not even differentiate to form the lumen in the chords.

Surprisingly, the model by Gamba and Serini was also able to catch all the features just described, reproducing at one extreme the percolative transition occurring about n_c $\sim$ 100 cell/mm$^2$, which discriminates the situation in which a functional network is formed and another in which it is not properly connected and therefore can not carry any blood, and the Swiss-cheese transition, which describes another non functional configuration containing cell carpets and lacunae.

In reality as already mentioned, as soon as the early migration stage ends, the cells adhere more strongly on the substratum and start to pull on the substratum, an aspect that is completely neglected in the model described above. In this second phase the cell–cell and cell-substrate mechanical interactions may become important, so that the stiffness of the substrate and the adhesive properties of the cell can influence the formation of the network. In order to take into account of such effects, several works proposed elasto-mechanical models (see, for instance [70, 72]). The main aim of such papers was to describe the phenomenon of pattern formation starting from monolayer initial conditions that can be re-proposed to study this second phase.

The paper by Murray and coworkers [72] was mainly devoted to mesenchymal morphogenesis on the basis of some experiments done by Harris, Stopak, and Wild

[73] on the interaction between ECM and fibroblasts, a cell well known for its strong pulling force. The essential features of the models developed in this paper and in the following ones [74, 75] are the following:

- Cells exert traction forces onto the extra-cellular matrix, which is a viscoelastic material;
- The Petri dish exerts a drag force on the matrix;
- Cells may move because of haptotaxis or chemotaxis (without any further specification on the chemoattractant).

The model does not include any specific signaling process, but takes into account mechanical issues. For this reason, it cannot describe phenomena which depend on cell signaling, but it can describe the dependence on the formation of the structure from the type of substratum.

It can be argued that a complete, realistic description of the diverse phases of in vitro vasculogenesis should connect the migration regime described by the model based on persistence and chemotaxis and the successive viscoelastic regime described by the mechanical model or by some modification of it. This aim was pursued by Tosin and coworkers [70]. They describe the system as made of two layers, the gel and the ensemble of cells, obeying force balance equations including a fundamental interaction term, which couples the motion of the cells and the substratum. Their final conjecture is that migration and traction are two different programs influenced by the local cell density, which lead toward a behavior that is more ameboid-like or mesenchymal-like according to the environmental conditions. In this view, persistence, chemotaxis, and mechanical traction are complementary effects. The former two are essential in the early migration-dominated phase of vasculogenesis, dictating the morphology of the network and in particular the typical size of the polygons. The latter activates in a later phase and after cell-to-cell contact and functions to stabilize the structure.

## 6 Concluding Remarks

In this chapter, we have briefly described different mathematical models related to tumor growth and angiogenesis. Starting from the historical and first attempt to describe tumor growth in vitro by using the Gompertz equation [4–7], we have followed the development of those models that are now about to impact the evaluation of oncological treatment efficacy in clinical trials [11–13, 25, 35]. Nowadays, attempts to complexify those models in order to take into account key biological process such as angiogenesis is under development. This will require the development of multiscale models integrating molecular signaling pathways together with tissue regulation and morphological information on tumor growth and regression when treated. Important methodological issues will have to be addressed such as the multi-integration of data coming from different sources (a priori knowledge from literature and data-driven knowledge from ad-hoc

experiments) since it does not seem feasible to setup a unique experiment to estimate the numerous parameters of those models. Hopefully, the development of new methodologies together with the better accessibility of relevant biological data will allow the development of an integrative platform to support research progresses in this area and the development of therapeutics.

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# Biomaterials for Cell-Based Therapeutic Angiogenesis

**Max H. Rich and Hyunjoon Kong**

**Abstract** Stem cells and endothelial progenitor cells are increasingly studied for use in therapeutic angiogenesis to treat ischemic tissues and critical-sized tissue defects because of their potential to sustainably express multiple angiogenic factors and also differentiate to endothelial cells. These cells are often incorporated into a variety of biomaterials which can function as a provisional matrix to localize or deploy cells and also to regulate cellular phenotypic activities at a transplantation site. This chapter will summarize previous and current efforts to design cell-laden biomaterials in order to improve therapeutic efficacy of transplanted cells to stimulate revascularization. Finally, it will discuss future strategies of biomaterial design that can further elevate the quality of the cell-based revascularization therapy.

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## 1 Introduction

Ischemia, characterized by the restriction of blood supply to various vital tissues and organs, is the leading cause of tissue morbidity, and ultimately death across the globe. There are a variety of causes of ischemia including acute injury, atherosclerosis, hypertension, and embolism, among others. Early diagnosis and subsequent surgical methods such as angioplasty or bypass grafting can prevent ischemia [1]. These methods are useful in preventing ischemia or treating patients in the early stages of ischemia. However, it is a challenging task to treat patients who are in the advanced stages of ischemia, necessitating the development of a method to treat such patients.

Efforts are separately being made to treat critical-sized wounds and tissue defects, as a result of acute and chronic injuries and surgery, via tissue regeneration. It is well agreed that success in these tissue regenerative therapies relies on the ability to facilitate the transportation of oxygen and nutrients to cells that are either transplanted in or migrate into the tissue defects [2]. It is also important to remove cellular metabolites and wastes from the tissue defects.

One promising approach that has emerged to treat ischemia and improve wound healing and tissue regeneration is to regenerate micro-sized blood vessels by stimulating angiogenesis in a controlled manner. Angiogenesis is characterized by the sprouting of capillaries from pre-existing blood vessels (Fig. 1). This branching process is active during development, self-healing, and tumorigenesis. Angiogenesis is activated by the binding of various angiogenic cytokines and growth factors with endothelial progenitor and precursor cells [3]. These soluble angiogenic factors include vascular endothelial growth factor (VEGF), fibroblast-like growth factors (FGFs), platelet derived growth factor (PDGF), and endothelin. The binding of angiogenic factors stimulates cells to proliferate, migrate, and form endothelial lumen, in concert with enzymatic degradation of interstitium driven by cell secreted matrix metalloproteinases [3]. Subsequently, smooth muscle cells and pericytes are mobilized to form mature blood vessels stabilized by smooth muscle layers and pericyte layers. The soluble factors involved with the maturation include PDGF, angiopoietin-1 (Ang1), and transforming growth factor (TGF)-$\beta$ [3].

Supported by in-depth understanding of the role of individual angiogenic cytokines and growth factors, these signaling factors have been tested in many preclinical and clinical trials for revascularization [4]. Specifically, significant advances in the recombinant techniques are expediting the use of these signaling proteins with minimal concerns of host inflammation and pathogenic infection. Single, dual, and multiple soluble factors are systemically or locally administered to target tissue and stimulate revascularization. In addition, these soluble factors are often encapsulated in nano-or micro-sized particles to extend their bioactivity in physiological conditions [3]. Alternatively, these factors are loaded into a micro porous scaffold or hydrogel typically used as a tissue engineering scaffold so that the factors sustainably stimulate host and transplanted cells towards blood vessel formation [5].

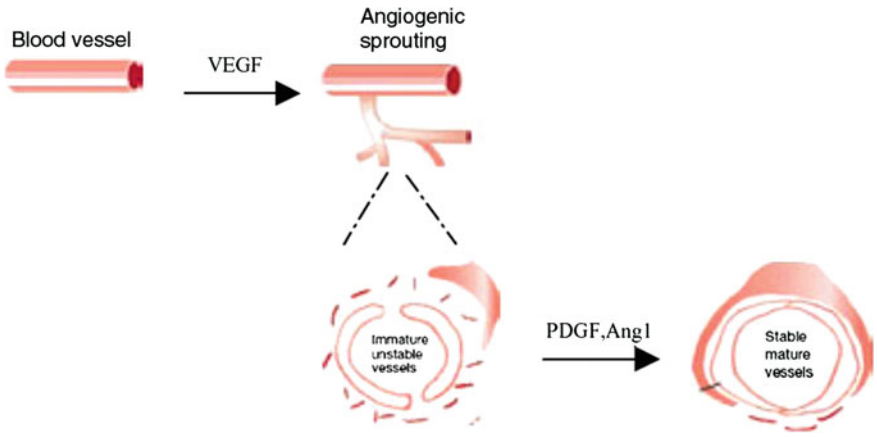


**Fig. 1** Schematic description of blood vessel formation through angiogenesis. Following vascular endothelial growth factor (VEGF) stimulation, immature new blood vessels sprout from existing vasculature lacking pericytes or smooth muscle cells. Stimulation via angiopoeitin 1 (Ang 1) and platelet derived growth factor (PDGF) promotes association of the neovessel with pericytes and smooth muscle cells. The result is a new stable and mature blood vessel [30]

However, these molecular therapies are often plagued by limited outcomes. For example, recreation of mature and functional blood vessels requires sequential activation of multiple signaling factors; however, it is still a challenging task to optimize the dose and duration of multiple drug molecules for revascularization [3]. In addition, the frequent and high dosage of angiogenic factors presents the potential risks of promoting inflammation and also producing leaky and dysfunctional neovessels [5].

To overcome these challenges encountered with molecular therapies for revascularization, cells which can endogenously express multiple angiogenic factors and also release them in response to external stimuli (e.g. hypoxia or inflammatory factors) are increasingly studied as an alternative vascular medicine. These cells include mesenchymal stem cells, endothelial progenitor cells, and fibroblasts [1]. Alternatively, cells transfected with non-viral or viral genes encoding angiogenic factors can produce exogenous angiogenic factors in a sustained manner [5]. These cells are also advantageous to delivering multiple angiogenic factors in a sustained manner.

In the beginning of cell therapies, it was common to locally transplant the cells via intramuscular injection [6]. However, transplanted cells without any physical barriers present the potential problem of initiating an immune response. Furthermore, cells injected into a patient may lose their bioactivity and also scatter due to extracellular chemical and mechanical factors at the transplantation sites. In order to enhance bioactivity and viability of cells, and further regulate their function to produce angiogenic factors, efforts are increasingly made to load cells into biomaterials with varied forms including microporous scaffolds, fibrous scaffolds and hydrogels.

Therefore, this chapter will discuss strategies for the biomaterial design of cell-based revascularization therapies, including control of material properties and microstructure and processes for loading cells into the materials. We will specifically focus on describing the design of porous polymer scaffolds, fibrous scaffolds, and hydrogels. In parallel, the influence of these biomaterial properties on improving cell viability, angiogenic function, and subsequent revascularization, both in vitro and in vivo, will be reviewed.

## 2 Biomaterial Design

### *2.1 Porous Scaffold*

Cell-laden porous scaffolds are increasingly used to enhance vascularization throughout implants because of their potential to facilitate the migration of blood vessel-forming cells into the scaffold and further stimulate endothelial lumen formation within the micro-sized pores. It is common to first assemble the scaffold with interconnected micropores and subsequently plate cells. The cells used in these scaffolds include cells that secrete angiogenesis factors to neighboring tissues, those that possess a potential to differentiate to endothelial cells, or both cells types.

These porous scaffolds are commonly prepared with biodegradable polymers, such as poly(lactide-co-glycolic acid) (PLGA), poly(ε-caprolactone), polyanhydrides, cross-linked collagen and glycosaminoglycan, and their derivatives [7]. Mechanical properties and degradation rates of these polymeric matrices are controlled by the fraction of hydrolyzable units, molecular weights, and hydrophilicity of the polymer. These polymers are co-polymerized with other polymeric units, such as poly(ethylene glycol) and polysaccharides, to further control material properties and interactions with host immune systems [7]. Alternatively, these polymers are physically mixed or chemically conjugated with cell adhesion peptides or proteins in order to control cellular adhesion to polymeric matrices.

It is common to incorporate the interconnected micropores into the polymeric matrices via porogen leaching, gas foaming, freeze drying, and phase separation (Fig. 2) [7]. Recently, diameters of micropores are controlled in a more sophisticated manner by incorporating microparticles with uniform diameters into the polymeric matrices and leaching them out using solvents (Fig. 2) [7]. Therefore, the biocompatibility of the porous matrices tends to significantly depend on types of porogen and microparticles used because they determine the solvents that should be used to create the interconnected pores [8]. In this regard, the gas foaming or freeze drying process may exhibit advantages with minimal concerns of cytoxic residues in the porous matrix, but these methods still present limited controllability of pore size [9].

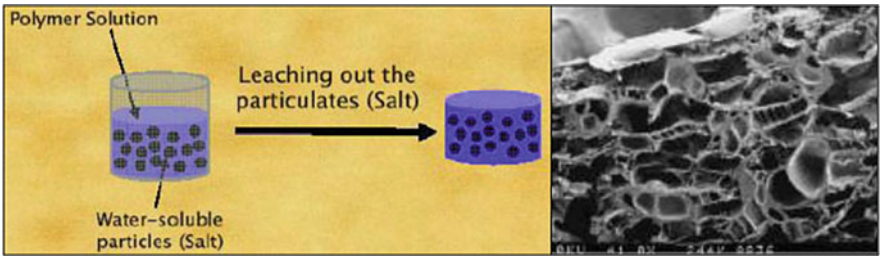


**Fig. 2** Schematic of particle leaching fabrication method. The polymer solution is placed in a mold with some sort of porogen (i.e. salt) and then a scaffold is formed. The porogen is then washed out of the scaffold, leaving a network of interconnected pores [31] (*left*). SEM images of porous ceramic scaffold fabricated through particle leaching method through dissolution of the porogen [32] (*right*)

Earlier studies discovered that pore diameter and biodegradability of the scaffold significantly influence the cellular efficacy to construct vascular networks within the scaffold and also bridge new blood vessels with pre-existing ones [10]. For example, human embryonic endothelial cells or human umbilical cord vein cells (HUVEC's) were incorporated into a myoblast-laden biodegradable PLGA scaffold, in order to crate vascularized muscle [11]. Micropores were incorporated into the scaffold using the salt leaching method [12]. The resulting scaffold pore diameters range from 225 to 500 μm with a porosity of 93 %. Endothelial cells secreted VEGF and PDGF within the pores and organized into tubular structures in vitro. Furthermore, in vivo studies, where the scaffold was implanted subcutaneously, showed that the prevasculature within the scaffolds integrated with host vasculature in SCID mice [11].

Alternatively, porous PLGA scaffolds were prepared using the gas foaming/particle leaching method. The copolymer, mixed with 100 mg of NaCl, was compressed into a disk form, and the disk was placed in a pressure chamber filled with carbon dioxide. After the gas was partially dissolved into the polymer disk, the chamber was depressurized to convert the carbon dioxide smeared into the matrix into a gaseous form and subsequently generate the pores in the polymer disks. Further leaching of NaCl in aqueous solution created the microporous scaffold porosity of 95 % with an average pore size of 190 μm. The human micro vascular endothelial cells (HMVECs) were incorporated into scaffolds by dropping cells in suspension into the pores. These scaffolds were capable of developing mature blood vessels via vasculogeneis within the scaffold (Fig. 3), as demonstrated with the scaffold subcutaneously implanted into SCID mice [13].

In addition, mesenchymal stem cells transfected with genes encoding VEGF and bFGF were used by loading them in a porous biodegradable collagen and glycosaminoglycan scaffold with the commercial name of the Integra® matrix. The matrix is composed of cross linked bovine collagen and glycosaminoglycans [14]. The resulting matrix has a porosity of 98 % and pore sizes ranging from 30 to 120 μm. Cells seeded in the matrix actively secreted exogenous VEGF and bFGF. The cell seeded scaffold was tested in vivo using a skin defect model where a


**Fig. 3** Schematic illustration of blood vessel formation mediated by seeding endothelial cells into a porous scaffold (**a**). Transplanted endothelial cells develop new blood vessels within the scaffold that grow towards host vasculature (**b**). Ultimately, host vasculature connects with new vasculature in order to form mature and functional blood vessels capable of carrying blood (**c**) [33]

15 mm hole was created on the back of athymic mice and the scaffold was placed over the wound. Wound healing was significantly improved by incorporating the drug-releasing cells into the scaffold, as seen with increased neovascular regeneration, when compared to the scaffold alone. In vitro studies indicated that the stable values of VEGF secreted into the media ranged from 1.5 to 2.1 ng/mL while bFGF stabilized at 0.2 ng/mL [14]. While this result is impressive, it is technically challenging to tune the cellular production of VEGF or FGF in terms of amount and sustainability.

## *2.2 Microfiber Scaffolds*

Microfibrous scaffolds are also used in cell-based revascularization therapies, because of their structural similarity to natural ECM. The fibrous structure of ECM is known to facilitate cell adhesion and also mass transport through a three dimensional matrix [15]. In native ECM, these fibers are typically formed from self-assembly between collagen and fibrin fibrils [15]. To mimic these structures, nano or microfibrous scaffolds are fabricated by electrospinning various biodegradable polymers including poly(L-lactic acid) and their copolymers to a substrate (Figs. 4 and 5) [15]. The diameter of resulting fiber ranges from 3 nm to 6 μm, depending on chemical structure of polymers and processing condition [16]. In addition, the resulting fibrous scaffolds exhibit the mechanical rigidity and fracture resistance superior to natural ECM [16].

Early studies have demonstrated that collagen-based microfiber scaffolds can be used to develop small diamater (<6 mm) blood vessels consisting of an endothelial lining and a smooth muscle layer. In this study, smooth muscle cells were plated onto a fibrous scaffold composed of collagen fibers and poly(lactic acid) (PLA) fibers. The scaffold was fabricated by placing collagen fibers on a stainless steel mandrel and then electrospinning PLA fibers on top of the collagen fibers. The collagen fibers were used to emulate the stress environment found in arteries. The PLA fiber mesh is designed to provide initial structure and as a place to seed the smooth muscle cells. Following 10 days of culture in vitro, smooth muscle cells adhered to the fibrous scaffold displayed a spindle shape and also alligned along the collagen fibers [17].

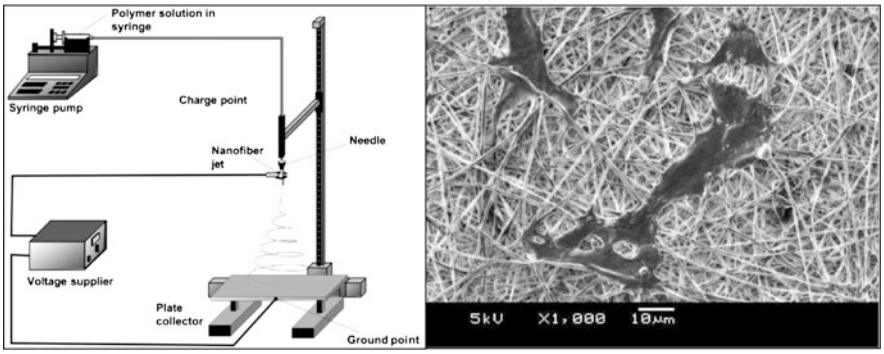


**Fig. 4** Schematic of electrospinning apparatus setup. (*Left image*) The polymer solution is pushed out through a syringe pump and then jetted out by an applied voltage. It is then collected on a grounded plate. (*Right image*) SEM image of endothelial cells adhered onto P(LLA-CL) nanofiber scaffold after 3 days of culture [18]

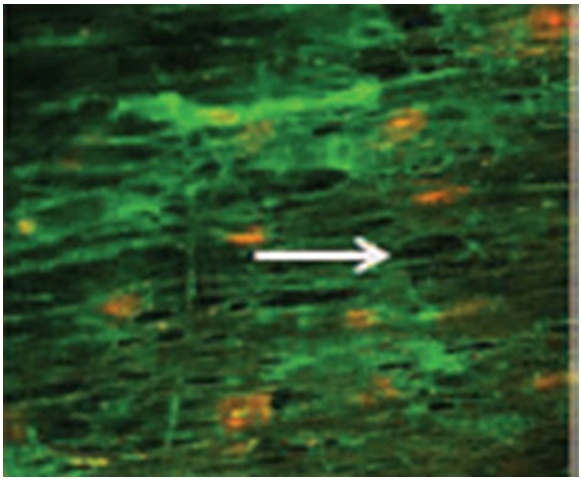

**Fig. 5** Fluorescent image of endothelial cells adhered onto aligned P(LLA-CL) scaffold. The endothelial cells were cultured for 3 days and stained for platelet endothelial cell adhesion molecule-1 (PECAM-1) (*green*) and the nucleus (*red*) [19]

Subsequent studies used fibrous scaffolds to guide the growth direction of engineered blood vessels. In one study, the endothelial cells and smooth muscle cells were seeded onto an electrospun poly(L-lactic acid)-co-poly(ε-caprolactone) nanofiber scaffold. The scaffold facilitated the adhesion of endothelial cells and smooth muscle cells, and further supported their natural phenotypes in vitro. The smooth muscle cells displayed the classic spindle shape and the endothelial cells displayed a cobblestone morphology. Furthermore, both the endothelial cells and smooth muscle cells migrated through the pores of the scaffold [18].

These microfiber surfaces were further coated with collagen in order to emulate the microstructure of artery walls more closely. Endothelial cells grew along the aligned nanofibers and also stimulated the smooth muscle cells to exhibit a spindle phenotype in vitro [19]. However, despite these impressive cell alignments on the

nanofiber mesh characterized in vitro, there has not been success in developing mature and functional blood vessels aligned along the fiber orientation in vivo.

## 2.3 Hydrogel

Hydrogels are commonly formed from chemical and physical cross-linking reactions between water soluble polymers [20]. These hydrogels have been increasingly used for cell encapsulation and transplantation because of several advantageous features including structural similarity to natural ECM and hydrated environments. Recently, efforts are being extensively made to regulate phenotypic activities of encapsulated cells with chemical and mechanical properties of hydrogels in concert with soluble factors. In addition, cell-encapsulated hydrogels can be injected into target sites, allowing for minimally invasive cell transplantation. Furthermore, coupled with various microfabrication techniques, cell-encapsulated hydrogels can be built into three dimensional constructs consisting of multiple layers with pre-defined spatial organization [21]. These merits of hydrogels have led to increasing exploration of the use of hydrogels in cell-based vascularization therapies.

One popular approach is to encapsulate cells which can endogenously produce multiple angiogenic factors. These cells were encapsulated into the hydrogel via mild in situ cross-linking reactions. Cell-secreted angiogenic factors are released from the hydrogel by diffusion. One example of such a system involves the encapsulation of fibroblasts in alginate gel beads. The cells encapsulated in the gel beads remained viable over the course of the study, as demonstrated by constant metabolic activity. After 17 days of in vitro culture, VEGF secreted into the media peaked at approximately 25 ng/mL. The cell-secreted VEGF was released from the gel beads via diffusion. Endothelial cells cultured with media conditioned from the alginate beads exhibited an increase in proliferation [22].

Recently, efforts are increasingly being made to control cellular production of angiogenic factors by tuning chemical and mechanical properties of the hydrogels. For example, using pendent polymer chains inserted between cross-linking junctions, the mechanical stiffness of poly(ethylene glycol) diacrylate (PEGDA) hydrogels was tailored without significantly altering gel permeability. Fibroblasts encapsulated in the hydrogels with varying stiffness displayed that the growth factor productions, specifically VEGF, become maximal when the elastic modulus of the hydrogel was comparable to the elastic of fibrous tissue (i.e. 10–12 kPa) (Fig. 6) [23]. Additionally, incorporating photo cross-linkable alginate methacrylates into the PEGDA hydrogels resulted in an increase in both rigidity and permeability of the hydrogel, so that the various cell types could remain viable and active to produce angiogenic factors in the 3D gel matrix [24, 25]. These important hydrogel properties can be controlled in a more sophisticated manner by assembling hydrogels with various microfabrication techniques including stereolithographic assembly processes (Figs. 7 and 8) [21].

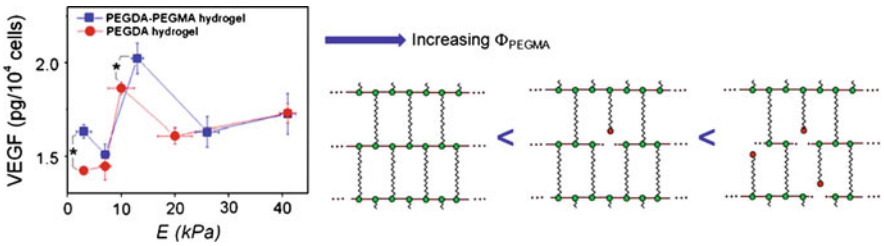


**Fig. 6** VEGF expression in encapsulated fibroblasts related to elastic modulus (E) of the PEGDA-PEGMA hydrogel and PEGDA hydrogel (*left*) Schematic of PEGDA-PEGMA hydrogels with varying weight percentages PEGMA (*right*) [23]

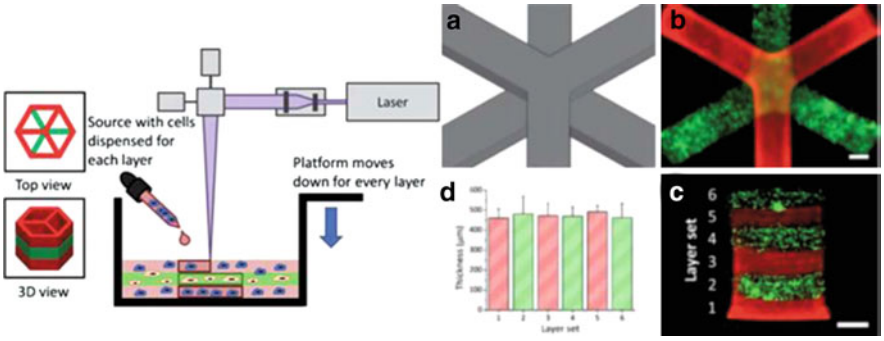


**Fig. 7** Schematic of assembling of cell-encapsulated hydrogels in a *top-down* manner using the SLA Apparatus. This process consists of a platform immersed just *below* the surface of a large tank of pre-polymer solution. After the layer is photopolymerized, the platform is lowered a specified distance to recoat the part with a new layer (*left*). Spatial 3D layer-by-layer cell patterning of viable cells at distinct layers. **a** CAD rendering of crosshatch pattern used for layer-by-layer spatial patterning. Each layer set was 1 mm thick (10 total layers, 100 μm each). **b** fluorescence image of NIH-3T3 cells encapsulated on different layer sets stained with either cell tracker® CMFDA (*green*) dye or CTMR (*orange*) dye. **c** cross-sectional view of a block pattern consisting of alternating layer sets (five total layers, 100 μm each). **d** quantitative analysis of (**c**) showing layer sets comparable to the desired 500 μm thickness. All values are mean ± standard deviation of n = 4. The scale bars are 1 mm [21]

Alternatively, hydrogels are encapsulated with endothelial progenitor or precursor cells which are capable of developing endothelial lumen. Fibrin gels have already been extensively used to examine endothelial sprouting in a three dimensional environment because of their fibril structure and enzymatic susceptibility which enables the fibrin gels to facilitate cellular reorganization to form endothelial lumen [26]. However, fibrin gels are too soft to be used as an implant for revascularization therapies. Therefore, there are several efforts to control the properties of fibrin gels or harness the structure of fibrin gels with gel-forming synthetic polymers. For example, fibrinogens are modified with methacrylic groups so that they can chemically cross-link with synthetic polymers such as

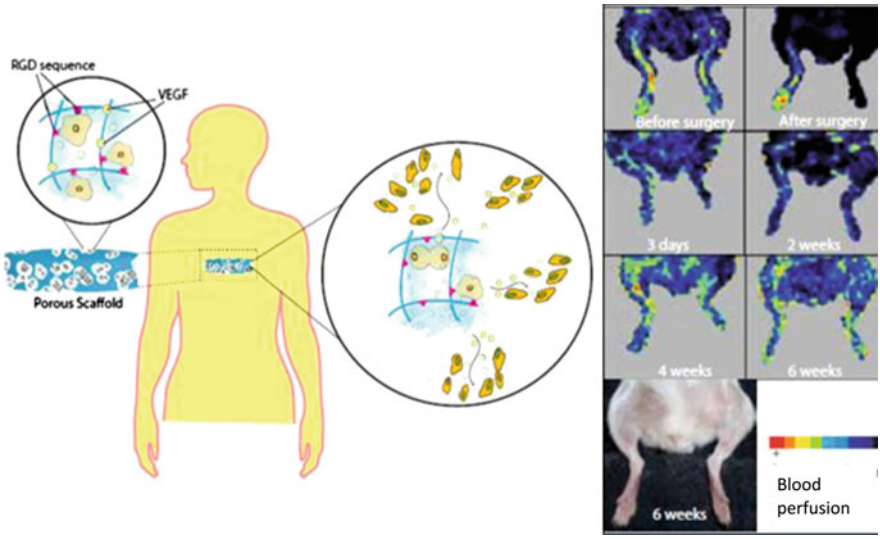


**Fig. 8** *Left* Schematic of therapeutic application of a hydrogel designed to deploy transplanted cells at an implantation site. *Right* Laser Doppler perfusion images of the recovery of blood flow in a mouse's *right* hindlimb damaged by ligation of femoral artery. The implantation of cell-deploying hydrogel consisting of porous alginate gel, $VEGF_{165}$, EPC and OEC significantly enhanced the recovery of blood perfusion [29]

PEGDA [27]. Alternatively, the PEG-based hydrogel is formed by cross-linking PEGDA with oligopeptides cleaved by cell-secreting proteinases such as matrix metalloproteinases (MMPs). Co-encapsulation of endothelial cells and mesenchymal progenitor cells in the MMP-sensitive synthetic hydrogel resulted in the formation of tubule-like structures. The mesenchymal progenitor cells acted as a source of MMPs to facilitate endothelial lumen formation.

These cell-encapsulating gels were further modified by encapsulating VEGF or VEGF-encapsulating microparticles to stimulate endothelial differentiation of encapsulated cells. In addition, these angiogenic factors may be essential to connect the new blood vessels formed within the hydrogel with host blood vessels neighboring the implanted gels. Implantation of these hydrogels encapsulated with cells and VEGF into mouse corneas sans cells resulted in a vascular network that penetrated the hydrogel implants within 7 days [28].

Conversely, hydrogels were used to dispatch cocktails of endothelial progenitor cells (EPCs) and outgrowth endothelial cells (OECs) into target tissues following implantation. EPCs and OECs produce multiple angiogenic growth factors and build new endothelial lumen, respectively. The alginate hydrogels encapsulated with $VEGF_{165}$ were lyophilized to introduce interconnected micropores and the cells were incorporated into the hydrogel during the rehydration process. Alginates modified with cells adhesion oligopeptides containing the Arg-Gly-Asp sequence were used to ensure cell adhesion to the gel matrix. The sustained release of $VEGF_{165}$ from the hydrogel stimulated the migration of EPCs and OECs from the hydrogel into

neighboring tissues at the implantation site. Further implantation of these cell-dispatching hydrogels into a mouse's ischemic hindlimb greatly improved the recovery of blood perfusion as a result of significant increases in the number of blood vessels [29].

## 3 Conclusion and Future Directions

Cell-laden polymeric biomaterials in forms of porous scaffold, fibrous scaffold, and hydrogels are increasingly being studied to enhance efficacy of cell-based revascularization therapies. These biomaterials are designed to release cell-secreted angiogenic factors, render the cells to form endothelial lumen in the matrix, or deploy the cells to the target tissue following implantation. In the past, the design of biomaterials used in this application focused on localizing transplanted cells in a target site and also retaining cell viability in order to sustain cellular production of angiogenic factors and also endothelial differentiation. These biomaterials are being further evolved as a device to regulate the cellular phenotypic activities essential to revascularization with chemical and mechanical properties of the matrix in concert with other supplemental soluble factors. In addition, biomaterials, specifically fibrous scaffold, were also assembled to control growth direction and spacing of new blood vessels at micrometer scale, but there have not been a significant success in recreating functional vascular network in vivo using the biomaterial. We expect that current efforts to integrate biomaterial design with various microfabrication technologies would resolve these challenges and further elevate the quality of revascularization therapies.

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# Translation of Pro-Angiogenic and Anti-Angiogenic Therapies into Clinical Use

**Sujata K. Bhatia**

**Abstract** Angiogenesis is a central physiological process that establishes blood supply and oxygen supply to tissues, thereby enabling the growth and maintenance of nascent bodily structures. Angiogenic signals function throughout the lifecycle to ensure perfusion, proliferation, and preservation of cells, tissues, and organs. During embryonic development, angiogenesis is absolutely critical; the generation of blood vessels is crucial to the formation of every organ. In adulthood, angiogenesis is necessary for wound healing, as well as recovery from ischemic insults; in such cases, it is beneficial to promote angiogenesis. However, angiogenesis is undesirable and pathological in the context of cancerous tumors, as well as diabetic retinopathy; in these cases, it is preferable to halt angiogenesis. Thus, pro-angiogenic and anti-angiogenic signals must operate in balance to assure physiological health. This chapter reviews current knowledge regarding biochemical regulators of angiogenesis, and highlights molecular targets of pro-angiogenic and anti-angiogenic therapies. The chapter additionally discusses current progress in translating both pro-angiogenic and anti-angiogenic therapeutics into clinical usage, and identifies potential barriers to the clinical introduction of such therapeutics. Finally, the chapter suggests future basic research and clinical research priorities for tailoring angiogenesis to address patient needs.

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## 1 Introduction

Angiogenesis plays a pivotal role in human health and human disease. Elucidated by the legendary biomedical scientist Judah Folkman [1], angiogenesis is the process of vascular generation which enables blood supply to tissues. Sufficient vascular supply is essential throughout life for the growth and maintenance of bodily structures. Angiogenesis not only establishes a vascular network during embryonic development, angiogenesis also operates following injuries to re-establish a vascular network. Deficient angiogenesis can lead to abnormalities in would healing, as well as insufficient recovery after an ischemic insult. Yet excessive angiogenesis can also be pathological; such is the case in retinopathies, malignancies, and inflammatory diseases [2]. Angiogenesis must therefore be tightly regulated to achieve an appropriate balance: either too much or too little angiogenesis results in disease (Fig. 1).

Indeed, imbalanced angiogenesis is a factor in several of the leading killers worldwide [3], including coronary artery disease, stroke, emphysema, cancer, and diabetes (Table 1). For instance, the clinical manifestations of diabetes mellitus demonstrate the centrality and complexity of angiogenesis: the diabetic patient simultaneously suffers inadequate angiogenesis in some tissues, along with excess angiogenesis in other tissues. The result is that such patients concomitantly experience impaired wound healing and ulcer formation due to insufficient angiogenesis, with retinopathy and nephropathy due to excess angiogenesis [4]. The ubiquity of angiogenesis makes this process a prime target for novel therapeutics. Based on the World Health Organization's report of the Global Burden of Disease [5], effective treatments that modulate angiogenesis could eliminate up to 31 % of all deaths globally, saving 18.3 million lives each year. Control of the angiogenic process, through pro-angiogenic and anti-angiogenic therapies, therefore represents a breakthrough treatment paradigm which could address many of the world's chronic diseases.

This chapter will provide an overview of the molecular biology and physiology of angiogenesis, and delineate the major molecular targets of pro-angiogenic and anti-angiogenic treatments. The chapter will then discuss the specific cases of pro-angiogenic therapy for coronary artery disease, and anti-angiogenic therapy for cancerous tumors; these cases are exemplary of the strategies and challenges of angiogenesis-targeting agents. The chapter will describe current progress in translating pro-angiogenic therapies and anti-angiogenic therapies into clinical usage for these specific diseases; the discussion will include pre-clinical trials, initial clinical trials, and large randomized trials. Finally, the chapter will identify shortcomings of contemporary approaches, and suggest future basic research and clinical research efforts for tuning angiogenesis to address clinical needs.

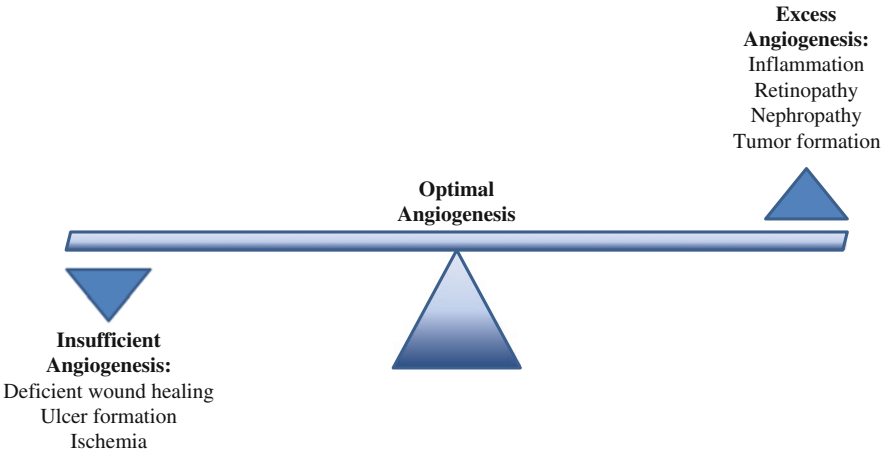


**Fig. 1** Implications of insufficient angiogenesis and excess angiogenesis for human disease

**Table 1** The role of angiogenesis in the global burden of disease

| Cause of death | Annual deaths worldwide, in millions | % of all deaths | Role of angiogenesis in therapy |
|---|---|---|---|
| Coronary heart disease | 7.20 | 12.2 | Improved angiogenesis may speed recovery of cardiac muscle |
| Stroke and other cerebrovascular disease | 5.71 | 9.7 | Improved angiogenesis in the brain may speed functional recovery |
| Chronic obstructive pulmonary disease | 3.02 | 5.1 | Improved angiogenesis of lung microvessels may allow alveolar regeneration |
| Trachea, bronchus, lung cancers | 1.32 | 2.3 | Blocking angiogenesis will halt growth of malignant tumors |
| Diabetes mellitus | 1.10 | 1.9 | Improved angiogenesis will enable wound healing; blocking angiogenesis will prevent retinopathy |
| TOTAL | 18.35 | 31.2 | |

## 2 Angiogenic and Angiostatic Regulators

Angiogenesis is finely controlled, both spatially and temporally, via an intricate system of molecular inducers and inhibitors. At least 50 different endogenous molecules are known to regulate angiogenesis [6]. The most important pro-angiogenic molecule is vascular endothelial growth factor (VEGF) [7], which serves as a master regulator of physiological and pathological angiogenesis [8]. VEGF is produced and secreted by cells in response to hypoxia; under conditions of low oxygen, a transcription factor known as hypoxia-inducible factor (HIF)

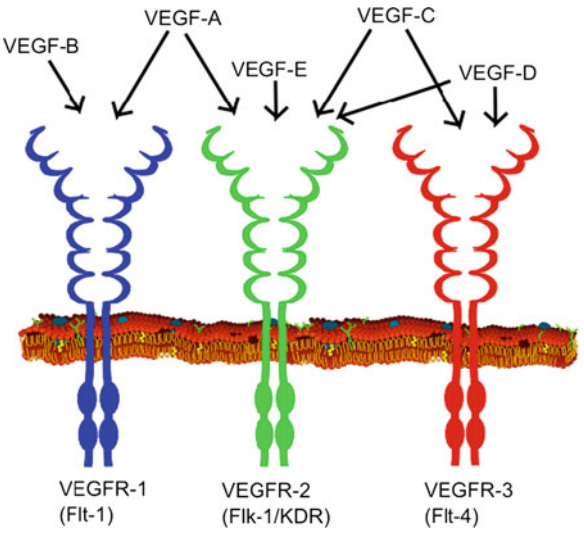


**Fig. 2** VEGF isoforms and receptors

promotes the transcription of genes encoding VEGF [9]. Upon translation and secretion, VEGF binds to receptor tyrosine kinases (VEGF receptors, also called VEGFR) located on the surfaces of neighboring endothelial cells [10]. Several isoforms of VEGF exist that bind to a family of different VEGF receptors (Fig. 2). The binding of VEGF to its receptors activates blood vessel formation, increases vascular permeability, and contributes to endothelial cell survival in blood vessels [11]. VEGF signaling is absolutely required for embryonic development [12]. In addition, upregulation of VEGF mRNA has been shown to occur during exercise in humans [13]. At the same time, VEGF is the most significant and potent survival factor in malignant tumor growth and metastasis [14].

Another important family of pro-angiogenic mediators is the fibroblast growth factors (FGF) [15]; the fibroblast growth factor family comprises 22 polypeptides that promote angiogenesis [16]. As with VEGF, the production and secretion of FGF are stimulated by HIF during hypoxic conditions. Once secreted, FGF binds to FGF receptors on endothelial cells, smooth muscle cells, and myoblasts. In ischemic tissue, FGF4 induces endothelial cell proliferation, as well as the secretion of metalloproteinases to carve out paths for new blood vessels [17]. FGF4 also stimulates secretion of VEGF, which subsequently stimulates angiogenesis. Moreover, it has recently been demonstrated that FGF9 orchestrates the wrapping of smooth muscle cells around nascent blood vessels during angiogenesis, to produce durable vasoresponsive blood vessels [18]. Other endogenous pro-angiogenic molecules include platelet-derived growth factor (PDGF), granulocyte colony-stimulating factor (GCSF), placental growth factor (PlGF), angiopoietin, and angiogenin [19, 20].

The actions of pro-angiogenic molecules are countered by anti-angiogenic, also known as angiostatic, regulatory molecules. Endogenous anti-angiogenic molecules include angiostatin and endostatin [21]. Angiostatin interferes with ATP production,

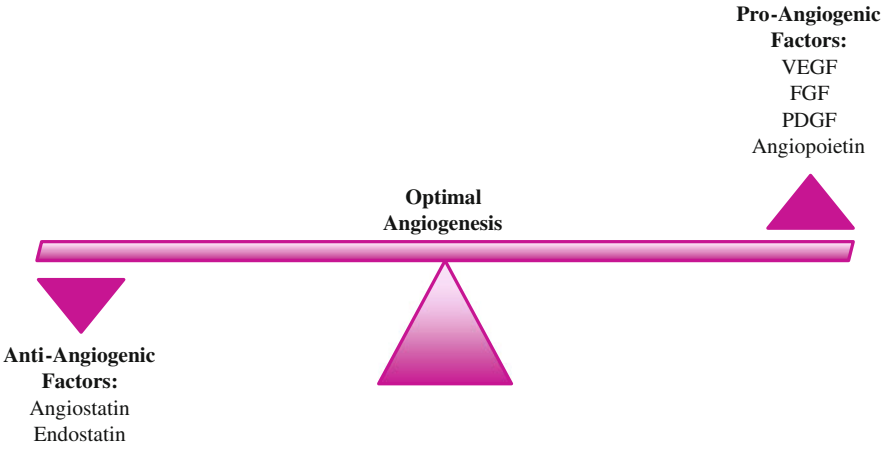


**Fig. 3** Endogenous pro-angiogenic and anti-angiogenic factors

thereby inhibiting endothelial cell growth, migration, and proliferation. Endostatin inhibits capillary endothelial cell proliferation. Both angiostatin and endostatin are potent suppressors of tumor growth and metastasis [22, 23]. For instance, angiostatin blocks angiogenesis induced by Kaposi's sarcoma cells; Kaposi's sarcoma is a highly angiogenic and inflammation-associated tumor [24]. Retinoids are another important class of molecules which oppose angiogenesis. Retinoids decrease VEGF expression in skin keratinocytes, and inhibit cell proliferation and differentiation [25]; retinoids are well-known teratogens due to their anti-angiogenic activity. Natural corticosteroids such as tetrahydrocortisol are also powerful inhibitors of angiogenesis [26].

Ultimately, the cocktail of pro-angiogenic and anti-angiogenic factors in a given tissue site determines whether blood vessel formation will take place (Fig. 3). Pro-angiogenic mediators such as VEGF and FGF, as well as anti-angiogenic mediators such as angiostatin and endostatin, are all potential targets for angiogenesis-modulating therapies. Both pro-angiogenic and anti-angiogenic pathways may be exploited to either encourage angiogenesis to treat injured and ischemic tissues, or discourage angiogenesis to treat cancerous tumors and inflammatory diseases. The next section will describe a prototypical pro-angiogenic approach: therapeutic angiogenesis for healing the heart.

## 3 Therapeutic Angiogenesis for Coronary Artery Disease

Coronary artery disease is the leading killer of men and women worldwide; it has been projected that deaths due to cardiovascular diseases will rise to 23.3 million per year by 2030 [27]. Congestive heart failure, the end stage of many heart diseases,

carries a 1 year mortality rate as high as 40 % and a 5 year mortality between 26 and 75 %; the prognosis for patients with congestive heart failure is worse than for those with most malignancies or AIDS [28]. Coronary artery disease results from occlusion of the coronary arteries supplying blood to the heart; complete occlusion leads to a myocardial infarction (heart attack). Acute myocardial infarction compromises the blood supply to the cardiac muscle, deprives the heart of oxygen and nutrients, and wreaks substantial cardiac tissue destruction. Coronary artery occlusion damages the large vessels of the heart, and also destroys endothelial cells of the microvasculature [29]. Further, a large number of cardiomyocytes (cardiac muscle cells), more than $10^9$ cells, can be lost following a myocardial infarction [30]. For these reasons, pro-angiogenic therapies are being developed to restore cardiac structure and function in patients with coronary artery disease.

The current standard of care for coronary artery disease is a combination of pharmacologic therapy and surgical intervention. Pharmacologic therapy includes angiotensin-converting enzyme (ACE) inhibitors and beta-blockers; both ACE inhibitors and beta-blockers improve long-term survival in coronary artery disease, but do not restore cardiac function [31]. The longtime surgical treatment for occluded coronary arteries has been coronary artery bypass surgery, in which a vein is harvested from another part of the body, and grafted onto the affected artery to bypass the blockage. However, bypass surgery is highly invasive, requiring the chest wall to be cracked open to expose the heart. Attendant complications include graft infection, chest wall dehiscence, and chest wound infection. Moreover, coronary artery bypass is technically difficult and costly, which limits patient access to the procedure. The contemporary interventional treatment for coronary artery disease is percutaneous angioplasty of occluded coronary arteries, using minimally invasive stents. Yet, even early reperfusion cannot completely reverse the effects of coronary artery occlusion, and the maximal benefit of early reperfusion has reached a level close to practical limits [32]. Therapeutic angiogenesis may be the next advance for overcoming coronary artery disease, enabling restoration of damaged heart tissues. Angiogenic therapy may also serve as a “biologic bypass” [33], providing a new treatment avenue for patients who are not candidates for mechanical revascularization or open bypass surgery.

### *3.1 VEGF Cytokines for Therapeutic Angiogenesis*

VEGF cytokines have been intensely pursued for therapeutic angiogenesis of ischemic heart tissues. In porcine models of chronic myocardial ischemia, VEGF infusion has been shown to improve collateral blood supply and decrease the ischemic zone. VEGF treatment induced natural bypass vessels in this pre-clinical model, and improved global and regional cardiac functioning: the cardiac ejection fraction increased (indicating stronger pumping function of the heart) and the regional cardiac wall thickened (indicating rebuilt cardiac muscle) [34]. In porcine

models subjected to left circumflex arterial constriction, VEGF treatment improved coronary flow and preserved regional hemodynamics [35]. Intracoronary treatment with VEGF, either by single intracoronary bolus or local delivery, stimulated significant angiogenesis in pigs [36]. In canine models subjected to left circumflex arterial occlusion, intracoronary VEGF infusion at the occlusion site was associated with a 40 % increase in collateral blood flow [37].

The safety of intracoronary infusion of recombinant human VEGF was assessed in a phase I clinical trial; the trial included 7 patients with coronary artery disease whose coronary anatomy was suboptimal for percutaneous or surgical revascularization [38]. Coronary infusion of VEGF was tolerated up to a maximal dose of 0.050 mg/kg/min; beyond this dose, patients experienced hypotension. VEGF gene transfer has also been attempted in phase I clinical trials. In one study of 5 patients who had failed conventional therapy for myocardial ischemia, naked plasmid DNA was injected directly into ischemic myocardium. The technique was safe, and there was objective evidence of reduced ischemia in all patients [39]. Direct myocardial injection of naked plasmid DNA encoding VEGF was subsequently shown to be safe in additional phase I trials [40, 41]. Further, the direct myocardial injection of an adenoviral vector expressing VEGF cDNA has demonstrated safety, in a phase I clinical trial of 21 patients with severe coronary artery disease [42].

Unfortunately, the successes of therapeutic angiogenesis with VEGF in pre-clinical and phase I clinical trials have not been reproduced in large phase II trials. In the VIVA (Vascular Endothelial Growth Factor in Ischemia for Vascular Angiogenesis) trial, a total of 178 patients with myocardial ischemia who were unsuitable for mechanical revascularization were randomized to receive placebo, low dose recombinant human VEGF, or high dose recombinant human VEGF [43]. Patients received an initial dose via intracoronary infusion on day 0, followed by peripheral infusions on days 3, 6, and 9. While the VEGF therapy was safe, the trial failed to meet its primary endpoint: VEGF offered no improvement beyond placebo in exercise treadmill time (the primary endpoint) at day 60. Direct myocardial injection of VEGF-encoding plasmid DNA has also been attempted in a randomized, double-blind placebo-controlled phase II study. In the Euroinject One trial, 80 “no-option” patients with severe stable coronary artery disease were randomized to receive either placebo or VEGF gene transfer [44]. This trial also failed to meet its primary endpoint: after 3 months, VEGF gene transfer did not significantly improve myocardial perfusion defects, though VEGF treatment was associated with improvements in local wall motion. Finally, the Kuopio Angiogenesis Trial assessed the efficacy of VEGF gene transfer during angioplasty and stenting [45]. This randomized, double-blind placebo-controlled phase II study involved 103 patients with coronary artery disease who were undergoing angioplasty. Patients were randomized to receive placebo, VEGF adenovirus, or VEGF plasmid liposome. Again, this trial did not meet its primary endpoint: after 6 months, there were no significant differences between study groups in clinical restenosis rates or minimal lumen diameter, though patients receiving VEGF adenovirus did demonstrate a significant increase in myocardial perfusion.

### 3.2 FGF Cytokines for Therapeutic Angiogenesis

The pro-angiogenic FGF cytokines have also been investigated for therapeutic angiogenesis of myocardial ischemia. In canine models of acute myocardial infarction, the intrapericardial injection of basic fibroblast growth factor (FGF2) induced angiogenesis in the infarcted area [46]. The result of FGF2 treatment was improved cardiac systolic function and reduced infarct size. Therapies based on FGF have subsequently been evaluated in clinical trials, with limited success. The AGENT (Angiogenic Gene Therapy) trial evaluated the safety of intracoronary delivery of an adenovirus encoding FGF4 [47]. Seventy-nine patients with coronary artery disease were randomized to receive a placebo or FGF4 adenovirus; the treatment was demonstrated to be safe. A phase II trial, the AGENT-2 study, then examined whether the FGF4 adenovirus treatment could improve myocardial perfusion [48]. In this randomized controlled double-blind trial, 52 patients with coronary artery disease were randomized to receive a placebo or FGF4 gene therapy. After 8 weeks, patients who received intracoronary injections of FGF4 gene therapy showed a significant reduction in ischemic defect size.

Two subsequent clinical trials, the AGENT-3 and AGENT-4 trials, were initiated as phase III studies of the FGF4 adenovirus for myocardial ischemia [49]. The two trials enrolled a total of 532 patients with coronary artery disease who were unsuitable for mechanical revascularization; both trials were randomized, double-blinded, and placebo-controlled. Patients were randomized to receive placebo, a low dose of FGF4 gene therapy, or a high dose of FGF4 gene therapy. The trials were intended to last 12 weeks. However, both trials were halted early, when an interim analysis revealed that the trials would fail to meet the primary endpoint: there would be no statistical significance between treatment groups in exercise treadmill time at 12 weeks.

There are several possible explanations for the failure of therapeutic angiogenesis utilizing VEGF or FGF in large-scale clinical trials. In terms of pharmacokinetics, VEGF and other growth factors have a relatively short half-life in the blood [50]; this may limit the ability of cytokines to act on target tissues. In terms of clinical trial design, the dosing concentrations and dosing schedules may have been suboptimal, and the routes of administration may have been ineffective [51]. In terms of cytokine biology, each cytokine has multiple physiological effects. Moreover, physiological angiogenesis involves the coordinated effort of multiple pro-angiogenic molecules. A single pro-angiogenic factor may be insufficient for lasting induction of angiogenesis. Therapeutic regimens that utilize combinations of cytokines may be required to achieve a clinically meaningful effect. Finally in terms of disease pathology, patients with coronary artery disease are likely to exhibit endothelial dysfunction, particularly in the context of severe atherosclerotic disease. This cellular dysfunction could limit the efficacy of therapeutic angiogenesis [52].

Novel biomaterials for therapeutic delivery could improve the clinical efficacy of pro-angiogenic therapies. Drug delivery vehicles may be useful for targeting cytokines to diseased tissue, thereby increasing the effective concentration of

pro-angiogenic cytokines at ischemic sites, and limiting the side effects of angiogenic growth factors (such as hypotension) outside the heart. For instance, the adhesion molecule P-selectin is upregulated on endothelial cells in myocardium, and may be targeted for delivery of angiogenic therapy. Anti-P-selectin coated liposomes have been designed as delivery vehicles for VEGF [53]. In a rat model of myocardial infarction, the VEGF-encapsulated immunoliposomes were injected systemically, and successfully concentrated VEGF in the ischemic heart. Treatment with VEGF-loaded immunoliposomes improved systolic function in rats, while systemic injection of non-targeted VEGF had no therapeutic effect. Targeted drug delivery is a promising approach for therapeutic angiogenesis, deserving of further investigation. In addition, the spatial and temporal presentation of VEGF has recently been demonstrated to influence angiogenesis [54]. Polymeric materials that control and regulate the spatial and temporal presentation of pro-angiogenic factors may be critical for optimizing therapeutic angiogenesis.

## 4 Suppression of Angiogenesis for Cancerous Tumors

Cancer is a disease of uncontrolled cellular proliferation and unchecked tissue growth. Tumor growth may lead to compression and disruption of local anatomical structures, as well as invasion of adjacent tissue, and infiltration beyond the primary tumor during metastasis. Cancerous tumors are caused by the accumulation of genetic alterations over time. Such alterations may be the result of inheritance or environmental exposures to carcinogens. As genetic alterations accumulate, cells transform from a normal phenotype into a malignant phenotype. Infiltration of malignant cells into the underlying stromal tissue signals the first sign of invasive cancer. The process of carcinogenesis may take 10–20 years to evolve [55]. Because of Judah Folkman's pioneering work [56], it is now well-known that a sustained level of tumor growth requires an adequate vascular supply. Tumors must have an ongoing blood supply to grow beyond a minimum size of 2–3 $mm^3$. Neovascularization of tumors is also a necessary prerequisite for escape and metastasis of cells; once metastatic cells arrive at a distant site, angiogenesis is again required to establish a new tumor [57]. For these reasons, the tumor vascular supply is a widely recognized target for anti-angiogenic treatments.

The chief objective of pharmacologic anti-angiogenic agents is to target disease-driven angiogenesis within cancerous tumors, while sparing healthy tissues from damage. Efforts toward anti-angiogenic therapies for cancer have yielded notable clinical successes; several anti-angiogenic agents are already part of standard treatment regimens for cancer. Yet these efforts have also been marked by clinical disappointment, as tumors tend to develop resistance to anti-angiogenic agents over time. The ultimate result is that patients experience a transitory improvement in cancer, followed by tumor recurrence and cancer progression. This phenomenon has been revealed during the development and clinical adoption of VEGF-targeting agents in cancer treatment.

### 4.1 Bevacizumab: the First Clinical Anti-Angiogenic Therapy

As the key regulator of angiogenesis, VEGF has been the prime focus of anti-angiogenic drug development for cancer. One of the first biologic VEGF-targeting agents was a mouse anti-VEGF antibody [58]. A recombinant humanized anti-VEGF antibody was then developed by Genentech. This antibody, named bevacizumab, targets all isoforms of human VEGF-A [59]. A phase III clinical trial was conducted in 813 patients with previously untreated metastatic colorectal cancer; patients were randomized to receive either combination chemotherapy plus bevacizumab, or combination chemotherapy alone [60]. The addition of bevacizumab to combination chemotherapy resulted in clinically significant improvements in progression-free survival and overall survival; bevacizumab boosted overall survival by a median time of 4.5 months when added to combination chemotherapy. In 2004, bevacizumab (Avastin®) gained Food and Drug Administration (FDA) approval as a first-line treatment for metastatic colorectal cancer, becoming the first FDA-approved anti-angiogenic treatment for cancer [61].

Bevacizumab has been evaluated for treatment of metastatic renal cell carcinoma. A multi-center, randomized, double-blind phase III trial was conducted in 649 patients with previously untreated renal cell carcinoma; patients were randomized to receive either interferon alfa plus bevacizumab, or interferon alfa alone [62]. Again, the results were clinically significant. The addition of bevacizumab to interferon alfa boosted progression-free survival by a median time of 4.8 months. Bevacizumab raised overall survival by a median time of 2 months when added to interferon alfa [63]. The combination of bevacizumab and interferon alfa is now FDA-approved as first-line treatment for metastatic renal cell carcinoma. Bevacizumab has also shown promise for treating a brain cancer, glioblastoma multiforme. A phase II clinical trial of bevacizumab plus irinotecan was conducted in 35 patients with recurrent glioblastome multiforme [64]. The prognosis for these patients has historically been very poor, but twenty of 35 patients in the bevacizumab trial had at least a partial response. Bevacizumab now carries FDA approval for recurrent glioblastoma multiforme.

The anti-VEGF antibody has demonstrated similar success for non-small-cell lung cancer. A randomized phase III clinical trial was conducted, to investigate whether the addition of bevacizumab to first-line chemotherapy with paclitaxel and carboplatin improves survival in patients with metastatic non-small-cell lung cancer [65]. The trial enrolled 878 patients with recurrent or advanced non-small-cell lung cancer; patients were randomized to receive either bevacizumab plus paclitaxel and carboplatin, or paclitaxel and carboplatin alone. The results were significant and clinically meaningful. Adding bevacizumab to first-line chemotherapy improved response rate and progression-free survival. Bevacizumab increased overall survival time by a median time of 2 months when added to first-line chemotherapy. The anti-VEGF antibody, in combination with carboplatin and paclitaxel, is FDA-approved for treatment of advanced non-squamous, non-small-cell lung cancer.

### 4.2 Limitations of Bevacizumab for Anti-Angiogenic Therapy

The limitations of bevacizumab became evident in clinical trials for metastatic breast cancer. An open-label, randomized phase III clinical trial was conducted to determine whether the addition of bevacizumab to paclitaxel is beneficial for metastatic breast cancer therapy [66]. A total of 722 patients were enrolled in the trial, and were randomized to receive either bevacizumab plus paclitaxel, or paclitaxel alone. Initial therapy of metastatic breast cancer with bevacizumab plus paclitaxel did prolong progression-free survival, as compared with paclitaxel alone. However, bevacizumab imparted no benefits for overall survival in metastatic breast cancer.

Bevacizumab also failed to demonstrate efficacy for pancreatic cancer. A randomized, double-blinded phase III clinical trial was conducted in 301 patients with metastatic pancreatic adenocarcinoma [67]. Patients were randomly assigned to receive either bevacizumab plus gemcitabine-erlotinib, or gemcitabine-erlotinib alone. The addition of bevacizumab to gemcitabine-erlotinib did prolong progression-free survival, but there was no improvement in overall survival. A subsequent phase III clinical trial was conducted in 602 patients with advanced pancreatic cancer; patients were randomly assigned to receive bevacizumab plus gemcitabine, or gemcitabine alone [68]. Again, there was no survival benefit associated with the addition of bevacizumab to gemcitabine.

### 4.3 Tumor Resistance to VEGF-Blocking Agents

VEGF-blocking agents not only bear limitations with regard to efficacy, they can also induce undesirable long-term effects. Notably, tumors can cultivate complete resistance to anti-angiogenic VEGF-targeting agents over time, leading to tumor resurgence. One mechanism for tumor resistance is the existence of alternative angiogenic signaling pathways. Even when VEGF-mediated signaling is entirely blocked, tumors can utilize other pro-angiogenic factors as substitutes for VEGF [69]. As a case in point, inhibition of VEGF signaling in late-stage pancreatic islet tumors leads to upregulation of other pro-angiogenic mediators, including molecules of the FGF family [70]. Inhibition of VEGF-mediated pathways can even lead to up-regulation of VEGF itself in glioblastoma multiforme [71]. Paradoxically, VEGF-blocking therapeutic agents increase tumor hypoxia, which leads to the activation of pro-angiogenic pathways. When malignant tumors are treated with VEGF-blocking agents, the tumors initially respond to treatment and stop growing; after this initial period of growth suppression, malignant tumors rebound aggressively as they re-vascularize and relapse via multiple signaling pathways.

For patients fighting cancer, the clinical result is that anti-angiogenic therapies have short-lived efficacy at best. Patients experience a transient clinical benefit, followed by tumor re-growth at a greatly accelerated pace. Therapies such as bevacizumab may effect a 3–6 month increase in progression-free survival, but

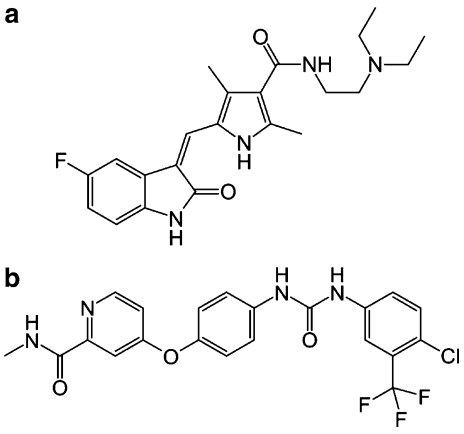


**Fig. 4** Chemical structures of receptor tyrosine kinase inhibitors. **a** Sunitinib. **b** Sorafenib

cannot provide lasting clinical responses. In fact, anti-angiogenic therapy can lead to heightened invasiveness and increased metastasis of tumors [72]. Indeed, a subset of glioblastoma patients experienced recurrence with more infiltrative tumors following treatment with bevacizumab [73].

## *4.4 Next-Generation Anti-Angiogenic Agents*

A second-generation of anti-angiogenic therapies, the receptor tyrosine kinase inhibitor class, is currently under development (Fig. 4). Unlike bevacizumab, these drugs are small molecules that inhibit multiple targets; the newer agents can thus be used as monotherapy for cancer. For example, sunitinib is a receptor tyrosine kinase inhibitor that blocks signaling through VEGF receptors and PDGF receptors; sunitib also targets other tyrosine kinases including, KIT, FLT3, colony-stimulating factor 1 (CSF-1), and RET, which are involved in several different malignancies [74]. Sunitinib has been shown to prolong progression-free survival for renal cell cancer. A multi-center randomized phase III clinical trial was conducted in 750 patients with previously untreated metastatic renal cell carcinoma; patients were randomized to receive either sunitinib or interferon alfa [75]. Sunitinib increased progression-free survival by a median time of 6 months as compared to interferon alfa. Sunitinib also demonstrates efficacy for gastrointestinal stromal tumors. A clinical trial was performed in 312 patients with unresectable gastrointestinal stromal tumors after failure of imatinib therapy; patients were randomized to receive either sunitinib or placebo [76]. Sunitinib significantly increased the time to tumor progression by more than 20 weeks. The first drug to simultaneously receive FDA approval for two different cancers, sunitinib is now FDA-approved for metastatic renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor [77].

Sorafenib is another second-generation anti-angiogenic agent; this receptor tyrosine kinase inhibitor blocks signaling through VEGF receptors, PDGF receptors,

and Raf [78]. Sorafenib has been shown to increase progression-free survival in renal cancer. A randomized, double-blind, placebo-controlled phase III clinical trial was conducted in 903 patients with advanced renal cell carcinoma that was resistant to standard therapy; patients were randomly assigned to receive either sorafenib or placebo [79]. Sorafenib was associated with an increase of 2.7 months in progression-free survival; however, sorafenib therapy was also associated with increased toxic effects, including hypertension and cardiac ischemia. Sorafenib has demonstrated definite efficacy for hepatocellular carcinoma, prolonging overall survival. A multi-center randomized, double-blind, placebo-controlled phase III clinical trial was conducted in 602 patients with advanced hepatocellular carcinoma who had not received previous systemic treatment; patients were randomized to receive either sorafenib or placebo [80]. Sorafenib therapy increased overall survival by a median time of nearly 3 months. Sorafenib is now FDA-approved for treatment of advanced renal cell carcinoma and advanced hepatocellular carcinoma.

Next-generation anti-angiogenic agents such as sunitinib and sorafenib do exhibit better efficacy than bevacizumab against certain forms of cancer, and these newer agents do target multiple angiogenic pathways. Yet, the newer drugs still have serious limitations; the receptor tyrosine kinase inhibitors have not proven to reduce tumor size or lengthen overall survival time in many types of cancer [81]. As with bevacizumab therapy, treatment with receptor tyrosine kinase inhibitors is characterized by an initial increase in progression-free survival, but there are few significant advantages for overall survival rate compared to patients not receiving the drug [82]. Indeed, the receptor tyrosine kinase inhibitor sunitinib has been shown to accelerate metastatic tumor growth and decrease overall survival in experimental metastasis models [83], as tumor cells metastasize to distant sites in search of oxygen and nutrients [84].

The tumor microenvironment, as well as the heterogeneity of endothelial cells and tumor cells, may contribute to tumor resistance to anti-angiogenic drugs [85]. Increased molecular and cellular characterization of tumor pathology may enable more efficacious anti-angiogenic therapies. In addition, feedback pathways and compensatory pathways influence tumor responses to therapy. The metabolic and signaling pathways that regulate tumor growth must therefore be elucidated. Finally, the pharmacokinetics of anti-angiogenic agents must be optimized. Maximal anti-angiogenic therapy requires prolonged exposures of tumor cells to anti-angiogenic drugs; it is more important to achieve a sustained optimal biological dose, rather than a maximal tolerated dose [86]. Novel drug delivery systems, which allow controlled release of anti-angiogenic therapeutics over extended time periods, could potentially boost clinical responses.

## 5 Conclusion and Future Research Directions

Because all living structures require an adequate oxygen supply to maintain viability, angiogenesis is a central process that determines the survival of cells, tissues, organs, and ultimately human beings. The modulation of angiogenesis is an emerging approach for attacking myriad chronic diseases. Pro-angiogenic

therapies hold promise for rebuilding tissues to treat wounds, ulcers, coronary artery disease, stroke, emphysema, and ischemic conditions in general. Anti-angiogenic therapies hold promise for treating cancer and inflammation. However, pro-angiogenic and anti-angiogenic approaches have not yet realized their potential in the clinic. As exemplified by therapeutic angiogenesis for coronary artery disease, the promotion of angiogenesis in human tissues is challenging, and successes in pre-clinical studies have not translated to large-scale clinical trials. As exemplified by angiogenesis inhibitors for cancer therapy, tissues often develop resistance to angiogenesis-blocking agents, and anti-angiogenic agents often lose their efficacy over time. Clearly, the administration of a single cytokine or a single cytokine-blocking agent is insufficient for achieving long-term pro-angiogenic or anti-angiogenic effects.

Future research must focus on refining the characterization of angiogenesis at the molecular, cellular, tissue, and organ levels. A detailed understanding of multiple cell signaling pathways will be necessary to the development of effective therapeutics, and may suggest novel avenues for therapy. Further, the properties of various cellular populations must be delineated, both in healthy and diseased tissues. For example, endothelial cells exhibit specific dysfunctions in both coronary artery disease and cancer; such abnormalities will most certainly influence cellular responses to therapy. The roles of cellular migration and cellular mechanics must be investigated, along with the interactions of cells with the extracellular matrix.

In regard to drug development, new methods for drug delivery which enable cell and tissue targeting, as well as controlled release, will improve therapeutic efficacy. The spatial and temporal delivery of pro-angiogenic and anti-angiogenic agents must be optimized. Finally, it is likely that a combination of agents will be required to achieve a lasting therapeutic effect. An urgent clinical need remains for novel pro-angiogenic and anti-angiogenic approaches. With continued research advances in angiogenesis, therapeutic strategies for angiogenesis will continually improve, and progressively alleviate the global burden of disease.

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# Erratum to: Reactive Oxygen Species in Physiologic and Pathologic Angiogenesis

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