Published in final edited form as:

Wiley Interdiscip Rev Syst Biol Med. 2013 September ; 5(5): 587–602. doi:10.1002/wsbm.1233.

Endothelial cell motility, coordination and pattern formation during vasculogenesis

Andras Czirok^{1,2,3}

¹Dept. of Anatomy and Cell Biology; University of Kansas Medical Center; Kansas City, KS

²Dept. of Biological Physics; Eotvos University; Budapest, Hungary

Abstract

How vascular networks assemble is a fundamental problem of developmental biology that also has medical importance. To explain the organizational principles behind vascular patterning, we must understand how can tissue level structures be controlled through cell behavior patterns like motility and adhesion that, in turn, are determined by biochemical signal transduction processes? We discuss the various ideas that have been proposed as mechanisms for vascular network assembly: cell motility guided by extracellular matrix alignment (contact guidance), chemotaxis guided by paracrine and autocrine morphogens and multicellular sprouting guided by cell-cell contacts. All of these processes yield emergent patterns, thus endothelial cells can form an interconnected structure autonomously, without guidance from an external pre-pattern.

1 Vasculogenesis and angiogenesis

Vascular network assembly is a morphogenetic problem that has attracted a great deal of attention for a number of reasons. Blood vessels are the earliest and simplest functioning organ system of the embryo. Vascular networks form so early in embryogenesis that investigators have reasonable access to specimens with which to observe and understand the emergence of a vascular pattern, its structures and associated functions. Furthermore, the assembly and remodeling of the vascular system is of vital importance in multiple pathologies – the premier examples being wound healing and tumor progression. A number of approved or planned medical interventions attempt to either promote or hinder tissue vascularization^{2,3}, or to correct an irregular vascular network^{4,5}. Finally, virtually all tissue engineering projects need to provide an adequate circulatory network in addition to the specific functions of the tissue or organ in question^{6,7}.

The special focus of this review is vasculogenesis; defined here as the assembly process resulting in the first embryonic blood vessel network before the onset of circulation⁸. Angiogenesis, in contrast, designates the formation of new vascular segments as sidebranches of existing, mature vessels containing blood – a process characteristic of later development and in adults. In avian (bird) and mammalian embryos vasculogenesis creates the primary system of tubes, via a process whereby thousands of essentially identical vascular endothelial cells assemble into an interconnected “polygonal” network (Fig. 1). The resulting transient primary vascular plexus has no similar counterpart in cold-blooded vertebrates like fish or frogs, where the main vessels assemble directly, without the adaptation of an intermediate network. While vascular precursors both proliferate and differentiate from the mesoderm during vasculogenesis⁸, the assembly of the primary network is completed in a few hours (between Hamburger and Hamilton⁹ stages 8 and 10),

³aczirok@kumc.edu

much shorter than the typical cell cycle time of 20 hours. The location of differentiating endothelial precursors also seem to be randomly scattered and not localized to the position of future vascular segments¹⁰. Therefore, the main cellular mechanism responsible for the early phase of vascular patterning is cell migration and its guidance. Interestingly, vasculogenesis, like angiogenesis, involves multicellular assemblies called “sprouts” that invade previously avascular areas^{11,12}, thus a basic cellular patterning mechanism (i.e. the formation, elongation and guidance of multicellular sprouts) appears to be shared to a great extent between vasculogenesis and angiogenesis.

The most obvious difference between the two processes is that vasculogenesis takes place in a simpler tissue environment and involves one cell type only, whereas during angiogenesis the pre-existing vessels are exposed to blood flow, consist of three tissue layers (*tunics*), multiple cell types (endothelium, smooth muscle, pericytes) and a specialized extracellular matrix (ECM). Thus, compared to angiogenesis, vasculogenesis is a conceptionally simpler and a more tractable problem to understand. An additional important aspect of vasculogenesis is that the underlying mechanism of “spouting” – i.e., the ability to form interconnected tubes or solid cords – is shared by a host of cell types. For example, highly malignant melanoma cells were reported to self-assemble into tubes to secure their blood supply¹³. Formation of cell chains is a widespread cellular behavior in culture^{14,15}. Thus the ability of cells to form interconnected cellular networks seems to be a quite generic property that is manifested to various extents by cell lines derived from all primary germ layers.

While the advances of molecular biology and imaging techniques have revealed aspects of the vessel assembly process, its guiding principles remained unsettled. The combinatorial complexity of the biological components participating in the process is daunting: growth factors, their receptors and co-receptors, the ECM and its degradation through various matrix metalloproteases, as well as the array of possible cell-cell interactions can all substantially influence the sprouting process¹⁶. The level of complexity is increased by the fact that in embryos and growing organs the surrounding tissues are expanding rapidly as the blood vessels form. The aim of this review is to discuss the ideas that have been proposed to explain the main mechanism of vascular patterning, especially during primary vasculogenesis. All explanations for the emergent morphogenetic patterning are in the form of a computational model – as the use of quantitative models is currently our only approach to understand complex systems.

Box on the Cellular Potts Model

The key concept of modeling multicellular assemblies is motivated by observations suggesting that cadherin-mediated cell-cell adhesion^{18–22} and – more recently – cell-exerted cortical tension²³ yield cell rearrangements that are analogous to surface tension driven dynamics of immiscible liquid droplets. Therefore, most computational models represent cells as fluid-like droplets^{24–26}, where their area and perimeter is restricted by a mechanism analogous to surface tension and elastic compressibility. The Cellular Potts Model (CPM)²⁴ captures the surface tension-like behavior of cell-cell adhesion through costs associated with free and intercellular cell boundaries. In the CPM approach a label σ is assigned to each lattice site $\mathbf{x}$ of a grid and cells are represented as simply connected domains, i.e., a set of adjacent lattice sites sharing the same label. In addition to identifying individual cells, specific labels are also used to mark cell free areas and ECM clusters. A goal function u (‘energy’) is assigned to each possible spatial configuration of cells and ECM, and it is used to distinguish favorable (low u) and unfavorable (high u) configurations. The simplest choice of u is

$$u = \frac{1}{2} \sum_{i,j} J_{i,j} \ell_{i,j} + \lambda \sum_i \Delta A_i^2, \quad (1)$$

where the indices i and j take all possible values of the label σ ²⁴. The first term in (1) enumerates and penalizes cell boundaries: $\ell_{i,j}$ denotes the boundary length between domains i and j ($\ell_{i,i} = 0$), and the sum enumerates all combinations of i and j where at least one of the indices annotate a cell. The second term is responsible for maintaining a preferred cell area: for each cell i the deviation of its area from a pre-set value is denoted by ΔA_i . Parameters, like the elements of the J matrix as well as the inverse compressibility λ are used as weights to tune model behavior such as the smoothness of cell boundaries or variation of cell sizes. For a homogenous population in an ECM environment elements of J can take only three values. For any cell i :

$$J_{i,j} = J_{j,i} = \begin{cases} \alpha, & \text{if } j \text{ is a cell} \\ \beta, & \text{if } j \text{ is an "empty" area} \\ \gamma, & \text{if } j \text{ is an ECM cluster} \end{cases} \quad (2)$$

Cell movement in the CPM is the result of a series of elementary steps, which are attempts to copy the spin value onto a random lattice site $\mathbf{b}$ from a randomly chosen adjacent site $\mathbf{a}$. This move can be thought of as a possible activity of the cell occupying site $\mathbf{a}$, and it is executed with a probability $p(\mathbf{a} \rightarrow \mathbf{b})$. The probability assignment rule ensures that the configuration of cells tend to improve according to the measure (1). For convenience and historical reasons p is given as

$$\ln p(\mathbf{a} \rightarrow \mathbf{b}) = \min[0, -\Delta u(\mathbf{a} \rightarrow \mathbf{b}) + w(\mathbf{a} \rightarrow \mathbf{b})], \quad (3)$$

where, $\Delta u(\mathbf{a} \rightarrow \mathbf{b})$ is the change in the goal function during the elementary step considered, and w represents a bias responsible for various cell-specific active behaviors. The latter, like persistent movement, rely on information that is not deducible from the current spatial configuration of the system. The morphologies which develop in a simple ($w = 0$) two dimensional system consisting of cells, ECM and cell free areas are demonstrated in Fig. 2.

2 Endothelial cell behavior

2.1 Motility of individual cells

The pioneering work of Stokes et al²⁷ revealed that the motility of individual endothelial cells can be well approximated as a persistent random walk. For a short period of time, cells move with a sustained speed along a straight line. In contrast, the motion is random (diffusive) if investigated during a long enough time frame. The speed of the individual cells varies with the culture conditions (such as the type of ECM and growth factors present), and was found to be in the range of 10 – 50 $\mu\text{m}/\text{h}$ ^{27–29}. In vivo, motile endocardial³⁰ and endothelial¹¹ cells also move with a mean speed of approximately 20 $\mu\text{m}/\text{h}$. The persistence time, the time scale separating the linear and diffusive regimes of the persistent random walk, was reported in the range of 0.6–3h in cell cultures^{27–29}.

2.2 Collective migration

Endothelial cells also exhibit a capacity for coordinated (collective) migration in culture. In the absence of directed expansion of the whole monolayer, these cells exhibit a globally undirected, but locally correlated streaming behavior. Statistical characterization of the spontaneous streaming motion within endothelial monolayers revealed that cells move in

locally anisotropic, 50–100 μm wide and 200–300 μm long streams, which form and disappear at random positions²⁹. Endothelial monolayers also exhibit collective flow patterns in the developing vasculature of the embryo. Studies imaging the vascularization of transgenic quail embryos – in which endothelial cell nuclei express a GFP variant – revealed vigorous motility within the inner lining of major vessels such as the aortae¹². While statistical characterization of the motion patterns is not yet available, the reported cell trajectories are in many aspects similar to those observed in monolayer cultures. A recent high-throughput study of the genes involved in endothelial sheet migration suggested that distinct “signaling circuits” are responsible for setting the speed of cell motility, its directionality and coordination with adjacent cells³¹.

To explain the flow that emerges within endothelial monolayers, a suitably extended cellular Potts model (CPM²⁵) was proposed²⁹. Spontaneous, persistent cell motility was introduced in the CPM through a postulated positive feedback between cell polarity and cell displacements²⁹. Thus, the model assumes that cell protrusions are more likely to occur at the front of the cell. In turn, the leading edge is stabilized by its continuous advance, a rule supported by empirical findings concerning actin polymerization and PI3K activity^{32,33}. As model simulations demonstrate, such a mechanism, together with steric constraints due to limited cell compressibility, can closely reproduce the observed spontaneous streaming behavior in endothelial monolayers²⁹.

2.3 Chemotaxis

A key characteristic of endothelial cells is their motility response to growth factors, and in particular to VEGF. In cultured endothelial cells, VEGF induces cell motility^{34,35} and a chemotactic response^{36,37}. Chemotactic response is often described as a drift (of the otherwise random cell motility) in the direction of the growth factor gradient³⁸. For a given concentration gradient, the chemotactic index compares the magnitude of the drift motion to the speed associated with the persistent random motion. Best characterized is the chemotactic response of microvessel endothelial cells to aFGF, where the chemotactic index was estimated as 1.5, thus chemotactic directed motion dominated over random motility²⁷. More recent studies using microfluidic chambers indicated that a VEGF gradient of approximately 20 (ng/ml)/mm is needed to elucidate directional migration of cells^{39,40}. To put this value in perspective, 40–100 ng/ml is the estimated bulk concentration of VEGF needed to saturate cell surface VEGF receptors *in vitro*³⁹. Thus, under optimal conditions endothelial cells could respond to distant VEGF sources, even to a source that is a few millimeters away. The ECM may also modulate the availability of growth factors by sequestering them. In particular, fibronectin was shown to control the availability of TGF beta^{41,42} and VEGF⁴³, in some cases acting in a synergistic manner with heparan sulfate⁴⁴.

The chemotactic response to VEGF is mediated by VEGF receptors. The two best characterized receptors are VEGFR1 (Flt1) and VEGFR2 (Flk1). The latter are monomeric receptors with cytoplasmic tyrosine kinase domains. VEGF is bivalent and initiates signaling by binding to two VEGFR2 receptors. As the first step of signal transduction, the two cytoplasmic receptor kinases in the VEGFR2 dimer complex phosphorylate each other⁴⁵. As VEGFR1 lacks the kinase domain, its presence in the receptor dimer complex renders it inert. In mouse embryonic development, VEGFR1 and VEGFR2 appear to compete for VEGF⁴⁶. As discussed in detail below, VEGFR1 also has a soluble isoform, which was reported to elicit profound effects on the vascular structure through VEGF sequestration^{47–50}.

2.4 Mechanosensing

Endothelial cell behavior is strongly influenced by the composition, structure and mechanical properties of the ECM microenvironment^{51–54}. In particular, collagen density can control vessel density (vessels per area) and vessel size (cross sectional area)⁵⁵. The motility and proliferation of ECs are enhanced at intermediate collagen densities of 1.2 – 1.9 mg/ml,⁵⁶. Endothelial cells can detect and respond to substrate strains created by the traction stresses of a neighboring cell, and this response was found to be dependent on matrix stiffness⁵⁷. In addition to the ECM environment's ability to modulate sprouting activity, endothelial cells actively follow micro-tracks (or channels) within the ECM environment^{58,59}.

2.5 Delta-notch signaling

A special type of cell-cell interaction, whereby a temporary lateral inhibition of motility exists between adjacent endothelial cells, was recently suggested to be operational within angiogenic sprouts⁶⁰. This process is mediated through delta and notch cell surface receptors, and is thought to be responsible for restricting the invasive phenotype to a few cells of the sprout⁶¹. The basic mechanism operates in the following fashion: VEGF-activated VEGFR2 receptors upregulate the expression of the Notch ligand Dll4. Dll4 binds to Notch receptors on neighbouring cells and renders these cells passive by down-regulation of VEGFR2 receptors^{62–64}. This interaction can lead to the specialization of cells within the sprouts. Empirical evidence suggests that during angiogenic sprouting the leading tip cells have a different intracellular signaling activity than the other (often termed “stalk”) cells⁶⁵. Tip cells are thought to be more motile, invasive and responsive to chemotactic signals. Invoking the delta-notch lateral inhibition mechanism, tip cells can temporarily restrict adjacent cells in the more inactive stalk state^{60,61}.

3 Mechanisms of vascular patterning

3.1 Cells guided by a pre-pattern

Early modeling attempts described vascular patterning as a random branching process, where each branch tip followed chemotactic cues, very similar to single-cell stochastic dynamics^{66,67}. Conventional explanations of vasculogenesis often assume that endothelial cells, like neural growth cones, migrate to pre- and well-defined positions following extracellular guidance cues or chemoattractants^{68–70} (Fig. 3a). The best documented example of such a process is angiogenesis within the retina. In the retina, the intricate structure of glial cell processes and the associated ECM, rich in VEGF, were shown to guide endothelial cells and organize the vasculature into a characteristic pattern^{65,71}. During this process fibronectin was shown to control the availability of VEGF⁴³, acting in a synergistic manner with heparan sulfate⁴⁴. Vasculogenesis in fish, where major vessels assemble directly (i.e. without forming an intermediate vascular plexus) also seems to be guided by a genetic pre-pattern, as specific vascular malformations are correlated with genetic defects⁷².

Yet, the applicability of VEGF-guided sprouting to generate the vascular plexus of warm blooded vertebrates is not clear. A VEGF prepattern, similar to the one in the retina, has not been demonstrated in the ECM associated with the lateral plate mesoderm⁷³. Furthermore, while stalk cells are supposedly less active than tip cells in angiogenesis, they are quite motile during vasculogenesis in avian embryos¹². Allantois explants, established *in vitro* models of primary vasculogenesis⁷⁴, also reveal vigorous cell displacements within the sprout, on a scale comparable to the sprout length⁷⁵. The capacity of endothelial cells to form a polygonal pattern is preserved in various *in vitro* systems, such as 3D collagen gels^{76,77}, where the presence of a genetic (or environmental) pre-pattern is either not

possible or highly unlikely. Thus, endothelial cells are clearly capable of self-assembling a network even in the absence of pre-existing environmental cues.

3.2 Contact guidance and mechanosensing

Cells embedded in an ECM gel can substantially remodel their environment. Groundbreaking experiments of Harris and Stoplak revealed that cell traction forces create aligned ECM bundles that radiate from, and may connect cell aggregates⁷⁸. Even individual cells can reorganize and align collagen fibers⁷⁹ and the oriented ECM structure can guide cell migration^{52,80}. Combining these observations, an early model of vasculogenesis proposed that angioblasts first segregate into compact clusters and exert traction forces on the surrounding ECM fibers⁸⁰. As a result, orientation develops within the ECM, which is able to route endothelial cells between clusters¹⁰.

A mathematical model (Fig. 3b) describing this system usually includes a viscoelastic constitutive equation for the ECM substrate^{81,82}, which relates the mechanical stress in the ECM, σ_{ECM} , to its strain ε and its strain rate $\dot{\varepsilon}$. For a simple, rubber-like isotropic material

$$\sigma_{ECM} = \mu_1 \partial_t \varepsilon + \mu_2 (\text{Tr} \partial_t \varepsilon) I + \frac{E}{1+\nu} \left[\varepsilon + \frac{\nu}{1-2\nu} (\text{Tr} \varepsilon) I \right] \quad (4)$$

where E , ν , μ_1 and μ_2 are material parameters and I is the unit tensor. The strain ε can be obtained from the ECM displacement field $\mathbf{u}$, which specifies the relocation of ECM particles relative to their initial, stress-free state. In its simplest (linear) form, valid for small displacements, the relation between ε and $\mathbf{u}$ is

$$\varepsilon = \frac{\partial_x \mathbf{u} + (\partial_x \mathbf{u})^T}{2}. \quad (5)$$

For large deformations more sophisticated strain measures are used^{83,84}. As cells exert traction forces, the requirement of mechanical equilibrium within the ECM is determined by the balance of forces

$$\nabla \cdot (\sigma_{ECM} + \sigma_{cells}) = \mathbf{F}_{ext}, \quad (6)$$

where $\mathbf{F}_{ext}$ represents possible additional external forces acting on the system, such as friction between the ECM and an underlying rigid substrate⁸¹. For a given spatial distribution of σ_{cell} and $\mathbf{F}_{ext}$, ECM movements can be derived from the set of differential equations (4)-(6).

As cells and the ECM form a composite material, displacements of both components are strongly related. Local conservation equations specify the changes in cell (n) and ECM (ρ) densities as

$$\partial_t n = -\nabla \cdot \mathbf{J}_{cell} \quad (7)$$

$$\partial_t \rho = -\nabla \cdot \mathbf{J}_{ECM} \quad (8)$$

where $\mathbf{J}$ denotes current densities ($\mathbf{J} \cdot \Delta \mathbf{A}$ being the amount of material moved across a small area of size ΔA per unit time). For the ECM, the current density is simply

$$\mathbf{J}_{ECM} = \rho \mathbf{v}. \quad (9)$$

For cells, we need to take into account that in addition to being convected with the ECM, cells also engage in active motility. If cell movements have a short persistence, cell motility can conveniently be formulated as a diffusion process that is being modulated by the state of the surrounding ECM as

$$\mathbf{J}_{cell} = n \mathbf{v} - D(\varepsilon) \nabla n. \quad (10)$$

Thus, contact guidance along oriented ECM filaments is modeled as an anisotropic diffusion, with greater diffusivity in directions parallel to the principal stretch strain. Finally, the biomechanical patterning framework assumes that traction force generation, σ_{cell} , is proportional to the local cell density, at least for low cell densities.

Similar models representing cell-ECM assemblies were analyzed and studied by computer simulations^{82,85,86}. These studies revealed a patterning mechanism in which a random initial inhomogeneity in cell density results in pattern coarsening: increasingly large cell free areas develop through the inflation and merger of pre-existing inhomogeneities, a process similar to the dynamics of foams⁸¹. Thus, cell free areas develop primarily by collecting both cells and the surrounding ECM into increasingly dense clusters. The coarsening process continues until the deformations in the ECM are so large that cell traction forces cannot compete with σ_{ECM} —the resulting balance sets the characteristic pattern size. This theory thus offers a reasonable explanation for a popular *in vitro* model of vascular assembly, in which endothelial cells are cultured on the surface of a highly malleable ECM gel, like Matrigel. Indeed, network assembly on Matrigel surfaces requires sub-confluent cell seeding densities and the main patterning mechanism involves progressive elimination of small cell-free areas. A confluent monolayer, without spatial variation in cell density, will not form a network by this mechanism. When the model is expanded with additional complexity, such as non-local elasticity⁸², sprouting-like solutions can appear, similar to those reported in Matrigel assays under optimized experimental conditions⁸⁷.

This biomechanical model suggests that a wide variety of cells can exhibit similar patterning dynamics – in fact all cells that both exert traction forces and are being guided by aligned ECM fibers. Indeed, fibroblasts, smooth muscle cells, and cells of the murine Leydig cell line TM3 form networks on Matrigel matrix in much the same fashion⁸⁸. Endothelial cells, however, are unlikely to utilize this biomechanical mechanism to create vascular networks and multicellular sprouts in the embryo. Neither ECM deformation pre-patterns nor comovement of cells and ECM into vascular polygons were reported either in embryos or in 3D collagen gel models. Furthermore, additional cellular mechanisms must be able to create multicellular sprouts as the process can also take place on unstructured (spatially uniform) rigid substrates¹⁵.

3.3 ECM “memory”

More recent models of contact guided patterning associate the ECM with local state variables such as the extent of crosslinking, or discontinuity of the composite ECM – instead of with variables characterizing strictly its mechanical state. These states are measures of irreversible changes that can act as local “memory”: present even after cells leave the area (Fig. 3c). This choice also allows greater flexibility to represent ECM reorganization by the cells^{17,89,90}. In these models, multicellular sprouts readily develop when a positive feedback between the direction of active cell movement (cell polarity) and ECM “memory” is assumed. This mechanism is thus very similar to the ones proposed to explain how ants

organize pheromone trails⁹¹. In the models, the ECM often has a dual role – it functions as a cell adhesion substrate, and it also restricts possible cell movements. In the CPM formalism, the preference of cells to adhere to the ECM is satisfied when the cost of a cell-ECM boundary is less than that of a free cell boundary ($\beta < \gamma$, i.e. parameters from the domain under the green line in Fig. 2). Furthermore, cells may actively spread along an ECM surface if the cost of extending the surface is balanced by the gain in reduced cost of cell-ECM contacts¹⁷:

$$\frac{3\gamma + \beta}{2} < \alpha, \quad (11)$$

i.e., for parameters below the blue line in Fig. 2. The ease of ECM degradation is controlled by the bias

$$w_{ECM}(\mathbf{a} \rightarrow \mathbf{b}) = \begin{cases} -Q, & \text{if the site } \mathbf{b} \text{ belongs to an ECM cluster} \\ 0 & \text{otherwise.} \end{cases} \quad (12)$$

Simulations demonstrated that when active cell motility is modeled by postulating a feedback between cell displacements and cell polarity²⁹, oriented adhesion substrates can increase the directional persistence of cells by a factor of 4–5 without effecting the cell speed¹⁷. Furthermore, in model simulations adhesion to and degradation of the ECM strongly biases cell motion and guides cells to invade the ECM. This bias is resulted from the asymmetry between extending and retracting a protrusion. The former increases the length of cell-ECM boundaries, which are preferred model configurations. The latter, in contrast, creates penalized free cell boundary segments. Hence, in the simulations, protrusions into the ECM occur more frequently than their retractions, creating an overall invasive bias of cell movements. This process also increases the persistence of a single cell, a behavior analogous to surface tension inhomogeneity-driven motion of liquid droplets⁹². These considerations are in good agreement with empirical data currently available on the persistence time of endothelial cells, being ≈ 2 h during unconstrained motion on 2D substrates²⁹ and ≈ 10 h for cells that leave an explant and invade 3D collagen I gels⁹³. Yet, if ECM-adherent cells are capable of degrading the ECM, this feature makes *all* cell-ECM contacts unstable – preventing cells from following ECM microchannels or forming multicellular sprouts. Thus, either ECM degradation needs to be restricted to a subpopulation of cells (tip cells), or the active spontaneous motility (and the associated cell polarity) must be persistent enough to “steer” cells away from invading the ECM *everywhere*¹⁷.

Models operating with various types of ECM “memory” are thus very promising to describe multicellular sprouts in situations where cells can degrade the ECM and the resulting cell tracks form “tunnels” which can guide other cells to follow^{58,94–96}. In fact, both endocardial⁹³ and endothelial⁵⁸ cells were reported to leave degraded ECM fragments or “channels” behind. Yet, their relevance for in vivo vasculogenesis has not yet been established.

3.4 Autocrine chemotaxis

Endothelial cell movements guided by *autocrine* chemotactic signaling were proposed as a potential mechanism for vascular pattern emergence^{97–99}. The mechanism relies on the secretion of a diffusing chemotactic morphogen, likely to be VEGF or a particular VEGF isoform (Fig. 3d). So, the chemoattractant concentration c is determined by its production, diffusion and a finite lifetime τ as

$$\partial_t c - D \nabla^2 c + \alpha n - c/\tau, \quad (13)$$

where the production, αn , is assumed to be proportional to the local cell density. For steady sources fixed in space, the concentration field reaches a steady state, which decays with distance according to the profile

$$c \sim e^{-d/\lambda}, \quad (14)$$

where d is the distance from a source and $\lambda = \sqrt{D\tau}$ is the diffusion length – the typical distance a secreted signaling molecule can reach before degradation or immobilization. Changes in cell density are described by the local balance equation (7) and the expression for the cell current density (10) and neglecting random cell movements ($D = 0$). The locally prevalent cell motion velocity is set by the chemoattractant gradient as

$$\partial_t \mathbf{v} + \mathbf{v} \cdot (\nabla \mathbf{v}) = \nabla c. \quad (15)$$

This choice of dynamics is unusual, as it assumes inertial motion of cells: once they gain polarity, they maintain it as a spontaneous polarity (see¹⁰⁰) even in the absence of additional external stimuli. Thus, according to this assumption cells can move along highly persistent trajectories as a response to an initial chemotactic stimulus. Without such assumptions an autocrine chemoattractant is expected to result in cell aggregation¹⁰¹.

Further modeling studies identified additional assumptions that can stabilize the network and steer the system from aggregation towards the creation of branching patterns. Of particular importance is the compressibility of the cells: cells are assumed to resist being compressed and behave as if an effective pressure developed within the aggregates¹⁰². To understand the patterning mechanism based on pressure and chemotaxis, consider the diffusion length of the chemoattractant is small enough and a smooth aggregate surface where the chemotactic gradient signal is the strongest (Fig. 5). In such an environment the further a cell manages to get from the aggregate surface, the less likely it is that chemotaxis will guide the cell back to its original position. At the same time, the pressure of the compressed cells inside the aggregate continues to push the cell further outwards – amplifying initial fluctuations of cell positions along the aggregate surface. Computer simulations of this system revealed that finite cell size, elongated cells, and increased chemotactic sensitivity at free cell surfaces all facilitate a multicellular sprouting process^{102,103}.

The obvious choice for a chemoattractant, VEGF₁₆₅, is unlikely to fit the model assumptions during embryonic vasculogenesis. VEGF₁₆₅ is expressed throughout the embryo except in angioblasts or early endothelial cells^{104,105}. Thus, even if early endothelial cells secrete some small amount of VEGF, the concentration of the autocrine VEGF molecules is likely to be too small compared to the amount already present in the ECM microenvironment. Similar objections can be raised when this explanation is applied to the *in vitro* 3D collagen invasion assays. In such experiments endothelial sprouts readily elongate even in the presence of relatively large concentrations of exogenous VEGF in the culture medium^{106,107}. Thus, while autocrine VEGF signaling may indeed contribute to the patterning of vascular sprouts⁹⁸, it is unlikely to be a required mechanism for sprouting activity. Still, a mathematically very similar patterning process can result from a related and more plausible hypothetical mechanism. If a secreted proteolytic agent increases the availability or “activates” ECM-bound VEGF, then a local gradient (of the bioavailable VEGF) may be produced in the microenvironment of an endothelial cell aggregate. Similarly, the binding of paracrine growth factors to angioblast-produced ECM can drive

patterning by creating spatially-restricted guidance cues required for directed cell migration¹⁰⁸. Unfortunately, it is difficult to visualize morphogen gradients *in vitro* and more so *in vivo*. Thus, experimental validation of the autocrine signaling mechanism remains an interesting challenge.

3.5 Secreted VEGF inhibitor

Recent studies utilizing mouse embryonic bodies suggested that soluble (secreted) sVEGFR1 may participate in the sprouting process (Fig. 3e). While the absence of sVEGFR1 does not fully eliminate vascular sprouts, if endothelial cells are deficient in expressing sVEGFR1, the direction of and distance between the sprouts becomes irregular^{49,50}. Introduction of sVEGFR1 constructs was reported to rescue sprout guidance parameters, and exogenous sVEGFR1 expression in endothelial cells immediately adjacent to the emerging sprout significantly improved their elongation.

These results, together with the available biochemical data on the function of VEGFR1, lead to the hypothesis that secreted VEGFR1 can antagonize the local VEGF bound to the ECM microenvironment. As the concentration of secreted VEGFR1 is high around the vessels, the bioactive (non-sequestered) VEGF may form a gradient pointing *away* from the endothelial cells. Thus, this patterning mechanism operates with a functional VEGF gradient that is the opposite of the one predicted by the autocrine VEGF models discussed in the previous section.

The dynamics of bioavailable VEGF concentration (c) and that of free (r) and ligand bound (r^*) VEGFR1 are given by the reaction-diffusion equations

$$\partial_t c = D_c \nabla^2 c + \alpha_c - c/\tau_c - k_{on} cr + k_{off} r^* \quad (16)$$

$$\partial_t r = D_r \nabla^2 r + \alpha_r n - r/\tau_r - k_{on} cr + k_{off} r^* \quad (17)$$

$$\partial_t r^* = D_r^* \nabla^2 r^* - r^*/\tau^* + k_{on} cr - k_{off} r^*, \quad (18)$$

where D , τ denotes the diffusivity and the lifetime of these molecular agents, respectively. The association and dissociation rates are denoted by k_{on} and k_{off} . The production of VEGFR1, $\alpha_r n$ is assumed to be proportional to the local cell density, while as a first approximation, the production of VEGF can be thought to be uniform, α_c . This latter assumption may reflect VEGF production by the adjacent endoderm during embryonic vasculogenesis⁸. A computational study¹⁰⁹ of the system (without including sprout dynamics) demonstrated that VEGFR1 secretion results in a gradient of VEGF-induced VEGFR2 signaling along the sprout surface (increasing towards the sprout tip), which could alter endothelial cell perception of directionality cues. The proximity of adjacent sprouts may also alter VEGF gradients, VEGFR2 binding, and thus alter the directionality of sprout growth. In particular, as sprout distances decrease, the probability that the sprouts will move in divergent directions increases.

The patterning process in which expansion of a structure is driven towards the gradient of an external diffusive factor has a well established theory. In the most basic scenario the propagation speed v_n of an interface (sprout boundary) is assumed to be proportional to the local gradient. More precisely, the interface displacement is perpendicular to the interface, thus it is directed towards the local normal vector $\mathbf{n}$. The local gradient is also evaluated along this direction, yielding

$$v_n \sim \mathbf{n} \cdot \nabla c. \quad (19)$$

The concentration of the diffusive substance c is kept near zero at the boundary and reaches a uniform positive value far from the interface (Fig. 6). In particular, if c at the interface is proportional to the local curvature k , then this arrangement results in the classic Mullins-Sekerka instability which renders a smooth interface unstable. The instability triggers a spontaneous tip-splitting process that will create a structure with a highly characteristic, dense branching morphology. The basic mechanism for the instability is shown in Fig. 6. A tip extending into the diffusive field senses increasingly steep gradients, which further enhances its growth. This process is balanced by the fact that a thin sprout (large curvature at the tip) may not reduce sufficiently c , resulting in shallower gradients. Thus, an optimal branch width develops: thicker branches split while thinner branches slow down and grow laterally. The Mullins-Sekerka mechanism was shown to be responsible for dense branching patterns in various, apparently unrelated physical systems such as crystal growth, electrochemical deposition or viscous fingering¹¹⁰ as well as for patterning of bacterial colonies under diffusion limited conditions¹¹¹. While rigorous analytical study has not yet been reported for sVEGFR1 guided sprouting, the main ingredients of the tip splitting and diffusive guidance mechanism seem to be present.

3.6 Cell-cell contacts

Finally, we consider a patterning mechanism for multicellular sprouts that utilizes cell-cell contacts only (Fig. 3f). This generic mechanism, like the one that involves traction forces and a malleable substrate, is motivated by the observation that the formation of linear segments and their interconnected network is not restricted to vascular endothelial cells: various glia-, muscle- or fibroblast-related cells also exhibit linear structures when grown on a rigid plastic tissue culture substrate¹⁵. Analysis of individual cell movements suggested that in each of these systems sprout expansion involved cell motility guided by contact with projections of other cells or elongated multicellular structures¹⁴. The speed of sprout expansion was found to be steady in time: longer sprouts were also able to recruit cells into the sprout and did not slow down its extension¹¹². Furthermore, the sprouts are often surprisingly “fluid”: cells of the sprout do not move with the same velocities and thus the order of cells within the sprout can be changed. In particular, expanding sprouts recruit cells from the aggregate. The newly recruited cells move along and may overtake or “leapfrog” the cells comprising the sprout⁷⁵.

Multicellular sprouting is often considered as a special case of sheet migration, the expansion process of a monolayer into an empty area or “wound”. During sheet migration cells at the boundary exert substantial traction forces¹¹³ and are thought to pull the passive bulk of the sheet forward¹¹⁴. Similarly, cells participating in sprout formation are often divided into two subpopulations and the process is explained as *leader cells* pulling a gliding bulk of passive, “*stalk*” cells by means of cell-cell adhesion¹¹⁵. The assumption that the stalk population is not participating in active cell motion is based on observations that these cells have few connections with the extracellular matrix (ECM) environment¹¹⁵, or lack filopodia⁶⁵. Theoretical considerations, however, suggest that stalk cells cannot be passively dragged by a motile tip cell: arguably this process is inconsistent with widely accepted models of cell-cell adhesion¹¹². In particular, cadherin-mediated cell-cell adhesion^{18–22} and – more recently – cell-exerted cortical tension²³ has been shown to be analogous to surface tension of immiscible liquid droplets, and has been modeled accordingly in theoretical studies^{24–26}. Surface tension-stabilized structures are, however, prone to the Plateau-Rayleigh instability: a liquid jet with a circular cross-section should break up into drops if its length exceeds its circumference^{116,117}. Due to this instability, a sprout pulled by a leader cell and held together by surface tension-like cell-cell adhesion should also break up.

Therefore, the presence of leader cells and cell-cell adhesion alone cannot fully account for the formation of long vascular sprouts that contain several cells¹¹². One proposed solution of this puzzle, somewhat incompatible with the strict tip/stalk phenotype differentiation, is the assumption that stalk cells are also driven by an external growth factor gradient¹¹⁸.

Analysis of cell movements within cell cultures exhibiting multicellular sprouts revealed that stalk (sprout) cell surfaces are more attractive migration targets than the surfaces of well-spread cells in the aggregate bulk¹⁵. As cells in the sprout tend to be elongated, a generic mechanism for multicellular sprout formation proposes that cells attach preferentially to elongated cells^{14,15}. In the framework of the CPM, this means that the J costs will be cell state dependent and asymmetric. In particular, when an elementary step represents the activity of cell i , the goal function (1) is evaluated with weights

$$J_{i,j} = \alpha - \mu \theta_j \quad (20)$$

when it contacts an other cell j . In expression (20) θ_j is a measure characterizing the shape (or some micromechanical status) of cell j , which is compared to the intercellular boundary penalty, α , by a weight parameter μ . Thus, in general $J_{i,j} \neq J_{j,i}$; hence one cell can move towards another without the need of the second cell being attracted to the first one. This asymmetry transforms the CPM simulation into a non-equilibrium system which is capable of forming interconnected networks. As simulations reveal, this assumption of a “preferential adhesion mechanism” is sufficient to stabilize sprouts: as a sprout elongates, its constituent cells become increasingly attractive migration targets. The influx of additional cells helps to restore normal cell shape, stabilize the sprout and allow its continued expansion. While the underlying molecular mechanism is unclear, micromechanical differences between sprout and well-spread cells¹¹⁹ and their detection through mechanosensing may represent a plausible potential mechanism. Utilizing computer simulations, preferential attraction to elongated cells has been shown to result in network assembly within a system of initially scattered cells. The resulting sprout patterns are, however, more irregular than vascular networks, they reflect better the behavior seen in non-endothelial cultures.

4 Conclusions

To test the validity of the various mechanisms proposed for vascular patterning, model specific predictions need to be compared with empirical data. As we indicated, all these mechanisms passed some of these tests and thus are biologically plausible, at least under special experimental settings. Furthermore, they were shown to be able to generate an interconnected cellular network in computer simulations. Thus, the most likely conclusion is that vasculogenic sprouts do not utilize a single patterning mechanism. Instead, endothelial cells are likely to combine contact guidance from the ECM, tip splitting instability from VEGF signaling, as well as cell-cell contacts to guide the active motion of “stalk” cells within the sprout. Furthermore, as vasculature forms in the most diverse tissue environments, the importance of the various mechanisms may be tissue dependent.

Dissecting the contribution of each of these mechanisms for a given tissue environment will require experiments that specifically interfere with interactions characteristic for only one of the mechanisms – like perturbing extracellular VEGF dynamics, micromechanical properties of the ECM environment, or the cell polarity apparatus of endothelial cells. Since these experiments are not expected to completely abolish vascular patterning, to analyze the resulting defects would also require a computational model in which most of the proposed mechanisms are integrated. Given the morphological variability of the early vascular network and the number of tunable model parameters, the comparison of model predictions

and empirical data should go beyond morphometric features (such as characteristic pattern size, vascular segment length and width, size distribution of avascular areas, vascular segment density, their persistence or tortuosity, branching angles) and should also consider additional dynamic information such as the motility statistics of endothelial cells, or the spatial distribution of chemotactic factors and ECM mechanical state. As an example, time lapse recordings of endothelial cell movements in vasculogenesis stage quail embryos revealed cell chains moving in opposite directions along the same vascular segment¹², a behavior that suggests reduced importance of an external directional guidance mechanism under these conditions. Thus, the synergy of further experimental and computational work will hopefully yield a predictive mechanistic model for vascular network assembly, which would be invaluable for understanding disease progression and could be used to evaluate personalized medical treatment options.

Acknowledgments

This work was supported by the NIH (grant HL087136), the Hungarian Research Fund (OTKA K72664) and the G. Harold & Leila Y. Mathers Charitable Foundation. I am most grateful for Drs Brenda Rongish, Charles Little and Andras Szabo for their comments on the manuscript.

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Figure 1.

Vasculogenesis in an avian embryo. Endothelial cells are visualized by a cell surface epitope (QH1¹, red) the ECM is labeled by an antibody against Fibronectin (green). The earliest vascular network is a transient structure, built up from multicellular sprouts –linear segments consisting of 3–10 cells.

Figure 2.

Morphology diagram of the equilibrium Cellular Potts Model ($w = 0$) where cells are within a cavity lined by immutable ECM-containing sites. Morphologies are shown as a function of the costs associated with free (β) and ECM-bound (γ) cell boundaries. These values are compared to α , the cost associated with intercellular boundaries. Configurations shown were obtained in the steady-state regime, with parameters marked by the blue dots and with $\alpha = 1$ and $\lambda = 1$. For each set of parameters, two configurations are shown – one with high and the other with low cell density. The red line divides the parameter space into two domains where cells are either adhesive (right) or non-adhesive (left). The green line indicates neutral ECM. Above and below this line the matrix is repulsive and attractive, respectively. The blue line demarcates an area where cells spread along the ECM, i.e., where an increase in cell perimeter is offset by the low expense associated with cell-ECM adhesion sites. After Fig. 4 of Ref ¹⁷.

Figure 3.

Schematic representations of the various mechanisms suggested for vascular pattern formation during vasculogenesis. Arrows represent causal links: changes in one component are directly influenced by the factors indicated by arrows. a) Conventional description of vascular pattern formation. Cells arrive to well defined spatial structures following extracellular guidance cues. b) Vascular patterning by ECM mechanics. Cells exert traction forces, that are balanced by mechanical stresses arising in the surrounding ECM microenvironment due to its deformation. The deformed ECM convects cells and also guides active cell motility. These processes alter the spatial distribution (density) of cells. c) Pattern formation utilizing ECM "memory". Cells irreversibly alter the state of the surrounding ECM, e.g., by creating microchannels or by changes in ECM bundling or

crosslinking. The altered ECM states persist even after the cells leave and thus are able to guide the motility of cells visiting the area at a later time point. The interplay between adhesion to and degradation of the ECM also has a profound influence on cell motion persistence. Most models also take into account that cell motility is restricted by the presence of other cells (dashed line). d) Schematic representation of autocrine chemoattractant signaling. Cells secrete a diffusing chemoattractant, which in turn guides their movements. Cell motility, or chemotactic response is restricted by contact with adjacent cells (dashed line). e) Schematic representation of chemoattractant signaling modulated by a secreted inhibitor. Cells secrete a diffusing chemoattractant inhibitor, such as sVEGFR1, which sequesters the VEGF available in the ECM microenvironment. The resulting concentration gradient of functional VEGF guides cell motility. f) Schematic representation of multicellular sprouts guided by cell-cell contacts. In these models cells are explicitly represented, and the shape and/or contact properties of adjacent cells serve as migration targets and also restrict the possible cell movements. Such cellular scale mechanisms can help to recruit additional cells into the expanding sprouts.

Figure 4.

Irreversible alteration of the ECM together with adhesivity increases the persistence of cell movements. In the absence of ECM (a), formation of a protrusion increases the length of free cell surface. Hence such a protrusion has a high probability to collapse. If cells can degrade the ECM (b), the collapse of the protrusion would result a free cell surface instead of the cell-ECM interface that was originally present. Moreover, depending on the parameters, the longer cell-ECM boundary can be preferred to the shorter free boundary. Hence, the stability of the protrusion is greatly increased. Arrows represent transitions between the depicted states. The size of the arrow indicates the likelihood of the transition.

Figure 5.

Mechanism for patterning instability in the autocrine chemotaxis model. If the diffusion length of the chemoattractant is small (concentration is indicated by orange color, and selected concentrations by black contour lines), then the strongest gradient (red arrows) develops at the surface of the aggregate (gray). Cells, however cannot follow this gradient due to the finite compressibility of the cells within the aggregate (green arrows). If a cell, however, leaves the aggregate and the adaptation of the concentration field is slow, then the sprout-forming cell encounters progressively smaller gradients and diminishing chemotactic bias to return.

Figure 6.

Mullins-Sekerka instability develops when the dynamics of a diffusive field is fast and a stronger gradient accelerates the movement of the interface. In such systems the tip of a “sprout” senses larger gradients in the “updated” concentration field, i.e., in the field that is adapted to the altered shape of interface. Hence the sprout elongates as long as it can effectively reduce the concentration of the chemoattractant at the tip. The symbols are the same as in Fig. 5.