Molecular Controls of Arterial Morphogenesis

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Abstract: Formation of arterial vasculature, here termed arteriogenesis, is a central process in embryonic vascular development as well as in adult tissues. Although the process of capillary formation, angiogenesis, is relatively well understood, much remains to be learned about arteriogenesis. Recent discoveries point to the key role played by vascular endothelial growth factor receptor 2 (VEGF) in control of this process and to newly identified control circuits that dramatically influence its activity. The latter can present particularly attractive targets for a new class of therapeutic arteriogenesis in diseased tissues. (Circ Res. 2015;116:1712-1724. DOI: 10.1161/CIRCRESAHA.116.302953.)

Key Words: angiogenesis factor □ arteries □ arteriogenesis □ vascular endothelial growth factor A

Arteriogenesis is a complex set of events that involve interactions among various cell types and signaling circuits. Recent studies have revealed many details of these processes, but much remains to be learned about how these vessels form and how arterial identity is acquired. One of the challenges has been in defining the term arteriogenesis itself, in part because so little about its biology is known. In developmental biology the term is used to describe formation of the arterial vessel network from the primary vascular plexus. This includes endothelial arterial fate specification, recruitment of smooth muscle cells (SMCs) and formation of the arterial vessel wall, and growth and branching of the forming arterial tree. The branching process also leads to the formation of artery-to-artery connections, termed collaterals.

In adult setting the term arteriogenesis refers to the formation of new (usually collateral) arteries after occlusion of an arterial trunk. Their origin is debated: one school of thought attributes their formation exclusively to the enlargement of a pre-existing collateral network, whereas the other allows for de novo formation of new arterial vessels by means of capillary arterialization. Semantics aside, these differences have potentially important clinical applications, as stimulation of enlargement of an existing artery may be different from induction of new artery formation.

Recent studies demonstrated that there are fundamental molecular differences between arterial and other vasculatures and that arterial fate is determined early in the course of development, in some settings even before the onset of blood circulation. Yet, little is known about arterial fate specification in the adult.

The importance of clear understanding of arteriogenesis cannot be overstated. Given the paucity of success in the therapeutic angiogenesis field during the past decade and realization that it is arterial and not the capillary growth that is the key to restoring effective circulation to compromised tissues, therapeutic arteriogenesis has emerged as a new concept. One driver, then, behind the desire to understand these events is the need for better therapeutic strategies to stimulate arterial growth that could benefit patients with a variety of illnesses compromised by defective or impaired arterial circulation. It is also becoming increasingly clear that many disorders of arterial circulation such as arteriovenous malformations (AVMs), aneurysms and similar syndromes have genetic origins rooted in arteriogenic signaling cascades. Thus, better understanding of arterial specification and arterial conduit formation promises to have a significant diagnostic and therapeutic impact in a variety of important disease conditions.

Arteriogenesis in Development

Early studies identified the key requirement for vascular endothelial growth factor (VEGF)/VEGFR receptor 2 (VEGFR) signaling during vascular development. The loss of a single allele of Vegfa is sufficient to induce early embryonic lethality because of failure of primitive vasculature formation. A homozygous disruption of Vegfr2, the principal signaling VEGF receptor in endothelial cells, induces a similar phenotype.

Arterial differentiation is thought to occur in angioblasts exposed to higher VEGF concentrations, whereas less exposed cells acquire venous fate. In mice, removal of nerve-derived VEGF in the embryonic skin prevents arterial differentiation of primitive vessels. In zebrafish embryos, VEGF expression is induced by the morphogen sonic hedgehog. Angioblasts lose to sonic hedgehog—expressing tissues receive high VEGF concentrations and subsequently differentiate into arterial cells that form the dorsal aorta. Angioblasts located further away differentiate as veins but can be converted into arterial cells by sonic hedgehog or VEGF overexpression.
In addition to VEGF, other pathways may be involved in arterial fate specification. The transforming growth factor-β superfamily receptor activin receptor-like kinase 1 (ALK1) is predominantly expressed in arterial cells. Heterozygous mutations in human ALK1 cause hereditary hemorrhagic telangiectasia, a disease characterized by focal AVMs in various tissues. Alk1-deficient vessels lack certain arterial genes, including Jagged1, UNC5B, and ephrinB2, suggesting that defective arterial differentiation could contribute to AVM formation. Furthermore, the Alk1 ligand BMP9 (bone morphogenic protein 9) induces expression of Jagged1, UNC5B, and EphrinB2 in a Smad-dependent manner, thereby linking Smad signaling to arterial fate specification.

Embryonic endothelial cells are not committed to the arterial fate: some dorsal aorta cells become incorporated into veins, and this fate switch is accompanied by the loss of arterial and gain of venous gene expression. Likewise, grafting of embryonic quail arteries and veins showed that arterial cells can colonize veins of the host and vice versa, again accompanied by switch in gene expression. Thus, reprogramming of arterial and venous endothelial cells occurs during normal development and can be induced experimentally. Factors governing fate switch are poorly understood but may involve repulsion between cells expressing ephrinB2 and its receptor EphB4 that trigger segregation of vein-fated endothelial cells from arteries.

Blood flow further contributes to arterial-venous specification and differentiation. In chick embryos, ligation of the extraembryonic artery induces a profound vascular remodeling and morphological and genetic transformation of arteries into veins and vice versa. In adult vasculature, positioning of a venous conduit in an artery leads to the loss of venous genes such as EphB4 without increased expression of Ephrin-B2, delta-like 4 (Dll4) or other markers of arterial identity.

Taken together, current evidence suggests that embryonic endothelial cells exhibit a significant degree of plasticity with respect to arterial-venous differentiation that is lost later in development. The switch in arterial and venous identity may be facilitated by a signaling system where threshold levels of morphogens such as VEGF-A activate arterial gene expression, whereas lowering VEGF-A input reverses the gene expression program to a venous one. Better understanding of arterial programming is relevant in clinical settings where vessels of different identity are grafted together, such as during bypass surgery or dialysis treatment. Changes in the transplanted vessels after grafting and the significant risk of graft failure involved in these therapies suggest a limited degree of plasticity in adults that could be improved by manipulation of pathways driving arterial-venous differentiation.

In contrast to the endothelial arterial identity acquisition, how SMCs in the arteries acquire identity remains poorly understood. Mature arterial tubes are surrounded by multiple concentric SMC rings, which are themselves surrounded by an adventitial layer of fibroblasts embedded in a collagen matrix. The size and pattern of the SMC layer depend on the arterial diameter and are developmentally controlled in a vessel-specific manner, with small diameter resistance arteries surrounded by 1 or 2 SMC layers, whereas larger diameter arteries can have a dozen or more SMC layers. How SMC assembly is controlled at the cellular and molecular levels is largely unknown but clinically important, because dysregulation of SMC development causes cardiovascular diseases such as aortic aneurysm, atherosclerosis, and pulmonary hypertension.

SMCs are derived from multiple embryonic tissue sources. Cellular and molecular mechanisms leading to arterial wall formation have been recently investigated in the pulmonary arteries in mice. Notch signaling is critically involved in SMC development, in addition to its role in arterial endothelial specification (see below). Among the 4 mammalian Notch receptors, Notch3 is the isoform most prominently expressed in arterial SMCs. It is required for arterial SMC development after activation by Jagged-1 on arterial endothelial cells. Mutations in Notch3 cause cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, a hereditary disease of the cerebral arteries that causes stroke and vascular dementia.

A few genes specifically expressed in arterial, but not venous SMCs are known. Intriguingly, these control development of sympathetic nerves and arterial innervation, which controls blood supply to organs. The glial-derived neurotrophic factor family member artemin, and the neurotrophins nerve growth factor and neurotrophin-3 are expressed in embryonic arterial SMCs, and inactivation of the genes encoding these molecules in mice leads to defects in sympathetic axon growth and extension along the arterial vasculature. Postnatal arterial SMCs acquire the expression of the axon guidance molecule netrin-1, which is required for innervation of arterial SMCs by sympathetic neurons.

**Arteriogenesis in Disease**

Abnormal arterial development can lead to several inherited disorders, including aortic arch malformations, aneurysms, and AVMs. In adults, several vasoocclusive diseases, ranging from sickle cell disease to polycythemia vera, involve abnormal angiogenesis.
from atherosclerotic coronary, peripheral and cerebral arterial disease to arteriosclerosis (transplant-related and unrelated), and systemic and pulmonary hypertension, among others, all reduce arterial blood flow to healthy tissues. This can result in outright cell death and organ damage as seen in myocardial infarction or stroke or may lead to a chronic impairment of various organs function as happens, for example, in pulmonary arterial hypertension, peripheral artery disease, or chronic stable angina. In all of these cases, restoration of blood inflow would be of considerable therapeutic benefit.

The current clinical strategies for the treatment of vasooocclusive diseases entail either some form of mechanical revascularization (eg, stenting, bypass grafting) or medications designed to limit end-organ oxygen demand (eg, β-blockers in patients with chronic stable angina). Despite numerous attempts, no strategy has evolved that allows restoration of arterial inflow either by reversing disease processes that lead to arterial narrowing and occlusion or by creating new arterial conduits.

Early studies using various angiogenic growth factors, mostly VEGF and fibroblast growth factors (FGFs), failed for a number of reasons including poor understanding of biology, inadequate drug delivery methodologies, and problems with clinical assessment of benefits. Recent advances in our understanding of the biology of arteriogenesis, discussed below, offer clues to what went wrong and provide a foundation for the development of different therapeutic strategies.

In discussion of the failure of the initial rounds of clinical trials and the literature dealing with arteriogenesis, it is important to consider animal models used to assess arteriogenesis in adult tissues. Almost all studies use a hindlimb ischemia model in which arteriogenesis is induced by femoral artery ligation. Several animal species, ranging from mice to pigs, and in patients in a not yet fully defined manner. The vast majority of studies using this model are performed in young pigs, other frequently used model is the ameroid occluder model usually performed in young pigs. Unlike the rodent models, most arterial growth in this setting is of the de novo variety and the gradual occlusion of a coronary artery trunk by the ameroid better mimics arteriogenesis seen in chronic coronary artery disease and peripheral artery disease.

In addition to the importance of arteriogenesis in adults with vasooocclusive diseases, abnormal vascular development, and in particular, abnormal arteriovenous fate specification can lead to a number of illnesses including AVM and cerebral cavernous malformations.

**Genetics of Arterial Collateral Circulation**

Key insights into arteriogenic signaling came from studies of genetic differences in the extent of collateral circulation in rodents and people. Numerous genes affecting native collateral density have been identified including CD44, chloride intracellular channel-4, gap junction proteins connexin-37 and connexin-40, platelet-endothelial cell adhesion molecule 1 (PECAM-1), nuclear factor-xB (NFκB), DII4, hypoxia-inducible factor (HIF)-2α, and regulator of G-protein signaling 5 among others (Table 1). Briefly speaking, these fall into 3 distinct categories: genes affecting endothelial extracellular receptor kinase (ERK) activation and Delta-Notch signaling, genes affecting shear stress and SMC G-protein signaling, and genes affecting monocyte/macrophage phagocytosis and inflammatory response.

VEGF signaling is clearly central to these differences. Thus, the extent of collateral density in various mouse strains correlates with the level of VEGF-A expression, whereas in the rat repetitive coronary occlusions model anti-VEGF antibody treatment significantly reduces collateral growth. Furthermore, a reduction in endothelial VEGF signaling input seen in synectin null, myosin-VI null and neuropilin-1 (Nrp1) mice also correlates with reduced collateral formation. It is further interesting to speculate that reduced arteriogenesis and decreased collateral extent in some patient populations may also reflect VEGF signaling abnormality. In particular, patients with diabetes mellitus, a population with well-established arteriogenic defects, demonstrate a dramatic reduction in VEGFR2 expression and activation despite increased VEGF-A expression while exhibiting a ligand-independent receptor activation.

**Molecular Mechanisms of Arteriogenesis**

Growth of the arterial vasculature requires coordinate action of several cell types including endothelial cells, SMCs, pericytes, and various auxiliary cells such as monocyte-derived macrophages, neurons, and others that regulate this process. These will be discussed in turn.

**Cellular Controls of Arteriogenesis: Endothelial Cells**

Endothelial cells play a critical role in developmental arteriogenesis as they establish the arterial identity of the forming vessel and form a backbone of what later becomes an artery. They play an equally important, if less understood, role in adult arteriogenesis. In both cases, VEGF-A seems to be the major driver.

VEGF-A binding to VEGFR2 activates several intracellular signaling cascades including mitogen-activated protein kinase (ERK1/2), phosphoinositol-3-kinase/Akt, Src, and Rac among others. Of these, VEGF-dependent ERK activation is particularly important in vascular development as a knockin of a VEGFR2 mutant carrying a single amino acid mutation in this site (Y1175F) results in a failure of vascular development that is virtually indistinguishable from Vegl2 knockout. Furthermore, ERK activation is critical to arteriogenesis as mice mutants carrying mutations that reduce VEGF-dependent ERK activation demonstrate reduced formation of arterial but not venous vasculature (Table 2). In contrast, stimulation of endothelial ERK signaling results in exuberant arteriogenesis (Table 2).

**Cellular Controls of Arteriogenesis: SMCs**

Although developmental arteriogenesis is clearly driven by the endothelium with SMCs coming into play later when a tube with arterial fate specification has already been established, the situation in adult arteriogenesis is complex and less well...
understood. In part, this is because of the existence of 2 distinct types of adult arteriogenesis-adaptive growth, a term that refers to enlargement of pre-existing arterial collaterals, and de novo arteriogenesis, the process of capillary bed arterialization. These have been difficult to distinguish experimentally and it is likely that SMCs play a much larger role in the former than in the latter.

Remodeling of the pre-existing collaterals is thought to be driven by biomechanical factors including shear stress–dependent activation of eNOS signaling leading to their dilatation. This in turn leads to increased circumferential wall stress that then stimulates growth and expansion of the media layer largely because of SMC proliferation and hypertrophy. SMC proliferation entails a switch in SMC phenotype from contractile to proliferative that can be activated directly by mechanical stresses as well as by nitric oxide. The critical role played by NO is demonstrated by a markedly reduced arteriogenesis and vessel rarefaction in eNOS null mice albeit the latter may also be because of the regulatory role of NO in regulation of angiopoietin-2 release from endothelial cells. At the same time, dysregulation of G protein signaling in SMC as, for example, seen with a knockout of regulator of G-protein signaling 5 also affects arteriogenesis.

Capillary arterialization is a poorly understood process that involves expansion of the capillary bed, change in endothelial cell fate, and acquisition of the medial layer. This is largely driven by endothelial cells but details are murky. A recent study demonstrated that thymosin-β4 stimulation of myocardin-related transcription factor-A in endothelial cells promotes capillary growth and pericyte maturation, thereby expanding the microcirculation bed that can then undergo arterialization. This, in turn, may augment flow of the pre-existing collaterals in the more proximal parts of the arterial tree thereby reducing peripheral resistance and enhancing perfusion of ischemic territories.

### Table 1. Genetics of Arteriogenic Defects

<table>
<thead>
<tr>
<th>Gene</th>
<th>Molecular Mechanism</th>
<th>Biological Processes</th>
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<tbody>
<tr>
<td>Synectin</td>
<td>EC VEGFR2 trafficking/ERK activation</td>
<td>Arterial branching and lumen size</td>
</tr>
<tr>
<td>Myosin-VI</td>
<td>EC VEGFR2 trafficking/ERK activation</td>
<td>Arterial branching and lumen size</td>
</tr>
<tr>
<td>Nrp1</td>
<td>EC VEGFR2 trafficking/ERK activation</td>
<td>Arterial branching and lumen size</td>
</tr>
<tr>
<td>PTP1b</td>
<td>VEGFR2 trafficking/ERK activation</td>
<td>Arterial branching and lumen size</td>
</tr>
<tr>
<td>Dil4</td>
<td>EC Delta-Notch signaling</td>
<td>Arterial branching and maturation</td>
</tr>
<tr>
<td>HIF2α</td>
<td>EC Delta-Notch signaling</td>
<td>Arterial branching and maturation</td>
</tr>
<tr>
<td>NFκB</td>
<td>EC Delta-Notch signaling</td>
<td>Arterial branching and maturation</td>
</tr>
<tr>
<td>Connexin-37</td>
<td>Gap junctions/EC–EC communication</td>
<td>Arterial branching and remodeling</td>
</tr>
<tr>
<td>Connexin-40</td>
<td>Gap junctions/EC–EC communication</td>
<td>Arterial branching and remodeling</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Shear stress signaling</td>
<td>Arterial branching, lumen size</td>
</tr>
<tr>
<td>Shc</td>
<td>Shear stress signaling</td>
<td>Arterial fate and remodeling</td>
</tr>
<tr>
<td>eNOS</td>
<td>SMC G protein signaling</td>
<td>Arteriolar density/vascular regression</td>
</tr>
<tr>
<td>RGS5</td>
<td>SMC G protein signaling</td>
<td>Arterial fate, branching and remodeling</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Monocytes recruitment</td>
<td>Perivascular inflammation</td>
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<tr>
<td>Mac-1</td>
<td>Monocytes recruitment</td>
<td>Perivascular inflammation</td>
</tr>
<tr>
<td>MCP1</td>
<td>Monocytes recruitment</td>
<td>Perivascular inflammation</td>
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<tr>
<td>CD44</td>
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<td>Perivascular inflammation</td>
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<tr>
<td>PHD2</td>
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<td>Perivascular inflammation</td>
</tr>
<tr>
<td>NFκB</td>
<td>Monocytes recruitment</td>
<td>Perivascular inflammation and Dil4 signaling</td>
</tr>
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<td>CD73</td>
<td>Monocytes recruitment</td>
<td>Perivascular inflammation</td>
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<tr>
<td>HuR</td>
<td>Monocyte VEGF-A stability</td>
<td>Monocyte VEGF production</td>
</tr>
<tr>
<td>Ang2</td>
<td>Inflammatory response modulation</td>
<td>Perivascular inflammation</td>
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<tr>
<td>CD180</td>
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<td>ADAMS 17</td>
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<td>Vessel stabilization</td>
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<td>Clec4</td>
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<tr>
<td>NPY</td>
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<td>Canq1 locus</td>
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<td>Unknown</td>
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<tr>
<td>BMX/ETK</td>
<td>Unknown</td>
<td>Unknown</td>
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Clic4 indicates chloride intracellular channel-4; Dil4, delta-like 4; ERK, extracellular receptor kinase; HIF, hypoxia-inducible factor; HuR, human antigen R; ICAM-1, intercellular adhesion molecule-1; MCP, monocyte chemoattractant protein-1; NFκB, nuclear factor-κ B; Nrp1, neuropilin-1; PECAM-1, platelet-endothelial cell adhesion molecule; PHD2, prolyl hydroxylase 2; PTP1b, phosphotyrosine phosphatase 1b; RGS5, regulator of G-protein signaling 5; VEGF, vascular endothelial growth factor; and VEGFR, VEGF receptor.

**Cellular Control of Arteriogenesis:**

**Extravascular Cells**

Given the key role VEGF-A plays in arteriogenesis, its source is an important question. During embryonic development,
Table 2. Endothelial ERK Activation and Arterial Morphogenesis

<table>
<thead>
<tr>
<th>Condition</th>
<th>Description</th>
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<tbody>
<tr>
<td>Absent or severely reduced ERK activation</td>
<td>Complete failure of vascular development</td>
</tr>
<tr>
<td>VEGFR2−/− or VEGFR2 1175Y→F knockin</td>
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<tr>
<td>PLCγ knockout</td>
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<tr>
<td>Ephrin B2 knockout</td>
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<tr>
<td>Moderately reduced ERK activation: impaired developmental and adult arteriogenesis</td>
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<tr>
<td>Synectin knockout</td>
<td></td>
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<tr>
<td>Myosin-VI knockout</td>
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<tr>
<td>Nrps1&lt;sup&gt;−/−&lt;/sup&gt; knockin</td>
<td></td>
</tr>
<tr>
<td>Atherosclerosis and diabetes mellitus</td>
<td></td>
</tr>
<tr>
<td>Increased ERK activation</td>
<td></td>
</tr>
<tr>
<td>Excessive embryonic arteriogenesis (reduced venous fate)</td>
<td></td>
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<tr>
<td>AVM and aneurysm formation</td>
<td></td>
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</tbody>
</table>

AVM indicates arteriovenous malformation; ERK, extracellular receptor kinase; Nrps1, neuropilin-1; PLC, phospholipase; and VEGFR, vascular endothelial growth factor receptor.

nerves serve as an important source of VEGF-A. Whether nerves contribute to VEGF production in adult arteriogenesis has not been clearly established. Although endothelial and SMCs have the ability to secrete VEGF, this is largely restricted to a hypoxic environment. As arteriogenesis takes place in tissues with normal oxygen content, it is unlikely that these cells are the main source of VEGF.

A substantial body of literature points to the role of blood (monocyte)-derived macrophages in arteriogenesis.

Early studies suggested that macrophage-secreted FGF1 or FGF2 are the key arteriogenic factors. However, mice deficient in FGF1, FGF2, or FGF5 do not demonstrate any arteriogenic defects and FGF signaling seems to be more important in maintenance of the vasculature than in its formation. However, macrophages with reduced VEGF-A expression demonstrate defective adult arteriogenesis, whereas expansion of macrophage population because of, for example, prolyl hydroxylase 2 haploinsufficiency significantly increases developmental and adult arteriogenesis. Similarly, activating macrophage HIF-1α expression promotes, whereas suppressing it inhibits, adult arteriogenesis.

Blood monocyte levels also correlate with collateral growth. Thus, mice lacking monocyte chemoattractant protein 1 receptor CCL2 demonstrate decreased arteriogenesis, whereas monocyte chemoattractant protein 1–driven increase in macrophage numbers at the site of arteriogenesis stimulates it. Taken together, these data point to macrophages as the key source of VEGF during arteriogenesis.

Regulation of Endothelial ERK Signaling

As foregoing discussions illustrate, VEGFR2-driven endothelial ERK activation is critical to the formation of arterial vasculature and to regulation of branching extent and lumen size. Indeed, ERK plays a central role during branching morphogenesis. Thus, manipulation of this signaling pathway may be of direct benefit for therapeutic arteriogenesis. The two principle themes that emerge from studies of VEGFR2-specific ERK activation are the role of the receptor’s endocytosis and trafficking and cross-talk with cellular signaling cascades. These will be discussed in turn.

Regulation of ERK Activation: VEGFR2 Endocytosis and Trafficking

Early studies of VEGFR2 signaling suggested that its internalization via a clathrin-dependent endocytic pathway is critical to its ability to signal. Subsequent experiments refined this concept. The endothelial deletion of one of VEGFR2 interacting proteins, EphrinB2, leads to a nearly complete lack of VEGFR2 endocytosis after VEGF-A binding. One consequence of this is the lack of ERK1/2 activation by VEGF-A. But VEGFR2 endocytosis by itself is not sufficient for a full ERK activation, On entering the cytoplasm via a clathrin-dependent endocytic pathway, VEGFR2 is found in Rab5+ early endosomes, shuttled to early endosome antigen 1+ endosomes, and then either recycled to the plasma membrane or delivered to lysosomes for cargo degradation. The movement to the EEA1+ compartment occurs via a protein complex that includes another VEGF-A165 receptor, Nrps1, as well as synectin and myosin-VI. As Rab5+ VEGFR2-containing endosomes traffic through the cytoplasm, they come in a close contact with an endoplasmic reticulum protein tyrosine phosphatase 1b (phosphotyrosine phosphatase [PTP]-1b) that specifically dephosphorylates VEGFR2 Y<sup>1175</sup> site thus leading to decreased ability of the receptor to bind phospholipase γ and activate ERK signaling (Figure 1). Any disruption of this complex (eg, knockout of synectin or myosin-VI or knockin of a Nrps1 mutant lacking its synectin binding site) increases the length of time VEGFR2 spends near PTP1b, thus increasing dephosphorylation of Y<sup>1175</sup> site and reducing ERK activation. The consequence of this is the reduction in the number of arterioles and arteriolar branching (Figure 2).

This phosphatase thus becomes a potential target for therapeutic interventions aimed to stimulate arteriogenesis. Indeed, suppression of PTP1b activity restores full ERK activation and normal arteriogenesis in synectin null mice. Genetically, this has been confirmed by crossing mice with endothelial-specific PTP1b knockout onto the synectin null strain, which fully restores abnormal arteriogenic phenotype in the latter.

Regulation of ERK Activation: Raf1-Centered Cross-Talk

VEGF activates ERK via a VEGFR2-dependent activation of phospholipase γ that in turn activates Raf1 (rapidly accelerating fibrosarcoma)-mitogen extracellular kinase (MEK)-ERK cascade. Despite the apparent simplicity of this pathway, it turned out to be elaborately regulated. Raf1 activity, which is critical to VEGF-dependent ERK activation, is controlled via a series of phosphorylation events that can affect different parts of the molecule. Activation of MEK involves dephosphorylation of the inhibitory Raf1 Ser<sup>259</sup> site and phosphorylation of the activating Ser<sup>218</sup> site that result in activation of Raf1 kinase activity and subsequent phosphorylation of MEK<sup>102–104</sup> (Figure 3A). The key event is Raf1 Ser<sup>218</sup> dephosphorylation as the site is phosphorylated under normal conditions, thereby repressing...
MEK-ERK activation. Although there is a debate as to the nature of the kinase(s) that phosphorylates Raf1 on this site, recent evidence points to LATS1 (large tumor suppressor kinase 1), a key member of the Yap/Hippo signaling pathway, although PKA and PKC have been also implicated (Figure 3A). Particularly interesting is the suggestion of Yap/Hippo-ERK cross-talk. Two key factors here are the aforementioned LATS1 phosphorylation of Raf1 Ser259 that makes Raf1 unable to phosphorylate MEK, and high Raf1 affinity for phosphorylated MST2 (Hippo) that reduces its availability to phosphorylate MEK. The later event is subjected to regulation by Akt that likely explains previously reported Akt/ERK cross-talk (Figure 3B).

Thus, phosphorylation of Raf1 Ser259 sites simultaneously regulated both Raf/MEK/ERK and Hippo pathways. This Raf1-dependent regulation of the Hippo pathway is particular interesting in the light of recent reports linking Hippo activation to early vascular development, angiogenesis, and arteriogenesis.

In agreement with these studies, endothelial expression of the Raf1 mutant Raf1S259A that is resistant to phosphorylation of this site results in constitutive increase of ERK phosphorylation in the absence of VEGF stimulation. Analysis of endothelial gene expression in this setting demonstrated increased expression of virtually all arterial fate markers. Consistent with these observations, endothelial activation of Raf1S259A expression during early development resulted in excessive development of arterial at the expense of venous circulation.

The arteries in the mutant mice were characterized by excessive branching and larger than normal lumen diameters (Figure 3C and 3D). Of note, human Raf1 mutations associated with decreased Raf1 Ser259 phosphorylation result in increased ERK activity and are found in several RASopathy conditions including Noonan’s and LEOPARD (lentigines, EKG, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, retardation of growth, deafness syndromes) characterized by the presence of AVMs. In addition, increased ERK signaling has been implicated in aneurysm development.

In agreement with the concept of signaling cross-talk discussed above, several studies have demonstrated that inhibition of phosphoinositol-3-kinase can be used to activate ERK signaling. The treatment of zebrafish embryos with a phosphoinositol-3-kinase chemical inhibitor resulted in increased ERK activation that leads to excessive formation of arterial vasculature including duplication of the dorsal aorta. Other demonstrated that inhibition of phosphoinositol-3-kinase activity can reverse decreased VEGF-dependent ERK activation in several settings including reduction of VEGFR2 Y phosphorylation by excessive contact with PTP1b in synectin null, myosin-VI, and Nrp1 knockin and decreased VEGFR2 activation by VEGF in hypercholesterolemic conditions. This restoration of ERK activation, in turn, resulted in increased arteriogenesis and functional blood flow improvement.

**Arteriogenesis Drivers**

The critical importance of VEGFR2-dependent ERK activation for arterial fate specification and arteriogenesis raises a critical question of the stimulus (or stimuli) inducing this activity. Although it is tacitly assumed that VEGF-A is that signal, the direct experimental evidence to that effect is fragmentary. VEGF knockout in mice induces a complete failure of endothelial cell formation that, by itself, cannot be used to deduce VEGF role in arterial fate specification. A decrease in VEGF levels (mouse VEGF hypomorphs, low-dose morpholino knockdown in zebrafish) leads to a partial (regional) loss of arterial marker expression.

A new level of complexity has arisen with the discovery of antiangiogenic form of VEGF-A, termed VEGF-A165b, likely generated by translational readthrough. The isoform has been detected in patients with peripheral artery disease and is capable of reducing blood flow recovery in the hindlimb ischemia model in mice.

However, experiments with constitutively active Raf1 (Raf1S259A) demonstrate that it is sufficient to activate ERK without any growth factor input. This form of Raf1 is resistant to phosphorylation on the Ser259 site that renders it inactive. The excessive activation of ERK induced by the introduction of Raf1S259A leads to increased arteriogenesis and increased arterial lumen diameter (Figure 3C and 3D). These data also suggest that the absence of ERK activation in endothelial cells required for arteriogenesis may well be the consequence of the presence of an inhibitory input (phosphorylation-dependent inactivation of Raf1) rather than the absence of a stimulatory (growth factor) input.

The situation is equally unclear in the case of adult arteriogenesis. Many growth factors, including VEGF-A, FGF2, and hepatocyte growth factor among others, have been suggested...
as possible drivers.\textsuperscript{42,120} In addition, physical forces such as shear stress play an important role.\textsuperscript{4,79} Shear stress is able to induce VEGFR2 activation in a ligand-independent manner.\textsuperscript{121}

Shear stress signal transduction in the endothelium involves the VE-cadherin-VEGFR2–PECAM complex.\textsuperscript{122} Mice with a homozygous disruption of global PECAM expression

\textbf{Figure 2.} Arteriogenic defects associated with delayed intracellular vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) trafficking. \textbf{A}, Micro-computed tomographic (CT) images of mouse coronary arteries from wild-type (WT; top) and Synectin null (bottom) mice. \textbf{B}, Micro-CT of mouse renal arterial circulation from WT (top) and myosin-VI null (bottom) mice. \textbf{C}, Micro-CT images of mouse heart (top), hindlimb (middle), and renal (bottom) arterial circulations from mice carrying a deletion of the neuropilin-1 (Nrp1) cytoplasmic domain. Adapted from Lanahan et al\textsuperscript{67,68} with permission of Cell Press (2013 and 2010).

\textbf{Figure 3.} Inhibition-dependent regulation of vascular endothelial growth factor receptor 2 (VEGFR2)–driven extracellular receptor kinase (ERK) activation. \textbf{A}, A schematic of rapidly accelerating fibrosarcoma (Raf1) phosphorylation control. Dephosphorylation of Ser\textsuperscript{259} allows phosphorylation of Ser338 and activation of Raf1 kinase activity. \textbf{B}, Raf1 regulation of ERK, AKT, and MST2 pathways cross-talk. VEGFR2–induced activation of Akt leads to MST2 phosphorylation and promotes formation of Raf1–MST2 complex that maintains Raf1 in an inactive (Ser\textsuperscript{259}-phosphorylated) state. Dephosphorylation of Raf1S\textsuperscript{259} site shifts MST2 to the RASSF1A (Ras association [RalGDS/AF-6] domain family member 1) complex thereby activating large tumor suppressor kinase 1 (LATS1) that subsequently acts on YAP (Yes-associated protein 1). At the same time this allows phosphorylation of Raf1Ser\textsuperscript{338} thereby activating mitogen extracellular kinase (MEK)/extracellular receptor kinase (ERK) signaling. \textbf{C}, Increase dorsal aorta (DA) diameter in Raf1S\textsuperscript{29A} (bottom) compared with wild type (WT; top) mice. \textbf{D}, Extraembryonic vasculature in control (WT) and Raf1S\textsuperscript{29A} mouse embryos. Note a marked increase in arterial size. For panel \textbf{B}, data derived from Romano et al.\textsuperscript{104} Panels \textbf{C} and \textbf{D} adapted from Deng et al\textsuperscript{78} with permission of the publisher (American Society of Hematology, 2013). Cx40 indicates Connexin-40.
demonstrate dramatically reduced arterial remodeling and arteriogenesis. However, whether this is primarily because of the loss of endothelial PECAM versus PECAM in other cell types has not been established.

Shear stress has several other effects on the endothelium including induction of endothelial VEGF expression and activation of NFkB signaling. One outcome is increased expression of adhesion proteins including ICAM-1 and VCAM leading to increased monocyte adhesion to the flow-activated endothelium. This factor seems critical to shear stress–induced accumulation of monocytes at the sites of arteriogenesis as suppression of NFkB activation in the endothelium leads to a profound reduction in monocytes/macrophages accumulation.

One of the consequences of shear stress signaling is activation of endothelial NFkB leading to expression of various adhesions molecules such as ICAM-1 and VCAM. This, in turn, facilitates accumulation of inflammatory cells, including blood-derived macrophages (Figure 4). Another factor driving macrophage accumulation is angiopoietin-1 that is
also produced by activated endothelial cells. The presence of macrophage at sites of arteriogenesis has been long appreciated. They are the primary source of VEGF and are also capable of producing other angiogenic growth factors including FGF2 and platelet growth factor among others.

Regulation of the Extent of Arteriogenesis and Arterial Branching

On par with induction of arterial fate specification and growth of the arterial tree, regulation of the extent of this growth is another key to effective arteriogenesis. Remarkably, virtually nothing is known on the signals that control the extent of vascular tree formation. Endothelial Notch activation induced by Dll4 is involved in regulation of arteriogenesis, whereas Dll4 binding is considered the principle mechanism controlling the extent of branching. Loss of a single Dll4 allele, or inhibition of Notch signaling, significantly augments the extent of arterial branching and artery-to-artery connections. Yet, despite the overall increase in arterial density and the number of collateral connections, tissue perfusion is not improved at baseline and is distinctly reduced in adult mice after a major arterial trunk ligation.

Despite an important role played by Dll4, regulation of its expression remains poorly understood. Among the known regulators are Sox7 (Sry-related HMG box 7) and Sox18 (Sry-related HMG box 18) transcription factors that may act via Sox17 (Sry-related HMG box 17). More directly linked to arteriogenesis is the recently described regulation of Dll4 expression by NFκB. Expression of a dominant-negative IκBα construct in endothelial cells results in a nearly complete suppression of inflammation-induced or shear stress–induced NFκB activation, thus eliminating signaling input of the major arteriogenesis triggers. This leads to reduction of expression of key molecules involved in arteriogenic response: Dll4, PDGF-BB (platelet-derived growth factor BB), and endothelial adhesion molecules such as ICAM-1 and VCAM-1 (Figure 4). Reduction in Dll4 levels reduced Notch signaling and hence increased branching, whereas decreased PDGF-BB levels likely account for reduced maturation of neovascularity because of impaired mural cell recruitment and differentiation. Finally, reduced adhesion molecule expression leads to a profound decrease in recruitment of blood-derived monocytes/macrophages thereby reducing local VEGF concentration. The resultant phenotype is characterized by reduced monocytes/macrophages thereby reducing local VEGF signaling and hence increased branching, whereas reduced PDGF-BB levels likely account for reduced maturation of neovascularity because of impaired mural cell recruitment and differentiation.

Summary and Practical Implications of the New Knowledge

The emerging data firmly identify endothelial ERK signaling as the key driver of arterial fate specification during development as well as developmental and adult arteriogenesis. The regulation of this signaling cascade (Figure 5) is complex and is still not fully understood.

At the level of an endothelial cell, expression of VEGFR2 and Nrp1 is required for arteriogenic signaling. Activation of the VEGFR2/Nrp1 complex, either by a ligand (e.g., VEGF-A) or in a non–ligand-dependent fashion (shear stress and perhaps other physical factors), leads to its endocytosis, a step required for activation of ERK signaling. Initial steps involved in VEGF-B/VEGFR2/Nrp1 complex entrance into the cell are poorly understood but involve ephrin B2, epsins 1 and 2, and polarity proteins atypical PKC and PAR3 (partitioning-defective 3) among others.

Once internalized, VEGF-containing endosomes undergo intracellular trafficking away from PTP1b-rich areas of the cytoplasm, allowing for a full phosphorylation of Y1175 site critical to ERK activation via the phospholipase γ/Raf1 cascade.

The state of Raf1 phosphorylation is another critical control point as phosphorylation of its Ser259 site leads to suppression of MEK-dependent ERK activation. Activation ERK signaling either by suppression of Raf1Ser259 phosphorylation or by introduction of constitutive-active MEK/ERK constructs promotes arteriogenesis.

This scheme suggests the presence of several critical checkpoints (Figure 5). One is the availability of VEGF-A. The other is the ability of the shear stress or another physical stimulus (e.g., radial wall stress) to activate VEGFR2 in a ligand-independent manner. Importantly, the relative contributions of these 2 factors to VEGFR2 activation are not understood.

During embryonic arteriogenesis nerves are likely the key source of VEGF, whereas adult arteriogenesis is largely dependent on the presence of blood-derived monocytes/macrophages. In the case of arteriogenesis in various disease settings it is doubtful that reduced VEGF levels are ever the key factors in impaired neovascular response. Therefore, therapies aimed at providing exogenous VEGF to stimulate arteriogenesis are unlikely to be effective. At the same time, non–ligand-dependent activation of VEGFR2 signaling in pathological conditions has not been fully explored.

Regardless of the stimulus, VEGFR2 becomes the central molecule driving all subsequent events. A decline in VEGFR2 levels seen in disease states such as diabetes mellitus and hypercholesterolemia may account for poor arteriogenesis in these settings. In addition, maintenance of endothelial VEGF2 expression is an active process that requires a continuous FGF signaling input acting via ETS (E26 transformation-specific) and Forkhead transcription factors.

Impaired VEGFR2 trafficking leads to a decline in its activity because of PTP1b-dependent dephosphorylation of the Y1175 site and inhibition of PTP1b activity seems effective in restoration of ERK activation in certain circumstances. An equally effective strategy may involve suppression of Akt or another kinases capable of Raf1 Ser259 phosphorylation, thereby also leading to ERK activation.

Finally, the effective size of the arterial tree is controlled via NFκB-dependent regulation of Dll4 expression and subsequent Notch signaling activation. Remarkably, in the absence of sufficient Notch activation, the increased number of arterial conduits and collaterals leads to ineffective...
circulation, suggesting that an optimal tree size and full maturation of the newly formed vasculature are required for effective tissue perfusion.

In a larger context, endothelial cells have emerged as central regulators of angiogenesis. The presence of inflammatory stimuli such as tumor necrosis factor-α or shear stress leads to activation of NFκB. This, in turn, leads to 3 key events: (1) induction of HIF2α expression that then stimulates Dll4 expression; (2) induction of HIF1α expression that leads to increased platelet-derived growth factor BB and VEGF-A production; and (3) expression of adhesion molecules such as vascular cell adhesion protein and intercellular adhesion molecule-1 that facilitate blood monocyte recruitment that subsequently become the key source of VEGF-A fully activating this cascade (Figure 5).

This emerging paradigm has direct implications with regard to potential therapies, as endothelial ERK signaling is clearly an appealing target. In the past efforts and therapeutic trials to potential therapies, as endothelial ERK signaling is likely unresponsive of the diseased endothelium to VEGF therapy. Although new data clearly points to its futility. The principle cause of failures is the large amount of VEGF-A therapy. When added to the data clearly an appealing target. In the past efforts and therapeutic trials to potential therapies, as endothelial ERK signaling is likely unresponsive of the diseased endothelium to VEGF therapy. Although new data clearly supports the biological importance of this molecule in adult angiogenesis, clinical trial experience to date equally clearly points to its futility. The principle cause of failures is the large amount of VEGF-A therapy. When added to the data clearly an appealing target.

Given this, the ability to stimulate angiogenesis by interfering with endogenous regulators of VEGF signaling such as Spp1b to enhance signaling of partially endocytosed VEGF/VEGFR2/Nrp1 complexes or to directly activate ERK activation via suppression of Raf1 Ser259 phosphorylation or kinases that phosphorylate this site may prove practically viable.

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References


Molecular Controls of Arterial Morphogenesis


