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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. While 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 (VEGFR2) 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 agents capable of activation of this signaling cascade in a ligand-independent manner, thereby promoting arteriogenesis in diseased tissues.

Keywords

Angiogenesis; arteries; vascular biology; animal model cardiovascular disease; vascular endothelial growth factor

Introduction

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[1], 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 formation of artery-to-artery connections, termed collaterals.

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DISCLOSURES

None.

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, while the other allows for de novo formation of new arterial vessels by means of capillary arterialization[1]. Semantics aside, these differences have potentially important clinical applications, as stimulation of enlargement of an existing artery may be quite different from induction of new artery formation.

Recent studies suggested 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[2, 3]. Yet very little is known about arterial fate specification in the adult.

The importance of clear understanding of arteriogenesis cannot be overestimated. Given the paucity of success in the therapeutic angiogenesis field over the last 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[4]. 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 arterio-venous malformations (AVMs), aneurysms and the like have genetic origins rooted in arteriogenic signaling cascades[5, 6]. 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 VEGF/VEGFR2 signaling during vascular development. The loss of a single allele of *Vegfa* is sufficient to induce early embryonic (~E8.5) lethality due to failure of primitive vasculature formation[7, 8]. A homozygous disruption of *Vegfr2*, the principal signaling VEGF receptor in endothelial cells, induces a similar phenotype[9].

Arterial differentiation is thought to occur in angioblasts exposed to higher VEGF concentrations, while less exposed cells acquire venous fate. In mice, removal of nerve-derived VEGF in the embryonic skin prevents arterial differentiation of primitive vessels[10, 11]. In zebrafish embryos, VEGF expression is induced by the morphogen sonic hedgehog (Shh)[12]. Angioblasts close to Shh 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 Shh or VEGF overexpression[12, 13].

In addition to VEGF, other pathways may be involved in arterial fate specification. The TGFβ superfamily receptor Activin receptor-like kinase 1 (ALK1) is predominantly expressed in arterial cells[14]. Heterozygous mutations in human ALK1 cause hereditary hemorrhagic telangiectasia (HHT), a disease characterized by focal AVMs in various

tissues[15]. Alk1-deficient vessels lack certain arterial genes, including Jagged1, UNC5B and ephrinB2, suggesting that defective arterial differentiation could contribute to AVM formation[16–18]. Furthermore, the Alk1 ligand BMP9[19–21] induces expression of Jagged1, UNC5B and EphrinB2 in a Smad-dependent manner, thereby linking Smad signaling to arterial fate specification[22, 23].

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[24, 25]. 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[26]. 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 involve repulsion between cells expressing ephrinB2 and its receptor EphB4 that trigger segregation of vein-fated endothelial cells from arteries[24, 27].

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[28, 29]. 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, Dll4 or other markers of arterial identity[30].

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, while 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 suggests a limited degree of plasticity in adults that could be improved by manipulation of pathways driving arterial-venous differentiation[31].

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 depends on the arterial diameter and is developmentally controlled in a vessel-specific manner, with small diameter resistance arteries surrounded by one or two SMC layers, while 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, since dysregulation of SMC development causes cardiovascular diseases such as aortic aneurysm, atherosclerosis and pulmonary hypertension[32].

SMCs are derived from multiple embryonic tissue sources[33, 34]. Cellular and molecular mechanisms leading to arterial wall formation have been recently investigated in the pulmonary arteries in mice[35]. 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 following activation by Jagged-1 on arterial endothelial cells.[36–38] Mutations in Notch3 cause cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), 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 (GDNF) family member artemin, and the neurotrophins Nerve growth factor (NGF) and neurotrophin-3 (NT-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[39, 40]. Postnatal arterial SMCs acquire the expression of the axon guidance molecule netrin-1, which is required for innervation of arterial SMCs by sympathetic neurons[41].

Arteriogenesis in disease

Abnormal arterial development can lead to several inherited disorders, including aortic arch malformations, aneurisms and arterio-venous malformations (AVMs). In adults, a number of vasoocclusive diseases, ranging 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 happens 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 treatment of vasoocclusive diseases entail either some form of mechanical revascularization (e.g. stenting, bypass grafting) or medications designed to limit end-organ oxygen demand (e.g. beta-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 FGFs, failed due to a number of reasons including poor understanding of biology, inadequate delivery methodologies and problems with clinical assessment of benefits[42]. 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 utilize a hindlimb ischemia model in which arteriogenesis is induced by femoral artery ligation[43]. A number of animal species, ranging from mice to pigs have been used in this fashion. Flow recovery in this model occurs by a combination of recruitment and expansion of pre-existing collaterals and by de novo arteriogenesis. Genetic factors play a major role in this response both in animals[44–46] and in patients[47] in a not yet fully defined manner[48]. The vast majority of studies using this model are performed in young and disease-free animals, with the important limitation that both age[49] and disease states such as diabetes[50, 51] and hypercholesterolemia[52] impair arteriogenic response. The other important model is the ameroid occluder model usually carried out 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 one of the main coronary trunks by the ameroid better mimics arteriogenesis in chronic CAD or PAD.

In addition to the importance of arteriogenesis in adults with vasoocclusive diseases, abnormal vascular development, and in particular, abnormal arterio-venous fate specification can lead to a number of other important illnesses including AVM and CCMs.

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[46, 47, 53–55]. Numerous genes affecting native collateral density have been identified including CD44[56], chloride intracellular channel-4[57], gap junction proteins connexin-37[58] and connexin-40[28], PECAM-1[59], NFkB[60], Delta-like-4 (Dll4)[61], HIF2α[62] and RGS5[63] among others (Table 1). Broadly speaking, these fall into three distinct categories: genes affecting endothelial ERK activation and Delta-Notch signaling, genes affecting shear stress and SMC G-protein signaling and genes affecting monocyte/macrophage recruitment and “inflammatory” response. Roles of a number of other genes have not been established.

VEGF signaling is clearly central to these differences. Thus, the extent of collateral density in mouse strains correlates with the level of VEGF-A expression[45] while in the rat repetitive coronary occlusions model anti-VEGF antibody treatment significantly reduces collateral growth[64]. Furthermore, a reduction in endothelial VEGF signaling input seen in synectin null, myosin-VI null and $Nrp1^{cyto}$ mice also correlates with reduced collateral formation[65–68]. It is further interesting to speculate that reduced arteriogenesis and/or collateral extent in some patient populations may be also due to the same. In particular, patients with diabetes mellitus, a population with well-established arteriogenic defects[69], demonstrate a dramatic reduction in VEGFR2 expression and activation despite increased VEGF-A expression[70] while exhibiting a ligand-independent receptor activation[71].

Molecular mechanisms of arteriogenesis

Growth of the arterial vasculature requires coordinate action of a number of cell types including endothelial cells, smooth muscle cells, 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 appears to be the major driver.

VEGF-A binding to VEGFR2 activates a number of intracellular signaling cascades including MAPK (ERK1/2), PI3K/Akt, Src and Rac among others[72, 73]. Of these, VEGF-dependent ERK activation is particularly important in vascular development as a knock-in of a VEGFR2 mutant carrying a single amino acid mutation in this site (Y1175F) results in a failure of vasculature development that is virtually indistinguishable from *Vegfr2* knockout[74]. Furthermore, ERK activation is critical to arteriogenesis as mice mutants carrying mutations that reduce VEGF-dependent ERK activation[65, 67, 68, 75] demonstrate reduced formation of arterial but not venous vasculature (Table 2). In contrast, stimulation of endothelial ERK signaling results in exuberant arteriogenesis[76–78] (Table 2).

Cellular controls of arteriogenesis: smooth muscle cells

While 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 more complex and less well understood. In part, this is due to the existence of two 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[1]. These have been difficult to distinguish experimentally and it is likely that smooth muscle cells 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[28, 79–81]. This in turn leads to increased circumferential wall stress that then stimulates growth and expansion of the media layer largely due to SMC proliferation and hypertrophy[82]. 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 rarefication in eNOS null mice[83, 84] albeit the latter may also be due to the regulatory role of NO in regulation of angiopoietin-2 release from endothelial cells[85]. At the same time, dysregulation of G protein signaling in SMC as, for example, seen with a knockout of RGS5 also affects arteriogenesis[63].

Capillary arterialization[86] is a very 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[87] but details are murky. A recent study demonstrated that thymosin-β4 stimulation of myocardin-related transcription factor-A (MRTF-A) in

endothelial cells promotes capillary growth and pericyte maturation[88] thereby expanding the microcirculation bed that can then undergo arterialization. This, in turn, may augment flow the pre-existing collaterals in the more proximal parts of the arterial tree thereby reducing peripheral resistance and enhancing perfusion of ischemic territories.

Cellular control of arteriogenesis: extravascular cells

Given the key role VEGF-A plays in arteriogenesis, its source is an important question. During embryonic development, nerves serve as an important source of VEGF-A[10, 11]. Whether nerves contribute to VEGF production in adult arteriogenesis has not been clearly established. While endothelial and smooth muscle cells 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[89]. However, mice deficient in FGF1, FGF2 or FGF5 do not demonstrate any arteriogenic defects and FGF signaling appears to be more important in maintenance of the vasculature than in its formation.[90] On the other hand, macrophages with reduced VEGF-A expression demonstrate defective adult arteriogenesis,[91] while expansion of macrophage population due to, for example, PHD2 haploinsufficiency, significantly increases developmental and adult arteriogenesis[92]. Similarly, activating macrophage HIF-1α expression promotes while suppressing it inhibits, adult arteriogenesis[85].

Blood monocyte levels also correlate with collateral growth[93]. Thus, mice lacking MCP-1 receptor CCL2 demonstrate decreased arteriogenesis[53] while MCP-1-driven increase in macrophage numbers at the site of arteriogenesis stimulates it[94, 95]. 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 formation of arterial vasculature and to regulation of branching extent and lumen size. Indeed, ERK plays a central role during branching morphogenesis[96–98]. 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 is 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[99]. Subsequent experiments refined this concept. The endothelial deletion of one of VEGFR2 interacting proteins, EphrinB2, leads to a complete lack of VEGFR2 endocytosis following VEGF-A binding[100]. 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. Upon entering the cytoplasm

via a clathrin-dependent endocytic pathway, VEGFR2 is found in Rab5+ early endosomes[68, 99], shuttled to EEA1+ 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, neuropilin-1, 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 (PTP1b) that specifically dephosphorylates VEGFR2 Y^{1175} site thus leading to decreased ability of the receptor to bind PLCγ and activate ERK signaling (Fig 1)[67, 101]. Any disruption of this complex (e.g. knockout of synectin or myosin-VI or knock-in of a Nrp1 mutant lacking its synectin biding site) increases the length of time VEGFR2 spends near PTP1b thus increasing dephosphorylation of Y^{1715} site and reducing ERK activation. The consequence of this is the reduction in the number of arterioles and arteriolar branching (Fig 2).

The 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[68]. 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[75].

Regulation of ERK activation: Raf1-centered cross-talk

VEGF activates ERK via a VEGFR2-dependent activation of PLCγ that in turn activates the Raf1-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^{259} site and phosphorylation of the activating Ser^{338} site that result in activation of Raf1 kinase activity and subsequent phosphorylation of MEK[102–104] (Fig 3A). The key event is Raf1 Ser^{259} dephosphorylation as the site is phosphorylated under normal conditions, thereby repressing MEK-ERK activation[104]. While there is a debate as to the nature of the kinase(s) that phosphorylates Raf1 on this site, recent evidence points to LATS1 (a key member of the Yap/Hippo signaling pathway) although PKA and PKC have been also implicated[104, 105] (Fig 3A). Particularly interesting is the suggestion of Yap/Hippo- MAPK crosstalk. Two key factors here are the aforementioned LATS1 phosphorylation of Raf1 Ser^{259} that makes Raf1 unable to phosphorylate MEK, and high Raf1 affinity for phosphorylated MST2 (Hippo) that reduces its availability to phosphorylate MEK[104, 106]. The later event is subjected to regulation by Akt that likely explains previously reported Akt/ERK cross-talk (Fig 3B)[77, 107].

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[108], angiogenesis[109] and arteriogenesis[110].

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[78]. The arteries in the mutant mice were characterized by excessive branching and larger than normal lumen diameters (Fig 3C,D). Of note, human Raf1 mutations associated with decreased Raf1 Ser259 phosphorylation result in increased ERK activity and are found in a number of "RASopathy"[111, 112] conditions including Noonan's and LEOPARD syndromes[113] characterized by presence of arterio-venous malformations. In addition, increased ERK signaling has been implicated in aneurysm development.[114, 115]

In agreement with the concept of signaling cross-talk discussed above, a number of studies have demonstrated that inhibition of PI3K can be used to activate ERK signaling. Thus, treatment of zebrafish embryos with a PI3K chemical inhibitor resulted in increased ERK activation that lead to excessive formation of arterial vasculature including duplication of the dorsal aorta[107]. Other demonstrated that inhibition of PI3K activity can reverse decreased VEGF-dependent ERK activation in a number of settings including reduction of VEGFR2 Y^{1175} phosphorylation by excessive contact with PTP1b in synectin null, myosin-VI and $Nrp1^{cyto}$ knock-in[67, 68] and decreased VEGFR2 activation by VEGF in hypercholesterolemic conditions[116]. 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. While 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[117].

A new level of complexity has arisen with the discovery of anti-angiogenic form of VEGF-A, termed VEGF-A_{165b}, likely generated by translational readthrough[118]. 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[119].

On the other hand, 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 Ser^{259} site that renders it inactive. The excessive activation of ERK induced by the introduction of Raf1S259SA leads to increased arteriogenesis and increased arterial lumen diameter (Fig 3C,D)[78]. 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 HGF among others have been suggested as possible drivers[42, 120]. In addition, physical forces such as shear stress play an important role[4, 79]. Shear stress is able to induce VEGFR2 activation in a ligand-independent manner[121]. Shear stress signal transduction in the endothelium involves the VE-cadherin-VEGFR2-PECAM complex[122]. Mice with a homozygous disruption of global PECAM expression demonstrate dramatically reduced arterial remodeling and arteriogenesis.[59] However, whether this is primarily due to the loss of endothelial PECAM vs. PECAM in other cell types has not been established.

Shear stress has a number of other effects on the endothelium including induction of endothelial VEGF expression[123] and activation of NFκB signaling[124]. One outcome is increased expression of adhesion proteins including ICAM-1 and VCAM[125] leading to increased monocyte adhesion to the flow-activated endothelium[126]. This factor appears critical to shear stress-induced accumulation of monocytes at the sites of arteriogenesis as suppression of NFκB activation in the endothelium leads to a profound reduction in monocytes/macrophages accumulation[60].

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-1[60, 127, 128]. This, in turn, facilitates accumulation of inflammatory cells, including blood-derived macrophages (Fig 4). Another factor driving macrophage accumulation is angiopoietin-1 that is also produced by activated endothelial cells[129]. The presence of macrophage at sites of arteriogenesis has been long appreciated[4, 93, 94]. They are the primary source of VEGF and are also capable of producing other “angiogenic” growth factors including FGF2 and PlGF among others[130, 131].

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 regarding the signals that control the extent of vascular tree formation. Endothelial Notch activation induced by Dll1 is involved in regulation of arteriogenesis[132] while Dll4 binding is considered the principle mechanism controlling the extent of branching[133–135]. Loss of a single Dll4 allele, or inhibition of Notch signaling, significantly augments the extent of arterial branching and artery-to-artery connections[61, 135]. 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 following a major arterial trunk ligation[61].

Despite an important role played by Dll4, regulation of its expression remains poorly understood. Among the known regulators are Sox7 and/or Sox18 transcription factors[136] and Wnt[137] that may act via Sox17[2]. More directly linked to arteriogenesis is the recently described regulation of Dll4 expression by NFκB[60]. Expression of a dominant-negative

IκBα construct in endothelial cells results in a nearly complete suppression of inflammation- 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 and endothelial adhesion molecules such as ICAM-1 and VCAM (Fig 4). Reduction in Dll4 levels reduced Notch signaling and hence increased branching, while decreased PDGF-BB levels likely account for reduced maturation of neovasculature due to 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[60]. The resultant phenotype is characterized by vastly excessive, hyperbranched and immature arterial vasculature and a dramatic reduction in tissue perfusion, similarly to the phenotype observed in Dll4 heterozygous mice[60, 61].

NFκB directly regulates VEGF-A and PDGF-BB expression via HIF1α[138, 139] and Dll4 via HIF2α[60]. The latter conclusion is supported by the observation of increased arterial branching and decreased tissue perfusion in mice with endothelial HIF2α deletion that resulted in decreased Dll4 expression[62].

Summary and practical implications of the new knowledge

The emerging data firmly identifies 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 (Fig 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/BVEGFR2/Nrp1 complex entrance into the cell are poorly understood but involve ephrin B2[100], epsins 1 and 2[140, 141], and polarity proteins aPKC and PAR3[142] among others.

Once internalized, VEGFR2-containing endosomes undergo intracellular trafficking away from PTP1b-rich areas of the cytoplasm, allowing for a full phosphorylation of Y^{1175} site critical to ERK activation via the PLCγ/Raf1 cascade[65, 67, 68, 75, 87, 143, 144].

The state of Raf1 phosphorylation is another critical control point as phosphorylation of its Ser^{259} 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[77, 78, 107].

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

During embryonic arteriogenesis nerves are likely the key source of VEGF[10, 11] while in the case of adult arteriogenesis, it is largely dependent on the presence of blood-derived monocytes/macrophages[4, 92]. In the case of adult arteriogenesis in disease settings it is doubtful that reduced VEGF levels are ever they key factors in impaired neovascular response. Therefore therapies aimed at providing exogenous VEGF in order 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[70] and hypercholesterolemia[52, 116] may account for poor arteriogenesis in these settings. In addition, maintenance of endothelial VEGFR2 expression is an active process that requires a continuous FGF signaling input[145] acting via ETS and Forkhead transcription factors[146, 147].

Impaired VEGFR2 trafficking leads to a decline in its activity due to PTP1b-dependent dephosphorylation of the Y^{1175} site and inhibition of PTP1b activity appears effective in restoration of ERK activation in certain circumstances[68, 75]. An equally effective strategy may involve suppression of Akt[77, 107] or another kinases capable of Raf1 Ser^{259} 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[60–62]. 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 arteriogenesis[87]. The presence of inflammatory stimuli such as TNFα or shear stress leads to activation of NFκB. This, in turn, leads to three key events: 1) induction of HIF2α expression that then stimulates Dll4 expression and activates Notch signaling; 2) induction of HIF1α expression that leads to increased PDGF-BB and VEGF-A production and 3) expression of adhesion molecules such as VCAM and ICAM-1 that facilitate blood monocyte recruitment that subsequently become the key source of VEGF-A fully activating this cascade (Fig 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 stimulation of neovascularization focused on growth factor (predominantly VEGF-A) therapy. While new data clearly supports the biological importance of this molecule in adult arteriogenesis, clinical trials experience to date equally clearly points to its futility[42, 148, 149]. The principle cause of failures is likely unresponsiveness of the diseased endothelium to VEGF stimulation that is due to either a reduction in VEGFR2 levels or a decrease in its cellular uptake upon stimulation.

Given this, the ability to stimulate arteriogenesis by interfering with endogenous regulators of VEGFR2 signaling such as PTP1b 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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Nonstandard Abbreviations and Acronyms

ALK1	activin receptor-like kinase 1
aPKC	atypical PKC
AVM	arterio-venous malformations
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CCM	cerebral cavernous malformation
Dll4	delta-like 4
EEA1	early endosome antigen 1
ERK	extracellular receptor kinase
FGF	fibroblast growth factor
GDNF	glial-derived neurotrophic factor
HIF	hypoxia-inducible factor
HGF	hepatocyte growth factor
ICAM-1	intercellular adhesion molecule-1
LATS1	large tumor suppressor kinase 1
LEOPARD	lentigenes, EKG, ocular hyperteleorism, pulmonary stenosis, abnormal genitalia, retardation of growth, deafness
MAPK	mitogen-activated protein kinase
MCP1	monocyte chemoattractant protein 1
MEK	mitogen extracellular kinase
MRTF-A	myocardin-related transcription factor-A
NFkB	nuclear factor kappa B
NGF	nerve growth factor
Nrp1	neuropilin-1
PECAM	platelet-endothelial cell adhesion molecule
PHD2	prolyl hydroxylase 2
PI3K	phosphoinositol-3-kinase
PLCγ	phospholipase gamma
PlGF	platelet growth factor

PDGF-BB	platelet-derived growth factor BB
PTP1b	phosphotyrosine phosphatase 1b
Raf1	rapidly accelerating fibrosarcoma
RGS5	regulator of G-protein signaling 5
Shh	sonic hedgehog
Sox	Sry-related HMG box
SMC	smooth muscle cells
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VCAM-1	vascular cell adhesion protein 1

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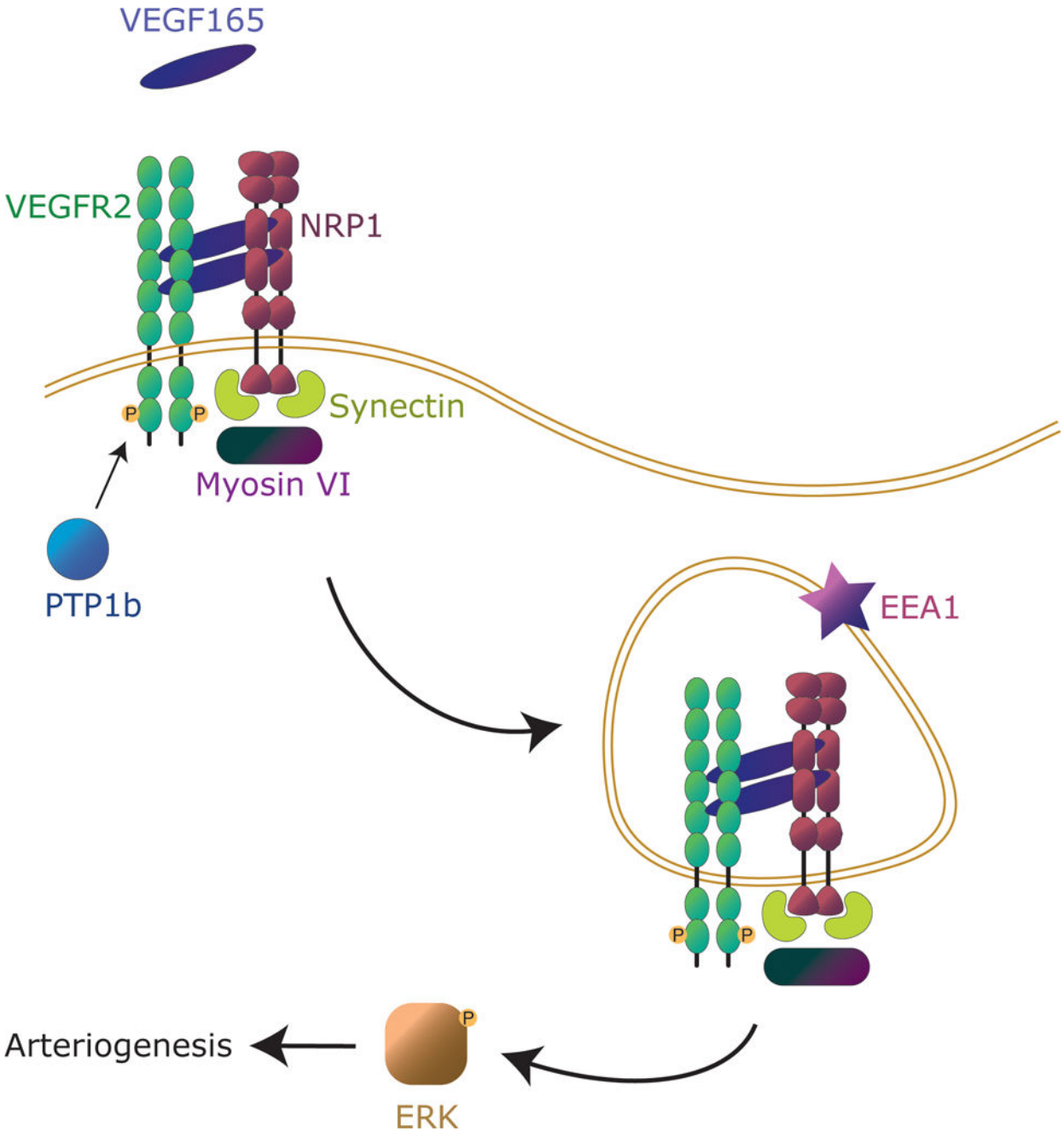

Figure 1.

Intracellular trafficking of VEGFR2.

VEGFR2 endocytosis and Neuropilin-1/synectin/myosin-VI-dependent trafficking. VEGF-A binding to VEGFR2 and Nrp1 creates a multiprotein complex and induces its endocytosis. A Nrp1-dependent trafficking that proceeds in a synectin/myosin-VI-dependent manner leads to VEGR2-driven activation of ERK signaling in EEA1+ endosomes.

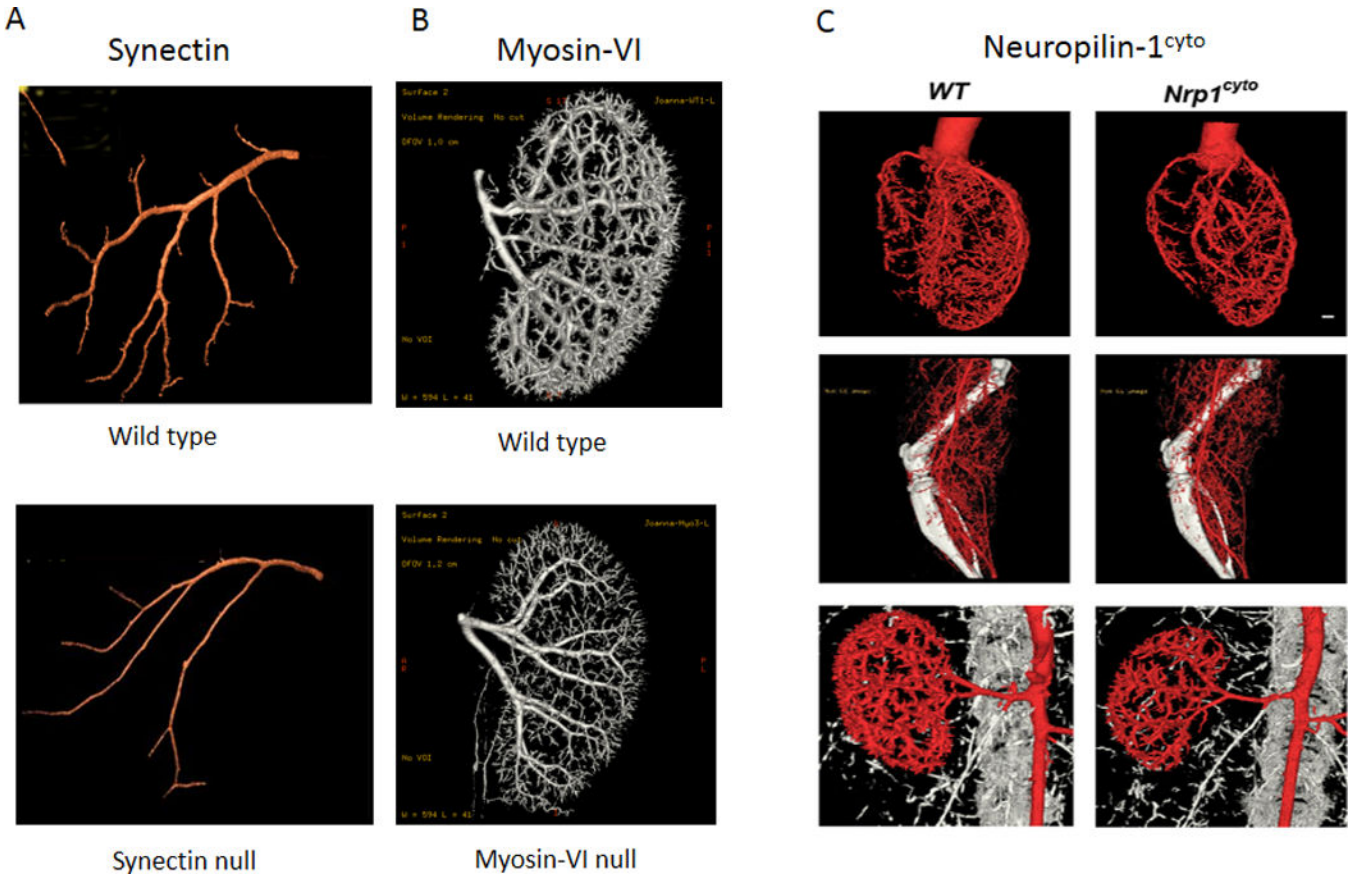

Figure 2.

Arteriogenic defects associated with delayed intracellular VEGFR2 trafficking.

A. Micro-CT images of mouse coronary arteries from wild-type (top) and Synectin null (bottom mice. B. Micro-CT of mouse renal arterial circulation from wild type (top) and myosin-VI null (bottom) mice. C. Micro-CT images of mouse heart (top), hindlimb (middle) and renal (bottom) arterial circulations from mice carrying a deletion of the Nrp1 cytoplasmic domain. Adapted from Lanahan et al Dev Cell 2010 and Lanahan et al Dev Cell 2013.

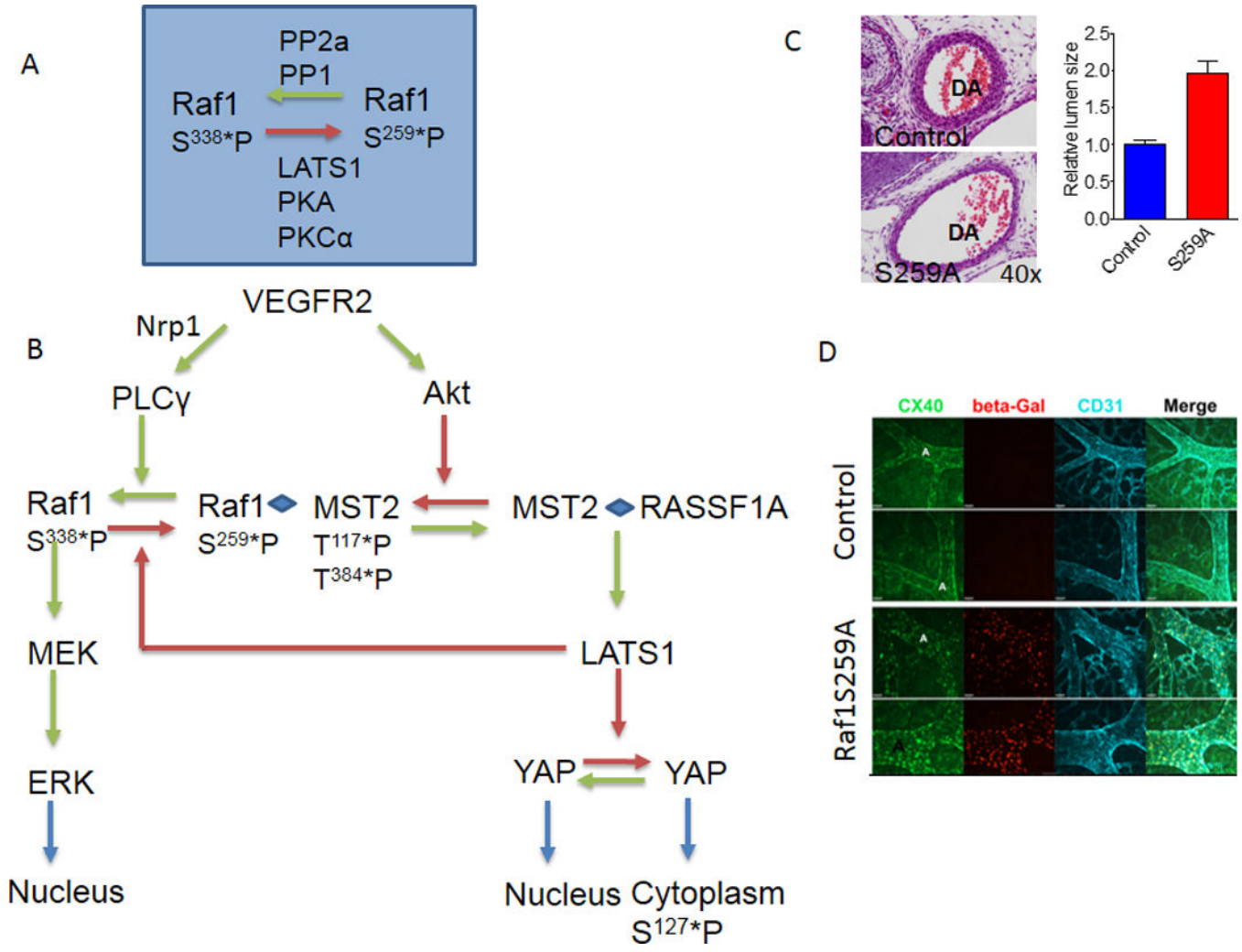

Figure 3.

Inhibition-dependent regulation of VEGFR2-driven ERK activation

A. A schematic of Raf1 phosphorylation control. Dephosphorylation of Ser^{259} allows phosphorylation of Ser338 and activation of Raf1 kinase activity. 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^{259}-phosphorylated) state. Dephosphorylation of $Raf1S^{259}$ site shifts MST2 to the RASSF1A complex thereby activating LATS1 that subsequently acts on YAP. At the same time this allows phosphorylation of $Raf1Ser^{338}$ thereby activating MEK/ERK signaling. Modified, in part, from[104].

C. Increase dorsal aorta diameter in Raf1S29A (bottom) compared to WT (top) mice. D. Extra-embryonic vasculature in control (wild type) and Raf1SA259 mouse embryos. Note a marked increase in arterial size. Cx40-Connexin-40. Panels C and D adapted from[78]

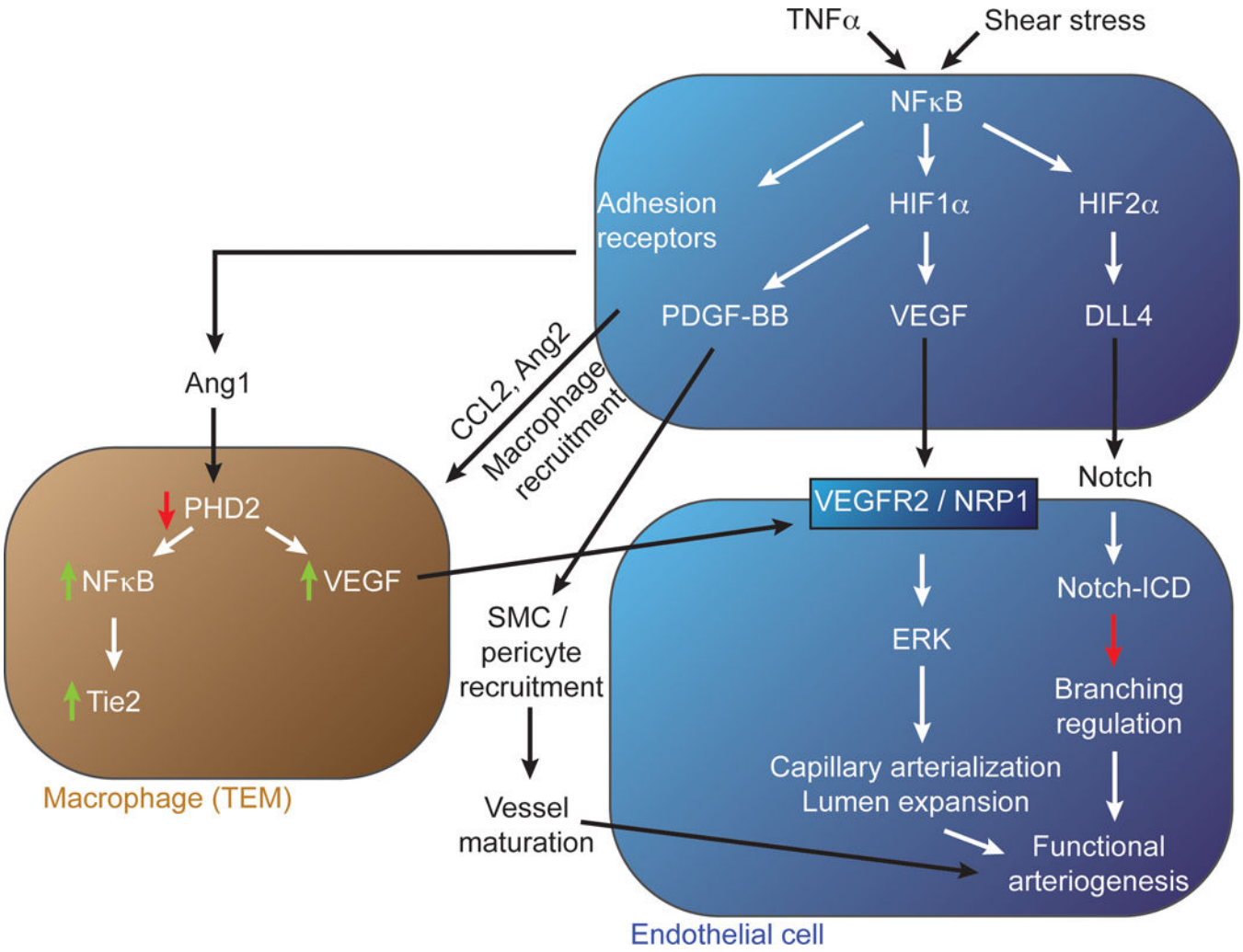

Figure 4. Regulation of arteriogenesis

Inflammatory (e.g. TNFα) and mechanical (e.g. shear stress) stimuli initiate arteriogenic signaling in a resting endothelial cell (top, blue). Activation of NFκB signaling by these stimuli leads to increase HIF1α and HIF2α levels, expression of adhesion receptors and production of PDGF-BB, Ang1 and Ang2. Ang2 in turn induces accumulation of a specific macrophage population that, under control of Ang1, reduce their PHD2 levels thereby increasing VEGF production. The macrophage-produced VEGF (and to a lesser extent endothelial-derived VEGF) activate arteriogenic signaling via VEGFR2/Nrp1 complex. HIF2α-induced expression of Dll4 activates Notch signaling in neighboring endothelial cells thereby controlling branching extent. PDGF-BB plays an important role in recruitment of mural cells and maturation of the new arterial network.

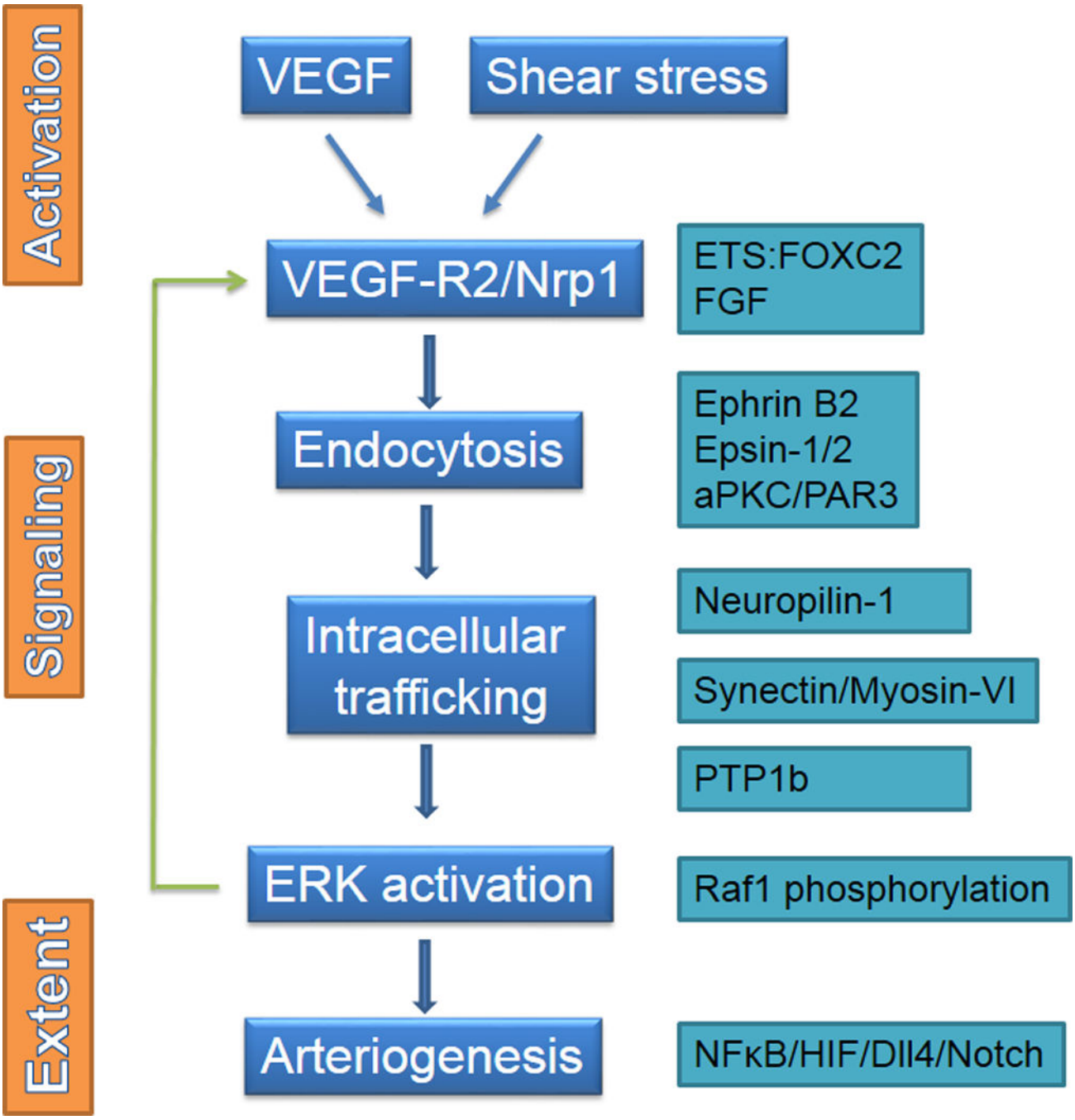

Figure 5. Key arteriogenic events

Key arteriogenic events including activation, regulation of signal transduction and arteriogenesis extent. See text for details.

Table 1

Genetics of arteriogenic defects

Gene	Molecular mechanism	Biological processes
Synectin	EC VEGFR2 trafficking/ERK activation	Arterial branching and lumen size
Myosin-VI	EC VEGFR2 trafficking/ERK activation	Arterial branching and lumen size
Nrp1	EC VEGFR2 trafficking/ERK activation	Arterial branching and lumen size
PTP1b	VEGFR2 trafficking/ERK activation	Arterial branching and lumen size
Dll4	EC Delta-Notch signaling	Arterial branching and maturation
HIF2α	EC Delta-Notch signaling	Arterial branching and maturation
NFkB	EC Delta-Notch signaling	Arterial branching and maturation
Connexin-37	gap junctions/EC-EC communication	Arterial branching and remodeling
Connexin-40	gap junctions/EC-EC communication	Arterial branching and remodeling
PECAM-1	shear stress signaling	Arterial branching, lumen size
Shc	shear stress signaling	Arterial fate and remodeling
eNOS	SMC G protein signaling	Arteriolar density/vascular regression
RGS5	SMC G protein signaling	Arterial fate, branching and remodeling
ICAM-1	monocytes recruitment	perivascular ‘inflammation”
Mac-1	monocytes recruitment	perivascular “inflammation”
MCP1	monocytes recruitment	perivascular “inflammation”
CD44	monocytes recruitment	perivascular “inflammation”
PHD2	monocytes recruitment	perivascular “inflammation”
NFkB	monocytes recruitment	perivascular “inflammation” and Dll4 signaling
CD73	monocytes recruitment	perivascular “inflammation”
HUR	monocyte VEGF-A stability	monocyte VEGF production
Ang2	inflammatory response modulation	perivascular “inflammation”
CD180	inflammatory response modulation	perivascular “inflammation”
ADAMS 17	vascular stabilization	vessel stabilization
Clic4	unknown	unknown
NPY	unknown	unknown
Canq1 locus	unknown	unknown
BMX/ETK	unknown	unknown

Clic4: chloride intracellular channel-4

NPY: Neuropeptide Y

Table 2

Endothelial ERK activation and arterial morphogenesis.

- Absent or severely reduced ERK activation: Complete failure of vascular development
  - VEGFR2$^{-/-}$ or VEGFR2 1175Y→F knock-in
  - PLCγ knockout
  - Ephrin B2 knockout
- Moderately reduced ERK activation: Impaired developmental and adult arteriogenesis
  - Synectin knockout
  - Myosin-VI knockout
  - Nrp1^{cyto} knockin
  - Atherosclerosis & diabetes
- Increased ERK activation
  - Excessive embryonic arteriogenesis (reduced venous fate)
  - AVM and aneurysm formation