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Role of Crosstalk Between Phosphatidylinositol 3-Kinase and Extracellular Signal-Regulated Kinase/Mitogen-Activated Protein Kinase Pathways in Artery–Vein Specification

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Arterial Guidance

Arterial–Venous Specification in Development

Michael Simons, Guest Editor

Role of Crosstalk Between Phosphatidylinositol 3-Kinase and Extracellular Signal-Regulated Kinase/Mitogen-Activated Protein Kinase Pathways in Artery–Vein Specification

Charles C. Hong, Tsutomu Kume, Randall T. Peterson

Abstract—Functional and structural differences between arteries and veins lie at the core of the circulatory system, both in health and disease. Therefore, understanding how artery and vein cell identities are established is a fundamental biological challenge with significant clinical implications. Molecular genetic studies in zebrafish and other vertebrates in the past decade have begun to reveal in detail the complex network of molecular pathways that specify artery and vein cell fates during embryonic development. Recently, a chemical genetic approach has revealed evidence that artery–vein specification is governed by cross talk between phosphoinositide 3-kinase and extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling in artery–vein specification. We discuss recent findings on the signaling pathways involved in artery–vein specification during zebrafish development and compare and contrast these results to those from mammalian systems. It is anticipated that the complementary approaches of genetics and chemical biology, involving a variety of model organisms and systems, will lead to a better understanding of artery–vein specification and possibly to novel therapeutic approaches to treat vascular diseases. (***Circ Res*****. 2008;103:573-579.)**

Key Words: artery–vein specification ■ signaling crosstalk ■ Shh ■ VEGF ■ Notch ■ Fox

Arteries and veins are functionally and structurally distinct, and disturbances in artery and vein specialization are thought to be responsible for many of human vascular pathologies. The arterial system, with its thick outer layers of elastic fibers and smooth muscle, is a high-pressure conduit that is susceptible to atherosclerosis. The venous system, with its relatively thin supporting layers, is a low-pressure/high-capacitance conduit that is relatively more prone to thrombosis. In the body, arteries and veins are typically found in close proximity to each other, but their distinct identifies are strictly maintained. Loss of this distinction because of improper establishment of artery–vein cell identity is thought to result in congenital arteriovenous malformations[1] and potentially devastating diseases such as hereditary hemorrhagic telangi-

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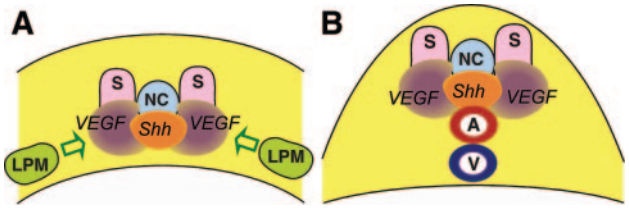

Figure 1. Schematic cross section of the trunk of zebrafish embryos showing endothelial development. A, Mid–somite stage embryo (12 to 14 hpf, 6 to 10 somites). At this stage, angioblasts are formed in the lateral plate mesoderm (LPM). In response to midline signaling by Shh, secreted by notochord (NC), and VEGF, secreted by somites (S), the angioblasts migrate medially. B, Twenty-four-hpf embryo. On completion of the migration, angioblasts situated dorsally adjacent to NC differentiate into the dorsal aorta (A), whereas ventral angioblast differentiate into the posterior cardinal vein (V). Dorsal is to the top. The schematic representation is based on findings by Gering and Patient.[14]

ectasia.[2] Thus, a better understanding of how distinct artery and vein identities arise and are maintained may lead to future therapies for vascular disease.

Here, we review recent findings in zebrafish that reveal the genetic program for establishing artery–vein cell identity during embryogenesis. In addition, we discuss relatively novel chemical genetic studies of zebrafish vascular development that have provided evidence that a crosstalk between 2 ubiquitous signaling pathways, the phosphoinositide 3-kinase (PI3K) and the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling pathways, plays a central antagonistic role in artery–vein specification during vasculogenesis.

Genetic Control of Artery–Vein Cell Fates in the Zebrafish Embryo

The zebrafish, which provides a unique complement of embryological and genetic tools for the study of vertebrate development, has had a major impact on the mechanistic understanding of artery–vein specification.[3,4] In zebrafish, endothelial progenitor cells, called angioblasts, first become evident in 2 parallel stripes of lateral plate mesoderm by 12 to 14 hours postfertilization (hpf) (Figure 1).[5] Within 2 hours of their formation, angioblasts begin their migration to the dorsal midline, and, by 18 hpf, angioblasts coalesce at the midline to form the 2 major axial vessels, the dorsal aorta and the posterior cardinal vein (Figure 1).[6,7] Before the commencement of circulation at 24 hours hpf, dorsal aorta expresses arterial endothelial markers, such as Ephrin-B2a, Notch5, and Gridlock, whereas the posterior cardinal vein expresses venous endothelial markers, such as EphB4 and Flt4 (fms-related tyrosine kinase 4).[8,9] In addition to the vessel-specific marker expression, the 2 axial vessels are clearly distinguishable before the onset of circulation by their spatial relationship within the developing embryo: the dorsal aorta always lies dorsal to the posterior cardinal vein (Figure 1).[8] Remarkably, the arterial–venous cell fate decision appears to be made very early, even before the angioblasts migrate to the midline. Cell-tracing experiments with a fluorescent lineage tracer show that individual angioblasts in the lateral plate mesoderm give rise to either arterial or venous endothelial cells but not both.[10]

Investigations in the zebrafish have made seminal contributions in defining the molecular pathways required for the acquisition of the arterial cell fate during embryogenesis.[11] This pathway involves many of the signaling components known to play important roles in endothelial cell biology, including Sonic hedgehog (Shh), vascular endothelial growth factor (VEGF), Notch, PI3K, and ERK/MAPK signaling.[12] Many of these have been shown to have pleiotropic effects essential to diverse aspects of vascular development. For example, Shh, which is expressed in the notochord (a dorsal midline structure),[11] and VEGF, which is expressed in somites (future trunk muscles just lateral to the notochord), also serve as extracellular signals critical for the midline migration of angioblasts (Figure 1).[13,14]

In a series of elegant zebrafish experiments using mRNA and morpholino antisense RNA injections, together with genetic mutants and transgenic embryos, Weinstein and colleagues revealed a genetic hierarchy of several signaling events that induce endothelial arterial fate (Figure 2).[11] At the top of this hierarchy is Shh, a member of the Hedgehog family of signaling molecules known to be an indirect angiogenic signal that regulates the expression of VEGF isoforms, and angiopoietin-1 and angiopoietin-2 in interstitial mesenchymal cells.[15] In zebrafish, loss of Shh signaling through genetic mutations in *syu* (sonic-you), encoding Shh, and *yot* (you-too), encoding its downstream activator Gli2a, or through pharmacological inhibition with cyclopamine results in the loss of the arterial marker Ephrin-B2a and the expansion of the venous marker Flt4 in the vasculature.[11] Conversely, overexpression of Shh caused formation of ectopic Ephrin-B2a–expressing presumptive arterial cells within the trunk vessels.

Induction of the arterial fate by Shh is mediated by VEGF. VEGF expression in the somites is induced by and dependent on Shh signals from the adjacent notochord. Knockdown of VEGF using morpholino antisense RNA results in the loss of arterial marker Ephrin-B2a expression.[11] Conversely, overexpression of VEGF results in upregulation of artery-specific markers and can rescue arterial marker expression in the absence of Shh signals. Although distinct combinations of multiple VEGF receptors are known to be required for the development of different endothelial types in zebrafish, the VEGF receptor-2 homolog Kdra appears to play a dominant role in artery differentiation.[16] A forward genetic screen has revealed the involvement of phospholipase C (PLC)γ1, a known downstream signaling component of various receptor tyrosine kinases including the VEGF receptors, in the arterial specification pathway. Like embryos lacking VEGF, PLCγ1 mutant embryos show specific defects in artery formation.[17] Overexpression of VEGF mRNA fails to restore arterial markers, suggesting that PLCγ1 is a key downstream transducer of VEGF signals for arterial development. Evidence in zebrafish indicates that Notch signaling acts downstream of both Hedgehog and VEGF signaling to induce the arterial differentiation by suppressing the venous fate. Disruption in Notch signaling through a mutation in *mib* (mindbomb), necessary for Notch activation, or via expression of

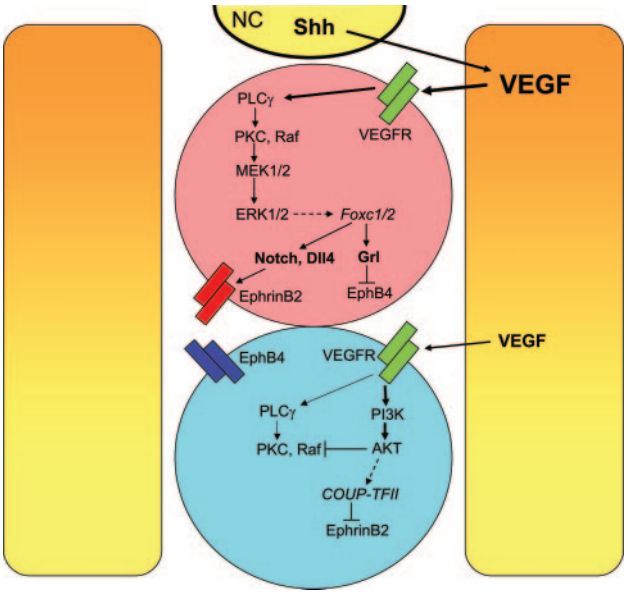

Figure 2. A model for molecular pathway for specification of artery and vein fates. This model is based largely on findings in zebrafish plus Foxc1/2 and COUP-TFII results in mice (noted by dashed arrows). Shh, expressed in the notochord and hypochord (on top), induces VEGF expression in nearby somites. By a process yet to be fully determined, VEGF activates the PLCγ-ERK/MAPK pathway only in the dorsal angioblasts (pink circle), which will develop into aortic endothelial cells. ERK activation results in Notch activation (indicated by Notch and Dll4 expression) via the transcriptional factors Foxc1/2. Notch signaling induces arterial differentiation (indicated by the arterial marker EphrinB2 expression). In the arterial progenitor cells, Grl, induced by Foxc1/2, blocks venous differentiation (indicated by the venous marker EphB4 expression). In the ventral angioblasts (blue circle), VEGF activates the PI3K/AKT pathway, which inhibits the PLCγ-ERK/MAPK pathway, possibly by a direct inhibition of Raf by AKT. AKT signaling is hypothesized to induce expression of COUP-TFII, which blocks arterial differentiation (also EphrinB2 expression). In this model, a gradient of VEGF along the dorsal–ventral axis is postulated to govern whether VEGF receptor activates PI3K or ERK signaling. For simplicity, the artery–vein specification is depicted here as occurring following the midline convergence of endothelial progenitors, but evidence suggests that this occurs earlier in zebrafish embryos. The schematic representation is based on discussions presented by Lamont and Childs.[12]

dominant-negative Su(H) (suppressor of hairless), a downstream mediator of Notch signaling, abrogates the expression of arterial makers and induces ectopic expression of venous markers.[18] Conversely, ectopic activation of Notch signaling by the overexpression of constitutively active Notch intracellular domain [NICD] induces ectopic arterial marker expression and blocks vein marker expression. In addition, activation of Notch signaling can induce ectopic arterial marker expression in the absence of VEGF signaling. However, forced expression of VEGF in *mib* mutants does not restore artery marker expression, suggesting that Notch acts downstream of VEGF signaling.

Gridlock, a member of the Hairy-related family of transcription factors that act downstream of the Notch signaling, is also involved in artery specification in zebrafish embryos.[10] A hypomorphic mutation in the *grl* (gridlock) (zebrafish *hey2* homolog) gene results in a focal defect in the dorsal aorta. Gridlock is first expressed in the lateral plate mesoderm, but its expression becomes restricted to the dorsal aorta. Morpholino knockdown of *grl* led to progressive loss of dorsal aorta and concomitant enlargement of posterior cardinal vein. Consistent with the role of Gridlock in artery–vein specification, overexpression of Gridlock suppressed venous markers. However, Gridlock overexpression did not result in ectopic expression of artery markers, suggesting that it functions specifically to block the venous state. Whether Gridlock is directly downstream of Notch signaling is unclear because its vascular expression remains intact following expression of dominant-negative Su(H) or in *mib* mutants.[11]

Artery–Vein Specification in Mammals: A Brief Comparative Overview

Studies in zebrafish have provided a crucial general framework for understanding the key aspects of mechanistic understanding of artery–vein specification during embryogenesis. Indeed, many signaling events in zebrafish are faithfully recapitulated in higher vertebrates.[19] Nonetheless, studies in mammalian systems have highlighted some differences between the 2 models, as well as provided novel insights into artery–vein specification that are yet to be confirmed in zebrafish.

Although the roles of Hedgehog and VEGF in artery–vein specification have been elegantly demonstrated in zebrafish, evidence for their involvement in mammalian artery–vein specification during development is less direct. In mice, disruption of Shh, which is expressed in the notochord, does not result in obvious vascular defects.[20] Nonetheless, Hedgehog signaling may play a role in arterial specification in mice, as mice lacking Smo (Smoothened), a transmembrane protein that transduces Hedgehog signal, exhibit defective dorsal aorta formation, although this could be attributable to a general defect in endothelial tube formation rather than a primary defect in artery–vein specification.[21] In addition, mice with disruptions in Ihh (Indian Hedgehog) or Smo exhibit blocks in remodeling of the yolk sac vasculature, but it is unclear whether this is a consequence of a primary defect in artery–vein specification.[22] Mice lacking VEGF or PLCγ1 show such a severe disruption in overall vascular development that a definitive conclusion about the direct role of VEGF or PLCγ1 in artery–vein specification cannot be made.[23,24] However, a more selective perturbation of VEGF expression did result in defective arterial development in the mouse retina.[25]

In mice, Notch1 and Notch4, as well as Notch ligands Jagged1, Jagged2, Delta-like (Dll)1, and Dll4 are selectively expressed in arterial endothelial cells,[26,27] suggesting that Notch signaling is required for arterial identity (reviewed by Roca and Adams[28]). In support of this notion, the Dll4 ligand is required for mouse artery development in a dose-dependent manner.[29] Studies in mice suggest that Gridlock/Hey2 and related Hey1 play an important and redundant role in mouse arterial specification. Although mice with a single knockout of Gridlock homolog Hey2 do not exhibit vascular defects, the compound Hey1:Hey2 double knockout mice show loss of the expression of arterial marker Ephrin-B2.[30] Moreover, 2 members of the forkhead/Fox transcription factor family Foxc1 and Foxc2 have recently been shown to be required for

artery specification in mice.[31] The compound Foxc1:Foxc2 knockout mice show various vascular defects including vascular fusions between the dorsal aorta and the anterior cardinal vein and loss of expression of the arterial markers, such as Dll4. Importantly, Foxc proteins were shown to activate the expression of Dll4, along with other arterial markers such as Hey2, in endothelial cells in vitro. Moreover, Foxc proteins were shown to directly bind to the Dll4 promoter. These and more recent studies, discussed below, demonstrate that Foxc factors play a critical role in linking VEGF signals to the Notch/Hey2 pathway to direct arterial specification.

Collective evidence from zebrafish studies led to a picture in which the venous fate is the default state for bipotent angioblasts and Notch signaling induces arterial development.[8] In fact, very little is known regarding the signaling components involved in establishing venous fate identity. Recently, an orphan nuclear receptor COUP-TFII (chicken ovalbumin upstream promoter–transcription factor II) was found to be expressed specifically in venous endothelial cells.[32] Endothelial-specific knockout of COUP-TFII led to ectopic expression of arterial markers, and endothelial-specific overexpression of COUP-TFII led to loss of arterial markers. Because manipulations of COUP-TFII expression perturbed expression of arterial-specific Notch pathway markers, it was hypothesized that COUP-TFII functions to maintain venous identity by repressing Notch signaling in venous angioblasts. However, the precise step targeted by COUP-TFII remains unclear because ectopic COUP-TFII expression also disrupted the expression of neuropilin-1 (NP1), a VEGF coreceptor that is normally expressed in arterial cells.[33] The role of COUP-TFII in zebrafish artery–vein specification is yet to be determined.

Chemical Genetic Analysis of Artery–Vein Specification Reveals Opposing Effects of MAPK and PI3K on Artery–Vein Specification

Although classic genetic analyses, such as those described above, have revealed much about the molecular pathways involved in artery–vein specification, such approaches have limitations when it comes to examining signaling pathways that function at multiple stages in development. With a typical nonconditional mutation, the primary role for a gene at later stages of development is often difficult to distinguish from indirect consequences of disrupting the gene at an earlier stage. The Hedgehog pathway, for example, plays a critical role in vasculogenesis, but is also involved in development of numerous other structures at various developmental time points.[34] Moreover, classic genetic approaches can be hampered by genetic redundancy.[35] A powerful alternative approach, chemical genetic analysis, can overcome the challenges posed by repeated utilization of a signaling pathway during development and by genetic redundancy. Recently, Hedgehog signaling inhibitor cyclopamine was instrumental in demonstrating that Hedgehog is required at 3 consecutive stages during vascular development[14]: for medial migration of angioblasts, for arterial gene expression, and for formation of intersomitic vessels.

To complement the genetics studies, small molecule screens were recently performed to identify compounds that suppressed absent trunk circulation because of reduced artery formation in *grl* mutant embryos.[36,37] The central rationale of a chemical suppressor study is analogous to that of classic genetic modifier screen: namely, if impairment in a genetic pathway for arterial development can be suppressed by small molecule, the cellular target of the "chemical suppressor" must be relevant to arterial specification. These screens identified 2 classes of gridlock suppressors. The first class, represented by the compound GS4012, appears to function by activating VEGF signaling. GS4012 induces VEGF expression in zebrafish and mimics the effects of VEGF in zebrafish and in endothelial tubule formation assays. The second class of gridlock suppressors, represented by GS4898, block the PI3K pathway. This was a surprising finding because PI3K is a well-known downstream component of VEGF signaling.[38]

The apparent paradox of rescue of the identical vascular phenotype by putative stimulators of VEGF signaling, as well as inhibitors of a downstream VEGF signaling component, was resolved with the recognition that 2 well-known signaling pathways activated by the VEGF receptor, the PLCγ1-ERK/MAPK pathway and the PI3K-ATK pathways, could play competing or antagonistic roles (Figure 2). In human umbilical vein endothelial cells, the PI3K-AKT pathway has been shown to antagonize ERK/MAPK signaling.[39] Moreover, in a human breast cancer cell line, similar PI3K-AKT and ERK/MAPK crosstalk occurred through direct inhibitory phosphorylation of Raf, a MAPK signaling component, by AKT, a key kinase acting downstream of PI3K.[40] In zebrafish embryos, incubation with GS4898 or known P3K inhibitors, LY294002 and wortmannin, results in activation of the ERK/MAPK and expansion of the arterial fate.[37] Conversely, inhibition of mitogen/extracellular signal-activated protein kinase kinase (MEK), an upstream activator of ERK, results in loss of arterial structures. To circumvent the pleiotropic effects of ERK/MAPK and PI3K/AKT in early development, mosaic transgenic expression of AKT in zebrafish were used to confirm chemical genetic findings. Expression of dominant negative AKT along with green fluorescent protein (GFP) resulted in preferential localization of GFP-positive cells in the dorsal aorta, whereas expression of constitutively active AKT resulted in localization of GFP-positive cells in the posterior cardinal vein. Together, these results suggest that ERK/MAPK signaling is required for the arterial cell fate, whereas PI3K signaling has an opposing effect of promoting the venous fate by inhibiting the ERK/MAPK pathway.

Importantly, during zebrafish development, activated/phosphorylated ERK is localized precisely to dorsal angioblasts that will develop into aortic endothelial cells but not ventral angioblasts destined to become venous endothelial cells (Figure 3).[37] Pharmacological blockade of VEGF receptor-1 and -2 signaling, and of Hedgehog signaling, both of which block arterial specification, were shown to prevent ERK activation in endothelial progenitors. Conversely, high concentrations of PI3K inhibitors were shown to expand ERK activation within endothelial progenitors. These results show that Hedgehog and VEGF signaling are required upstream of ERK/MAPK pathway to activate it specifically in arterial

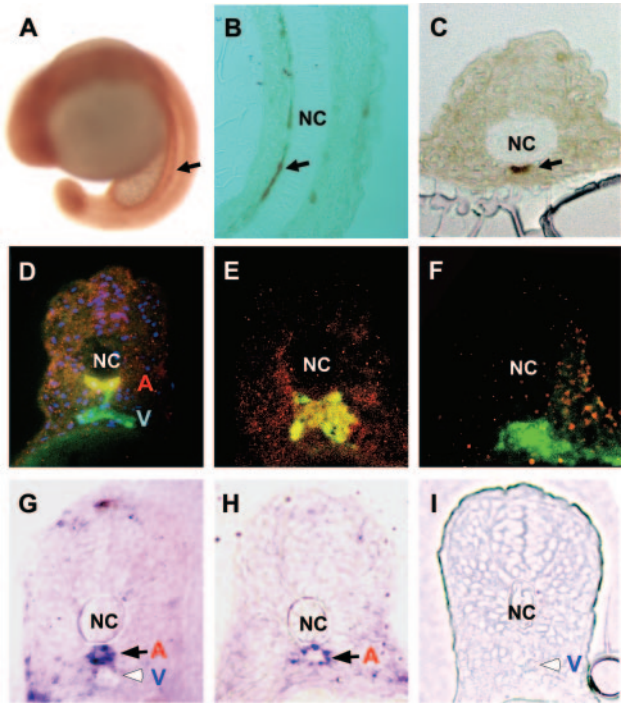

Figure 3. ERK activation is an early marker and determinant of arterial fate. A, Whole-mount staining of 20-somite-stage (ss) zebrafish embryo with antibody that recognizes diphosphorylated (activated) ERK indicates high levels of ERK activation (indicated by arrows) in developing vasculature. B and C, Longitudinal section (B) and cross-section (C) demonstrate the same. D, In this cross-section of a wild-type 20-ss embryo, ventral, venous angioblasts (denoted as V) are immunostained for vascular-specific GFP (green), and dorsal, arterial angioblasts (denoted as A), which immunostain for activated ERK, are shown in yellow (green GFP merged with red phospho-ERK immunostaining). Nuclei are marked with DAPI (blue). E, In embryos treated with a high dose of PI3K inhibitors, the proportion of angioblasts that immunostain for activated ERK (yellow) is greatly expanded. F, In an embryo treated with VEGF receptor inhibitor 676475, ERK activation in angioblasts is lost. G, In this cross-section of an in situ hybridization of 24-hpf wild-type embryo, the arterial marker ephrinB2a is clearly expressed in the dorsal aorta (denoted as A), but not in posterior cardinal vein (denoted as V). H, In embryos treated with a high dose of PI3K inhibitors, the Ephrin-B2a–expressing dorsal aorta (denoted as A) is prominent, but the posterior cardinal vein is not observed. I, In embryos treated with MEK inhibitor SL327, neither Ephrin-B2a expression nor dorsal aorta is observed. In A and B, the dorsal side is to the right. In C through I, the dorsal side is to the top. NC indicates notochord. Adapted Hong et al[37] by permission of Elsevier Ltd.

progenitors and that ERK activation is among the earliest known markers and determinants of artery cell fate in zebrafish embryos. Of note, strikingly analogous aorta-specific ERK activation has also been observed in mouse embryos,[41] suggesting a similar role for ERK signaling in mouse arterial development.

Interestingly, the earliest detection of ERK activation occurs even before the completion of angioblast migration to the midline (15 hpf; Figure 4).[37] During angioblast migration, activated ERK is preferentially localized to a distinct subset of angioblasts on the leading edge (Figure 4), which, on time-lapse micrography, appear to contribute to the nascent dorsal aorta (C. Hong and R.T. Peterson, unpublished observations, 2008). Such early emergence of distinct arterial and venous angioblasts, which exhibit different migratory timing, is consistent with the findings in synectin-deficient zebrafish, in which selective disturbance in angioblast migration precede the deficient artery differentiation.[42] These results are consistent with cell lineage tracing experiments that suggest that artery–vein fate is already established when angioblasts begin their midline migration from their origins in the lateral plate mesoderm.[10] Lastly, activated ERK is not detected after the circulation is established, suggesting that ERK activation is not required for maintenance of the arterial phenotype in zebrafish.

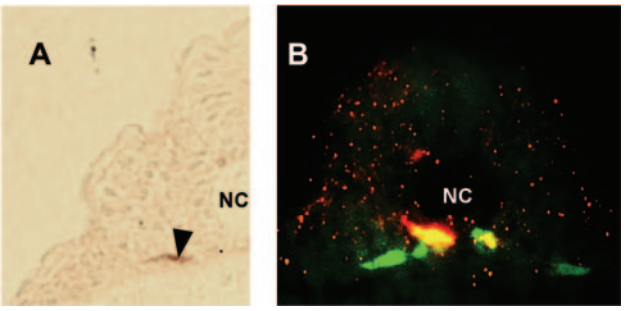

Figure 4. Activated ERK is found in a subset of angioblasts before the completion of their midline migration. A and B, Cross-section of immunostain for activated ERK in 12-ss (15-hpf) embryo. A, Activated ERK can be found in angioblast in the process of midline migration. B, Merged view of migrating angioblasts, marked by GFP (green) and by activated ERK (yellow). By 12-ss, activated ERK is found preferentially in the "leading edge" angioblast subpopulation (yellow) that reaches the midline earlier than the rest (green). NC indicates notochord. Dorsal side is to the top. Adapted from Hong et al[37] by permission of Elsevier Ltd.

Crosstalk Between PI3K and MAPK in Mammalian Systems: Comparison to Zebrafish Findings

In cultured mammalian endothelial cells, stimulation with VEGF results in immediate activation of PI3K and ERK/MAPK signaling.[38] An important distinction from in vivo findings in zebrafish embryos is that ERK/MAPK activation in cultured cells is transient (typically lasting only a few minutes)[43] versus persistent detectable levels of activated ERK over several hours in zebrafish embryos. Such dramatic differences in signaling kinetics may have important functional implications when comparing biological responses in vitro and in vivo. In cultured cells, the VEGF receptor activates 2 downstream signaling branches, each of which elicits a distinct set of biological responses.[38] In the first branch, PI3K activation leads to AKT activation, which promotes endothelial cell migration, survival, and nitric oxide production. In the second branch, the VEGF receptor activates PLCγ, resulting in activation of protein kinase C and Raf, which then triggers a kinase activation cascade leading to ERK/MAPK activation and promoting endothelial cell proliferation. In most in vitro contexts, the 2 branches are stimulated by VEGF together and often act in a synergistic manner. Nonetheless, in certain endothelial culture systems, the PI3K branch has been shown to antagonize the PLCγ-

ERK/MAPK branch, similar to the observations in zebrafish embryos.[39]

In vitro mammalian cell studies have uncovered important differences from in vivo zebrafish studies regarding interactions between VEGF, Notch, and ERK signaling. In cultured human arterial endothelial cells, VEGF signal induces expression of Notch1 and Dll4.[44] However, in contrast to the zebrafish data, VEGF-induced Notch activation is mediated by PI3K, rather than ERK/MAPK.[44] Additional studies in bovine aortic endothelial cells have demonstrated that Foxc transcription factors mediate VEGF signaling by directly activating Dll4 and Hey2 promoters.[45] Moreover, VEGF-activated PI3K and ERK pathways were found to modulate the transcriptional activation of Dll4 and Hey2 genes by Foxc proteins. Again in contrast to the zebrafish data, the PI3K pathway was found to be necessary for inducing Dll4 and Hey2 expression. Interestingly, the ERK pathway was found to repress Dll4 and Hey2 expression, supporting the presence of a functional ERK-PI3K crosstalk in aortic endothelial cells. Reasons for the discrepancy between in vitro and in vivo data regarding the functional effects of ERK and PI3K crosstalk are unclear. One possible explanation is that these in vitro experiments were all done in endothelial cells with a well-established arterial cell identify, not in uncommitted bipotent endothelial progenitor cells. In addition, as mentioned earlier, ERK activation in arterial progenitor cells is noted over the course of several hours, a far longer time frame than the transient ERK activation observed in cultured endothelial cells following VEGF stimulation. Thus, there may be important fundamental differences in ERK activation between in vivo and in vitro models. Finally, in a mouse ES cell model of in vitro differentiation of artery and vein cell types, higher concentrations of VEGF promoted expression of arterial marker genes, whereas low and intermediate levels of VEGF preferentially induced expression of the venous marker COUP-TFII.[46] Moreover, this VEGF-dependent arterial development could be blocked by Notch signal inhibition. Whether graded VEGF signaling could govern preferential activation of either PI3K or ERK pathways was not tested in this model. In summary, although interactions between VEGF, Notch, and ERK signaling in isolated endothelial cells in culture may not fully recapitulate complex multicellular interactions that occur in the developing embryo, recent evidence supports the existence of a crosstalk between PI3K and ERK, with functional implications for artery–vein specification.

Concluding Remarks

As discussed here, studies in zebrafish have made important contributions to the genetic control of artery–vein specification during development. Nonetheless, there remain numerous challenges to achieving a more complete understanding of this complex process, involving many cells types and signaling molecules characterized by both genetic redundancy and pleiotropic functions. For example, the detailed mechanistic understanding of how Shh and VEGF specify arterial cell fate is still a challenge, particularly because both Shh and VEGF modulate diverse cellular processes, from cell fate decisions, cell division/arrest decisions, chemotaxis, and interpretation of positional information. Another important question involves specific components that link VEGF signaling and downstream Notch signaling in the artery-specification pathway.

The chemical genetic approach was recently used to reveal previously unsuspected roles of PI3K and ERK, 2 well-known VEGF signaling branches, in artery–vein specification. These findings raise additional interesting questions. For example, what determines whether an angioblast activates ERK or PI3K signaling? Could particular VEGF receptor subtypes or VEGF gradient govern which signaling branch is activated? What are the downstream targets of the ERK and PI3K signaling involved in artery–vein specification? Another important question regards whether the crosstalk between PI3K and ERK also plays an analogous role in specification of artery–vein fates in mammals. Although studies in cultured mammalian cells have yielded contradictory results, an earlier finding of localized activation of ERK in the developing aorta in mouse embryos[41] suggests a conserved role for ERK in mammalian arterial specification. Further studies using classic and chemical genetics in zebrafish, as well as in vivo and in vitro mammalian models, will be necessary to resolve whether the discrepancy is attributable to a fundamental species difference or to a difference between in vivo and in vitro models. Finally, although genetic programs play a critical role in artery–vein specification during development, local environmental factors such as shear stress are also known to modify artery and vein cell identity.[47–49] Curiously, in the setting of adaptation after femoral artery occlusion, sustained shear stress promotes collateral artery growth, in part, by activating the ERK pathway.[50] Thus, it will be interesting, and clinically relevant, to examine whether the influence of environmental factors on artery–vein plasticity involves the signaling components implicated in artery–vein specification during development.

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Disclosures

None.

References

1. Shovlin CL, Scott J. Inherited diseases of the vasculature. *Annu Rev Physiol*. 1996;58:483–507.
2. Guttmacher AE, Marchuk DA, White RIJ. Hereditary hemorrhagic telangiectasia. *N Engl J Med*. 1995;333:918–924.
3. Grundwald DJ, Eisen JS. Headwaters of the zebrafish - emergence of a new model vertebrate. *Nat Rev Genet*. 2002;3:717–724.
4. Thisse C, Zon LI. Organogenesis - heart and blood formation from the zebrafish point of view. *Science*. 2002;295:457–462.
5. Thompson MA, Ransom DG, Pratt SJ, MacLennan H, Kieran MW, Detrich HW, Gates MA, Amores A, Bahary N, Talbot WS, Her H, Beier DR, Postlethwait JH, Zon LI. The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. *Dev Biol*. 1998;197:248–269.
6. Eriksson J, Lofberg J. Development of the hypochord and dorsal aorta in the zebrafish embryo *(Danio rerio)*. *J Morphol*. 2000;244:167–176.
7. Fourquet B, Weinstein BM, Serluca FC, Fishman MC. Vessel patterning in the embryo of the zebrafish: guidance by notochord. *Dev Biol*. 1997;183:37–48.

8. Lawson ND, Weinstein BM. Arteries and veins: making a difference with zebrafish. *Nat Rev Genet*. 2002;3:674–682.
9. Mukhopadhyay A, Peterson RT. Deciphering arterial identity through gene expression, genetics, and chemical biology. *Curr Opin Hematol*. 2008;15:221–227.
10. Zhong TP, Childs S, Leu JP, Fishman MC. Gridlock signalling pathway fashions the first embryonic artery. *Nature*. 2001;414:216–220.
11. Lawson ND, Vogel AM, Weinstein BM. Sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Dev Cell*. 2002;3:127–136.
12. Lamont RE, Childs S. MAPping out arteries and veins. *Sci STKE*. 2006; 2006:pe39.
13. Cleaver O, Krieg PA. VEGF mediates angioblast migration during development of the dorsal aorta. *Development*. 1998;125:3905–3914.
14. Gering M, Patient R. Hedgehog signaling is required for adult blood stem cell formation in zebrafish embryos. *Dev Cell*. 2005;8:389–400.
15. Pola R, Ling LE, Silver M, Corbley MJ, Kearney M, Blake Pepinsky R, Shapiro R, Taylor FR, Baker DP, Asahara T, Isner JM. The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nat Med*. 2001;7:706–711.
16. Covassin LD, Villefranc JA, Kacergis MC, Weinstein BM, Lawson ND. Distinct genetic interactions between multiple Vegf receptors are required for development of different blood vessel types in zebrafish. *Proc Natl Acad Sci U S A*. 2006;103:6554–6559.
17. Lawson ND, Mugford JW, Diamond BA, Weinstein BM. phospholipase C gamma-1 is required downstream of vascular endothelial growth factor during arterial development. *Genes Dev*. 2003;7:1346–1351.
18. Lawson ND, Scheer N, Pham V, Kim C-H, Chitnie AB, Campos-Ortega J, Weinstein BM. Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development*. 2001; 128:3675–3683.
19. Coultas L, Chawengsaksophak K, Rossant J. Endothelial cells and VEGF in vascular development. *Nature*. 2005;438:937–945.
20. Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature*. 1996;383:407–413.
21. Vokes SA, Yatskievych TA, Heimark RL, McMahon J, McMahon AP, Antin PB, Krieg PA. Hedgehog signaling is essential for endothelial tube formation during vasculogenesis. *Development*. 2004;131:4371–4380.
22. Byrd N, Becker S, Maye P, Narasimhaiah R, St-Jacques B, Zhang X, McMahon J, McMahon A, Grabel L. Hedgehog is required for murine yolk sac angiogenesis. *Development*. 2002;129:361–372.
23. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*. 1996;380:435–439.
24. Liao HJ, Kume T, McKay C, Xu MJ, Ihle JN, Carpenter G. Absence of erythrogenesis and vasculogenesis in Plcg1-deficient mice. *J Biol Chem*. 2002;277:9335–9341.
25. Stalmans I, Ng YS, Rohan R, Fruttiger M, Bouche A, Yuce A, Fujisawa H, Hermans B, Shani M, Jansen S, Hicklin D, Anderson DJ, Gardiner T, Hammes HP, Moons L, Dewerchin M, Collen D, Carmeliet P, D'Amore PA. Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. *J Clin Invest*. 2002;109:327–336.
26. Villa N, Walker L, Lindsell CE, Gasson J, Iruela-Arispe ML, Weinmaster G. Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. *Mech Dev*. 2001;108:161–164.
27. Limbourg A, Ploom M, Elligsen D, Sorensen I, Ziegelhoeffer T, Gossler A, Drexler H, Limbourg FP. Notch ligand Delta-like 1 is essential for postnatal arteriogenesis. *Circ Res*. 2007;100:363–371.
28. Roca C, Adams RH. Regulation of vascular morphogenesis by Notch signaling. *Genes Dev*. 2007;21:2511–2524.
29. Duarte A, Hirashima M, Benedito R, Trindade A, Diniz P, Bekman E, Costa L, Henrique D, Rossant J. Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev*. 2004;18:2474–2478.
30. Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes Dev*. 2004;18:901–911.
31. Seo S, Fujita H, Nakano A, Kang M, Duarte A, Kume T. The forkhead transcription factors, Foxc1 and Foxc2, are required for arterial specification and lymphatic sprouting during vascular development. *Dev Biol*. 2006;294:458–470.
32. You LR, Lin FJ, Lee CT, DeMayo FJ, Tsai MJ, Tsai SY. Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. *Nature*. 2005;435:98–104.
33. Mukouyama YS, Gerber HP, Ferrara N, Gu C, Anderson DJ. Peripheral nerve-derived VEGF promotes arterial differentiation via neuropilin 1-mediated positive feedback. *Development*. 2005;132:941–952.
34. McMahon AP, Ingham PW, Tabin CJ. Developmental roles and clinical significance of hedgehog signaling. *Curr Top Dev Biol*. 2003;53:1–114.
35. Ihle JN. The challenges of translating knockout phenotypes into gene function. *Cell*. 2000;102:131–134.
36. Peterson RT, Shaw SY, Peterson TA, Milan DJ, Zhong TP, Schreiber SL, MacRae CA, Fishman MC. Chemical suppression of a genetic mutation in a zebrafish model of aortic coarctation. *Nat Biotechnol*. 2004;22: 595–599.
37. Hong CC, Peterson QP, Hong JY, Peterson RT. Artery/vein specification is governed by opposing phosphatidylinositol-3 kinase and MAP kinase/ERK signaling. *Curr Biol*. 2006;16:1366–1372.
38. Zachary I, Gliki G. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. *Cardiovasc Res*. 2001;49:568–581.
39. Blum S, Issbruker K, Willuweit A, Hehlgans S, Lucerna M, Mechtcheriakova D, Walsh K, von der Ahe D, Hofer E, Clauss M. An inhibitory role for the phosphatidylinositol 3-kinase-signaling pathway in vascular endothelial growth factor-induced tissue factor expression. *J Biol Chem*. 2001;276:33428–33434.
40. Zimmermann S, Moelling K. Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science*. 1999;286:1741–1744.
41. Corson LB, Yamanaka Y, Lai KM, Rossant J. Spatial and temporal patterns of ERK signaling during mouse embryogenesis. *Development*. 2003;130:4527–4537.
42. Chittenden TW, Claes F, Lanahan AA, Autiero M, Palac RT, Tkachenko EV, Elfenbein A, Ruiz de Almodovar C, Dedkov E, Tomanek R, Li W, Westmore M, Singh JP, Horowitz A, Mulligan-Kehoe MJ, Moodie KL, Zhuang ZW, Carmeliet P, Simons M. Selective regulation of arterial branching morphogenesis by synectin. *Dev Cell*. 2006;10:783–795.
43. Takahashi T, Ueno H, Shibuya M. VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. *Oncogene*. 1999;18: 2221–2230.
44. Liu ZJ, Shirakawa T, Li Y, Soma A, Oka M, Dotto GP, Fairman RM, Velazquez OC, Herlyn M. Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. *Mol Cell Biol*. 2003;23: 14–25.
45. Hayashi H, Kume T. Foxc transcription factors directly regulate Dll4 and Hey2 expression by interacting with the VEGF-Notch signaling pathways in endothelial cells. *PLoS ONE*. 2008;3:e2401.
46. Lanner F, Sohl M, Farnebo F. Functional arterial and venous fate is determined by graded VEGF signaling and notch status during embryonic stem cell differentiation. *Arterioscler Thromb Vasc Biol*. 2007;27: 487–493.
47. Moyon D, Pardanaud L, Yuan L, Breant C, Eichmann A. Plasticity of endothelial cells during arterial-venous differentiation in the avian embryo. *Development*. 2001;128:3359–3370.
48. le Noble F, Moyon D, Pardanaud L, Yuan L, Djonov V, Matthijsen R, Breant C, Fleury V, Eichmann A. Flow regulates arterial-venous differentiation in the chick embryo yolk sac. *Development*. 2004;131:361–375.
49. Othman-Hassan K, Patel K, Papoutsi M, Rodriguez-Niedenfuhr M, Christ B, Wilting J. Arterial identity of endothelial cells is controlled by local cues. *Dev Biol*. 2001;237:398–409.
50. Eitenmuller I, Volger O, Kluge A, Troidl K, Barancik M, Cai WJ, Heil M, Pipp F, Fischer S, Horrevoets AJ, Schmitz-Rixen T, Schaper W. The range of adaptation by collateral vessels after femoral artery occlusion. *Circ Res*. 2006;99:656–662.

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