Signaling Pathways in the Specification of Arteries and Veins

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Abstract—The establishment of arterial and venous identity of endothelial cells is critical for the proper anatomic configuration and function of the vascular tree. Arterial and venous specification of endothelial cells is determined by genetic factors, although surrounding cells and hemodynamic forces may also contribute to vascular remodeling. This review provides an overview of the signaling pathways and related transcription factors implicated in differentiation of endothelial cells. We will discuss, in particular, the role of upstream and downstream effectors of Wnt, Sox, and Notch pathways. The understanding of the molecular mechanisms that orchestrate endothelial differentiation may have therapeutic relevance for diseases such as atherosclerosis, arteriovenous malformations, aneurysms, and others. (Arterioscler Thromb Vasc Biol. 2014;34:2372-2377.)

Key Word: endothelial cells

During early stages of embryo development, endothelial cell progenitors differentiate from mesoderm and form a fragile and irregular tubular network, called primitive vascular plexus. These early vascular structures then undergo intense remodeling forming different types of vessels such as arteries, veins, and capillaries. Vascular remodeling is a complex process largely determined by genetic factors and occurs in absence of blood flow.1,2

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However, when the heart starts to beat, endothelial cells are exposed to hemodynamic forces that strongly influence their functional properties such as matrix organization, cytoskeletal reshaping, cell-to-cell junctions, and others. Furthermore, endothelial cells contribute to the recruitment of smooth muscle cells and pericytes that protect the endothelium from the impact of the hemodynamic forces and modulate blood flow.1,4

At a subsequent stage, lymphatics originate from the cardinal vein, thus completing the general anatomic configuration of the vascular network.3 It is also important to point out that even if arteries, veins, and lymphatics are equally necessary for the proper functioning of the cardiovascular system, they perform different tasks, and this is mirrored by their structural features (Figure [A]).1,6 Although arteries have to cope with the high pressure of blood exiting from the heart and are thus equipped with thick layers of tunica media and adventitia, veins are functionally and structurally different, having much thinner vessel walls to convey low-pressure blood flow. Veins are the preferred site for leukocyte extravasation as well.4 The role of lymphatics, instead, is 2-fold: to facilitate immune cell patrolling and to drain extravasated fluids, proteins, and cells from tissues bringing them back in the venous circulation. The specification of lymphatics occurs from a subset of venous endothelial cells in the embryo as it has been already thoroughly reviewed elsewhere.1,5 A crucial and further step of vascular differentiation is organ specification (ie, endothelial cells acquire specialized properties to meet the specific needs of the organ where they are located).9 For instance, the brain microvasculature has specific properties to strictly control permeability to plasma solutes and inflammatory cells. Contrariwise, in other organs, such as the liver or bone marrow, sinusoidal vessels are fenestrated and allow dynamic permeability between blood cells and proteins.7 Postcapillary venules express a complex system of adhesive proteins, chemokines, and specialized cell-to-cell junctions that regulate the passage of immune cells to the areas of infection. The organ-specific endothelial phenotype is induced and maintained by a continuous cross talk with the surrounding tissues, although we still know little about the different growth and differentiation factors implicated.2,7,10 On the other side, we know more on the signals that induce arteriovenous and lymphatic differentiation of endothelial cells. In this brief review, we shortly discuss the most important signaling pathways and downstream transcription factors responsible for arteriovenous differentiation of endothelial cells. The resulting picture shows a complex hierarchical network of different types of signals (Figure [B]) and underlines the plasticity of endothelial cells that retain, to some extent, the capacity to switch to an arterial or venous phenotype depending on the stimuli to which they are exposed.

Arterial Differentiation

Upstream Regulators

As mentioned above, arterial and venous specification is genetically determined early in the course of development.
This complex developmental program requires the correct spatial and temporal expression of many genes orchestrated by a relatively large set of transcription factors. The micro-environment and the hemodynamic forces further shape the vasculature to adapt the blood flow to the requirements of the surrounding tissues.

Endothelial progenitors can be detected early in embryo development and express several endothelial-specific markers which are present, in general, in all type of endothelium, including vascular endothelial-cadherin (VE-cadherin), platelet endothelial cell adhesion molecule (PECAM), vascular endothelial growth factor receptor (VEGFR-2), endothelial-specific receptor tyrosine kinase (Tie-2), and others. The complex process of endothelial differentiation is not directed by a single transcription factor but occurs through the coordinated action of members of different families such as the E-twenty six (ETS), GATA, Kruppel-like factor (KIF), homeobox, forkhead (FOX), and others. ETS transcription factors play an essential role in endothelial cell specification. To date, all the identified endothelial promoter and enhancer regions contain ETS-binding motifs. Among the 30 members of the mammalian ETS family, 19 are expressed in human endothelial cells and 12 different ETS factors are expressed in zebrafish endothelial and hematopoietic progenitors.

Furthermore, De Val et al identified a transcriptional code consisting of FOX and Ets factors, which direct endothelial gene expression through the combinatorial activation of a composite cis-acting element. In more details, the authors discovered a highly conserved 44 bp element in Mef2c enhancer consisting of 2 Ets-binding sites, one of which flanked by a noncanonical FOX-binding site. This module, once bound by Ets2 and FoxC transcription factors, is able to drive endothelial-specific gene expression during mouse, Xenopus, and zebrafish development. Most importantly, they also found that most endothelial-specific enhancers are characterized by the presence of this FOX:ETS motif, suggesting a widespread role in driving the expression of vascular specification genes.

Overall, these data introduce the concept that 2 broadly expressed transcription factor families may induce tissue-specific differentiation by combinatorial activity on a single composite cis-acting element.

A further step of differentiation of endothelial cells is the acquisition of specific arterial and venous properties. The hierarchical organization of the signaling pathways that participate in arterial determination is synthetically reported in Figure B.

Shh is a member of the Hedgehog family that plays a general role in embryo development and is an early trigger of arterial differentiation in the fish. Shh acts as a secreted molecule through the binding to the transmembrane receptor patched (Ptc) and the G protein–coupled receptor smoothened (Smo). In zebrafish, Shh is secreted by the notochord at the midline of the developing embryo and induces the arterial fate of endothelial progenitors. Shh acts indirectly by inducing VEGF in the adjacent somites that, in turn, activates Notch signaling to promote the arterial program. However, recent findings show that in zebrafish Shh signaling can induce arterial differentiation also directly, via calcitonin receptor-like receptor, and independently from VEGF signaling.

Although demonstration of the role of Shh signaling in arterial specification is rather strong in the fish, it remains unclear in mammals. Shh mutant mice do not exhibit severe vascular defects, and it is, therefore, possible that murine Shh signaling is dispensable for arterialvenous specification.

VEGF is an important inducer of arteriogenesis. Several reports showed that there is an antagonistic cross talk between VEGF-activated intracellular signaling pathways. More specifically, the phosphoinositide 3-kinase (PI3K) and the
extracellular signal–regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathways have opposite effects in vascular specification.\textsuperscript{21–24} It was found that a strong stimulation of the ERK pathway directs endothelial differentiation to the arterial phenotype while activation of PI3K/AKT/Akt pathway inhibits arteriogenesis in favor of venous differentiation. Moreover, Deng et al.\textsuperscript{21} uncovered a central role for RAF1, a kinase acting upstream of ERK, in the induction of arterial-specific genes such as \textit{Ephb2}, \textit{Dll4}, \textit{Notch4}, \textit{Nrp1}, and the downstream Notch targets \textit{Hey2}, \textit{Hey1}, and \textit{Hes1}.

These data increase the complexity of the system introducing the concept that the same ligand (VEGF) and receptor (VEGFR-2) may induce 2 different differentiation pathways depending on the type and intensity of the activation signal.

Consistent with these findings, neuropilin-1 (Nrp1), a coreceptor that facilitates VEGF signaling in concert with VEGFR-2, is expressed in arterial endothelial cells specifically.\textsuperscript{25–28} and \textit{Nrp1} mutant mouse embryos show impaired arterial differentiation, independent of blood flow patterns.\textsuperscript{29}

Moreover, it has been recently demonstrated that Nrp1 plays an important role in developmental and adult arteriogenesis.\textsuperscript{30} In more details, with a knockin mutation (that ablates the Nrp1 cytoplasmic tail), the authors demonstrate that the cytoplasmic tail of Nrp1 mediates the interaction between Nrp1/VEGFR-2 and synectin, resulting in a delayed trafficking of endocytosed VEGFR-2. This leads to an increased dephosphorylation of VEGFR-2 at tyrosine Y1175, a residue involved in activating ERK signaling. These findings establish Nrp1 as a specific regulator of VEGF-A–induced VEGFR-2 trafficking and ERK signaling, both crucial for arterial morphogenesis.

VEGF induces arteriogenesis through activation of the Notch pathway, which has a central role in vascular development. From one side, Notch signaling reduces vascular sprouting and branching but on the other is a key inducer of the arterial phenotype in endothelial cells both in the fish and the mouse.

\textbf{Downstream Effectors}

Besides VEGF, other stimuli converge to the Notch pathway in inducing arterial differentiation of endothelial cells. We have recently reported that a member of the \textit{Y-box subgroup F (SoxF)} of transcription factors is selectively activated in arteries but not in veins and is able to bind to the \textit{Dll4} promoter activating Notch signaling. In absence of \textit{Sox17}, endothelial cells do not express arterial markers and are unable to recruit pericytes and smooth muscle cells correctly.

These observations are in agreement with Sacilotto et al.\textsuperscript{31} who showed that the arterial-specific upregulation of \textit{Dll4} is because of a combinatorial binding of recombination signal binding protein for immunoglobulin kappa \textit{J} region (RBPJ)/Notch intracellular domain (NICD) and the SoxF transcription factors to the gene enhancer located in \textit{Dll4} intron 3.\textsuperscript{31}

Importantly, a multistage, genome-wide association study that included a few thousand patients with intracranial aneurysm identified a susceptibility locus that contains the \textit{Sox17} gene.\textsuperscript{32–34}

The intracranial aneurysms predominated at arterial branch points and sites of shear stress. It is tempting to speculate that altered Sox17 expression can affect arterial differentiation and increase the vascular fragility to hemodynamic stress.

During embryo development, gain-of-function mutation of \textit{β}-catenin, the downstream signaling partner of canonical Wnt signaling, upregulated \textit{Dll4} by direct interaction with the promoter. This induced arterial markers in veins suggesting a role for this pathway in arterial differentiation of endothelial cells.\textsuperscript{35} Furthermore, another group found that \textit{β}-catenin can form an activation complex with NICD and RBPJ, the transcriptional effectors of Notch, and synergize with this pathway in induction of the arterial fate of the vessels.\textsuperscript{36} Other evidence in the literature suggests also that in confluent endothelial cells the stimulation of Tie-2 receptor with its ligand angiopoietin1 (Ang1) results in a \textit{β}-catenin–dependent upregulation of \textit{Dll4} and Notch signaling activation,\textsuperscript{37} further substantiating the complexity of the interactions between Notch and Wnt signaling pathways.

Canonical Wnt signaling upregulates \textit{Sox17},\textsuperscript{38} and this transcription factor may mediate the arterial differentiation of the vasculature downstream of canonical Wnt signaling. Importantly, \textit{β}-catenin signaling is detectable during the early phases of embryo development only, whereas it is strongly reduced postnatally. Sox17 is induced in the embryo by \textit{β}-catenin but remains high in the arteries of the adult mouse even in absence of Wnt signaling, suggesting a role of this transcription factor in the maintenance of arterial properties at postnatal stages.\textsuperscript{39}

Members of the Fox transcription factor family also play an important role in the arterial cell fate during embryonic development. Mice deficient in either \textit{Foxc1} or \textit{Foxc2} present an abnormal vasculature that lacks arteries but displays normal venous marker expression.\textsuperscript{39} Foxc1 and Foxc2 can act downstream of VEGF to activate directly the expression of \textit{Dll4} and \textit{Hey2}.\textsuperscript{39,40}

An arterial-specific and VEGF-dependent enhancer of \textit{Dll4} has been recently described.\textsuperscript{41} The analysis of this enhancer defined a minimal region that can drive arterial expression. In contrast with the previous observations discussed above, these authors could not find \textit{Foxc1}/2, \textit{β}-catenin–dependent regulation of \textit{Dll4}. They also did not find active Wnt/\textit{β}-catenin signaling in the early arteries, and in the absence of \textit{β}-catenin in the endothelium, \textit{Dll4} mRNA levels in the dorsal aorta are not modified. Furthermore, although the \textit{Dll4} enhancer contains binding site for RBPJ, Notch signaling was not required for the initial arterial expression of \textit{Dll4}. Differences in the stage of embryo development analyzed may explain these discrepancies. Similarly, the lack of detection of \textit{β}-catenin signaling in the embryo vasculature in contrast with what has been observed by others\textsuperscript{35,42} may be because of the sensitivity of the detection assay used. Further work should be done comparing the different systems and stages of vascular development to clarify these different results.

\textbf{Venous Specification}

We still know little about how veins are specified. Evidence of a direct transcriptional induction of venous differentiation came from the identification of the chicken ovalbumin upstream promoter transcription factor II (COUP-TFII, also
known as nuclear receptor subfamily 2, group F, member 2, NR2F2). COUP-TFII has been reported to be expressed only in venous and not arterial endothelial cells. More recently, it was found that lymphatics express COUP-TFII as well and that COUP-TFII heterodimerization with Prox1 trancription factor is essential for lymphatic specification.8,46-47

You et al elegantly demonstrated that COUP-TFII plays a cell autonomous role in venous specification. Venous endothelial cells lacking COUP-TFII display a partial reduction of venous markers but a strong upregulation of several arterial markers. Conversely, endothelial-specific COUP-TFII overexpression leads to a lethal vascular phenotype and hampers the expression of several arterial markers with the simultaneous acquisition of the venous marker ephrin receptor B4 (EphB4) by arteries.45

As discussed above, plasticity is not an uncommon feature of endothelial cells, and alterations in genes and pathways involved in arterial or venous specification often lead to the acquisition of opposite fate characteristics.16,45,48

Several laboratories, using different approaches, reported that the mechanism of action of COUP-TFII is to exert an inhibitory activity on Notch signaling, thus promoting venous differentiation by repressing arterial specification.49 On the other way around, Notch activation was found to repress COUP-TFII levels in different systems.50,51

Despite its important role for venous (and lymphatic) specification, the molecular determinants driving the expression pattern of COUP-TFII have been elusive until recently. The mammalian SWITCH/sucrose nonfermentable (SWI/SNF)-like brahma-related gene 1 (BRG1), encoding an ATPase involved in chromatin remodeling, was found to promote the expression of COUP-TFII, and its inactivation resulted in the induction of arterial markers in veins.52

In the fish, COUP-TFII has been recently reported to be a target of Sox7 and 18.51 This, however, is in conflict with what was independently reported, whereby the knockdown of Sox7 and Sox18 would reduce arterial differentiation.53-55 Finally, it is worth pointing out that COUP-TFII and Sox18 have also been involved in the upregulation of Prox1 in a subset of venous endothelial cells that will further differentiate to lymphatics.8,56,57

Origins of Arterial and Venous Progenitors
Recent articles shed new light on the anatomic origin of arterial and venous endothelial progenitors. The formation of the big vessels, such as the dorsal aorta and cardinal vein, has been studied in different model systems as chick, zebrafish, and mice. Although it is agreed that the dorsal aorta forms before the cardinal vein by the coalescence of endothelial progenitors,56 the origin of the cardinal vein was elucidated only recently. In zebrafish, 2 distinct populations of Ent2-positive endothelial cell progenitors arise in the lateral plate mesoderm. The medial one arises at 4-somite stage, whereas the second one appears later from 10-somite stage onwards and is located laterally. Using elegant photoconversion experiments, Kohli et al demonstrated that the most medially located progenitors migrate toward the midline before the lateral populations. After this migratory step, the 2 populations of endothelial progenitors do not mix up. On the contrary, the medial progenitors migrate dorsally in a VEGF- and Shh-dependent manner. The signals from these morphogens dictate the arterial fate of medial progenitors that locate closer to the morphogen source and will form the dorsal aorta. Conversely, the lateral progenitors will migrate ventrally to form the posterior cardinal vein.59

Unexpectedly, studies on mice have revealed also an arterial origin of venous endothelium. It was found that the dorsal aorta contains a small population of endothelial cells that express COUP-TFII and other venous markers. These venous progenitors relocate from the dorsal aorta to the cardinal vein thanks to the repulsive signals mediated by the EphrinB2/EphB4 interaction that dictate the segregation of arterial from venous endothelial cells.60

Overall, these observations underline the heterogeneity of endothelial cells even on the same vessel and suggest that at least part of venous endothelial cells in the cardinal vein may have an arterial ancestry.

Concluding Remarks
The data summarized in this brief review underline the progress made in the field of vascular differentiation. The identification of arterial and venous markers helped the field tremendously in following the fate of the endothelium and the activity of the specific arterial and venous transcription factors.

However, several questions remain open. For instance, although the mechanisms of induction of arterial or venous specification of endothelial cells have been analyzed in detail, much less is known on the signals that maintain arterio/venous identity of the cells. This is not a trivial point because maintenance of correct arterio/venous phenotype is important to prevent the development of vascular shunts and malformations and to induce a correct development of new arteries in case of tissue ischemia.

Another important aspect is the definition of how postnatal hemodynamic factors are able to modify the functions of arteries and veins. Transcription factors including KLF2 and 4 play an important role on morphological reshaping of blood vessels and control basic endothelial functions. Moreover, recent articles suggest an important role for KLF4 in the maintenance of vascular health and its deficiency in aggravating vascular disease. Deletion of KLF4 in ECs and hematopoietic cells affects injury-induced neointimal formation by repressing arterial inflammation.66

KLFs have potent effects on a broad range of vascular processes that contribute to atherogenesis, and all these experimental observations may lead to new clinical applications considering KLFs as therapeutic targets.67

However, how endothelial cells adapt to flow changes in terms of maintenance of expression of specific arterial and venous properties remains to be defined in more detail both in healthy or pathological conditions.

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**Significance**

There are morphological and physiological distinction between arteries and veins, and although some molecular differences are, at least in part, genetically predetermined, the maintenance of arterial and venous identity is susceptible to some degree of plasticity. Endothelial differentiation requires the orchestrated activity of many transcription factors and the activation of several signaling pathways to create a functional vascular network of arteries, veins, and lymphatics. Although we have made significant progress toward the identification of the molecular basis of arteriovenous determination, much less is known on the signals that maintain the phenotype. Alteration of the correct specification of endothelial cells may have pathological consequences such as atherosclerosis, vessel fragility, arteriovenous malformations, aneurysms, and disturbance of blood flow. Furthermore, in proliferative diseases, such as cancer, the disruption of the normal architecture of the vascular network may cause major problems in tissue perfusion and, eventually, a disturbed access of chemotherapeutics.
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