Neural guidance molecules, tip cells, and mechanical factors in vascular development

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The vascular system is generated and maintained by reactions of blood vessels to stimuli of several types. The basic outline of the vascular system is determined during development by genetic programming, guided by the unique temporal and spatial patterns of structural and molecular features available in the embryo. With establishment of blood flow, control of vascular development is increasingly taken over by feedback signals derived from vascular function, including blood flow and pressure, in addition to those derived from the metabolic state of the tissue. Mechanical and molecular signals also govern the post-natal structural adaptation of vascular beds in response to functional requirements, both during normal, physiological conditions (growth, exercise) and during pathophysiological conditions including ischaemic diseases and tumour growth. The orderly structure of vascular beds emerges as each vessel segment reacts to the local conditions and stimuli that it experiences, according to a common set of genetically determined responses. In this process of angioadaptation, the properties and architecture of vascular beds are determined by the continuous interplay between vascular and cellular reactions to haemodynamic and molecular signals and the functional implications of these reactions, constituting a complex feedback system. Here, studies on vascular development and adaptation in response to haemodynamic and molecular factors are integrated, with emphasis on arterial-venous network development and structural adaptation of vessels.

KEYWORDS
Angiogenesis; Arteriogenesis; Haemodynamics; Shear stress; Wall stress; Tip cells; neural guidance molecules; Mechanosensing; Vascular development; Embryo

1. Embryonic development

The formation of a properly branched vascular system is critical for embryo development and survival. Formation of the vascular system involves vasculogenesis, the in situ differentiation of angioblasts and endothelial cells from mesenchyme,1,2 expansion of the primitive vascular system through sprouting and intussusception angiogenesis,3 and the subsequent differentiation of capillaries into branched arteries and veins.4 Evidence is emerging showing that branching morphogenesis and establishment of vascular identity, depending on the stage of development and the vascular bed investigated, can be regulated by two distinct mechanisms: (i) genetic hardwiring of vessel positioning and identity, (ii) flow-controlled vascular patterning and maintenance of vessel identity.5

2. Vessel positioning and arterial-venous identity

2.1 Formation of a primary vascular system in embryonic development

The genetic hardwiring of vascular development involves the expression of neural guidance genes in the vascular system.6 Neurogenesis and angiogenesis share common ligands and receptors. It is now well established that the vascular system has co-opted control mechanisms from the neural system to mediate vascular guidance events and establish vessel identity (for extensive review, see Carmeliet and Tessier-Lavigne6 and Eichmann et al.7). In the neural system, growing axons are guided by axonal growth cones. The functional equivalent in the vascular system is the endothelial tip cell.8 Endothelial tip cells have numerous filopodia that extend into the extracellular matrix (ECM). They express receptors including vascular endothelial growth factor (VEGF)-R28 and UNC5B,9 which allow them to sense gradients of guidance cues provided by their local environment. Similar to the
nervous system, the guidance cues in the vascular system can also be attractive or repulsive. The balance between attractant and repulsive cues determines in which direction the tip cell guides the angiogenic sprout through the ECM and establishes the final position of the vessel sprout.

At present, VEGF is the most prominent attractor. The magnitude of attraction, short or long range, varies between the VEGF isoforms and is related to the extracellular-matrix-binding capacity and affinity for the VEGF co-receptor and guidance molecule neuropilin-1. The diffusible form of VEGF seems to act as a short-range attractor, whereas the extracellular-matrix-bound VEGF isoforms act as long-range attractors. Blockade of VEGF-R2 receptors using neutralizing antibodies, or scavenging VEGF using soluble VEGF-R1, induces filopodia retraction and loss of tip cell number. It is postulated that the continuous presence of a VEGF gradient is necessary to maintain filopodia extensions, allowing guidance of the tip cell towards the source of VEGF, and appropriate branching (Figure 1).

In both mouse and zebrafish, the receptor UNC5B can mediate repulsive guidance events controlling vessel branching morphogenesis. Activation of the endothelial tip cell UNC5B receptor with the ligand netrin-1 causes strong filopodia retraction, and loss of tip cell number. In zebrafish, loss of UNC5B or netrin-1 function causes intersomitic vessels to deviate from their normal stereo-typed path and branch into the horizontal myoseptum. Interestingly, loss of function studies in mouse and zebrafish indicate that the plexinD1–semaphorin3E signalling pathway may act in a similar way, albeit at different positions in the growing vascular tree. It is therefore postulated that the developing embryo has several tissue-specific guidance checkpoints that control local branching morphogenesis. At present, the full repertoire of guidance genes is not known.

Recently, a series of studies addressed the molecular mechanism controlling differentiation of endothelial cells into tip cells. They all showed that endothelial expression of Delta-like 4 (Dll4), a ligand of the notch signalling pathway is crucial for negatively regulating tip cell identity (Table 1). In mouse, loss of just a single allele of Dll4 resulted in a massive increase in tip cell number. Similar observations were made in Dll4 morphant zebrafish embryos. The general concept that arises from these studies is that endothelial cells in response to a VEGF gradient can differentiate into a tip cell. This tip cell now starts to express Dll4 on its cell membrane. Dll4 subsequently activates Notch-1 receptors on the adjacent stalk cell. The activation of Notch-1 suppresses tip cell differentiation in these stalk cells. As a result, only the cell that initially differentiated into a tip cell will stay tip cell, whereas the surrounding endothelial cells remain undifferentiated or stalk cells. The principle in which a differentiating cell represses the differentiation of adjacent cells is called lateral inhibition, a growth principle well established in the developing nervous system. The physiological implication of such molecular cross-talk mechanism between endothelial

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**Table 1** Molecular markers of tip cell identity and regulators of tip cell behaviour and differentiation

<table>
<thead>
<tr>
<th>Marker</th>
<th>Function</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-R2</td>
<td>Tip cell attraction</td>
<td>Receptor</td>
</tr>
<tr>
<td>UNC5B</td>
<td>Tip cell repulsion</td>
<td>Receptor</td>
</tr>
<tr>
<td>PlexinD1</td>
<td>Tip cell repulsion</td>
<td>Receptor</td>
</tr>
<tr>
<td>Neuropilin-1</td>
<td>Tip cell attraction</td>
<td>Receptor</td>
</tr>
<tr>
<td>Dll4</td>
<td>Tip cell differentiation</td>
<td>Ligand</td>
</tr>
<tr>
<td>Notch1</td>
<td>Tip cell differentiation</td>
<td>Receptor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Tip cell attraction</td>
<td>Ligand</td>
</tr>
<tr>
<td>Sema3E</td>
<td>Tip cell repulsion</td>
<td>Ligand</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>Tip cell marker</td>
<td>Ligand</td>
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</table>
cells is that only one sprout will form and grow towards the gradient of VEGF, hence, a sophisticated molecular pathway to control the efficiency of sprouting angiogenesis.

2.2 Determination of vessel identity

After the formation of the initial vascular plexus through vasculogenesis and angiogenesis, vessels have to differentiate into arteries and veins in order to allow proper perfusion. Arteries and veins in adult networks differ considerably in morphology and function. Evidence is emerging showing that these differences already arise during embryogenesis and involve neural guidance genes expressed in the vascular system. However, it is not clear how the molecular markers for arterial-venous identity are regulated, and what the functional and structural consequences are upon activation (Table 2). At present, it is debated whether arterial-venous differentiation is genetically predetermined or plastic and regulated by epigenetic cues including haemodynamics and hypoxia. Several signalling molecules have been discovered, which differentially label arterial or venous endothelial cells from early developmental stages onward, prior to the onset of circulation. Many of these molecules are also expressed in the nervous system, where they regulate cell fate decisions and guide migration of neuronal precursors as well as of developing axons.

Arterial EC in chick, mouse, and zebrafish selectively express ephrin-B2, neuropilin-1 (NRP-1), and members of the notch pathway, including notch3, DLL4, and gridlock. Other molecules are specifically expressed in the venous system, most notably EphB4, the receptor for arterial ephrin-B2, neuropilin-2 (NRP-2), and Coup-TFII. Mutations of the ephrinB2 tyrosine kinase and its receptor, Hey1/2 (gridlock), neuropilin-1, and Coup-TFII in arterial and venous endothelial cells are upon activation (Table 2). At present, it is debated whether arterial-venous differentiation is genetically predetermined or plastic and regulated by epigenetic cues including haemodynamics and hypoxia. Several signalling molecules have been discovered, which differentially label arterial or venous endothelial cells from early developmental stages onward, prior to the onset of circulation. Many of these molecules are also expressed in the nervous system, where they regulate cell fate decisions and guide migration of neuronal precursors as well as of developing axons.

Table 2  Molecular markers of arterial and venous identity in endothelium

<table>
<thead>
<tr>
<th>Ephrin-B2</th>
<th>Arterial</th>
<th>Ligand</th>
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<tbody>
<tr>
<td>Neuruplin-1</td>
<td>Arterial</td>
<td>Receptor</td>
</tr>
<tr>
<td>UNC5B</td>
<td>Arterial</td>
<td>Receptor</td>
</tr>
<tr>
<td>Notch1</td>
<td>Arterial</td>
<td>Receptor</td>
</tr>
<tr>
<td>Notch3</td>
<td>Arterial</td>
<td>Receptor</td>
</tr>
<tr>
<td>Notch4</td>
<td>Arterial</td>
<td>Receptor</td>
</tr>
<tr>
<td>Delta-like 4</td>
<td>Arterial</td>
<td>Ligand</td>
</tr>
<tr>
<td>Jagged-1</td>
<td>Arterial</td>
<td>Ligand</td>
</tr>
<tr>
<td>Jagged-2</td>
<td>Arterial</td>
<td>Ligand</td>
</tr>
<tr>
<td>Gja5</td>
<td>Arterial</td>
<td>Gap junction</td>
</tr>
<tr>
<td>Hes1/2</td>
<td>Arterial</td>
<td>Transcriptional regulator</td>
</tr>
<tr>
<td>Hey1/2 (gridlock)</td>
<td>Arterial</td>
<td>Transcriptional regulator</td>
</tr>
<tr>
<td>EphB4</td>
<td>Venous</td>
<td>Receptor</td>
</tr>
<tr>
<td>Neuruplin-2</td>
<td>Venous</td>
<td>Receptor</td>
</tr>
<tr>
<td>Coup-TFII</td>
<td>Venous</td>
<td>Receptor</td>
</tr>
</tbody>
</table>

During normal development, expression of Coup-TFII in venous endothelium suppresses neuropilin-1 and inhibits Notch signalling and the expression of artery specific genes. Without neuropilin-1 and Notch signalling, venous markers, such as EphB4, are expressed and venous identity maintained. Ablation of Coup-TFII in endothelial cells enables veins to acquire arterial characteristics including expression of ephrinB2, neuropilin-1, and Notch signalling molecules. However, it was noted that the acquisition of the arterial identity in veins of Coup-TFII mutant mice was not sufficient to convert mutant veins fully into arteries suggesting additional control mechanisms including cells in the vessel wall. Chick chimaera studies and in vitro endothelial-smooth muscle spheroid cell culture experiments indeed indicate a potential role for vascular smooth muscle cells in molecular instruction of endothelial cells with an arterial identity. In the chimaera study, the ability for the vessel wall to instruct endothelial cells was dependent on both age and anatomical location (aorta vs. jugular vein) of the host vessel. Until day 7 of embryo development, endothelial cells derived from grafted aortas colonized both arteries and veins, whereas from embryonic day 11 onwards, the endothelial cells mainly colonized arteries. This effect was lost when the cells were isolated from the vessel wall before grafting, suggesting a critical role for the E11 arterial vessel wall. Interestingly, after grafting during the subsequent migration process, the migrating endothelial cells are not in close contact anymore with the vascular smooth muscle cells from the grafted vessel wall. This indicates that in this setting the vessel wall may have given a permanent ‘arterial identity’ instruction to the migrating endothelium that allows integration in arteries only. Alternatively, such cues may also come from the perivascular nerves innervating the grafted artery as has been demonstrated for sensory nerves in the developing mouse skin vasculature. However, not all arteries become innervated by autonomic nerves, and most arteries differentiate before development of nervous innervation.

Taken together, these data indicate that, before the onset of heart beat, genetic hardwiring of arterial-venous differentiation is essential to allow proper vascular development. Genetic hardwiring of arterial-venous identity is essential for the proper development of the in- and outflow tract of the heart, and the establishment of the aorta and cardinal vein which will allow delivery of cardiac output to the developing organs once the heart starts beating.

2.3 Flow-controlled vascular patterning and maintenance of vessel identity

After the start of heartbeat and generation of cardiac output, the developing vascular system shows a high degree of endothelial cell plasticity. Using a combination of in vivo molecular imaging and vascular grafting studies, we showed that endothelial cells are not genetically committed to their initial phenotype but plastic and adapt their identity continuously depending on local epigenetic cues including haemodynamics. In addition, we observed evidence showing that the global patterning of arteries and veins in peripheral organs such as the yolk sac depends on haemodynamics and local tissue compliance.
We observed several novel morphological events essential for arterial-venous differentiation and branching during embryogenesis. These included (i) flow-driven fusion of small calibre vessels into large tubes, a process accounting for the formation of the large vitelline arteries and veins, (ii) selective disconnection of endothelial cells from the arterial system and reuse of these endothelial cells to fashion growth of the venous system, and (iii) shear stress driven guidance of lumenized vessel sprouts. We could show that in the absence of cardiac output and active flow perfusion of the yolk sac, despite the expression of arterial markers such as ephrinB2, AV differentiation and patterning would not take place. In addition, perfusing an embryonic artery with venous blood transformed this artery genetically and morphologically into a vein. Arterial markers such as neuropilin-1 and ephrinB2 were downregulated, whereas venous markers including neuropilin-2 were upregulated.

From these observations, we concluded that haemodynamics control arterial-venous differentiation and are essential for the maintenance of AV vessel identity. The outstanding questions are: which physical signals exerted by flowing blood account for AV differentiation in the embryo, is it restricted to embryogenesis or does it also play a role in post-natal development and pathophysiological adaptations.

Arterial-venous vessels plasticity is also observed in the developing trunk vasculature of the zebrafish embryo. In early stage embryos, primary vessel sprouts grow between the somites to form the intersomitic vessel. This vessel subsequently branches laterally into the caudal and cranial regions to from the dorsal-lateral anastomosing vessel. At this stage, the intersomitic vessel is connected to the aorta and is considered arterial, carrying arterial flow. During subsequent stage, the so-called secondary sprouts originate from the cardinal vein form. These venous sprouts grow parallel and in close proximity to the intersomitic arterial vessel. Occasionally, these venous sprouts fuse with the intersomitic arterial segment, which progressively loses its connection with the aorta as the intersegmental vessel turns into a vein. As a consequence of the vein fusing with the arterial segment process, the flow direction in the intersomitic arterial segment is reversed. The exact cellular and molecular mechanism accounting for this process is not known. It is suggested that the fusion of veins with arteries is the result of a stochastic process and essential to balance local perfusion. Similar to the morphological events observed in the developing chick embryo, plasticity of arterial segments seems to be important to obtain functional perfused networks. To what extent such plasticity events occur in other organs during development remains to be elucidated.

Recent evidence shows that blood flow is also essential for driving vascular remodelling in the mouse embryo yolk-sac. Using Mlc2a–/– mutant mice, which display a selective cardiac contractility defect impeding proper control of cardiac output, it was clearly demonstrated that without adequate flow, yolk sac remodelling does not occur. This could be attributed to the mechanical properties of the early blood, not the presence of circulation angiogenic factors or changes in oxygen-carrying capacity. Using elaborate erythroblast trapping techniques and injection of high molecular weight synthetic sugars, it was demonstrated that the mechanical force, normally imparted by the flow of circulating erythrocytes, is necessary and sufficient to induce vessel remodelling in the yolk sac of early mouse embryo. It remains to be tested whether erythrocyte-mediated changes in viscosity also affect vascular remodelling in other model systems and pathological conditions.

3. Translation of haemodynamic forces: mechanisms of mechanosensing

3.1 General concept of mechanosensing

The next obvious question is which vascular components or elements act as mechanosensor (Figure 2). Vascular cells are subjected to physical forces originating either from tension created by cells themselves or from the local environment including blood flow (shear stress) and pressure (circumferential wall stress). Physical stimuli must be sensed by cells and transmitted through intracellular transduction pathways to the nucleus, resulting in altered gene expression and (patho)physiological vessel adaptation. It is well established that changes in blood flow and pressure can activate specific molecular signalling cascades that are translated into lumen diameter and wall thickness adaptations. However, how cells sense biomechanical stimuli is still under debate. The outstanding question in the field is, does a single or specific mechanoreceptor exist, or is the cell itself through cytoskeleton rearrangements, the concept of tensility, able to sense physical stimuli. Many kinds of molecules, including receptors, ion channels, caveolins, Src, FAK, G proteins, transcriptional factors, and specific shear-responsive elements in the promoter region, associate with sensing mechanical stimulation. It is postulated that mechanical forces can modify the conformation of proteins directly, leading to functional switches (opening of ion channels), or changes in phosphorylation status, resulting in activation of downstream signalling cascades.

3.2 Flow-driven transcriptional activation

In a classical study by Resnick et al., it was shown that shear stress activates gene expression via transcription factors interaction with a common promoter element. They noted that shear stress induced PDGF-B transcription requires an essential 12 bp component containing a core binding sequence that allows binding of transcription factors. This common promoter element was called shear-stress-responsive element (SSRE) which was subsequently identified in other shear-stress-regulated genes including ICAM-1 and TGF-beta. Such SSREs were also identified in eNOS, another factor essential for flow-mediated vasodilation.

The transcription factor Kruppel-like factor 2 (KLF2) is rapidly upregulated by shear stress. A substantial number of in vitro and in vivo studies have shown that KLF2 is important in haemodynamic regulation in response to arterial shear stress, both in the embryo and in the adult, as well as pathophysiological conditions including atherosclerosis. Using a genetic approach in both zebrafish and mice, it was shown that loss of KLF2 results in heart failure. Detailed haemodynamic analysis revealed that this heart failure is caused by a high cardiac output state, in the absence of structural vascular defects, indicating loss of...
Peripheral resistance. Consistent with this hypothesis, in both KLF2a-morphant zebrafish and in KLF2-deficient mice, addition of phenylephrine (a potent alpha-adrenergic vasoconstrictor) could rescue from lethal high-output heart failure. Based on these observations, it is hypothesized that fluid shear forces drive the expression of endothelial KLF2 that regulates smooth muscle tone in the developing embryo.

3.3 Structural components involved in mechanosensing

At the structural level, it is suggested that adhesive receptors that physically link extracellular support scaffolds (the ECM) to the internal cytoskeleton may function as mechanoreceptors (Table 3). The endothelial cytoskeleton is a candidate for shear stress transduction because of its fast and dramatic response to shear.

At the protein level, an elaborate work by the group of Ingber and others has shown that integrins and adhesion receptors (including PECAM-1, E-selectin, cadherins) can link the internal cytoskeleton with the ECM and provide mechanical coupling across the cell surface. Genetic deletion of fibronectin, alpha5beta1 integrin, and components of the ECM including thrombospondin 1,2 results in vascular remodelling and angiogenesis defects. Detailed analysis indicated that in these models, vasculogenesis occurred normally but subsequent steps in network formation including lumenization of vessels and establishment of a functionally perfused vasculature were disturbed.

Signals from the ECM must be transduced to the signalling machinery inside the cell. Integrins accomplish this by recruiting multimolecular complexes of cytoskeletal and signalling molecules at focal adhesion sites. The activation of non-receptor tyrosine kinase focal adhesion kinase (FAK) depends on this integrin clustering. Cells exposed to cyclic stretch or shear stress show enhanced FAK tyrosine phosphorylation indicating that FAK activity links to mechanical stimuli. Endothelial cells derived from mutant FAK−/− mice show an inability to organize into vascular networks. Targeted disruption of the fak gene in mice leads to severely impaired blood vessel development and is lethal around embryonic day 8.5. The in vitro results suggest that this is not due to deficient endothelial differentiation, but flow-driven tubulogenesis.

The endothelial cell–cell adhesion site is also implicated as a potential site for mechanosensing. VE-cadherin is a major adhesive protein for the adherens junction and expressed specifically in vascular endothelial cells. It interacts with the cytoskeleton via anchoring molecules...
including beta-catenin. In mice, genetic deletion of VE-cadherin results in severe vascular remodelling defects and abolishes VEGFR-2 signalling. Short intervals of shear stress stimulate the formation of the VEGFR-2, VE-cadherin, and beta-catenin complex in vascular endothelial cells. Exposure of arterial endothelial cells in vitro to laminar shear stress results in induction of VEGFR-2 and promotes the formation of a complex containing VEGFR-2, VE-cadherin, and beta-catenin. This intact complex appears essential for activating the downstream SSRE-dependent gene transcription. In promoter-reporter assays containing the PDGF-A SSRE-driving luciferase, genetic ablation of VE-cadherin resulted in abolishment of shear-induced PDGF-A/SSRE luciferase activity. In addition, phosphorylation of Akt1 and p38 did not occur in mutant VE-cadherin–/– endothelial cells exposed to laminar shear. Based on these observations, it was concluded that the formation of the VE-cadherin–beta-catenin–VEGFR2 complex is important in shear sensing and that the adherens junctions can serve as a shear stress transducer.

Adhesion molecules play a pivotal role in mediating mechanosensing upstream of the integrins. It could be demonstrated that the PECAM-1–VEGFR2–VE-cadherin complex acts as a mechanosensory complex in vitro. The PI(3)kinase is essential for shear-driven activation of alpha-5, beta-3 integrin involving c-Src. PECAM-1 is required for Src activation while VE-cadherin is required for transmitting this signal to PI(3)kinase. Neither VE-cadherin–/– nor PECAM-1–/– cells showed alignment of actin filaments in response to flow. Interestingly, in this setting, the involvement of VE-cadherin in shear-stress-dependent signalling appeared independent of cell–cell signalling. In response to flow, VEGFR2 is activated via an Src-dependent pathway, which was absent in PECAM-1 and VE-cadherin mutant cells indicating that VEGFR2 activation is downstream. Detailed analysis showed that PI(3)kinase activation involved direct binding to VEGFR2 and associated with the presence of VE-cadherin and beta-catenin suggesting that VE-cadherin acts as an adaptor protein. Using artificial COS-7 cells transfected with plasmids for PECAM-1, VE-cadherin, and VEGFR2, it could be demonstrated that only cells transfected with the complex of these three receptors were able to transduce shear-stress-dependent changes in cell alignment. In PECAM-1 mutant mice, this mechano-signalling complex was associated with susceptibility for atherosclerosis. Interestingly, PECAM-1–/– mice are viable and can reproduce indicating that additional mechanosensing pathways must exist. Taken together, these studies suggest that the PECAM-1, VE-cadherin, beta-catenin, and VEGFR2 pathway is critical for mediating shear stress responses in endothelial cells.

Another outstanding question is when do cells become responsive to mechanical factors exerted by blood flow. Several studies using mouse ES cells indicate that the differentiation potential towards cardiovascular cells can be stimulated by shear, indicating that responsiveness to mechanical factors may be already present during early differentiation stages.

Taken together, these studies indicate that many factors are involved in mechanosensing. Given the specific regulation of arterial and venous marker molecules in the respective vascular domains, it remains to be established whether specific mechanosensing mechanisms exist in arteries and veins.

### 4. Implication for vascular remodelling in the adult

#### 4.1 Implications of arterial-venous differentiation in post-natal vascular remodelling

After the initial observations on the involvement of neural guidance genes in embryonic vascular development, current studies also indicate a prominent role in post-natal angiogenesis and arteriogenesis and pathological angiogenesis. EphrinB2 is expressed and upregulated in the arterial vasculature after the initial angiogenic responses in tissue ischaemia. In the cornea assay, addition of ephrinB2 was associated with enhanced vascular growth mainly through venous angiogenesis. More recently, activation of circulating endothelial progenitor cells (EPC) with ephrinB2-Fc chimaeric protein enhanced, in an EphB4-dependent manner, therapeutic neovascularization in the hind limb ischaemia model. In this setting, treatment with ephrinB2-Fc, stimulated adhesion of EPCs to activated endothelium and their arrest at foci of neovascularization. Interestingly, ex vivo treatment with ephrinB2-Fc did not induce EPC specification towards an arterial or venous
phenotype in vitro. Preferential incorporation of treated EPCs in the arterial or venous compartment was not reported. Several attempts have been made to direct specification of circulating progenitors in vitro. Human multipotent adult progenitor cells (hMAPCs) and AC113+ EPCs show distinct AV differentiation responses. When exposed to VEGF165, hMAPCs differentiate in both arterial and venous endothelial cells, whereas human AC133+ cells only adopted a venous phenotype. This difference was attributed to differential expression of Notch1 and 3 receptors in these cell types. Inhibition of Notch signalling attenuated arterial differentiation, whereas stimulation of Notch signalling using DI4 as a ligand stimulated arterial differentiation in hMAPCs. This study provides evidence that it is feasible to instruct progenitor cells with AV identity, which has obvious importance for optimization of revascularization strategies in ischaemic cardiovascular diseases.

During post-natal development, overexpression of EphB4 results in two fundamental effects on vascular organization and function. First, morphogenesis is switched from sprouting angiogenesis towards circumferential vessel growth (outward remodelling of existing vessels), and second, it leads to a reduction in vascular permeability. Similar effects on enhanced outward remodelling were observed in the E4 chick embryo allantois vasculature treated with clustered ephrinB2-Fc. Although dominance of EphB4 reverse or forward signalling may depend on the context, the data indicate that one function of ephrin signalling could be regulation of lumen diameter. Given the observation that ephrinB2 expression, at least in embryos, is regulated by flow, ephrin-Eph signalling may be involved in transduction of haemodynamic signals into lumen-diameter growth.

The interposition of a venous segment into the arterial circulation occurring during coronary bypass surgery results in adaptation of the vascular wall to the new haemodynamic conditions. This process is referred to as ‘arterialization’ of the vein graft. The adaptive response includes thickening of the vascular wall, leading to lumen-diameter reduction, frequently evolving into lumen occlusion and graft failure. It is well established that exposure of the vein segment to arterial flow and pressure triggers these responses. Another clinically relevant example of haemodynamics evoked aberrant vascular remodelling potentially involving ‘vessel identity problems’ is the AV-shunt in renal dialysis patients.

Since developmental biology studies suggest genetic predetermination of arterial and venous identity, failure to genetically instruct the vein graft with a new arterial identity may associate with the observed aberrant vessel wall remodelling. In a rat vein graft model in which the jugular vein was interpositioned into the common carotid artery, expression of EphB4 mRNA and protein were downregulated in endothelial cells and smooth muscle cells of patent grafts, thus consistent with adaptation to their new arterial environment. However, expression of ephrinB2 was not induced in the arterialized graft. Other markers of arterial identity, including dll4, Jagged 1-2, and notch 1, 3, and 4, were also not significantly upregulated. Interestingly, expression of the arterial marker neuropilin-1 and the venous marker neuropilin-2 increased. From these observations, it was concluded that during vein graft adaptation, venous identity is lost, but arterial identity not acquired. The developmental biology studies suggest that VEGF through Notch signalling activates ephrinB2 expression inducing arterial identity. In the graft model, this mechanism did not appear functional. Careful examination of the VEGF expression in the graft model shows upregulation of VEGF in the early phase after implantation and downregulation in later stages after 72 h onwards. This associated with unaltered ephrinB2 expression and a continued downregulation of EphB4. siRNA directed against VEGF in this setting completely abolished EphB4 expression. Interestingly, intima media thickness greatly increased after VEGF siRNA knockdown. From these data, it may be concluded that venous grafts fail to turn on the molecular machinery that regulates arterial identity and that adult vessels may lose the plasticity that is commonly observed in embryonic vessels. The questions that arise from this study are: does genetically reprogramming of veins with arterial identity through forced overexpression of arterial markers including ephrinB2 and notch partners result in patent grafts, and what is the molecular mechanism accounting for loss of plasticity. For clinical practice it will be important to determine which molecular conditions provoke the pathological neo-intima formation. It remains to be tested whether expression of the appropriate arterial identity genes indeed prevents pathological inward remodelling or whether it is sufficient to just target smooth muscle proliferation, for example, through cell-cycle blockade.

4.2 Haemodynamics and post-natal network remodelling

In adult networks, haemodynamic factors play an essential role in adaptation to arterial occlusion in the context of peripheral and cardiac ischaemia. In case of chronic or acute arterial occlusion, pre-existing (collateral) arterial networks may provide a significant compensation for the deficit of a nutrient arterial inflow. These collateral networks are the only arteriolar/arterial conduits connecting the pre-stenotic region with the low-pressure peripheral territories and these arteries are able to increase their lumen diameter efficiently allowing flow bypass around the site of occlusion. This process is termed arteriogenesis and is defined as the positive outward remodelling of pre-existing collateral arteries. It is nature’s most efficient mechanism to compensate for a deficit in arterial inflow upon arterial stenosis or occlusion. At present, it is believed that the contribution of other forms of neovascularization such as angiogenesis or de novo growth of capillaries via circulating endothelial cells distal to the site of stenosis do not significantly solve the problem of tissue hypoperfusion due to the unaltered deficit of physiological arterial inflow. Angiogenic responses appear to be more restricted to the distal parts of the occluded vascular bed driven by the ischaemia in these areas. In terms of collateral artery biology, three important features appear fundamental and interestingly share in part common features with arterial network development during embryogenesis: (i) the dependency on a pre-existing vascular network, (ii) biomechanical forces as the primary molding force, (iii) independence of ischaemia.

Post-natally arteriogenesis depends on the existence of pre-existing collateral arteries. Our current knowledge indicates that local de novo formation of collateral arteries at the site of the occlusion is unlikely to explain the rapid velocity at which collateral flow can be established.
As previously shown, all recruitable arterial networks of collaterals are present before ligation. In several studies, it was first believed that these arterioles are new vessels; however, with the development of new imaging techniques including high resolution angiographies via micro-CT, the opposite has been demonstrated. Just like in the embryological setting, existing vascular networks are being recruited rather than the de novo formation of complete branched vessel structures.

Blood flow and hence biomechanical forces as indispensible factors during arteriogenesis: collateral recruitment and proliferation do not take place without pressure gradients and hence increased shear forces. The latter are the first activatory steps in early phases of collateral artery growth: Besides morphological signs of initial collateral 'activation' due to the opening of endothelial chloride channels controlling cell volume with subsequent endothelial cell swelling, the endothelial cytoskeleton transduces shear stress and thus selectively induces the transcription of several endothelial genes. In detail, the activation of NFκappaB with further binding of its subunits p50 and p65 to the GAGACC sequence is found to be an initial event in collateral arterial activation. SSRE responsive genes are being upregulated in the proliferating collateral wall (MCP-1, ICAM-1, eNOS, PDGF, etc.) and lead to a pro-inflammatory state of the vessel wall.

The further upregulation of cell adhesion molecule such as ICAM-1 (described earlier) initiates the transendothelial migration of circulating monocytes into the site of adaptive arterial proliferation. Upon deficiency of this CAM, arteriogenesis is significantly reduced; furthermore, monocyte deficiency—as observed in osteopetrotic mice—leads to a significant reduction of adaptive proliferation. This concept of circulating non-endothelial cells as initially described by Schaper et al. in 1976 resembles the recent observations on arteriogenesis in zebrafish embryos.

In zebrafish, arteriogenesis can occur in response to occlusion of the aorta. The gridlock mutant zebrafish embryo is characterized by permanent occlusion of the proximal aorta caused by a mutation in the Hey2 transcription factor that is involved in arterial specification. In early development, as a result of this occlusion, the distal aorta does not carry flow. With time, gridlock mutant embryos recover blood flow in the distal aorta via communications with the intestinal vasculature. This arteriogenesis response can be phenocopied by laser-induced proximal aortic occlusion. Pharmacological inhibition of nitric oxide synthesis using L-NAME attenuated collateral formation in gridlock mutants indicating involvement of nitric oxide. Morpholine knockdown of pu.1 to prevent myeloid development also reduced the restoration of distal aorta flow. Analysis of hypoxia markers including HIF-1α revealed that in gridlock mutants collateral flow develops independently of tissue hypoxia or ischaemia. Thus, collateral aortic blood flow in gridlock mutants was dependent on both nitric oxide and myeloid cells. Comparable to adult arteriogenesis in mammals, the presence of myeloid cells accelerates arteriogenesis by matrix degradation to allow vessel expansion, or by local delivery of vasoactive molecules and growth factors (e.g. GM-CSF). Taken together, increased levels of shear stress, followed by a controlled inflammatory milieu and the independence from ischaemia are three unique features in arteriogenesis, which are present in both mammals as well as zebrafish, during embryogenesis, and in the adult. These observations suggest that flow-driven arteriogenesis is highly conserved.

The outstanding question in the arteriogenesis-collateral vessel formation field is how and when the number of pre-existing collaterals is determined. Based on the embry study, it can be hypothesized that both guidance-mediated arterial vascular branching events and haemodynamics may contribute to establishing pre-existing collaterals. The number of pre-existing collaterals would be the result of fine-tuning of haemodynamic responses and genetically provided patterning cues, as well as genetic determinants in arterial wall remodelling including VEGF-Notch signalling. Clinical experience shows differences between patients with regard to collateral vessel development in response to arterial occlusion. Given the profound role of mechanical factors in both embryonic and adult vascular network remodelling, we postulate that genetic differences in mechanosensing regulating both number of pre-existing collaterals (embryo-early post-natal) and the velocity at which collaterals are recruited (adult) upon arterial stenosis account for the clinical differences in collateral development.

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