The quiescent endothelium: signalling pathways regulating organ-specific endothelial normalcy

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Abstract | Endothelial cells are at the interface between circulating blood and tissues. This position confers on them a crucial role in controlling oxygen and nutrient exchange and cellular trafficking between blood and the perfused organs. The endothelium adopts a structure that is specific to the needs and function of each tissue and organ and is subject to tissue-specific signalling input. In adults, endothelial cells are quiescent, meaning that they are not proliferating. Quiescence was considered to be a state in which endothelial cells are not stimulated but are instead slumbering and awaiting activating signals. However, new evidence shows that quiescent endothelium is fully awake, that it constantly receives and initiates functionally important signalling inputs and that this state is actively regulated. Signalling pathways involved in the maintenance of functionally quiescent endothelia are starting to be identified and are a combination of endocrine, autocrine, paracrine and mechanical inputs. The paracrine pathways confer a microenvironment on the endothelial cells that is specific to the perfused organs and tissues. In this Review, we present the current knowledge of organ-specific signalling pathways involved in the maintenance of endothelial quiescence and the pathologies associated with their disruption. Linking organ-specific pathways and human vascular pathologies will pave the way towards the development of innovative preventive strategies and the identification of new therapeutic targets.

The endothelium forms the innermost layer of blood vessels and lymphatic vessels and is best viewed as a multi-functional organ with both systemic and tissue-specific roles. At the whole-organism level, the endothelium regulates oxygen and nutrient supply, immune cell trafficking and inflammation1, haemostasis and coagulation2, vasomotor tone3, blood vessel permeability4 and angiogenesis5. In addition, the endothelium has a number of organ-specific functions including regulation of organ size and function (myocardial hypertrophy6, liver size and function7, pulmonary alveolar repair8 and kidney function9,10). Given this heterogeneity of endothelial cell function, it is not surprising that studies show a remarkable heterogeneity of gene expression profiles in endothelial cells from different organs11. Interestingly, these expression profiles are functionally matched to local tissue needs. Microenvironment stimuli (shear stress, hypoxia and the presence of specific growth factors, cytokines and hormones) and epigenetics define and continuously optimize local characteristics of endothelial cells. Epigenetic signatures that regulate the basal expression of endothelial-specific genes in different organs are specified during embryonic development and conserved during mitotic cycles12. Transcriptome analysis of endothelial cells from different tissues revealed heterogeneous gene expression signatures even after several passages in cell culture, indicating that tissue-specific epigenetic modifications participate in the regulation of organotypic transcriptomic profiles13,14. However, after long-term cell culture, which removes endothelial cells from their in vivo microenvironment, approximately 50% of gene expression patterns are lost15, and major architectural characteristics, such as fenestrae, also disappear16.

To characterize organotypic endothelial specificity as close to in vivo conditions as possible, many groups have utilized microarray or RNA sequencing (RNA-seq) of endothelial cells isolated by flow cytometry without the cell culture step17,18. Single-cell RNA-seq of endothelial cells isolated from adult male mice identified transcriptomic signatures of quiescent arterial, venous, capillary and lymphatic endothelial cells in 11 different tissues19. Interestingly, lymphatic endothelial cells...
from all the tissues cluster together, suggesting that the molecular signature of lymphatic endothelial cells is not tissue-specific. By contrast, arterial and venous endothelial cells from a specific tissue clustered together, showing that vascular endothelial cell heterogeneity comes mainly from tissue specificity rather than arterial, capillary or venous identity. Moreover, capillary endothelial cells that are involved in gas, ion, metabolite and hormone exchange between the blood and tissues have the highest heterogeneity among tissues.

Structural differences in the capillary endothelium were first described in the 1960s with the use of electron microscopy. Three major types of capillaries exist (continuous, fenestrated and discontinuous). The capillary type of an organ is related to its functions. Most organs have barrier-forming, continuous capillaries (lungs, brain, skin and heart) with tightly connected endothelial cells surrounded by a continuous basement membrane. This architecture permits diffusion of water, small solutes and lipid-soluble materials, while precluding the passage of cells or pathogens. By contrast, fenestrated capillaries have intracellular pores (windows) with a diaphragm and are found in renal glomeruli, exocrine glands, endocrine glands and intestinal mucosa. These fenestrae increase permeability to fluids and solutes, but not macromolecules. Sinusoids are fenestrated capillaries with gaps instead of pores between endothelial cells and a thinner basement membrane than in continuous or fenestrated endothelia and are present in the liver, spleen and bone marrow. The gaps found in sinusoids facilitate selective exchange of materials.

Structural differences notwithstanding, normal endothelial cells everywhere are quiescent. This quiescent state is defined by minimal or absent endothelial proliferation and migration, minimal or no vascular leakage across the endothelial barrier and minimal (or fully absent) expression of leukocyte adhesion molecules. Indeed, the half-life of a normal endothelial cell is an estimated 6 years in the heart as measured by ¹⁴C incorporation, and proliferative activity is absent (except in the liver and spleen, where about 1% of endothelial cells proliferate in a quiescent state).

However, this ‘quiescent’ endothelium performs lots of active work, from secretion of paracrine and endocrine factors to active support of barrier maintenance for cell survival. Remarkably, little attention has been paid to what controls this ‘active quiescence’ and what maintains vascular normalcy under physiological conditions. Although much effort has been expended on exploration of signalling pathways underlying endothelial cell activation and proliferation, almost no attention has been given to the signalling events that maintain endothelial normalcy and quiescence. The latest advances in this area are the subject of this Review.

**FGF signalling**

The fibroblast growth factor (FGF) signalling cascade includes a family of 18 ligands, four receptor tyrosine kinases (RTK) (FGFR1–FGFR4) and several accessory molecules such as Klotho proteins and syndecans. After FGFs bind to their high-affinity RTKs, several intracellular pathways are activated, including the phosphoinositide 3-kinase (PI3K)–AKT pathway and mitogen-activated protein kinase (MAPK) pathways mediated by extracellular signal-regulated kinase 1 (ERK1) and ERK2.

The extensive structural overlap and cross-reactivity among FGF ligands and receptors represent a real challenge to identifying the function of FGF signalling in the endothelium. Results from studies in mice with knockout of individual Fgf genes or individual Fgfr genes are hard to interpret because of functional redundancy, whereas attempts to use FGF chemical inhibitors are hampered by the low specificity and cross-reactivity of these compounds. Successful strategies to circumvent the redundancy in the FGF family and investigate FGF signalling include mice with knockout of multiple Fgf genes (Fgfr1−/−Fgfr2−/−) and Fgfr1−/−Fgfr3−/−, the use of soluble FGFR traps that target various FGF family members, and endothelial cell-specific expression of a dominant-negative FGFR1 construct that can inactivate all four FGF receptors. Mice with conditional endothelial cell-specific deletion of Fgfr1 and Fgfr2 are viable, with no vascular developmental defects and no alterations in vascular homeostasis. However, postnatal endothelial cell-specific knockout of Fgfr1 in mice with global knockout of Fgfr3 results in impaired development of blood and lymphatic vessels. A soluble receptor trap strategy was tested with the use of a soluble FGF1 trap (sFGFR1) that binds to a large number of FGFs. In this study, transient FGF inhibition was achieved in vivo in mice via adenovirus-mediated systemic expression of sFGFR1. This FGF inhibition led to an increase in vascular permeability and, eventually, pulmonary and myocardial haemorrhages, demonstrating the necessity for FGF signalling in the maintenance of vascular integrity (Table 1). A particularly interesting finding was the disrupted endothelial cell–cell junctions in large vessels, such as the femoral artery, carotid artery and jugular vein (Table 1). One possible explanation for the disrupted endothelial cell–cell junctions is that the loss of FGF signalling decreases the expression of the phosphatase SHP2 (also known as PTPN11), thereby increasing phosphorylation of the junctional protein VE-cadherin on tyrosine 658, which, in turn, results in loss of the VE-cadherin–β-catenin interaction.

The intracellular kinase SRC can also phosphorylate VE-cadherin, especially in venous endothelial cells. Phosphorylated VE-cadherin is internalized and ubiquitinated in response to inflammatory mediators. However, phosphorylation of VE-cadherin in the absence of inflammatory mediators is not sufficient for induction of vascular permeability.
The FGF signalling cascade also maintains endothelial cell identity. FGF2 stimulation decreases expression of the transforming growth factor-β (TGFβ) receptors (TGFβRs), TGFβ ligands (especially TGFβ2) and the major intracellular TGFβ signal transducer SMAD2 (REFS 26,27). FGF-driven suppression of TGFβ signalling reduces endothelial-to-mesenchymal transition (EndMT)11,12 (TABLE 1), a state in which endothelial cells lose the expression of important endothelial genes and acquire mesenchymal cell-like characteristics, including increased migration and proliferation25. Mechanistically, FGF signalling controls the cellular levels of the let-7 microRNA family26, which targets a number of TGFβ family members. A reduction in let-7 expression results in impaired alignment of endothelial cells under disturbed flow18. In vitro studies have confirmed the relationship between the level of endothelial FGF1 expression and the magnitude and type of shear stress19. In vitro studies have confirmed the relationship between the level of endothelial FGF1 expression and the magnitude and type of shear stress19.

Loss of FGF signalling in endothelial cells in areas of high shear stress has been linked to increased atherosclerotic plaque growth19 (TABLE 2). Atherosclerosis is a progressive disease characterized by gradual intracellular lipid deposition in the vasculature leading to the formation of atherosclerotic plaques20,21. At these sites, endothelial cells acquire a pro-inflammatory phenotype, which predisposes to atherosclerotic plaque development21. Disturbed flow-induced expression of pro-inflammatory genes in endothelial cells affects the magnitude and type of shear stress19. In vitro studies have confirmed the relationship between the level of endothelial FGF1 expression and the magnitude and type of shear stress19.
EndMT\textsuperscript{39}, TGFβ signalling further promotes an inflammatory phenotype in endothelial cells, thereby establishing a feed-forward loop between inflammation and TGFβ pathway activation\textsuperscript{39,42}. In patients with coronary artery disease, a strong correlation exists between disease progression and loss of FGFR1 expression and activation of TGFβ signalling in endothelial cells of the left main coronary artery, with up to 70% of endothelial cells overlying atherosclerotic plaques expressing mesenchymal markers\textsuperscript{39} (TABLE 2). Syndecan 4, a proteoglycan that increases FGF2 signalling, protects against atherosclerotic plaque formation in mice\textsuperscript{35}, highlighting the protective role of endothelial FGF signalling against atherosclerosis.

**VEGF signalling**

VEGF is the most-studied angiogenic growth factor. All members of the VEGF family have important functions in the endothelium. VEGFA and VEGFC are key drivers of angiogenesis and lymphangiogenesis, respectively\textsuperscript{43}. VEGFB is involved in the regulation of endothelial cell metabolism\textsuperscript{44}, whereas the function of VEGFD is less clear\textsuperscript{45}. A closely related growth factor, placental growth factor, is crucial for placental angiogenesis\textsuperscript{46}. All the

### Table 1 | Phenotypes associated with dysfunction of quiescent endothelial cells

| Signalling pathway | Animal model | Age of induction | Phenotype | Refs |
|--------------------|--------------|-----------------|-----------|------|
| FGF                | Soluble Fgfr1 overexpression | Adult | Increased vascular permeability, pulmonary and cardiac haemorrhages, disrupted endothelial cell interaction | 24 |
|                    | Frs2 knockout in endothelial cells | Postnatal day 5 | Induced endothelial-to-mesenchymal transition | 39 |
| VEGF               | Vegfa heterozygous knockout in podocytes | Not inducible | Endotheliosis, glomerular basement membrane thickening, loss of endothelial cell fenestrations, necrotic syndrome | 49 |
|                    | Overexpression of Vegfa in podocytes | Not inducible | Collapsing glomerulopathy (at postnatal day 5) | 49 |
|                    | Adult | Proteinuria, glomerulonegaly, glomerular basement membrane thickening, loss of slit diaphragms, podocyte effacement, no endotheliosis, no loss of endothelial fenestration | 50 |
|                    | Deletion of Vegfa in pancreatic β-cells | Not inducible | Loss of endothelial fenestration | 52 |
|                    | Overexpression of a soluble form of Vegfr1 (decoy receptor) in pancreatic β-cells | 8–12 weeks | Loss of endothelial fenestration | 53 |
|                    | Vegfr2 deletion in endothelial cells | 6–7 weeks | Loss of endothelial fenestration | 54 |
|                    | Vegfa knockout in endothelial cells | Not inducible | Haemorrhages, intestinal perforations, myocardial infarction, endothelial cell apoptosis, 25% lethality in adults | 55 |
|                    | Treatment with a VEGFR2 inhibitor (SU5416) | Adult (rat) | Pruning of pulmonary arterial vasculature, emphysema | 72 |
| VEGF–Notch         | Dll4 heterozygous knockout | Not inducible | Pulmonary haemorrhages | 75 |
|                    | Erk2 knockout in endothelial cells on an Erk1 global knockout background | 8 weeks | Renal failure, endothelial-to-mesenchymal transition, loss of endothelial fenestration, premature death | 10 |
|                    | Ctnnb1 (encoding β-catenin) knockout in endothelial cells | 10–12 weeks | Seizures, brain haemorrhages, death | 96 |
|                    | Fzd4 knockout in endothelial cells | Adult | Increased PV1 expression, decreased claudin 5 expression (in retina and cerebellum) | 98 |
| SHH                | Smo knockout in endothelial cells | Not inducible | Increased blood–brain barrier permeability (at 8 weeks of age) | 101 |
| Angiopoietin       | Angpt2 knockout | Not inducible | Loss of endothelial cell inflammatory response to TNF stimulation | 112 |
|                    | Tie2 heterozygous knockout | Not inducible | Increased sepsis-induced disseminated intravascular coagulation | 117 |
| AKT                | Akt1 knockout in endothelial cells on an Akt2 global knockout background | Adult | Loss of mural cells by decrease of the Jagged 1–Notch pathway, mural cell apoptosis | 123 |
| BMP                | Acvrl1 (encoding ALK1) knockout in endothelial cells | 2 months | Arteriovenous malformations in the gastrointestinal tract and uterus, pulmonary haemorrhages, death | 139 |
|                    | Eng knockout in endothelial cells | >8 weeks | Pelvic arteriovenous malformations | 141 |
|                    | Bmpr2 endothelial knockout | Not inducible | Spontaneous pulmonary hypertension in 40% of adult animals | 108 |

All studies were in mice except where indicated. ALK1, activin receptor-like kinase 1; BMP, bone morphogenetic protein; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; PV1, plasmalemma vesicle protein 1; SHH, sonic hedgehog; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.
VEGF ligands function via three related high-affinity tyrosine kinase receptors (VEGFR1–VEGFR3) and a host of auxiliary signalling molecules including neuropilins and syndecans43. VEGFR2 is the principal signalling VEGFR in blood endothelial cells, whereas both VEGFR2 and VEGFR3 are involved in lymphatic endothelial cell VEGF signalling45. Similar to FGF ligand binding to FGFRs, VEGF ligand binding to VEGFR2 activates several intracellular pathways, including PI3K–AKT and MAPK (including ERK1, ERK2 and p38 MAPK) pathways, among a number of others43. Of these VEGF-induced pathways, VEGF-mediated activation of ERK1 and ERK2 is thought to be crucial during embryonic vascular development and in angiogenic settings in adult tissues43,47.

The role of VEGF–VEGFR2 signalling in angiogenesis is well established. However, the role of this signalling cascade in the quiescent endothelium remains unclear. Interestingly, VEGF ligands are expressed by a number of specialized cell types, such as podocytes, choroid plexus epithelium and hepatocytes in adult mice48. Studies using mice expressing the VEGF–lacZ reporter construct showed that VEGF is expressed in cells overlying fenestrated and sinusoidal blood vessels, such as podocytes in the kidney and hepatocytes in the liver, as well as in tissues with secretory functions48. Furthermore, VEGFR2 in the adjacent endothelial cells was phosphorylated, demonstrating paracrine activity of the non-endothelial VEGF in these specialized environments.

Local effects. The importance of paracrine VEGFA signalling in quiescent endothelium is well documented in glomerulus endothelium FIG. 1; TABLE 1. Renal glomeruli are composed of fenestrated capillary endothelial cells and highly specialized epithelial cells (podocytes) separated by a glomerular basement membrane. Glomerular podocytes continuously express high levels of VEGFA. Constitutive heterozygous deletion of Vegfa in podocytes in mice leads to endotheliosis (swelling of endothelial cells with a partial loss of fenestrations) by 2.5 weeks of age49. By 6.5 weeks of age, mice

| Table 2 | Pathologies associated with endothelial dysfunction in adult patients |
|-----------------------------------------------|--------------------------------------------------------------------------------------------------|
| Affected signalling pathway | Pathological condition | Mechanism | Clinical findings | Refs |
|-----------------------------------------------|--------------------------------------------------------------------------------------------------|
| FGF | Atherosclerosis | Decreased FGF signalling due to high shear stress or TGFβ activation | Increased atherosclerotic plaque formation | 39,42 |
| VEGF | Anti-VEGF therapy | Neutralization of VEGF signalling | Hypertension, renal failure | 54–55 |
| | Anti-VEGFR2 therapy | Inhibition of VEGFR2 | Hypertension, renal failure, haemorrhages | 64 |
| | Tyrosine kinase inhibitors | Inhibition of VEGFR2, Notch signalling, ephrin receptor | Pulmonary hypertension | 78–70,12,24 |
| | Pre-eclampsia | High levels of soluble VEGFR1 or endoglin in plasma | Hypertension, renal failure | 50–67 |
| | Adult respiratory distress syndrome | High levels of soluble VEGFR1 in plasma | Acute respiratory distress syndrome | 86 |
| | Oedema, inflammation | Increased VEGF signalling in pulmonary endothelial cells | Pulmonary oedema and inflammation | 93,98–92 |
| WNT | Norrie disease | NDP (which encodes Norrin) loss-of-function variants | Blood–retina barrier defect, blindness | 109,107 |
| Angiopoietin | Venous malformation | TEK gain-of-function variants | Soft, blue, compressive, localized lesions | 126–128 |
| BMP | Hereditary haemorrhagic telangiectasia type 2 | ACVR1L1 (which encodes ALK1) loss-of-function variants | Arteriovenous malformation (in liver and lungs), epistaxis, telangiectasia | 157 |
| | Pulmonary arterial hypertension | BMPR2 loss-of-function variants | Pulmonary arterial hypertension | 106 |
| | BMP9 and BMP10 loss-of-function variants | Pulmonary arterial hypertension | 170–171 |
| TGFβ | Atherosclerosis | Endothelial cell activation of the TGFβ pathway | Increased endothelial-to-mesenchymal transition, increased atherosclerotic plaque formation | 39,42 |
| | Fibrosis | Activation of the TGFβ pathway | Increased extracellular matrix deposition, endothelial-to-mesenchymal transition? | 155 |

ALK1, activin receptor-like kinase 1; BMP, bone morphogenetic protein; FGF, fibroblast growth factor; TGFβ, transforming growth factor-β; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.
with heterozygous deletion of Vegfa have an expanded glomerular basement membrane and a near-complete loss of endothelial fenestration. The outcome is terminal renal failure with nephrotic syndrome at 9 weeks of age (TABLE 1). Interestingly, constitutive overexpression of Vegfa in podocytes in mice leads to a different kidney disease7. In this case, by 5 days of age, mice have a collapsing glomerulopathy characterized by kidney haemorrhages, proteinuria and complete collapse of the capillary loops, with no or few multinucleated endothelial cells (TABLE 1). Induction of Vegfa overexpression in podocytes of adult mice induces a different phenotype from that of mice with non-inducible Vegfa overexpression9,10. Inducible Vegfa overexpression in podocytes in adult mice also leads to kidney failure, proteinuria, glomerular basement membrane thickening, slit diaphragm loss and podocyte effacement, although endothelial cells are mostly unaffected (no endotheliosis or loss of fenestration9,10 (TABLE 1). Together, these data show the crucial requirement for finely balanced VEGF signalling in the filtration barrier of the glomeruli at different developmental stages.

Similar to the kidney, ablation of VEGF in endocrine glands leads to the loss of endothelial fenestrations11. Deletion of Vegfα2 or overexpression of a soluble form of Vegfr1 (REF.79) in pancreatic β-cells in mice results in a loss of endothelial fenestrations in pancreatic islets (TABLE 1). A similar loss of endothelial fenestrations is observed in thyroid capillaries following pan-endothelial Vegfa2 deletion in mice (TABLE 1).

In addition to paracrine activation of VEGF signalling, an autocrine VEGF loop is also crucial to the integrity of the quiescent endothelium74. VEGF expression in pulmonary, aortic and intestinal blood vessels in adult mice is patchy75,76. Constitutive homozygous deletion of Vegfa in endothelial cells results in lethality at any age, including around 25% lethality in adult mice, with a peak in death at 20–25 weeks after birth, as a result of multiorgan haemorrhage (in the spleen, kidney, brain, intestines, heart and lungs), intestinal perforations and myocardial infarction77. These haemorrhages and thrombotic events are the consequence of endothelial cell apoptosis78. Interestingly, endothelial-specific knockout of Vegfa in mice does not induce a loss of endothelial fenestrations in renal glomeruli79, unlike in mice with loss of paracrine VEGFA signalling. These differences in phenotype imply differences in VEGF signalling circuits when activated in an autocrine versus paracrine manner. However, the identity of these circuits remains unknown. Finally, small amounts of circulating VEGF ligands are found in blood. Whether the presence of VEGFs in blood implies the existence of endocrine signalling and what that might entail has not been established.

The VEGF pathway is also activated by shear stress. VEGFR2 is part of a complex with VE-cadherin and platelet endothelial cell adhesion molecule80, which allows endothelial cells to sense shear stress. Increased shear stress leads to ligand-independent activation of VEGFR2 by SRC, activation of AKT and then activation of endothelial nitric oxide synthase (eNOS; also known as NOS3) to induce vasodilatation90.

**Systemic hypertension.** In addition to specific effects in various organs, VEGFs have a number of systemic effects that became evident with the widespread clinical use of VEGF-neutralizing agents and tyrosine kinase inhibitors that target VEGFR2. Use of anti-VEGF therapies can often trigger the development of systemic hypertension owing to decreased synthesis of the vasodilator nitric oxide (NO) and increased expression of the vasoconstrictor endothelin 1 (ET1)81-84 (TABLE 2). The decrease in blood NO levels is due to a reduction in the expression of eNOS, which catalyses the synthesis of NO from L-arginine. However, specifically how decreased VEGF signalling leads to increased ET1 expression and whether a specific vascular bed is predominantly responsible for NO or ET1 synthesis is unclear. Other adverse effects of anti-VEGF therapies include proteinuria and membranous glomerulonephropathy, which can potentially advance to renal failure85 and are attributable to the requirement for VEGF in the maintenance of the renal vasculature and filtration units (TABLE 2). Finally, haemorrhages have been described in patients with cancer who were treated with sunitinib or sorafenib, two tyrosine kinase inhibitors targeting VEGFR2 signalling86 (TABLE 2).

Lack of VEGF signalling is responsible for the hypertension in women with pre-eclampsia, a common maternal complication of pregnancy associated with oedema, renal failure and systemic hypertension (TABLE 2). Syncytiotrophoblasts, a placental cell type, secrete a soluble form of VEGFR1 (sVEGFR1) and if this secretion is abnormally elevated, increased amounts of circulating sVEGFR1 sequestrate VEGFA, initiating the disease process87-90. Adenovirus-mediated overexpression of sVEGFR1 in pregnant rats induced systemic hypertension, proteinuria and glomerular endotheliosis, similar to what is observed in patients with pre-eclampsia91, and validating the requirement for sVEGFR1 in the pathogenesis of pre-eclampsia. Similar to the hypertension induced by the use of VEGF inhibitors, systemic hypertension in patients with pre-eclampsia is also driven by decreased NO production and increased ET1 levels in plasma90,91. Interestingly, elevated plasma TGFβ2 levels in patients with systemic hypertension92 and the changes in renal histology in patients with pre-eclampsia93 are similar to findings in mice with a systemic deletion of Erk1 and an inducible, endothelial-specific deletion of Erk2 (Erk1−/−Erk2βC−/−)94 (TABLE 1).

Both VEGF and VEGF signalling cascades activate endothelial ERK1 and ERK2 signalling95. A study from 2019 elucidated the function of ERK1 and ERK2 in the quiescent endothelium96. Inducible, endothelial-specific deletion of Erk2 in adult Erk1-null mice led to universal lethality within 4 weeks97,98. Interestingly, the phenotypes of these mice are a combination of the phenotypes found after inhibition of the FGF pathway and the VEGF pathway (TABLE 1). Erk1−/−Erk2β−/− mice have increased TGFβ signalling as a result of a decrease in let-7 microRNA expression, leading to EndMT. This TGFβ-induced EndMT has previously been observed after endothelial FGF pathway inhibition99. These Erk1−/−Erk2β−/− mice also develop systemic hypertension due to a loss of eNOS expression and increased ET1 expression, lose
fenestrations in endocrine gland and kidney endothelium, and develop kidney failure with proteinuria and endotheliolysis of the glomerulus endothelium. These phenotypes are very similar to those found in mice with VEGF pathway inhibition. Taken together, these results demonstrate the crucial function of the ERK1–ERK2 pathway in the maintenance of quiescent vasculature integrity and highlight the differences between VEGF-mediated and FGF-mediated activation of ERK1–ERK2.

**Pulmonary vasculature.** The function of VEGF in the pulmonary vasculature is less well-defined. Pulmonary capillaries are continuous, which means they do not have fenestrations. Nevertheless, VEGF is important for the maintenance of the pulmonary vasculature. Inhibition of VEGF signalling in rats by treatment with the VEGFR blocker SU5416 leads to pruning of the pulmonary arterial vasculature, which, in turn, induces alveolar cell apoptosis and emphysema at high doses. Interestingly, Notch signalling can attenuate the angiogenic sprouting effect of VEGF signalling. Consistent with this notion, mice with a heterozygous deletion ofDll4 (which encodes the Delta-like protein 4, a ligand of the Notch pathway), which is highly expressed in arterial endothelial cells and is a target gene of VEGF signalling, have pulmonary haemorrhages, suggesting a crucial role of VEGF signalling and the downstream Notch pathway in pulmonary endothelial cells. Furthermore, some patients treated with neutralizing antibodies against DLL4 (demcizumab or enoticumab) or a bispecific antibody against DLL4 and VEGF (navicixizumab) develop pulmonary hypertension. The cancer therapy drug dasatinib inhibits ephrin receptor signalling, which is directly connected to the VEGF signalling pathway by modulating VEGFR2 endocytosis and activation of downstream pathways. The interaction of these signalling pathways and the fact that multiple intracellular and cell-surface kinases are simultaneously inhibited by dasatinib might explain why this therapy is associated with the development of pulmonary hypertension in some patients and aggravates pulmonary hypertension in rats (Table 2). VEGF signalling also has an important role in vascular protection in patients with acute respiratory distress syndrome (ARDS). ARDS is characterized by diffuse alveolar damage leading to impaired gas exchange and is common in several pulmonary diseases, including viral pneumonitis (such as those caused by H1N1 influenza virus infection), severe acute respiratory syndrome and coronavirus disease 2019 (COVID-19), in which ARDS is associated with intense bronchial and lung parenchyma inflammation. The early, exudative phase of ARDS is characterized by diffuse alveolar damage, disruption and loss of epithelial and endothelial cell–cell junctions, and alveolar oedema. The exudative phase is followed by a proliferative phase that involves the formation of hyaline membranes on the epithelial side of the basement membrane and extensive cellular infiltrates in the alveolar spaces. VEGFs are present in the normal alveolar space and probably serve to maintain alveolar function. The loss of this protection, such as occurs with increased levels of VEGFA trap sVEGFR1 in the lungs, is predictive of the development of ARDS and of an increased risk of adverse outcomes among patients with ARDS. At the same time, these protective effects of VEGF signalling are counterbalanced by the capacity of the same VEGFA to induce oedema and promote inflammation. Indeed, studies of patients with ARDS associated with viral vasculitis, such as those caused by hantavirus infection, have demonstrated a strong association between VEGF levels in the lungs and pulmonary oedema.

**WNT and Hedgehog signalling**

Whereas the VEGF–ERK signalling axis is central to the maintenance of endothelial fenestrae of the kidney and endocrine glands, WNT signalling has an equally important role in the maintenance of tight junctions and the continuous endothelium of the central nervous system. The WNT family is composed of ten WNT receptors (Frizzled 1–10), four WNT co-receptors (LDL receptor-related protein 5 (LRP5), LRP6, RTK-like orphan receptor 2 and receptor-like tyrosine kinase), and 16 WNT ligands (WNT1–WNT16). Canonical WNT signalling involves the binding of WNT ligands to Frizzled receptors and the co-receptors LRP5 and LRP6. Phosphorylation of LRP5 and LRP6 recruits the β-catenin destruction complex (Axin, casein kinase 1, glycin synthase kinase 3 and adenomatous polyposis coli protein) from the cytoplasm to the plasma membrane, where the complex cannot degrade β-catenin. This stabilized form of β-catenin accumulates and translocates to the nucleus to activate the transcription of WNT target genes. Glia and neurons produce WNT7a and WNT7b ligands (Figure 1), and binding of these WNT ligands to the receptor Frizzled 4 activates canonical WNT signalling in endothelial cells of the central nervous system. Uregulation of β-catenin in endothelial cells leads to increased expression of genes encoding tight junction components (claudin 1, claudin 3 and claudin 5) and the glucose transporter 1 (GLUT1). Simultaneously, expression of Plvap, the gene encoding plasmalemma vesicle protein 1 (PV1), which is the principal component of endothelial fenestrae, is repressed in endothelial cells. This combined effect of WNT signalling is crucial to the integrity of the blood-brain barrier (BBB). Indeed, endothelial cell-specific deletion of Ctnnb1, the gene encoding β-catenin, in adult mice leads to severe seizures, brain haemorrhages and death (Table 1). Endothelial deletion of Fzd4, which encodes the receptor Frizzled 4, in adult mice leads to increased PV1 levels and decreased claudin 5 levels in retinal, cerebellar, spinal cord and olfactory bulb endothelial cells. Therefore, the WNT pathway is a perfect example of how the cellular...
microenvironment can induce the final differentiation step of endothelial cells, leading to a highly specialized organotypic endothelium.

Similar to the paracrine WNT signalling pathway, a paracrine sonic hedgehog (SHH) signalling pathway is also activated in the BBB\(^2\,01\). Astrocytes in the BBB express the ligand SHH, which binds to and inactivates the receptor protein patched homologue 1 (PTCH1), which is expressed in brain endothelial cells\(^2\,01\) (TABLE 1). Inactivation of PTCH1, in turn, results in the inactivation of the G protein-coupled receptor Smoothened (SMO), which leads to the activation of the glioma-associated oncogene (GLI1). Genetic endothelial-specific deletion of Smo specifically led to an increase in BBB permeability in adult mice, manifested by plasma protein leakage and decreased expression of junctional proteins (occludin, claudin 3, claudin 5 and tight junction protein ZO1)\(^1\,11\). This increased BBB permeability induces a pro-inflammatory phenotype in BBB endothelial cells with upregulation of intercellular adhesion molecule 1 (ICAM1) and recruitment of circulating inflammatory cells\(^9\,11\). Furthermore, SHH signalling also induces the expression of netrin 1 in the BBB endothelial cells, a laminin-related protein that is critical for cell–cell junction and cell–substrate adhesion\(^1\,02\).

Disregulation of the WNT-β-catenin pathway has been implicated in various central nervous system disorders that involve BBB breakdown, including multiple sclerosis\(^9\,13\), Alzheimer disease\(^9\,14\) and Huntington disease\(^9\,15\). The blood–retina barrier shares high similarity with the BBB. Mutations in NDP, a gene encoding the WNT ligand Norrin and expressed in the blood–retina barrier, are linked to Norrie disease, a condition in which the blood–retina barrier integrity is compromised, leading to blindness\(^9\,10\,07\) (TABLE 2). Dysregulation of the SHH pathway is found in HIV-associated neurocognitive disorders with disruption of BBB integrity\(^9\,18\).

**Angiopoietin signalling**
Angiopoetins (ANGs) are a family of secreted factors comprising ANG1, ANG2 and ANG3 (ANG4 in humans). Unlike the FGF and VEGF signalling pathways, which are involved in both pro-angiogenic processes and maintenance of endothelial cell quiescence signalling, ANG1 signalling is purely a quiescence signalling pathway in endothelial cells. ANG1 is expressed in mural cells and binds to the angiopoietin 1 receptor

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**Fig. 2 | Signalling crosstalk in quiescent and activated endothelial cells.**

**a** Regulation of endothelial fenestration is achieved by a combination of signalling pathways regulating Plvap expression. Inhibition of the transforming growth factor-β (TGFβ) signalling is controlled by several signalling pathways including bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) signalling circuits.

**b** Pathological endothelium. Decreased FGF or VEGF signalling input leads to activation of an autocrine TGFβ signalling loop that, in turn, induces inflammation, hypertension and endothelial-to-mesenchymal transition (EndMT). Excessive VEGF signalling and autocrine angiopoietin 2 (ANG2) signalling induce pathological angiogenesis. ACVR2A, activin A receptor type 2A; ALK, activin receptor-like kinase; BMPR2, bone morphogenetic protein receptor type 2; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; FGFR, fibroblast growth factor receptor; GLUT1, glucose transporter 1; LRP, lipoprotein receptor-related protein; Plvap, plasmalemma vesicle-associated protein; SMAD, mothers against decapentaplegic homologue; TIE2, angiopoietin 1 receptor; VEGFR, vascular endothelial growth factor receptor.
(TIE2) expressed on the surface of endothelial cells. After ANG1 binding, TIE2 clusters and autophosphorylates to activate downstream pathways. Phosphorylated TIE2 is found in all adult vasculatures. The main pathway activated downstream of TIE2 is the AKT pathway. During inflammation or hypoxia, endothelial cells can deactivate the quiescence signal of TIE2 by expressing ANG2 that competes with ANG1 for binding to TIE2 and predominantly functions as an antagonist of TIE2 (REF. (2)) (FIG. 2; TABLE 1). The loss of TIE2 signalling reactivates the endothelium by weakening endothelial cell–cell junctions, inducing the expression of pro-inflammatory adhesion molecules, including ICAM1 and vascular cell adhesion molecule 1 (VCAM1), and increasing the levels of procoagulant proteins on the luminal surface of endothelial cells (16,17) (TABLE 1). Intriguingly, ANG2–TIE2 interactions are context-dependent — ANG2 acts as a TIE2 agonist under pathogen-free conditions but as an antagonist under inflammatory conditions (such as infection and tumour necrosis factor (TNF) or lipopolysaccharide stimulation) (18). TIE1 is an orphan receptor unable to bind any angiopoietin or other known ligands. TIE1 and TIE2 interact in the absence of a ligand. Endothelial TIE1 is required for the agonist effects of paracrine ANG1 and autocrine ANG2 on TIE2 activation. During inflammation, the ectodomain of TIE1 in endothelial cells is cleaved, resulting in the loss of ANG2 agonist activity, thereby promoting vascular remodelling and leakiness (19). TIE1 cleavage reduces, but does not abolish, ANG1 agonist activity.

Despite AKT signalling functioning in different aspects of cellular regulation in multiple cell types, in the quiescent endothelium, AKT is considered to function as a survival pathway. In vitro transduction of endothelial cells with a dominant-negative Akt variant decreases endothelial viability by opposing the pro-survival effects of VEGF (20). However, a 2016 study in mice showed that the main function of the endothelial AKT pathway is not endothelial cell survival but maintenance of adequate interactions between endothelial cells, pericytes and vascular smooth muscle cells (21) (TABLE 1). Indeed, endothelial cell-specific deletion of Akt1 in mice with global Akt2 deletion alters Jagged 1–Notch signalling between endothelial and mural cells, leading to apoptosis of vascular smooth muscle cells and pericytes and subsequent vessel regression, particularly in coronary arteries (22). However, endothelial cell apoptosis was not detected in these Akt2−/− mice with endothelial cell-specific Akt1 deletion. The regulation of Notch signalling by AKT following ANG1 stimulation in endothelial cells is mediated by the endothelial transcriptional regulator ERG (23). Inducible, endothelial cell-specific deletion of Erg in mice leads to a phenotype similar to that produced by the deletion of Akt1 and Akt2, with a loss of vascular smooth muscle cell coverage and vascular regression (24).

Clinical data have added a layer of complexity to our understanding of ANG–TIE signalling. Multiple cutaneous and mucosal venous malformations have been reported in patients carrying gain-of-function genetic variants in TEK (encoding TIE2) (25,26). This vascular malformation is characterized by the development of soft, blue, compressive and localized lesions (27). Histological features of these venous lesions include uneven endothelial cell lining, disorganized extracellular matrix structure, enlarged lumen and sparse mural cell coverage (28). These lesions can be present at birth or present around puberty (29). These gain-of-function TEK genetic variants result in autophosphorylation of TIE2 and excessive activation of the downstream AKT pathway in endothelial cells (30). At the same time, secretion by endothelial cells of platelet-derived growth factor B, which is responsible for mural cell recruitment, is downregulated (31). These observations suggest that, although TIE2 signalling is important for the switch from an activated to a quiescent endothelium, overactivation of this pathway is deleterious. The mechanism responsible for ensuring the proper extent of TIE2 activation is currently unclear. Vascular endothelial protein tyrosine phosphatase (VEPTP), a vascular phosphatase, seems to be crucial in limiting TIE2 activation, because neutralization of VEPTP in vivo in mice results in vascular lesions similar to those seen in mice with gain-of-function TEK genetic variants (30,31).

BMP signalling

The TGFβ superfamily includes a large pool of ligands, such as TGFβ1–TGFβ3, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), activins and inhibins and nodal. These ligands bind to a complex of two dimers of a combination of type I receptors named activin receptor-like kinase 1 (ALK1)–ALK7 and two dimers of type II receptors (BMP receptor type 2 (BMPR2), TGFβR, activin receptor type 2A and activin receptor type 2B), leading to the activation of a number of different canonical (SMAD-dependent) and non-canonical signalling cascades (32). The major distinction between canonical BMP and TGF pathways is the phosphorylation of SMAD2–SMAD3 by TGFβ, activins, inhibins and nodal, whereas BMPs and certain GDFs phosphorylate SMAD1, SMAD5 and SMAD8. BMP9 and BMP10 are circulating BMPs produced by the liver and the heart, respectively (33). Both ligands bind the high-affinity receptor ALK1, which is specifically expressed by endothelial cells (34). ALK1 signalling is crucial for developmental angiogenesis (35–38). In addition, ALK1 also has a crucial role in the quiescent endothelium. Endothelial deletion of Alk1 in adult mice (aged 2 months) is lethal within 9–21 days of deletion, although the exact cause of death is still unknown (39) (TABLE 1). Major autopsy findings included cardiac enlargement and haemorrhages in the lungs and the gastrointestinal tract (39). Deletion of Alk1 in endothelial cells in adult mice also led to the spontaneous formation of arteriovenous malformations (AVMs) — direct shunts between arteries and veins — in the gastrointestinal tract and uterus (39). AVMs were also suspected to be present in the lungs, but were difficult to assess because of the multiple haemorrhages in the lungs (39). Furthermore, wounding can also induce de novo AVM formation in the skin of adult mice with Alk1 deletion (39). Endoglin, the co-receptor for ALK1, is also important for vascular quiescence. Endothelial-specific deletion of
Eng (which encodes endoglin) in adult mice resulted in wound-induced AVMs in the skin, but no visceral AVMs were found\(^{46}\). However, spontaneous pelvic AVMs were shown in another study of mice with endothelial-specific Eng deletion\(^{41}\) (TABLE 1). Interestingly, the pelvic area where the AVMs form after Eng deletion has high VEGFA levels\(^{41}\). Anti-VEGF treatment blocked the formation and maturation of AVMs in Alk1-knockout mice and Eng-knockout mice\(^{41,42}\). Together, these data support the concept that ALK1 and endoglin are required for the maintenance of endothelial quiescence in adult life to counteract an over-exuberant endothelial proliferative response to VEGF signalling.

The molecular mechanism by which ALK1–endoglin signalling maintains the integrity of the quiescent endothelium is still unknown. BMP9 and BMP10 induce endothelial quiescence by inhibiting endothelial cell migration and proliferation in microvascular endothelial cells\(^{134,144}\). Moreover, the BMP9–ALK1 signalling pathway inhibits the pro-angiogenic VEGF–AKT1 pathway\(^{144}\). BMP9, which is produced by hepatic stellate cells, induces the fenestration of the sinusoidal endothelial cells\(^{145}\) (FIGS 1, 2). Deletion of Bmp9 in 129/Ola mice triggers hepatic fibrosis following sinusoidal capillarization (transformation of the fenestrated hepatic sinusoids into continuous capillaries, with synthesis of a basement membrane of collagen between the endothelial cells and the hepatocytes)\(^{146}\) (TABLE 1).

The BMP9–ALK1 pathway is also modulated by shear stress. Endoglin increases BMP9 signalling through ALK1 in endothelial cells during shear stress\(^{42}\). This increased BMP9 activity is accomplished by an increase in the association between endoglin and ALK1 before their binding to the ligand\(^{42}\). Loss of eng in zebrafish leads to defective blood flow-induced cell shape changes, resulting in enlarged vessels\(^{148}\). The primary cilia, which can function as a sensor of blood flow-induced mechanical forces on endothelial cells, can also regulate BMP signalling. In vitro, the loss of BMP9 signalling through the cilium was shown to increase endothelial cell migration\(^{49}\). Together, these studies show that shear stress increases BMP9 signalling in quiescent endothelial cells.

Interestingly, stimulation of human pulmonary endothelial cells with BMP9 in vitro inhibits the TGFβ pathway by inducing the expression of inhibitory SMADS (SMAD6 and SMAD7) and by decreasing TGFβR2 expression\(^{154}\). These results identify a second pathway that inhibits TGFβ signalling in endothelial cells\(^{150,152}\) (FIG. 2). Further evidence supporting the importance of TGFβ pathway inhibition in quiescent endothelium is the increased expression of SMAD6 and SMAD7 observed in pulmonary endothelial cells of adult mice compared with pulmonary endothelial cells from infant mice, which are proliferative cells\(^{155}\). These studies firmly support the notion that BMP9–ALK1 signalling inhibits the TGFβ pathway in quiescent pulmonary endothelial cells. The endothelial-specific transcription factor ERG can also activate the BMP pathway by upregulating SMAD1 as well as inhibiting the TGFβ pathway by downregulating SMAD3 expression in the quiescent endothelium of the hepatic vasculature\(^{157}\).

Disruption of normal BMP signalling in quiescent endothelial cells is the molecular basis for hereditary haemorrhagic telangiectasia (HHT), a rare genetic vascular disease (TABLE 2). HHT is characterized by recurrent nosebleeds, mucous telangiectasia and formation of AVMs\(^{153,154}\). In most patients, HHT is caused by loss-of-function genetic variants in ENG or ACVR1 (encoding ALK1)\(^{153,158}\), and these variants decrease BMP9 signalling\(^{157,158}\). Interestingly, a second variant on the somatically non-mutated allele of ACVR1 or ENG can be found in some lesions in patients with HHT\(^{159}\). These results establish HHT as a disease of decreased BMP9 and BMP10 signalling. AVMs in patients with HHT are predominantly found in the liver and lungs and, to a lesser extent, in the brain\(^{160}\). Interestingly, AVMs in the liver are more frequent in patients with HHT type 2 (patients carrying an ACVR1 variant) than in patients with HHT type 1 (carrying an ENG variant), whereas the opposite is true for AVMs in the lung\(^{160}\). Whether AVMs are congenital or acquired during adult life is unclear. Given that most AVMs are asymptomatic, sparse data exist on AVM frequency in children and younger adults. One study found that, in patients with HHT type 2, hepatic AVMs were present in 67% of patients aged <45 years and in 93% of patients aged >45 years\(^{161}\). A similar difference was found in patients with HHT type 1 (hepatic AVMs were present in 46% of patients aged <45 years and in 78% of patients aged >45 years)\(^{161}\). With regard to pulmonary AVMs, a Canadian study compared the frequency of pulmonary AVMs in children (aged <18 years) and their parents\(^{162}\). In patients with HHT type 1, the frequency of pulmonary AVMs was similar in both groups, whereas among patients with HHT type 2, 8.3% of children had pulmonary AVMs compared with 25.9% of the parents\(^{162}\). Moreover, the incidence of HHT symptoms increases with age, and symptomatic AVMs in the liver are found in adult patients (aged >30 years)\(^{163,164}\). Taken together, these data suggest that at least some AVMs can develop in adulthood because of alterations in endothelial quiescence and that AVM size increases with age leading to symptomatic AVMs in older patients. The hypothesis of an increase in VEGF signalling as a result of the loss of BMP signalling owing to ACVR1 or ENG variants was validated by data from a clinical trial showing that inhibition of VEGF in patients with HHT decreases the symptoms of HHT\(^{165}\).

The BMP pathway is also involved in the development and progression of pulmonary arterial hypertension (PAH) (TABLE 2). Heterozygous germline variants in BMPR2 underlie the main genetic susceptibility for PAH, found in 53–86% of patients with a family history of PAH and 14–35% of patients with idiopathic PAH\(^{166}\). Although the presence of a BMPR2 variation is neither necessary nor sufficient to cause PAH, a reduction in BMPR2 activity is currently viewed as the major molecular defect conferring a predisposition to develop PAH as well as an increased risk of progression of the disease\(^{166,167}\). Constitutive deletion of Bmpr2 in endothelial cells in mice predisposes the animals to develop spontaneous PAH\(^{168}\), supporting the notion that disrupting BMP signalling in the endothelium is a risk factor for PAH. However, given the potential
inhibitory role of BMPR2 in BMP signalling\textsuperscript{169}, the exact effect of BMPR2 variants on BMP signalling in the pulmonary endothelium is unclear. The discovery in some patients with PAH of variants in GDF2 (encoding BMP9), leading to decreased circulating BMP9 level, and variants in BMP10 revealed another layer of complexity of the regulation of the pulmonary endothelium and PAH pathogenesis\textsuperscript{170–173}. Therapy with BMP9 has been proposed as a strategy to compensate for the loss of one BMPR2 allele in patients with PAH\textsuperscript{150}. However, Bmp9-null mice do not develop spontaneous pulmonary hypertension, and these mice were even protected in experimental models of pulmonary hypertension\textsuperscript{174}. Given these contradictory findings, further research is needed to clarify the role of BMP signalling in PAH. In particular, understanding how the reduction in the BMPR2 activity predisposes to PAH and how the BMP9–BMP10–BMPR2 axis contributes to the pathophysiology of PAH is essential. An early event that seems to be facilitated by dysfunction in the BMP9–BMP10–BMPR2 axis is the pro-inflammatory phenotype of endothelial cells in PAH\textsuperscript{174}. In PAH, during the process of vascular remodelling, quiescent pulmonary endothelial cells become activated and express high levels of adhesion molecules, such as VCAM1 and ICAM1, and secrete high levels of chemokines, such as IL-6 and CC-chemokine ligand 2 (CCL2; also known as MCP1)\textsuperscript{175}.

### TGFβ signalling

TGFβ ligands bind to TGFβR1 (also known as ALK5) and TGFβR2. Type III receptors (TGFβR3) increase ligand binding to their cognate receptors. Although endothelial ALK5 and TGFβR2 are crucial for cerebral vascular development\textsuperscript{176} and endothelial TGFβR3 for coronary vessel development\textsuperscript{177} in mouse embryos, Alk5 or Tgfb2 deletion in endothelial cells in adult mice does not affect vascular morphogenesis\textsuperscript{178}. Activation of the TGFβ pathway in endothelial cells in adult mice and humans is associated with a change in endothelial cell identity referred to as EndMT, a cell fate change event underlying a number of pathological processes\textsuperscript{32} (FIGS 1, 2). When endothelial cells undergo EndMT, they acquire mesenchymal characteristics including fibroblast-like morphology, cell junction rearrangement, increased mobility and proliferation, a thrombogenic and inflammatory phenotype, and increased secretion of the extracellular matrix proteins fibronectin and collagen\textsuperscript{179}. To date, at least three pathways that inhibit TGFβ signalling in quiescent endothelia have been identified: the VEGF/FGF–ERK–let-7 pathway and the BMP9–ALK1 pathway discussed above, and the cerebral cavernous malformation (CCM)–MEKK3 pathway\textsuperscript{179}. Postnatal deletion of any of the three known CCM genes in mice leads to overactivation of the MEKK3 pathway\textsuperscript{180,181}, which induces the expression of Klf4 (which encodes the transcription factor Krüppel-like factor 4 (KLF4))\textsuperscript{182} and Klf2 (which encodes KLF2)\textsuperscript{183}. The exact mechanism of how KLF2 and KLF4 induce CCM lesion formation is unclear. One study showed that KLF4 induces an autocrine loop that involves BMP6, which activates the TGFβ pathway leading to EndMT\textsuperscript{184}. However, another study found that CCM gene deletion induces MEKK3-mediated overactivation of KLF2 and KLF4, but that this overactivation does not induce EndMT\textsuperscript{185}. Interestingly, an ensemble computational intelligence strategy, comprising deep learning and probabilistic programming of RNA-seq data, causally linked the loss of ERK1 and ERK2 in human endothelial cells in vitro to the activation of an autocrine loop driven by TGFβ2 [REF.\textsuperscript{186}]. Verified in mice, this autocrine loop resulted not only in the induction of EndMT (seen in vitro and in vivo in Erk1\textsuperscript{−/−}–Erk2\textsuperscript{−/−} mice), but also in systemic hypertension. The latter was induced by suppression of eNOS expression (and therefore NO production) and induction of vasoconstrictor ET1 expression. A decrease in endothelial fenestration was also observed (caused by decreased PV1 expression as seen in vitro and in vivo in Erk1\textsuperscript{−/−}–Erk2\textsuperscript{−/−} mice)\textsuperscript{187} (FIG. 2). Systemic hypertension and the loss of endothelial fenestrations are features of VEGF–ERK pathway inhibition. This in silico analysis suggested that the phenotypes seen after the loss of VEGF signalling are, at least partially, due to increased TGFβ signalling.

Fibrosis is a devastating process characterized by myofibroblast cell proliferation and abnormal extracellular matrix accumulation, leading to organ failure. Endothelial cell injury often precedes the development of fibrosis and is suspected to be an initiating event\textsuperscript{188} (TABLE 2). Endothelial cells produce profibrotic mediators, such as TGFβ, plasminogen activator inhibitor 1 and connective tissue growth factor, which induce fibroblast growth and differentiation and collagen synthesis by fibroblasts\textsuperscript{189}. In addition, endothelial cells can also differentiate into fibroblast-like cells and secrete collagen as a result of EndMT\textsuperscript{190}. However, the exact contribution of EndMT as the source of myofibroblasts is controversial, and lineage tracing experiments in animal models of cardiac and renal fibrosis show that EndMT is not a major source of myofibroblasts\textsuperscript{191–193}. Liver sinusoidal endothelial cells (LSECs) have a major role in liver fibrosis\textsuperscript{194,195}. After liver injury, LSECs rapidly switch from a fenestrated to a capillarized phenotype and acquire a pro-vasoconstrictive, pro-inflammatory, pro-angiogenic and pro-fibrotic phenotype, which induces hepatic stellate cell activation that leads to liver fibrosis. VEGF and BMP9 can both function to maintain the fenestrated quiescent state of LSECs\textsuperscript{196,197}. Inflammatory cells also contribute to the development of fibrosis. Activated endothelial cells provide important signals, such as the expression of adhesion molecules (for example, ICAM1 and VCAM1) and secretion of various cytokines and chemokines (such as IL-6, CCL2 and

**Box 1 | Unknowns in quiescent endothelium biology**

- What is the genetic and metabolic basis of endothelial heterogeneity?
- What determines disease-prone versus disease-resistant endothelial cell subsets?
- What are the organ-specific signals governing endothelial cell specialization and final differentiation?
- What are the main organ-specific interactions between endothelial cells and non-endothelial cells?
CXC-chemokine ligand 12), to recruit leukocytes and perpetuate inflammation. This pro-inflammatory phenotype of endothelial cells and the recruitment of inflammatory cells contribute to the pro-fibrotic environment by inducing the secretion of collagen. Activation of the TGFβ pathway in endothelial cells triggers an endothelial inflammatory phenotype. In addition, TGFβ secreted by endothelial cells can induce resident fibroblasts to become myofibroblasts. Finally, activation of the TGFβ pathway is also a major trigger of plaque formation in atherosclerosis, as a consequence of decreased FGF signalling (see the section on FGF signalling).

**Conclusions**

Endothelial quiescence has emerged as an important area of investigation in the field of vascular biology research. The vascular endothelium is central to the regulation of tissue and organ homeostasis and is crucial for disease resilience. Understanding the signalling pathways that regulate the numerous functions of the endothelium, including immune regulation, glucose transport, blood-brain barrier, and others, is crucial for developing targeted therapies for vascular diseases.
Endothelial heterogeneity in health and disease.

a Quiescent endothelial cell (EC) heterogeneity in structure, function, immune regulation (interferon response and leukocyte adhesion molecule expression) and metabolism between tissues and within tissues. The information shown in this panel is from Refs. 1, 2. | Heterogeneity in healthy capillary ECs between organs. c) Development of endothelial dysfunction. This is a stepwise process, progressing from activation of ECs to the development of endothelial-to-mesenchymal transition to the full-blown pathological end state. This sequence of events leads to ECs losing their normal fate and acquiring features of mesenchymal cell types, including fibroblasts, smooth muscle cells and macrophages, in a process known as endothelial-to-mesenchymal transition. These events result in the initiation and propagation of inflammation, loss of normal endothelial structures and function, increased vascular permeability and formation of pathological lesions, such as atherosclerotic plaques.

Endothelial heterogeneity in different organs is central to the understanding of normal physiology as well as the pathophysiology of numerous diseases, and addressing important knowledge gaps is the current challenge in the field of vascular biology (Box 1).

Although many functions of the quiescent endothelium are organ-specific (Fig. 1), other functions are general to all quiescent endothelial cells. Thus, the TGFβ signalling pathway is inhibited in the healthy quiescent endothelium regardless of organ or location, and activation of TGFβ is linked to the loss of normal endothelial cell fate (via EndMT) and to the development and progression of disease states (RGS 1, 2). Indeed, the capacity of an endothelial bed to avoid the activation of TGFβ signalling is closely linked to its capacity to resist disease development and might account for different disease susceptibilities in different patient populations. It is interesting to speculate that an increased susceptibility of endothelial cells to TGFβ activation might be a risk factor for some of the most common vascular diseases, such as atherosclerosis. The ability to understand and assess this endothelial cell susceptibility, both genetically and functionally, would allow better risk assessment and the development of therapies aimed at disease prevention. The importance of keeping TGFβ signalling in check is further illustrated by the variety of signalling cascades that inhibit TGFβ signalling in endothelial cells, including VEGF–ERK, FGF–ERK, BMP9–ALK1 and CCM–MEKK3 (Fig. 2). The existence of these signalling cascades suggests that control of TGFβ signalling is crucial for maintaining cell homeostasis and that abnormalities in this pathway can trigger specific diseases.

Endothelial cell senescence and ageing are crucially linked to endothelial cell quiescence, and endothelial normalcy is probably one of the most crucial factors contributing to a healthy lifespan. Examples of such a link include arterial stiffness and hypertension, two hallmarks of the ageing process. Although this subject is outside the scope of this Review, the mechanisms of ageing-related endothelial cell senescence have been well described previously.

A thorough knowledge of the dynamic control of endothelial quiescence is required. To this end, we need to understand how a signalling pathway that is involved in angiogenic stimulation, such as VEGF signalling, can also maintain endothelial quiescence. This dichotomy could be a result of different VEGF dosages, differential VEGF signalling through different co-receptors, such as neuropilin 1 (Ref. 19) and syndecan 2 (Ref. 20), alterations in the duration of VEGF stimulation, paracrine versus endocrine versus autocrine activation of VEGF signalling, or crosstalk with other signalling pathways. All these factors might differentially affect VEGF stimulation and point towards the existence of regulators that modulate the effects of VEGF signalling to achieve the desired physiological objective.

We also need to understand the molecular basis of the organotypic effects of endothelial cell signalling. For example, although CCM proteins are expressed in all endothelial cells, variants in CCM genes seem to affect only the central nervous system vasculature. Another related problem is that organ-specific mutation of genes in a given signalling pathway does not have a consistent phenotype across organs. Genetic variants in ACVRL1 and ENG lead to the development of HHT (with AVM mainly in the lungs and liver), whereas variants in BMPR2 predispose to the development of pulmonary hypertension, with no effect on the vasculature of other organs.

Advances in research into endothelial cell metabolism show a difference in the metabolic signature between quiescent and activated endothelial cells (Ref. 21). Of note, alterations in endothelial cell metabolism could be very important to regulate cell quiescence and warrant further investigation.

Another important unknown is the link between endothelial cell quiescence and disease resilience. Emerging data from single-cell RNA-seq studies highlight the heterogeneity of endothelial cells between tissues but also within each tissue (Fig. 3a, b). These single-cell RNA-seq data suggest that in many cases, disease progression is due to the expansion of a single population of normal cells (for example, endothelial or smooth muscle cells) that are susceptible to a particular disease stimulus (Ref. 22). These findings also suggest that other normal populations of these cell types are disease-resistant. An in-depth understanding of this phenomenon is crucially important. Further studies to characterize the genetic, molecular and metabolic signatures of disease-resistant versus disease-prone cell populations are also needed (Box 1; Fig. 3c). To summarize, the understanding of the active regulation of the organotypic endothelial quiescence is currently one of the biggest challenges in vascular biology research.

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