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BASIC AND THERAPEUTIC ASPECTS OF ANGIOGENESIS UPDATED

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ABSTRACT

All organisms growing beyond the oxygen diffusion limit critically depend on a functional vasculature for survival. Yet blood vessels are far more than passive, uniform conduits for oxygen and nutrient supply. A remarkable organotypic heterogeneity is brought about by tissue-specific differentiated endothelial cells (ECs – lining the blood vessels' lumen) and allows blood vessels to deal with organ-specific demands for homeostasis. On the flipside, when blood vessels go awry, they promote life-threatening diseases characterized by ECs inappropriately adopting an angiogenic state (*eg* tumor vascularization) or becoming dysfunctional (*eg* diabetic microvasculopathies), calling respectively for anti-angiogenic therapies (AATs) and pro-angiogenic/vascular regenerative strategies. In solid tumors, despite initial enthusiasm, growth factor-based (mostly anti-VEGF) AATs do not sufficiently live up to the expectations in terms of efficiency and patient survival, in part due to intrinsic and acquired therapy resistance. Tumors cunningly deploy alternative growth factors than the ones targeted by the AAT to re-instigate angiogenesis or revert to other ways of securing blood flow, independently of the targeted growth factors. In trying to alleviate tissue ischemia and to 'repair' dysfunctional or damaged endothelium, local in-tissue administration of (genes encoding) pro-angiogenic factors or endothelial (stem) cells harnessing regenerative potential have been explored. Notwithstanding evaluation in clinical trials, these approaches are often hampered by dosing issues and limited half-life or local retention of the administered agents. Here, without intending to provide an all-encompassing historical overview, we focus on some recent advances in understanding EC behavior in health and disease, and identify novel molecular players and concepts that could eventually be considered for therapeutic targeting.

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NON-STANDARD ABBREVIATIONS AND ACRONYMS

AAT	anti-angiogenic therapy
BM	basement membrane
BST1	bone marrow stromal antigen-1
Cdc42	cell division cycle 42
Dll4	delta like canonical NOTCH ligand 4
EC	endothelial cell
ECM	extracellular matrix
EMP	erythro-myeloid progenitor
EPC	endothelial progenitor cell
FAO	fatty acid oxidation
FGF	fibroblast growth factor
HEV	high endothelial venule
IA	intussusceptive angiogenesis
NICD	NOTCH intracellular domain
OXPHOS	oxidative phosphorylation
PVN	perivascular niche
SA	sprouting angiogenesis
scRNA-seq	single cell RNA sequencing
SUMO	small ubiquitin-like modifier
TAZ	transcriptional coactivator with PDZ-binding motif
TCA	tricarboxylic acid
TEC	tumor endothelial cell
VC	vessel cooption
VM	vascular mimicry
VEGF(R)	vascular endothelial growth factor (receptor)
YAP	yes associated protein

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THE BASIC ARCHITECTURE OF THE VASCULAR TREE

The vascular system is a closed network of arteries, veins and interconnecting capillaries, organized into tree-like structures. Endothelial cells (ECs) line the lumen of blood vessels and regulate the dynamic passage of nutrients and cells. ECs exhibit organ-specific molecular and functional properties that provide heterogeneity to the vascular tree. As such, the endothelium of arteries and veins forms a continuous monolayer, whereas the endothelium of capillaries can be continuous, fenestrated or discontinuous depending on their (tissue-specific) function [1]. ECs are supported by a basement membrane with a different composition across vascular beds.

In the adult organism, ECs rarely proliferate and remain in a dormant, quiescent state for protracted periods of time, yet they retain the ability to rapidly initiate the formation of new vessels from pre-existing ones, a tightly controlled process termed angiogenesis. This process of new blood vessel formation is for example initiated in nutrient deprived, ischemic/hypoxic environment, where restoration of blood flow (in order to provide oxygen and nutrients) is required to support the growth and function of a tissue. Remodeling of the vascular network occurs throughout life as capillaries grow and regress according to functional demands [2].

MAKING NEW BLOOD VESSELS: SPROUT AND SPLIT

At early embryonic stages, a primitive vascular plexus is formed during vasculogenesis, a process involving angioblast aggregation into vessel-like tubes (reviewed in [3]). The further angiogenic growth of the vascular systems is an example of tight coordination of cell proliferation, differentiation, migration, matrix adhesion and cell–cell signaling processes. Vessel growth by *sprouting angiogenesis* (SA) is initiated by a gradient of pro-angiogenic growth factors (e.g. vascular endothelial growth factor (VEGF); see further below) released by oxygen- and nutrient-deprived microenvironments, which trigger quiescent ECs to become activated (Figure 1). Just to highlight a few underlying mechanisms, activated ECs secrete matrix metalloproteinases (MMPs) to degrade the basement membrane (BM) and become invasive [4]. This allows endothelial tip cells to protrude and migrate towards the source of the angio-

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genic signal. Tip ECs minimally proliferate, are highly polarized and extend filopodia and lamellipodia to guide the nascent sprout towards hypoxic and nutrient deprived areas. Moreover, they are rich in cell-surface receptors and molecules involved in extracellular matrix (ECM) degradation and BM deposition [5-8]. Tip cells are followed by stalk ECs, which proliferate to ensure sprout elongation and mediate lumen formation [9] (Figure 1). Specification in migratory tip *versus* proliferating stalk cell is highly dynamic, and ECs continuously compete for the lead position, presumably to ensure that the fittest EC leads the sprout [10]. Once two tip cells from neighboring sprouts meet, they anastomose to form a perfused new vessel. In addition to other mechanisms (reviewed in [11]), blood flow itself by virtue of its hemodynamic force drives lumen formation in a process dubbed 'inverse blebbing' [12]. Perfusion then induces vascular maturation by re-establishing pericyte recruitment and BM deposition to promote vessel stabilization [8]; in contrast, non-perfused segments regress. Once tissue vascularization is restored, the levels of pro-angiogenic factors diminish and ECs re-establish their quiescent phenotype. Quiescent ECs (dubbed 'phalanx' ECs based on their typical structured appearance [8]) become highly interconnected through junctional molecules as VE-cadherin, claudins and tight junction proteins that mechanically strengthen the vessels wall and create a permeable barrier [8].

During *intussusceptive angiogenesis* (IA, aka 'vessel splitting' or 'nonsprouting angiogenesis') pre-existing vessels are longitudinally split into new ones to expand and remodel an immature capillary plexus. ECs of opposing inner vessel walls make a 'kissing' contact to form a transluminal bridge. Within this structure, intracellular junctions are reorganized to centrally perforate the endothelial bilayer resulting in the formation of an interstitial pillar core. Finally, myofibroblasts and pericytes invade the pillar to cover its interstitial wall, the pillar increases in circumference and fuses with adjacent pillars to split one capillary into two parallel capillaries [13, 14] (Figure 1). IA is a faster mechanism to expand a plexus than SA and has been postulated to be less energetically demanding, given its minimal dependency on EC migration and proliferation [15]; however, the exact metabolic costs of structurally 'reshaping' ECs into interstitial pillars remains largely unexplored. Finally, IA comes in different flavors: intussusceptive microvascular growth rapidly expands the vascular network, intus-

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susceptive arborization reshapes the plexus into a hierarchical vascular tree and intussusceptive branching remodeling optimizes the number of vessels [15]. Finally, other modes of vascular expansion exist (*eg* artery reassembly [16]) but fall outside the scope of this review.

TUMOR BLOOD VESSELS: DEVIOUS WAYS TO SECURE BLOOD SUPPLY

The vascular network connects to all tissues (with a few exceptions e.g. cartilage) to perform both basic household and highly tissue-specific chores; it is therefore not surprising that vessel abnormalities or dysfunctionality and excessive vascular growth contribute to many life-threatening diseases including tumor growth. Indeed, solid tumors exceeding a few mm^3 in size rely on a sustained vascular supply for oxygen and nutrient delivery to fuel rapid growth. The elevated production of pro-angiogenic factors (e.g. VEGF) in the hostile tumor microenvironment paradoxically incites a self-reinforcing loop of non-productive SA. Indeed, tumor vessels are disorganized, tortuous, lack normal EC and mural cell lining and are leaky (reviewed in [17]). SA has been considered a hallmark of cancer for several decades [18], but the occurrence of IA in solid tumors has also been illustrated, though limitedly [19]. Additionally, cancer cells cunningly deploy non-angiogenic strategies to secure blood supply. In *vessel co-option* (VC), cancer cells hijack the pre-existing vasculature of the surrounding normal tissue that they colonize by growing around and incorporating the vessels into the tumor (Figure 1). VC occurs in a broad range of human tumors at various anatomical sites and determines patient outcomes (reviewed in [20]), yet remains an often overlooked/understudied phenomenon. ECs in coopted vessels display limited proliferation in comparison to ECs in angiogenic tumors and coopted vessels lowly express angiogenic markers (*eg* $\alpha v\beta 3$ integrin (lung co-opted vessels) or CD34 (liver coopted vessels)). In contrast, expression of EC markers in non-malignant tissues is maintained as is the pattern of BM marker expression (reviewed in [20]).

Besides hijacking, cancer cells can also sneakily act like ECs, a phenomenon first described in human uveal melanoma, coined *vascular mimicry* (VM) [21]. VM allows highly aggressive cancer cells to construct, without the need for ECs, their own blood-conducting channel network (discernible as periodic acid-Schiff (PAS)-positive patterns), which serves as an irrigation system to meet the high metabolic demands of a rapidly growing tumor (Figure

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1). VM involves three features: (i) enhanced malignant cancer cell plasticity characterized by epithelial-to-mesenchymal transition (EMT) and the acquisition of a cancer stem cell-like behavior, both allowing cancer cells to adopt an EC-like phenotype [22-25]; (ii) formation by the EC-like cancer cells of a tubular network rich in ECM proteins (*eg* collagen IV and VI, laminin, proteoglycans, heparan sulfate) (reviewed in [26]); and (iii) connection of the VM network to the surrounding microcirculation resulting in a mosaic endothelial/non-endothelial vasculature [27]. VM occurrence correlates with increased metastasis risk and poor clinical outcome [27], though further study is required to confirm this phenomenon. Finally, in addition to acting like them, cancer stem cells can differentiate into *bona fide* ECs (Figure 1). This vasculogenic phenomenon has been observed in several cancer types, yet its exact clinical impact remains to be determined (reviewed in [28]). The overall impact of the latter two phenomena, including of the role of circulating endothelial progenitors [29] and resident endothelial stem cells [30] in new vessel formation, requires further characterization.

ENDOTHELIAL CELL HETEROGENEITY: NO SUCH THING AS 'THE' ENDOTHELIAL CELL

Trillions of ECs build the vasculature into a functional system that encompasses large vessels (artery and vein) and a widespread network of small branched capillaries [31]. These ECs are not uniform passive building blocks but are characterized by a specific function depending on the organ type. In this section, we discuss some of the most recent molecular insights into EC heterogeneity in physiological and pathological conditions as well as related technological developments.

The subdivision of the functional blood circulatory system into interconnected networks of arteries and veins marks the first level of EC heterogeneity. Arteriovenous specification involves key players such as neuropilins (Nrp), VEGF/VEGF receptor 2 (VEGFR2, see below), Notch and its ligand Delta-like 4 (Dll4, see below), Ephrin B2 and Eph receptor B4 (EphB4, see below) and chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) as reviewed elsewhere [32, 33]. However, with the transcriptional programs driving either artery or vein formation being largely antagonistic, it remained unclear how new arteries can form from pre-existing veins during development. Single-cell RNA sequencing

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(scRNA-seq) and lineage tracing of mouse cardiac ECs isolated from the sinus venosus (a vein known to give rise to coronary arteries) identified a new subset of "pre-artery" ECs [34] based on the fact that these cells have a gene expression profile typical of mature arteries and displayed decreased cell cycling – cell cycle inhibition is crucial for arterial gene expression [35] (Figure 2). In this setting, entry into the pre-artery state only occurs when a threshold in loss of venous identity coupled to a gain in arterial identity is reached. It is the venous marker COUP-TFII that blocks ECs from overcoming this threshold by activating cell cycle genes [34]. Whether these findings apply to artery development in other organs too will be important to clarify.

The above pre-artery ECs were detected at a stage where the plexus had not yet received blood flow – contrary to the notion that arteriovenous remodelling of the plexus occurs after connection with the aorta [34]. Indeed, ECs exposed to high (pulsatile) shear stress induce the expression of arterial markers (GJA5, EFNB2, NRP1) [36, 37] and blood flow activates the NOTCH-GJA4-CDKN1B axis to promote cell cycle arrest and enable arterial gene expression [35]. A study in zebrafish explained how the establishment of venous identity occurs during intersegmental vasculature development. This process involves blood flow-induced high Notch expression in arteries, which hampers vein anastomosis to a pre-existing artery, and subsequent venous transformation [38]. Given the role for blood flow in the above, it is worth mentioning that Kruppel-like factors (KLFs) are important transcriptional regulators and mechanotransducers translating blood flow into EC responses. KLF2 deficient mice die at early stage between embryonic day (E)12.5 and E14.5 due to haemorrhaging and cardiovascular failure [39]. More specifically, EC specific ablation of KLF2 and KLF4 compromises EC identity and vascular integrity [40]. Finally, flow-induced endothelial KLF4 increases expression of the arterial marker GJA5 to maintain arterial endothelial quiescence [41].

EC heterogeneity also pertains to organ-specific vascular beds and intra-vessel heterogeneity between neighboring ECs, allegedly driven by microenvironmental changes and demands. Indeed, bulk profiling of organ-specific ECs from mice revealed a remarkable heterogeneity in their angiocrine profile [42]. This is particularly important in instructing and influencing the homeostasis of healthy tissues (reviewed in [31, 43]). Global transcriptomic analy-

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sis of human fetal heart-, lung-, liver- and kidney-derived ECs shows distinct organ-specific expression profiles, suggesting organ-specific barrier properties, angiogenic potential and metabolic demands (cultured heart ECs have the highest metabolic rate reflected by high glycolytic rate and mitochondrial respiration) [44]. Likewise, bulk transcriptomics signatures of mouse ECs from different tissues revealed tissue-specific EC characteristics [45] (Figure 3A). *CD36* and *FABP4*, encoding transporters for fatty acid (FA) uptake from the circulation, together with their upstream regulating transcription factors TCF15 and MEOX2, are enriched in cardiac (and muscle) ECs [45] (Figure 3B), in line with Meox2/Tcf15 heterodimers programming heart capillary endothelium for cardiac FA uptake [46]. Hence, bulk RNA sequencing generates insights into vascular bed heterogeneity but fails to faithfully and minutely depict intravascular EC heterogeneity, which requires single cell resolution.

A single cell RNA sequencing (scRNA-seq)-based atlas of 20 mouse organs confirms profound differences at the single cell level in overall gene expression and transcription factor expression levels [47]. While ECs were present in this and in other scRNA-seq-based mouse or human tissue atlases [48-57], they were generally too low in abundance to allow comprehensive analysis. However, an EC-dedicated atlas [58] identified a staggering number of different EC subclusters across healthy mouse tissues with the tissue type of origin rather than the vessel type predominantly contributing to the observed EC heterogeneity – in line with previous bulk analyses [42, 59]. Whereas arterial, venous and lymphatic EC markers were found that were shared by most tissues (implying cross-tissue phenotypic conservation of these tissues), capillary ECs exhibited a tissue type-dependent phenotypic variation, in line with their higher degree of plasticity to meet tissue-specific physiological needs. Analysis of metabolic gene signatures revealed inter- as well as intra-tissue EC heterogeneity with heart/muscle ECs, for example, upregulating the expression of distinct sets of metabolic genes involved in lipid uptake/metabolism (Figure 4). This further illustrates the potential of ECs to adapt, also at the metabolic level, to the physiological/metabolic needs and settings of their tissue compartment of origin. Future layering of scRNA-seq data from diseased ECs on top of this atlas, will confirm how EC heterogeneity co-determines disease (see below). Lastly, attempts to unravel single EC heterogeneity in the kidney revealed novel renal EC

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phenotypes [60, 61], identified specific molecular pathways that dictate renal vascular zonation from embryos to adulthood [60], and uncovered EC metabolic adaptations (involving OXPHOS regulation) to acute renal stress such as dehydration [61].

A current limitation of scRNA-seq is the lack of information on intra-tissue localization of each individually sequenced cell. While still in relative infancy, top-notch technologies are arising such as microscopy combined with spatial sequential fluorescence *in situ* hybridization (seqFISH+) [62] and spatial transcriptomics, which spatially resolves expression data at near individual cell size resolution [63]. In the future, these may enable distinction of the transcriptome of each individual single cell and provide information about neighboring surrounding cells to better understand the characteristics of organotypic and intra-organ EC (sub)types and may pave the way for tailored vascular bed-specific therapeutics.

CURRENT LIMITATIONS AND NEEDS OF ANTI-ANGIOGENIC THERAPIES

The initial postulate that growth of solid tumors depends on angiogenesis had set high expectations for the therapeutic potential of AATs, which, due to the importance of the VEGF pathway in developmental angiogenesis and the abundance of VEGF in tumors, were almost exclusively directed towards inhibiting the VEGF pathway [64, 65]. The initial enthusiasm was tempered by observations that such AATs are not uniformly active across all tumor types. Indeed, overall survival (OS) did not increase in prostate cancer, breast cancer, pancreatic adenocarcinoma or melanoma patients. Increases in OS and progression-free survival (PFS), however, do occur in renal cell carcinoma, colorectal carcinoma and hepatocellular carcinoma. However, in the adjuvant (micrometastatic) disease setting, out of 15 recent phase III clinical trials, none showed a significant increase in disease-free or recurrence-free survival (reviewed in [20]). Taken together, current AATs are not overly successful to provide systematic and durable disease control.

Which mechanisms limit the efficacy of AATs? A large body of literature deals with resistance to AATs in preclinical mouse models [64-67]. Anti-angiogenic drugs can induce hypoxia by pruning tumor blood vessels. This then leads to a plethora of escape mechanisms including upregulation of pro-angiogenic cytokines besides the VEGF axis and increased infil-

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tration with resistance-confirming myeloid cells such as myeloid-derived suppressor cells or M2 macrophages, amongst other mechanisms [64-69]. Notably, the upregulation of alternative pro-angiogenic factors including placental growth factor (PlGF) and fibroblast growth factor (FGF) has been detected in cancer patients upon treatment with AATs [69-71] – clinical relevance of strategies tackling this growth factor redundancy is yet to be confirmed [20]. Also, the above described armamentarium of a tumor to secure blood supply without involving angiogenesis (but rather relying on other modes of vascularization) defies the initial dogma and may provide another explanation for the limited efficiency of current AATs. Finally, as described above, ECs in different tissues display distinct phenotypic characteristics (heterogeneity) as do tumor endothelial cells (TECs) in the tumor microenvironment [72, 73]. Dogma claims that anti-VEGF-based AATs hit all TECs indiscriminately, however more recent scRNA-seq-based findings indicate that specifically tip/breach (see further below) TECs were most sensitive to anti-VEGF blockade [73]. Conversely, the pool of immunomodulatory postcapillary (see further below) TECs expanded, which might imply an effect on tumor immunity. Importantly, with tip and proliferating TECs representing only <10% of all TECs in human lung tumors [73], concerns about expected efficiency of current AATs are further justified. The field is only starting to uncover the different TEC subpopulations within and across tumors and the phenotypic peculiarities that determine their responsiveness to AATs. TEC heterogeneity might therefore be a far more prominent issue in AAT resistance than previously anticipated.

In summary, AATs were approved in different cancer entities and many cancer patients have benefitted from this approach. However, the benefit is not durable and efficacy is hampered by multifaceted mechanisms of resistance. Therefore, novel lines of thought are warranted in order to move the field forward. In this respect, we summarized below newly identified as well as conceptually repurposed established key molecules and emerging concepts in both angiogenic and non-angiogenic blood vessel formation.

MOLECULAR DRIVERS OF BLOOD VESSEL FORMATION – 'OLD' *VERSUS* 'NEW'

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The activation of ECs and specification into tip and stalk cells is tightly orchestrated by pro-angiogenic growth factors that are released by hypoxic and nutrient-deprived cells. An extensively studied proangiogenic protein is vascular endothelial growth factor (VEGF). VEGF signaling through EC-expressed VEGF receptors (VEGFRs) induces new blood vessel formation. VEGFR2 activation initiates an intracellular signaling cascade in tip cells that promotes migration and tip cell formation[74]. The VEGF family members [VEGF-A, -B, -C, -D, -E and placental growth factor (PlGF)] and their complex signaling biology have been reviewed [74, 75]. Binding of the tip cell-derived Notch ligand Delta-like 4 (Dll4) to stalk cell-localized Notch and subsequent γ-secretase-mediated release of the Notch intracellular domain (NICD1) prevents stalk cells from acquiring tip cell characteristics by suppression of the VEGF receptor VEGFR2 and the concomitant upregulation of the VEGF trap VEGFR1, a lateral inhibitory mechanism [8]. Rather than reiterating established knowledge, due to space limitations, we aim here to highlight some of the more recent insights on key angiogenesis regulators (restricted to VEGFR2 and Dll4/NOTCH) and to identify new molecular players (focus on Yap/Taz signaling) and mechanisms (EC metabolism) that can become potential targets for novel AAT development.

OLD PLAYERS – NEW TRICKS

VEGFR2: VEGFR2's signaling activity is key in angiogenesis and known to be controlled by post-translational modifications such as acetylation, methylation and ubiquitination [74, 76]. EC-specific deletion of the SUMO endopeptidase SENP1 illustrated the importance of VEGFR2 SUMOylation [77]. ECs with SENP1 deficiency have increased levels of SUMOylated VEGFR2, which aberrantly accumulates in the Golgi rather than at the cell surface, thereby impairing proper VEGFR2 signaling. Hence, deletion of SENP1 in ECs reduces pathological angiogenesis (Figure 5A). VEGFR2 also bears an α(2,6)-linked sialic acid required for VEGF binding. Binding this sialic acid with a *Sambucus nigra* lectin reduces VEGF-induced VEGFR2 signaling and attenuates angiogenesis in *in vitro* and *in vivo* models [78]. Not only at the post-translational but also at the transcriptional level, VEGFR2 is subject to intricate regulation. As such, the focal adhesion kinase (FAK) emerged as a transcriptional (co-)regulator of

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VEGFR2 expression in ECs. Indeed, while FAK was already established as a downstream VEGFR2 signaling mediator (mainly directed towards EC migration) [79], it now became clear that FAK translocates to the nucleus to take part in the RNA polymerase II complex on the VEGFR2 promoter (Figure 5B). Both genetic EC-specific FAK deletion and inactivation of its kinase activity caused angiogenesis defects *in vivo*, implying that this novel mechanism relies on FAK's kinase activity [80]. The cullin3 – speckle type POZ protein – death domain associated protein (CUL3-SPOP-DAXX) axis represents another novel route via which VEGFR2 expression in ECs can be regulated. Via yet to be defined (epi-)genetic mechanisms, DAXX suppresses the expression of VEGFR2 (but also of other key angiogenesis regulators such as Notch1, Dll4 and NRP1). DAXX degradation by the CUL3-SPOP complex reinstalls VEGFR2 expression and consequently angiogenesis [81]. Finally, VEGF-induced intracellular VEGFR2 trafficking from and to the plasma membrane is key to proper signaling. Urokinase plasminogen activator receptor (uPAR) knock-out mice fail to increase the complexity of their retinal vascular plexus upon intraocular administration of VEGF [82]. Analyses revealed that upon VEGF stimulation, endothelial uPAR is crucial to recruit the low-density lipoprotein receptor-related protein 1 (LRP-1) to VEGFR2 in order to induce VEGFR2 internalization (Figure 5C). Ultimately, while long considered a mainly clathrin-mediated phenomenon, VEGF-driven VEGFR2 internalization has now been shown to preferentially occur through CDC42-mediated macropinocytosis [83] (Figure 5D).

<u>*NOTCH1/NICD1-Dll4*</u>: The Dll4-NOTCH1 interaction is at the nexus of vascular patterning during vessel sprouting with optimal NICD1 levels required to ensure normal vascular outgrowth. The deacetylase SIRT1 for example regulates EC NOTCH responses by counteracting acetylation-induced NICD stabilization [84]. To further fine-tune NOTCH1 responses in ECs, NICD1 interacts with the ubiquitin-specific peptidase 10 (USP10), which slows down its rapid ubiquitin-dependent degradation. Mice with an EC-specific deletion of USP10 feature aberrantly increased vessel sprouting [85]. Interestingly, USP10 itself is activated by AMPK [86], possibly linking hypoxia and nutrient scarcity in the avascular tissue to new vessel formation, in order to offer temporal fine-tuning of NICD1 activity. Spatio-temporal fine-tuning of Dll4

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levels too prove crucial in mediating the correct angiogenic response. As such, the multiple PDZ domain protein (MPDZ) interacts with Dll4 to promote interaction with the junction protein nectin-2 and subsequent stabilization of Dll4 at the cell membrane, which increases NOTCH signaling activity [87]. During normal vessel sprouting, Dll4 levels fluctuate asynchronously in individual neighboring ECs. High VEGF and Dll4 expression levels synchronize these dynamics and thereby induce a switch from vessel branching (retraction of the new sprout) to vessel expansion (widening of the pre-existing vessel) [88]. Deletion of the G-protein-coupled receptor-2-interacting protein-1 (GIT1), which is highly expressed in stalk cells during sprouting, increases Dll4 levels in stalk cells and impairs retinal vascular sprouting [89]. Whether this coincides with vessel widening remains unstudied. Mechanistically, GIT1 inhibits Dll4/NOTCH signaling by competing with NICD for binding to the recombination signal binding protein for immunoglobulin kappa J region (RBP-J) that drives Dll4 expression. Functionally, endothelial NOTCH is involved in mechanosensing in arteries [90] and regulates adherens junctions and barrier function. In particular, flow-induced Dll4 endocytosis favors the formation of a non-canonical NOTCH complex at adherens junctions comprising the NOTCH transmembrane domain, the leukocyte antigen-related (LAR) protein tyrosine phosphatase, the RAC1 guanidine-exchange factor TRIO and VE-cadherin. RAC1 is activated by this complex leading to stabilized junctions and improved barrier function [91]. Finally, the classic view on tip/stalk specification via Dll4/NOTCH-driven lateral inhibition (see above and Figure 6A) might need to be expanded to integrate NOTCH's signaling role in *stalk* cells (to prevent excessive tip cell formation and thereby restricting angiogenesis) with its role in inducing artery formation. Studies in zebrafish now show that NOTCH activation in a subset of *tips* (as opposed to the 'classic view' signaling in trailing *stalk* ECs) induces expression of the chemokine receptor cxcr4a, allowing tip ECs to migrate towards and connect to an existing artery (involving the presence of the cxcr4 chemotactic ligand cxcl12 in the artery's vicinity) to establish arterio-venous blood flow [92]. Likewise, in mice, NOTCH activation in tip ECs directs them into developing arteries [93] (Figure 6B). While this suggests zebrafish to mouse conservation for this phenomenon, its possible context-specificity might need further investigation.

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NEW PLAYERS

YAP-TAZ: The Hippo signaling pathway is critical for development by restricting cellular proliferation and controlling organ size [94]. Inhibition of this pathway results in the activation of Yes-associated protein (YAP) and its paralog transcription activator with PDZ binding motif (TAZ), both transcriptional coactivators that regulate the expression of numerous target genes, including cysteine-rich angiogenic inducer 61 (CYR61) and angiopoietin-2 (Ang-2), involved in angiogenesis [95]. This has prompted the field to study the involvement of YAP/TAZ in (sprouting) angiogenesis. VEGF activates YAP/TAZ in ECs and YAP/TAZ deletion impaired VEGFR2 trafficking from the Golgi to the plasma membrane thereby reducing angiogenesis. Mechanistically, endothelial YAP and TAZ run a transcriptional program to ensure cytoskeletal organization for VEGFR2 trafficking (among other processes) [96]. Parallel studies showed that the retinal vasculature in mice with EC-specific YAP/TAZ deletion has blunt-ended tip ECs with fewer and dysmorphic filopodia and is overall hyper-pruned [97]. The proposed mechanism is that YAP/TAZ control CDC42 and MLC2 activity to endow the tip EC with adequate actomyosin contractility required for filopodia formation and migration – the simultaneous sustainment of MYC-driven metabolism particularly serves stalk cell proliferation [97]. Conversely, EC-specific YAP overexpression enhances retinal angiogenesis by favoring the physical interaction between nuclear YAP and the signal transducer and activator of transcription 3 (STAT3) leading to increased expression of the vascular growth factor angiopoietin-2 [98]. Endothelial YAP/TAZ signaling presents a remarkable inter-organ heterogeneity: postnatal YAP/TAZ inactivation in ECs reduces angiogenesis in the retinal vasculature whereas it induces vessel growth in long bones where YAP/TAZ limit HIF1α signaling in ECs. This organ-specificity is presumably brought about by differences in oxygenation/hypoxia levels [99].

EC METABOLISM: EC metabolism is another key regulator of angiogenesis that has been increasingly in the spotlight for the last ten years now and can be of particular value for novel therapy development given that most metabolic enzymes are druggable and EC metabolism increasingly appears to differ from the metabolism in other cell types [100]. In sprouting angi-

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ogenesis, the growth factor-induced 'angiogenic switch' from a quiescent phalanx phenotype to an angiogenic state is co-determined by a 'metabolic switch'. While quiescent ECs mainly use their metabolism to maintain redox homeostasis, angiogenic ECs particularly need efficient ways of biomass and ATP production. Remarkably, with oxygen levels in the bloodstream being high in the mammalian body, glycolysis is the major metabolic pathway in ECs to generate over 85% of their ATP [101, 102]. This, together with other aspects of EC metabolism such as the peculiar use by angiogenic ECs of fatty acid oxidation (FAO) for nucleotide synthesis (in conjunction with the use of other anaplerotic substrates; for more complete explanation, see ref. [103]) and of glutamine for tricarboxylic acid (TCA) cycle anaplerosis and asparagine synthesis, has been reviewed [102].

More recently, the role of pyruvate kinase (PK) in EC metabolism was elucidated. PK catalyses the final step in glycolysis, generating pyruvate and ATP from phosphoenolpyruvate and ADP. Of the different isoforms, ECs express PKM2 almost exclusively over PKM1 and knockdown of PKM2 in ECs reduces proliferation and migration *in vitro* and *in vivo* [104-106]. Loss of PKM2 in proliferating ECs leads to TCA cycle dysfunction and p53-mediated cell growth arrest at multiple points in the cell cycle [105, 106]. Surprisingly, in quiescent ECs, PKM2 is critical to maintain barrier function *in vitro* and *in vivo*, by supressing the NF-κB -mediated induction of Ang-2, a potent inducer of vascular leak [105]. Additionally, PKM2 contributes to delivering the necessary ATP for maintaining optimal VE-cadherin dynamics at EC junctions [107].

With glycolysis being a major energy generating pathway and a mitochondrial volume of a mere 2-5% of their cytoplasmic volume, ECs were considered to only minimally rely on oxidative phosphorylation (OXPHOS). Nonetheless, genetic deletion of a subunit of the respiratory chain complex III in the endothelium shows that interfering with OXPHOS compromises developmental and pathological angiogenesis, due to reduced NAD^+/NADH ratios [108, 109]. Likewise, EC-specific deletion of phosphoglycerate dehydrogenase (PHGDH, key enzyme in serine biosynthesis) depletes the intracellular heme pool causing reduced activity of the heme-containing OXPHOS complexes III and IV and subsequent defective mitochondrial respiration, leading to vascular defects and neonatal lethality [110]. Finally, silencing FAH do-

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main containing protein 1 (FAHD1) in ECs *in vitro* reduces mitochondrial ATP-coupled respiration and leads to senescence [111].

Proliferating ECs rely on FAO for nucleotide synthesis, not by replenishing the TCA cycle with a net contribution of fatty acid carbons via anaplerosis, but by sustaining, in conjunction with anaplerotic substrates, the TCA cycle [103, 112]. Briefly, even though ^{13}C-labelled acetyl-CoA from fatty acids enters the TCA cycle and labels oxaloacetate, there is no net formation of oxaloacetate from fatty acid-derived acetyl-CoA. Rather, under conditions of adequate availability of anaplerotic substrates (glucose, glutamine), FAO generates acetyl-CoA, which helps to sustain the TCA cycle to produce precursors of dNTPs for proliferation. In addition to its role in nucleotide synthesis in proliferating ECs, FAO sustains redox homeostasis in quiescent ECs by sustaining the TCA cycle and production of the intermediates isocitrate and malate, which drive NADPH production through malic enzyme and isocitrate dehydrogenase IDH2 respectively [113]. Remarkably, deletion of 3-oxoacid-CoA-transferase-1 (OXCT1, rate-limiting in ketone body oxidation) in lymphatic ECs (LEC), impairs lymphangiogenesis in development and disease by lowering acetyl-CoA levels and pools of TCA cycle intermediates, dNTP precursors and dNTPs affecting LEC proliferation. Ketone body (KB) supplementation rescues these defects [114]. A ketogenic diet – raising ketone body levels – improves the growth and function of lymphatic vessels and reduces oedema in a pre-clinical mouse model of lymphoedema, opening perspectives for a diet-based treatment for this currently incurable disorder [114]. An ongoing clinical trial will test the possible beneficial effect of a ketogenic diet for lymphoedema patients (NCT03991897 on ClinicalTrials.gov).

ECs are equipped with molecular mechanisms for glutamine and fatty acid uptake from the blood/interstitium, therefore, the expression in ECs of enzymes involved in their *de novo* synthesis – glutamine synthetase (GS) and fatty acid synthase (FASN) respectively – remained enigmatic. Yet loss of GS or FASN in ECs leads to severe angiogenic defects. Unexpectedly, GS interacts with the EC enriched GTPase RHOJ to sustain palmitoylation, membrane localization and activity of RHOJ required for EC migration during sprouting [115]. Endothelial FASN on the other hand is crucial in maintaining optimal levels of its substrate malonyl-CoA – deleting FASN causes cytosolic malonyl-CoA levels to rise leading to post-

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translational malonylation of mTOR, reduction of mTOR's activity and impaired angiogenesis [116].

DIFFERENT GAME, BETTER PLAYERS?

Whereas the above description of novel players and concepts mostly relates to SA, we will discuss recent progress in understanding non-SA related blood vessel formation too, even more so because those co-determine resistance to current AATs [117].

Intussusceptive angiogenesis: Previous findings established that NOTCH inhibition (in pre-existing vascular beds) can induce IA by recruitment and extravasation of mononuclear cells, which help build and stabilize transluminal pillars [118]. By combining *in vivo* and *in vitro* approaches, this model has now been further refined: NOTCH inhibition induces endothelial stromal cell-derived factor 1 (SDF1) expression to interact with CXCR4 on mononuclear cells to promote their recruitment [119]. Ephrin B2/EphB4 signaling, otherwise known to be critical for arteriovenous differentiation [120], mediates VEGF-induced IA too. By modulating VEGFR2 signaling, EphB4 keeps VEGF-induced EC proliferation necessary for circumferential vessel enlargement in check in order to allow IA [121]; a similar role was attributed to platelet-derived growth factor-BB (PDGF-BB) [122]. Of note, COUP-TFII, another molecule involved in arteriovenous differentiation [123], also induces IA [124]. Most recently, targeting MT1-MMP in ECs was found to reduce inflammation-driven IA and to ameliorate colitis in a pre-clinical model for inflammatory bowel disease [125]. However, an unbiased global analysis of possible molecular mediators of IA has not been performed in sufficient detail.

Vessel cooption (VC): Despite increasing evidence showing the importance of VC in solid tumors, the molecular and cellular mechanisms driving this phenomenon remain poorly understood. Indeed, whether the endothelium runs specific (signalling) programs to elicit VC or merely passively undergoes being hijacked by cancer cells, remains incompletely characterized – as do the key endothelial molecular players involved [20]. Given that cancer cells need to be motile to find a target vessel in normal tissue, identified key players at the side of the cancer cells are, not entirely unexpected, mostly involved in cellular motility such as

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the actin-related protein 2/3 (ARP2/3), silencing of which suppresses co-option in a liver metastasis model in mice [126]. Remarkably, in a model of breast cancer metastasis to the brain, coopted vessels have an elliptical shape and an unusually small lumen – indicative of being mechanically compressed by the cancer cells. This renders the coopted vessel dysfunctional, causes hypoxia and instigates secondary angiogenesis [127]. Although it is currently unclear whether and to which extent this phenomenon occurs across different cancer types, it can have clear implications for VC-oriented therapies. Conceptually, VC probably brings more to cancer cells than only nutrients and oxygen. It is not unthinkable that the perivascular niche (PVN) is actually what cancer cells are after (with supply of oxygen and nutrients as a surplus). The PVN features (i) high local levels of angiocrine factors that can act as trophogens for cancer cells [128], (ii) EC- and BM-derived factors that favor cancer cell stemness and control cancer cell dormancy [129, 130], and (iii) immune escape [131]. Whether vessel-coopted TECs differ from angiogenic TECs, and whether peri-vascular cells co-regulate the vessel coopted TECs requires further work. Single cell studies might help to shed light onto these outstanding issues.

Vascular mimicry: Given the growing evidence that with the appearance of VM, patients' survival chances plummet, efforts have been undertaken to identify molecular drivers and stimuli in the formation of these ersatz tumor vessels – as reviewed elsewhere [27, 132]. Additionally, interleukin 33 (IL-33), which is commonly detected in the tumor microenvironment, was hypothesized to promote VM in melanoma. This was corroborated by elevated expression levels of the IL-33 receptor ST2 in melanoma patient samples. Mechanistically, via ERK1/2 phosphorylation, IL-33 instigates expression of matrix metalloproteinase (MMP)-2/9, which have been previously involved in VM formation [132, 133]. In hepatocellular carcinoma (HCC), cytoplasmic expression of lysyl oxidase-like 2 (LOXL2) correlates with VM and poor patient prognosis [134]. Interestingly, LOXL2 has a role in establishing a functional EC-built vasculature by remodelling the ECM [135] – its role in VM though seems intertwined with the expression of the epithelial-to-mesenchymal (EMT) transcription factor Snail and of VE-cadherin [134]. While VM conduits were previously dubbed the tumor's "do-it-yourself' blood

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vessels [136], it now became clear that cancer cells call in the help of pericytes to support their VM network. Apparently, they use PDGF-B to instruct pericytes, as blocking PDGF-B signalling reduces VM and tumor growth in mouse models [137]. Along the same lines, the concept of VM was expanded by the observation that not only cancer cells but also tumor-associated macrophages can form perfused VM channels [138]. A phase II clinical trial testing the combined effect of Sorafenib and CVM-1118 (a small molecule targeting VM by curbing the activity of Nodal (a known driver of VM [132])) is currently ongoing (NCT03582618 on ClinicalTrials.gov).

EC HETEROGENEITY AS DISEASE MODIFIER?

In solid tumors, blood vessels are characterized by an abnormal structure and heterogeneity, but the characterization of individual EC subtypes is missing. Single cell analysis of a xenograft colorectal cancer model clustered into three distinct subpopulations named tip-like, stalk-like and transition ECs [72]. The latter allegedly represented a population at a stage in-between those two phenotypes, although no validation or further characterization was performed. In human control *versus* malignant lung tissue (without prior EC enrichment), 5 endothelial clusters were identified: lymphatic ECs, two tumor-derived blood EC clusters and two others deriving predominantly from non-malignant lung [139]. Tumor *versus* normal ECs comparison revealed overexpression of Myc targets and nucleotide metabolism (purine and pyrimidine biosynthesis), while genes involved in immune activation and immune cell homing were downregulated in tumor ECs [139]. These intriguing results may indicate a hyperproliferative phenotype of TECs (also shown in mouse TECs [140]) as well as (unexpected) EC subtype-specific contribution to tumor immuno-tolerance.

Remarkably, a more recent scRNA-seq analysis of normal (peritumoral) ECs and TECs from human non-small-cell lung carcinoma (NSCLC) patients and mouse lung cancer models found only a small fraction of TECs to represent the traditional angiogenic phenotypes (proliferative and tip cell) [73]. TEC subpopulations with a transcriptomic signature reminiscent of immune cell recruitment (TECs expressing marker genes shared with high endothelial venules (HEVs - involved in leukocyte trafficking and correlating with good prognosis in several

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cancers [141]) or semi-professional antigen presentation were newly discovered. The tip TEC phenotype correlated with patient survival and was further broken down into known migratory tip ECs and putative BM 'breaching' ECs (opening up the BM to allow invasive sprouting) [73]. These findings suggest a far more complex TEC landscape than initially anticipated and provide several novel insights to be further explored in AAT research. Another unique finding is that only the tip TEC phenotype was conserved across species (mouse and human) and models (cultured and freshly isolated). An integrated meta-analysis with proteomics and transcriptomics from human cancer patients allowed the establishment of conserved tip cell markers in tumors, which could be highly valuable to find new alternative – and more effective – anti-angiogenic targets [73].

CURRENT LIMITATIONS AND NEEDS OF PRO-ANGIOGENIC THERAPIES

Pathologies such as coronary artery disease, limb ischemia, myocardial infarction and ischemic stroke feature hampered perfusion, causing the affected tissue to suffer from oxygen shortage and metabolic waste accumulation. Also, chronic exposure to hyperlipidemia and diabetic hyperglycemia promotes EC dysfunction, contributing to the morbidity and mortality of diseases such as atherosclerosis. While this damage has been proposed to be repaired by locally derived ECs and circulating endothelial progenitor cells (EPCs; their exact role and defining characteristics remain subject of debate [142]), this is a slow (weeks to months) rather inefficient process – re-endothelialization often remains incomplete and even upon complete restoration, EC function might remain impaired [143]. In addition, the severe disparity between demand and supply of transplantable organs drives the regenerative medicine field to *in vitro* generate 'mini-organs' or 'organoids' for transplantation purposes. However, establishing a functional, organ type-specific vasculature in such organoids – the key to successful in-host perfusion and survival – remains a formidable challenge. There is thus an unmet medical need for tailored and efficient pro-angiogenic strategies.

LOCAL ADMINISTRATION OF PRO-ANGIOGENIC AGENTS

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A battery of locally administered pro-angiogenic strategies have been developed to alleviate ischemic damage and/or to replace dysfunctional, damaged endothelium [144]. In general, three main lines of pro-angiogenic therapy can be distinguished based on the agent being administered. Most common is the delivery of therapeutic molecules such as growth factors and cytokines (*eg* VEGF, FGF2, TGF-ß, PlGF). Accurate *in situ* dosing problems (overdosing *versus* short half-life of the administered proteins), poor local retention and high cost to produce the necessary amounts of growth factor currently limit these approaches [145]. This is probably why the extensive testing *in vitro* and in preclinical models is not matched (yet) by clinical data. Local administration (both plasmid-based and viral) of genes rather than recombinant pro-angiogenic proteins partially circumvents the local retention and half-life issues and has been explored in clinical trials mostly involving genes encoding VEGF (isoforms) but suffers from obtaining adequate levels over appropriate time periods [144].

While (combinations of) single genes/proteins fall short in capturing whole-cell signalling and cross-talk events, a second approach entails local administration of a relevant cell type to the ischemic tissue site. Cell types to be used are not limited to ECs and EPCs but also include mononuclear cells and bone marrow- and adipose-derived stem cells. An added value of using cells as a pro-angiogenic therapy is the option to pre-condition the cells (*eg* hypoxia priming to increase local, in-tissue survival) or to genetically modify them (though the potential adverse effects of such approach need to be carefully examined). Having single cells first aggregate in 3D spheroids might offer advantages in local retention and attachment after administration. An overview of phase I-III clinical trials involving cell delivery in ischemic disorders and the observed outcome is summarized elsewhere [144].

Finally, local administration not of cells but rather of their secretome is being explored – either as standardisable conditioned medium or as extracellular vesicles (EVs, comprising exosomes and microvesicles (ranging from 40 nm to 1 µm in size)). The cargo of EVs might not be fully characterized but they are known to carry proteins, nucleic acids, lipids, membrane associated channels and receptors [146, 147]. The lipid bilayer of EVs is an advantage in terms of protecting the cargo from (proteolytic) degradation. EPC-derived exosomes have been shown in *in vitro* and in preclinical models to enhance angiogenic behavior of ECs in an

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ERK1/2 signalling-dependent manner [148] and to alter EC expression of angiogenesis modulators like for example FGF1, VEGFR-2, ANG-1 and eNOS [149]. In a rat model of myocardial ischaemia, sustained delivery of EPC-secreted EVs via an injectable hydrogel system ameliorates peri-infarct remodelling by improving haemodynamics and increasing angiogenesis and scar recovery [150]. While these preclinical findings are promising, they await clinical validation. A phase I clinical trial using mesenchymal stromal cell-derived EVs in ischemic stroke examining functional recovery and enhanced neurogenesis and angiogenesis, is currently listed as NCT03384433 at www.clinicaltrials.gov.

WHAT ABOUT THE FORCE FROM WITHIN?

Specialized EC subtypes can be found in adult stem cell niches where they control the stem cells' behavior towards tissue regeneration [151]. Whether the vasculature contains stem or progenitor cells for repair/regeneration is less well established. In an *in vivo* model of aortic endothelial injury, regeneration of the EC lining initiates from resident quiescent ECs flanking the injury, which proliferate to regenerate the denuded area [152]. Performing these assays in Rainbow mice (allowing multi-color clonal tracing) revealed that this phenomenon is biphasic and involves at least two EC populations with different proliferative activity. The observed regeneration did not rely on a contribution of circulating cells or cells from a non-endothelial lineage – a matter of continued controversy. This phenomenon likely does not depend on (sprouting) angiogenesis-inducing factors but might rely on junction disruption following the initial EC injury [153]. This could cause transcription factors that are connected to adherens junctions (*eg* FosL2 and Hippo pathway members) to instigate a transcriptional program for growth and migration [152, 153]. Anyhow, (sc)RNA sequencing revealed that induction of stress response genes such as ATF3 is required to unravel the molecular signature of endothelial regeneration [152].

Of note, ECs lining blood vessels have been discovered to originate from an additional source next to their known developmental path from the mesoderm. Indeed, in the developing mouse embryo, erythro-myeloid progenitors (EMPs) derive from the endothelium lining the yolk sac, travel into the embryo and revert back to their initial EC fate to get inter-

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spersed into the otherwise mesoderm-derived endothelium of blood vessels in different organs (up to 60% in the liver) [154]. This dual-origin, mosaic build-up of vessels – which was maintained into adulthood – might explain why in the above described regeneration after injury, both ECs with high and low proliferative capacity have been observed in the same vessel [155].

While these findings point to the existence of resident endothelial stem/progenitor cells within the vascular wall implying vascular regeneration potential in (peripheral) blood vessels, they beg the question for further characterization and eventually for use in regenerative medicine. As such, bone marrow stromal antigen-1 (BST1, also named CD157) was described as a cell surface marker for a liver endothelial side-cell population that self-renews and is able to regenerate vascular structures upon vascular injury. Subsequent immunostainings showed CD157 expression in ECs of (large) arteries and veins in different mouse tissues [30]. Finally, transplanted $CD157^{+}$ liver ECs were incorporated into functional vessels – underscoring their translational potential [30].

Targeting the endothelium could be a way forward for bone regeneration as well. A particular subset of $CD31^{hi}$ endomucin $(EMCN)^{hi}$ endothelium resides in the bone marrow near the growth plate [156] and is controlled by PDGF-BB, possibly linking this $CD31^{hi}$ $EMCN^{hi}$ endothelium to osteoclasts' resorptive action [157]. Osteoblasts cross-talk to this EC subpopulation to incite the microvasculature to initiate bone formation – the signal sent by osteoblasts was identified as the axon guidance cue Slit homolog R protein (SLIT3) with the roundabout homolog ROBO1 being its main receptor at the endothelial side [158]. Strikingly, systemic and local administration of SLIT3 enhanced bone fracture healing in mice (without affecting vascular morphology in other tissues) and i.v. injection of recombinant SLIT3 in the mouse ovariectomy model of postmenopausal osteoporosis performed as well as parathyroid hormone in reversing bone-loss effects [158].

VASCULARIZATION OF TISSUE CONSTRUCTS AND ORGANOIDS FOR REGENERATIVE MEDICINE

Regenerative medicine seeks to deploy (engineered) tissue-based transplant strategies for the treatment of (whole) organ dysfunction, yet they face challenges in terms of correct

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spatial and timely development of an organ type-specific vasculature in the transplantable tissue – a prerequisite for efficient engraftment [159]. Indeed, much like a growing solid tumor, above a certain size, bio-printed tissue constructs and (stem cell-derived) organoids (see below) defy the limits of simple diffusion of oxygen and nutrients, resulting in a necrotic core [160]. In bio-printing of tissue constructs, several strategies have been developed to *ab initio* provide the tissue construct with a tubular network to accommodate a vasculature. A cell-laden matrix can for example be printed onto a sacrificial filament lattice, which after being dissolved leaves perfusable hollow channels into which ECs can be seeded to form a vascular network (reviewed in [160, 161]). Several shortcomings and uncertainties come with these approaches. The network topology is pre-defined and somewhat rigid, implying it cannot dynamically respond to changes in oxygen consumption and tissue growth. Oxygen supply occurs at the level of the microcirculation (capillaries) with µm scale diameter, dimensions that are difficult to realize with current techniques. Finally, which type of ECs is best suited to seed into the network voids to in the end provide the much-required organ-type specific characteristics (see above under heterogeneity)?

Organoids are 3D-organized cell clusters harbouring multiple organ-specific cell types that resemble the tissue by function and/or morphology. Even though organoids have been generated for different tissues, the flow-chart seldom differs: a sequence of stimuli recapitulating the normal developmental pathways of a given tissue are administered to a stem cell population *in vitro* to drive differentiation towards that tissue. Given that they resemble the target tissue (the different organ-specific cell types are mostly intrinsically present in an organoid), organoids hold promise for regenerative medicine for therapy screening and transplantation. Even for drug screening and disease modelling purposes, organoids need a perfused vasculature. Some nephrotoxic drugs for example – with AATs being among the most common culprits – cause glomerulopathy by affecting endothelial-podocyte interaction [162]. A functionally vascularized kidney organoid (*ie* featuring a glomerular filtration unit) would then more faithfully allow screening for nephrotoxicity. However, it is incompletely understood whether organoids from different organs require different vascular signals to become adequately vascularized. Charting transcriptome signatures of each organ during

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development, health and disease to identify such tissue-specific drivers will be essential yet challenging. The impact of tissue-specificity in this context is further illustrated by the finding that in 3D co-cultures of ECs with distal lung stem cells, the tissue-origin of the ECs co-defines the differentiation outcome: in comparison with primary lung ECs, co-culturing with primary liver ECs increased bronchiolar and reduced alveolar colony formation [163].

Towards transplantation, scalability is an issue as, already mentioned above, bringing organoids *in vitro* to a size amenable for transplantation would require a perfused vasculature. Transplanting kidney organoids in mice promotes the formation of a perfused vasculature and the increased epithelial maturation with vascularized glomerular structures whereas time-matched non-transplanted organoids remained disorganized and immature [164]. These findings show that blood flow through a perfused vascular network is key to scale up organoids to transplantable size. Given that small animal models are not the preferred platform for upscaling, several *in vitro* microfluidic devices are used in which purified ECs are being flushed into open cast channels where – induced by the microfluidic flow – these ECs will adhere to the channel wall to form patent surrogate vessels. A disadvantage is that capillary dimensions are difficult to obtain and (large numbers of) purified EC sources are needed [159].

In contrast, human pluripotent stem cell (hPSC)-derived kidney organoids undergoing flow in millifluidic chips show enhanced vascularization and maturation of nephron components when compared to static controls [165]. The vasculature features perfusable lumen and is covered by mural cells (Figure 7). The vasculature formed under flow originates from an expansion of the endogenous endothelial progenitor cell pool. Strikingly, in this setting, addition of 'exogenous' human glomerular microvascular endothelial cells (GMEC) or human umbilical venous endothelial cells (HUVEC) did not enhance vascularization. The in-organoid vascular-tubular interactions depend on balanced endogenous VEGF signalling as addition of both VEGF or a VEGF inhibitor (bevacizumab) to the flow medium reverts these interactions to the level observed in the static counterparts [165]. Which other vessel formation promoting molecules are involved, is an outstanding question.

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While the above describes efforts and achievements in promoting intra-organoid vascularization, the formation of self-organizing 3D human blood vessel organoids directly from human pluripotent stem cells has now also been further established. Treatment of stem cell aggregates with a defined sequence of stimuli and growth factors (including the glycogen synthase kinase 3 inhibitor CHIR99021, bone morphogenetic protein (BMP), VEGFA and FGF2) leads to the formation of organoids existing of a network of $CD31^+$ ECs and PDG-FRß$^+$ pericytes enveloped by a BM, and minor mesenchymal stem-like and hematopoietic cell populations. When transplanted into mice, these blood vessel organoids turn into a perfused vascular tree [166]. The blood vessel organoids proved valuable as an *in vitro* model to study diabetic microvasculopathy, still a largely unknown yet important phenomenon in light of the worldwide increasing prevalence of diabetes. As such, Dll4-NOTCH3 signalling was identified as implicated in the pathomechanism underlying this diabetic complication [166]. It goes without saying that blood vessel organoids will also need to be explored as study tools and therapeutic platform for other diseases with a vasculopathy component.

CONSIDERATIONS AND PERSPECTIVES

Blood vessel homeostasis is a complex multi-player game by itself. Additional layers of complexity are added in disease conditions where the vasculature either excessively yet abnormally grows, gets hijacked by cancer cells or becomes dysfunctional. On top is the extensive heterogeneity in ECs – probably more imperative than previously anticipated. On the other hand, current vascular-oriented therapies, pro- or anti-angiogenic, to some extent fail to capture this complexity causing them to not reach full and expected efficacy. With this review, we have tried to highlight some of the striking new (molecular) players and concepts in blood vessel formation and maintenance, some of which bear potential to be explored as therapeutic targets. Still, a number of considerations and perspectives should be discussed.

As well thought-out a novel therapy is, if it does not reach its target – in this case the EC – or affects other tissues in addition to its primary target, efficiency will be low and toxicity will be an issue. Furthermore, should all ECs be targeted indiscriminately? Much alike healthy endothelium, diseased or dysfunctional endothelium can be heterogeneous in na-

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ture too (see above). Single-cell profiling techniques are revealing this EC heterogeneity in pathological conditions at a remarkable pace [58, 61, 72, 73, 139], yet more power is currently required to exhaustively chart all EC subpopulations in diseased *versus* healthy tissues, and across different tissues to determine which EC phenotypes are congruent between tissues and which are not. Increasingly powerful single cell approaches will enable listing of highest ranking marker genes/proteins per EC subpopulation – preferably targetable membrane associated proteins – which will allow therapeutic targeting of one specific EC subpopulation over the other. Inevitably, this will lead to the question of 'pruning *versus* tuning' where an educated decision will need to be taken to either totally ablate a disease propelling EC population (prune) or to enhance disease fighting capacities in other EC populations (tune). This decision will need to be made on a per disease, or even on a per patient, basis eventually requiring a high degree of versatility in generating the tools to efficiently target populations of choice. The recently developed self-assembly platform (ASSET) for the production of antibody-coupled cargo-delivering lipid-based nanoparticles (LNPs) might deliver such versatility as it circumvents problems related to chemical conjugation [167].

The concept of EC heterogeneity within and between (diseased) organs summits at the level of the individual patient and pertains to striving for 'personalized medicine'. It is highly likely that the proportional contribution of specific EC subpopulations within the abnormalized vasculature of the same tumor type differs between patients. It remains to be determined to which extent this can be explored as a prognostic marker for therapy outcome. Along the same lines, the newly identified molecular players in alternative vascularization modes such as cooption and vascular mimicry (see above) could be of potential value as biomarkers for the development of novel AATs.

Finally, the endothelium is increasingly being considered a key, non-hematopoietic component of the immune system [168]. Moreover, the tumor vasculature is a major barrier for lymphocytes as it upregulates PD-L1, downregulates the lymphocyte adhesion molecules ICAM-1 and VCAM-1 and expresses the death mediator FasL (killing CD8+ T effector cells), all together hindering tumor-reactive T cell activation and infiltration into the tumor and fostering an immunosuppressive environment that allows tumors to evade host immunosur-

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veillance [169]. In light of immunotherapy being highly promising in cancer treatment yet facing resistance issues, the role of the endothelium therein should also be further explored and exploited.

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DISCLOSURES

None

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FIGURE LEGENDS

Figure 1: Vascularization of tissues/tumors comes in different flavors. Apart from sprouting angiogenesis (upper left) where in response to pro-angiogenic factors ECs in a pre-existing vessel specify into proliferating stalk cells and migrating tip cells to elongate and spatially guide the nascent sprout respectively, other modes of vascularization can take place. Intussusceptive angiogenesis (upper right) features the formation of hollow transcapillary pillars which grow in circumference and eventually split the vessel in two parallel capillaries – hence the name 'splitting angiogenesis'. In vessel cooption (middle left), cancer cells surround and literally hijack the pre-existing vasculature of normal tissue to incorporate the vessels into the tumor for blood supply. Additionally, cancer cells themselves can start forming a non-endothelial blood irrigation system, a phenomenon dubbed vascular mimicry (lower right) or transdifferentiate into ECs (lower left).

Figure 2: Cardiac blood vessel specification. Simplified scheme showing arterial *versus* venous specification in cardiac vessels as an example of EC heterogeneity. During development, the venous transcription factor COUP-TFII controls the specification from venous ECs into pre-artery ECs which eventually segregates the plexus into veins and arteries. See main text for details.

Figure 3: Inter-organ and intra-vessel heterogeneity. A) To support their highly specific function, different organs impose different demands on their vasculature leading to a high degree of EC heterogeneity (for more details see main text). With the advent of increasingly more powerful (single-cell) analyses techniques, molecular characterization of such heterogeneity is rapidly expanding. Fictitious principal component analysis (PCA) shows how (transcriptomic) markers cluster in an organ-specific manner. B) In comparison with ECs form other tissues, cardiac ECs express higher levels of CD36 and FABP4 together with their upstream transcriptional regulators TCF15 and MEOX2 to prime the heart capillary endothelium for cardiac fatty acid uptake.

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Figure 4: Tissue-specific metabolic wiring in ECs. Schematic overview of how ECs present differential metabolic wiring in relation to their tissue of origin according to scRNA-seq data. For testis and intestine, the same metabolic characteristics hold as for brain and heart/muscle respectively, albeit to a lesser extent. EDL, extensor digitorum longus.

Figure 5: New adventures in VEGFR2 regulation. A) The SUMO endopeptidase SENP1 keeps VEGFR2 in a de-SUMOylated state, allowing normal VEGFR2 ER/Golgi to plasma membrane trafficking and angiogenic signaling. In diabetic hyperglycemia SENP1 activity is reduced, leading to VEGFR2 hyper-sumoylation, Golgi accumulation and impaired VEGFR2 signaling. B) The focal adhesion kinase (FAK), otherwise known as a downstream VEGFR2 signaling mediator, translocates to the nucleus to participate in the RNA polymerase II complex on the VEGFR2 promoter. C) Endothelial urokinase plasminogen activator receptor (uPAR) recruits the low-density lipoprotein receptor-related protein 1 (LRP-1) to VEGFR2 in order to induce VEGFR2 internalization and angiogenic signaling. D) Upon VEGF stimulation, VEGFR2 internalization occurs preferentially by macropinocytosis next to the long established clathrin-mediated way. Question mark indicates unknown player. ß1, ß1 integrin.

Figure 6: Connecting tip cells with arterial circulation via Notch: A) The current model of sprouting angiogenesis in which tip cell-derived Dll4 binds NOTCH in stalk cells to prevent overproduction of tip cells through a lateral inhibitory mechanism. B) Extended model incorporating the role of NOTCH in artery formation illustrating that following Dll4 expression in tip cells, stalks cells start expressing Dll4 too. Consequently, first tip and later stalk cells undergo NOTCH activation. NOTCH activation in tip cells induces cxcr4a expression allowing migration to and ultimately connection with a pre-existing artery.

Figure 7: Creating vasculature with flow. Embedding hPSC-derived kidney organoids in extracellular matrix and subjecting them to flow in a millifluidic device causes the endogenous endothelial progenitor cell pool in the organoid to expand and to form a vasculature with perfusable lumens and mural cell coverage.

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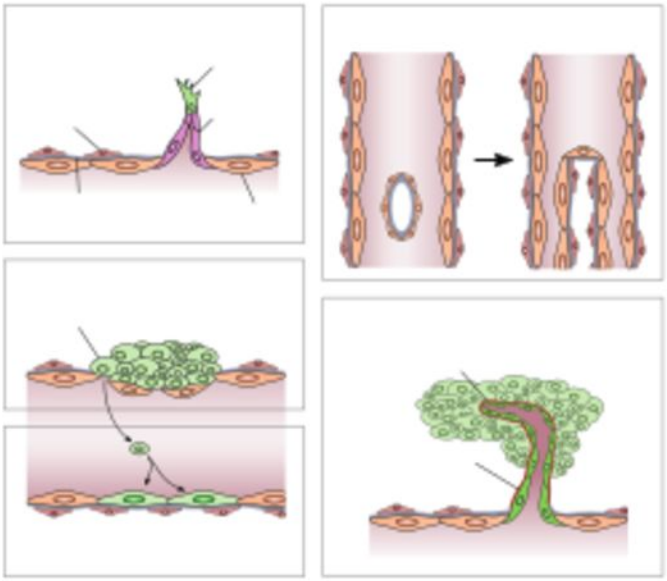
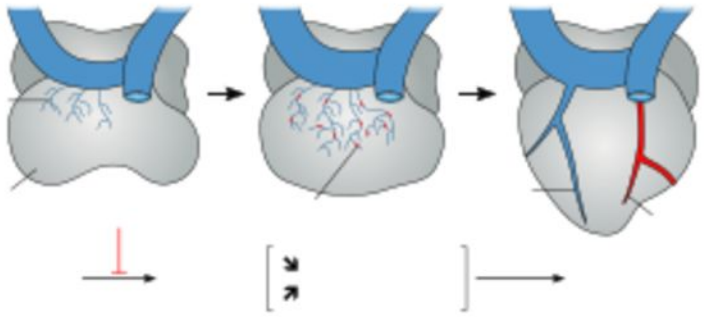
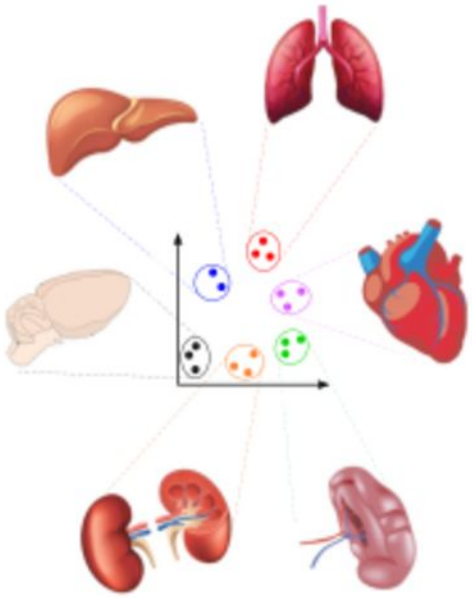
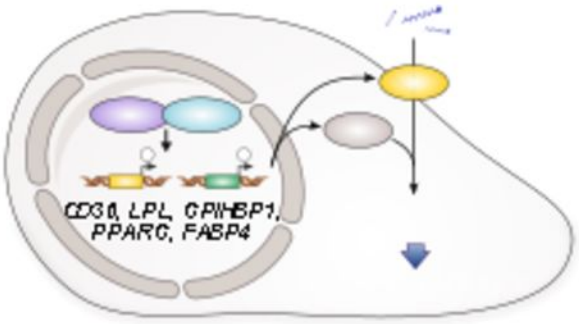
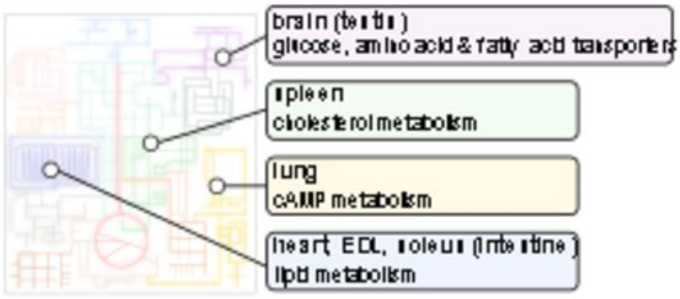







CD36, LPL, GPIHBP1,
PPARG, FABP4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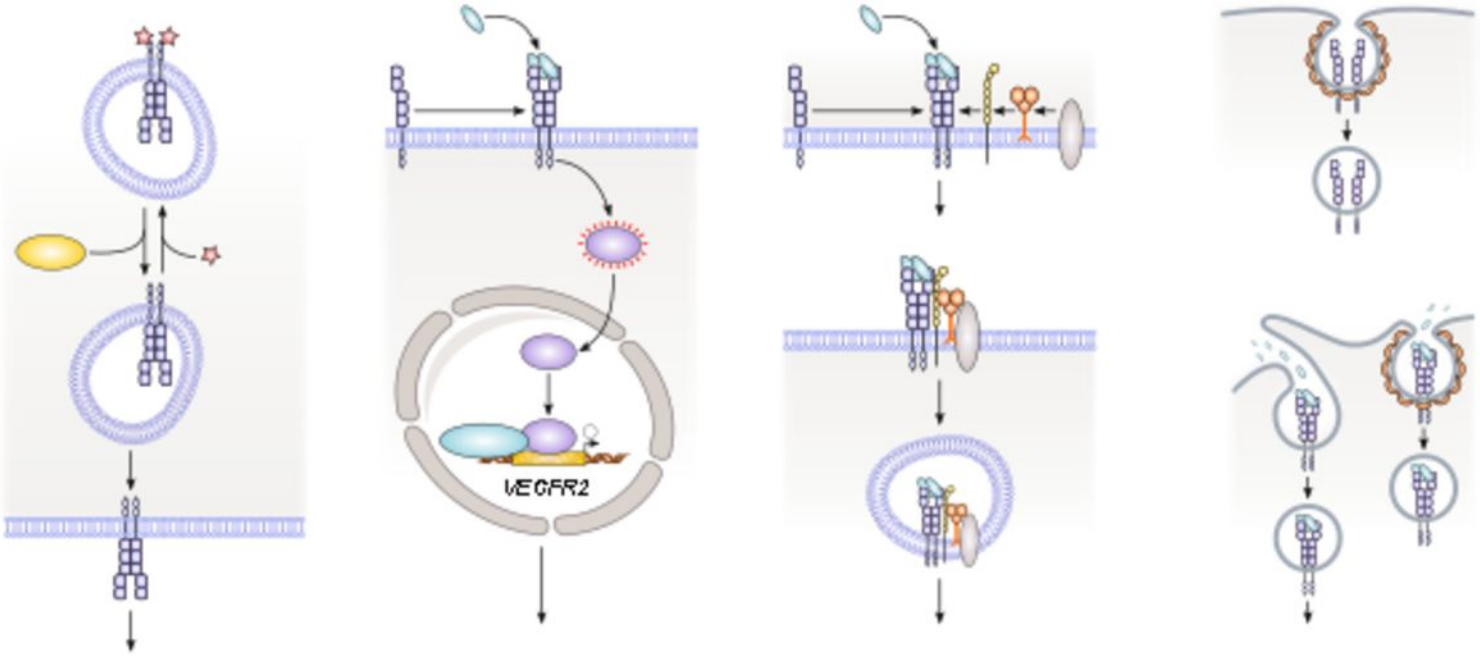
glucose, amino acid & fatty acid transporters
spleen
cholesterol metabolism
lung
cAMP metabolism
heart, EDL, soleus (intestine)
lipid metabolism


VEGFR2

