Deciphering endothelial heterogeneity in health and disease at single cell resolution: progress and perspectives

DECIPHERING ENDOTHELIAL HETEROGENEITY IN HEALTH AND DISEASE AT SINGLE CELL RESOLUTION: PROGRESS AND PERSPECTIVES

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ABSTRACT

Endothelial cells (ECs) constitute the inner lining of vascular beds in mammals and are crucial for homeostatic regulation of blood vessel physiology, but also play a key role in pathogenesis of many diseases, thereby representing realistic therapeutic targets. However, it has become evident that ECs are heterogeneous, encompassing several subtypes with distinct functions, which makes EC targeting and modulation in the disease-context challenging. The rise of the new single cell era has led to an emergence of studies aimed at interrogating transcriptome diversity along the vascular tree, and has revolutionized our understanding of EC heterogeneity from both a physiological and pathophysiological context. Here, we discuss recent landmark studies aimed at teasing apart the heterogeneous nature of ECs. We cover driving (epi)genetic, transcriptomic and metabolic forces underlying EC heterogeneity in health and disease, as well as current strategies used to combat disease-enriched EC phenotypes, and propose strategies to transcend largely descriptive heterogeneity towards prioritization and functional validation of therapeutically targetable drivers of EC diversity. Lastly, we provide an overview of the most recent advances and hurdles in single EC OMICs.

INTRODUCTION

Endothelial cells (ECs) line the interior surface of blood and lymph vessels. The endothelium plays a crucial role in maintaining tissue homeostasis in normal health[1], but also contributes to the progression of many diseases[2]. ECs respond to various physical and chemical stimuli and interact with other cells in the vessel wall, such as smooth muscle cells or pericytes, to regulate vascular tone, blood flow, inflammation, permeability of solutes, and cellular adhesion[1]. Blood vessel overgrowth promotes diseases like cancer[3], while EC dysfunction contributes to vascular complications in diabetes, cardiovascular disease, and ageing-associated pathologies (including neurological diseases with a vascular component, such as Alzheimer's disease[4]). Hence, understanding the basic function and dysfunction of the endothelium in health and disease has broad reaching implications.

Despite their common characteristics[5], ECs are heterogeneous under physiological and disease conditions[6-8] (see Box 1 for definition of heterogeneity). Whilst they are present throughout the whole body, ECs are highly specialised to meet the distinct needs of the organs and sites they reside in. Within each organ, this heterogeneity is evident between different vascular beds (arteries, veins, capillaries, lymphatics), between different segments of the same vessel type, and even between neighbouring ECs[8]. EC phenotypes in disease are equally diverse, exemplified by their ability to activate or inhibit angiogenesis, metabolic switching or the release of vasodilators, reflecting varying responses to different stimuli and changes in the pathological microenvironment[3]. While EC heterogeneity was highlighted in other reviews[6-8], recent advances in single cell technologies brought new resolution and new insights into this heterogeneity. Characterising the different heterogeneity levels and their functional relevance is crucial[5], albeit dependent on the technologies used to measure and quantify heterogeneity[9].

In this review, after a brief historical perspective on the methods used to study EC heterogeneity, we will focus on novel discoveries regarding EC heterogeneity in health, disease and under therapeutic intervention, made based on single-cell OMICs, and discuss the current challenges and perspectives in the field. Rather than providing an all-encompassing overview, we discuss key principles and examples.

HISTORICAL PERSPECTIVE ON METHODS TO UNRAVEL EC HETEROGENEITY

Prior to the advent of single-cell technologies, various *in situ* and *in vivo* methods were developed to identify organ- and vessel type-specific endothelial markers whilst circumventing difficulties faced in isolating pure populations of ECs from tissues and the loss of *in vivo* phenotype of ECs cultured *in vitro* (reviewed in[10-12]). The concept that ECs from different organs and vascular beds express different molecular markers was fueled by early evidence of cancer and immune cells, preferentially migrating to specific organs – likewise, peptides with a particular sequence homed to specific vascular beds[13-16]. For instance, the Stamper-Woodruff assay was designed to study lymphocyte-endothelial binding in lymph nodes[13], later modified for use in other tissues[14,15], and alongside an emerging monoclonal antibody technology, led to the identification of L-selectin as the receptor responsible for selective homing of lymphocytes to high endothelial venules (HEVs) in lymph nodes[16].

Phage display peptide libraries were used to unbiasedly screen peptide sequences that home to particular organs[17] or vascular beds *in vivo*[18]. These approaches, as well as SAGE analysis[19,20] and microarrays[21-23], contributed to the mapping of endothelial markers across different organs and vascular beds within organs[24-30], the development of tissue-targeted pharmacodelivery[18,31,32], and to increasing our understanding of baseline EC phenotypes in different organs[33]. However, these methods suffer from relatively low throughput and

parallel processing capabilities, and some of these strategies also require prior knowledge of the cellular states and markers of the subpopulations of interest, limiting their use in identifying novel EC subtypes. In addition, these techniques allow us to study EC heterogeneity only at the bulk, not at the single cell, level. Hence, the advance in single cell technologies has had unparalleled influence on the study of ECs.

SINGLE-CELL STUDIES IN ENDOTHELIAL CELLS

Our characterisation and understanding of EC heterogeneity has advanced considerably in the past years, due to the development of single-cell OMICs approaches (Figure 1A). These techniques offer simultaneous analysis of hundreds to thousands of cells from complex samples, such as tissues and heterogeneous cell populations, often without any prior knowledge of cell markers[34]. In particular, single-cell transcriptomics have been used to identify and study EC populations in health and disease, across virtually all stages of life (e.g. development, adulthood, aging), as detailed hereafter. While initial studies often described a single organ in healthy condition[35-40], recent studies now provide multi-organ analysis[41-44] or focus on a specific disease, allowing to study ECs in physiological and pathological conditions. Most single-cell RNA-sequencing (scRNA-seq) experiments relied on a droplet-based approach, with the majority using the 3' end sequencing Chromium 10X technology. The main characteristics and advantages of the two major scRNA-seq platforms (10X Genomics and Smart-Seq) are described in Figure 1B, and more details on these technologies and applications can be found in several reviews[45,46]. Some scRNA-seq studies were also accompanied by single-cell ATAC-seq (Assay for Transposase-Accessible Chromatin sequencing), revealing the (epigenetic) chromatin accessibility landscape in ECs[47-50]. scRNA-seq studies of ECs using mouse tissues/models took advantage of tissue availability, allowing

a more in-depth study of development and/or early disease stages, for which only late-stage human disease samples are available, such as pulmonary arterial hypertension[51]. scRNA-seq was also performed in healthy and diseased human tissues, such as types of lung[52-58], liver[59], heart[60-63] or brain[64] diseases. Only a few studies combined both human and mouse analysis[53,65,66], allowing a cross-species comparison. Of note, the interpretation and findings of the EC scRNA-seq studies described below are also limited by the study design and chosen data analysis pipelines. Some of these limitations, as well as the general caveats of scRNA-seq analyses, have been highlighted in Box 2.

Endothelial Heterogeneity in Health

In the healthy adult, phenotypic and structural diversity of ECs are a reflection of the breadth of functions they perform to maintain tissue homeostasis, and are highly dependent on the organs and microenvironment in which they reside. Given that different organs have different needs[8], dissecting the functional specialisation of ECs in healthy organs is key to understanding EC health and behaviour, and essential for identifying how and why they become dysfunctional in disease.

Organotypic heterogeneity

Initial single-cell transcriptomic studies characterising EC heterogeneity focused largely on single tissues[35,36,39,67]. Although these studies highlighted organotypic diversity in EC populations, and increased our understanding of the EC subtypes in individual organs, a robust comparison across different tissues requires multi-organ studies. A recent study performed scRNA-seq on 32,567 ECs from 11 different murine organs to create a

comprehensive single-EC transcriptome atlas (EC atlas)[41]. Across different organs, inter-tissue heterogeneity in EC transcriptional states was detected. ECs from different organs expressed distinct transcriptional signatures, although ECs from certain organs had overlapping gene signatures, suggesting shared biological processes[41]. Higher expression of gene sets involved in immune modulation and scavenging were for example shared by liver and spleen ECs, while an enrichment of genes involved in membrane transport was detected in heart and skeletal muscle ECs[41] (Figure 2). Different transcription factor networks were moreover up-regulated in ECs from different tissues, which may drive organotypic diversity of ECs. Regulons of the *Gata* family were for example enriched in liver and spleen ECs, while spleen ECs additionally showed up-regulation of *Nr5a1* (Figure 2). Skeletal muscle ECs displayed enriched expression of the *Pparg* network, while pulmonary ECs showed higher expression of the *Foxf1* network. Another multi-organ study extracted mouse EC transcriptomes across 12 different organs from the single-cell dataset generated by the Tabula Muris consortium[42]. Largely similar overall findings were reported in both studies regarding unique EC molecular profiles across different organs and overlapping gene expression between certain organs, as well as enrichment of similar gene sets in the same organs (e.g. up-regulation of transporter-related genes in brain ECs).

Vascular bed heterogeneity

Apart from organotypic heterogeneity of ECs, endothelial diversity also exists within the vascular bed (artery, vein, capillary, lymphatic). In the aforementioned multi-organ study[41], conservation of EC vascular diversity across different organs was reported, as arterial, venous, capillary and lymphatic ECs clustered together, regardless of the organ they originated from. The vasculature in all organs displayed an arteriovenous hierarchy and the topography of

various endothelial subclusters along the vascular tree paralleled differences in blood flow, pressure, and chemical composition in the circulation[41].

scRNA-seq allowed finetuning of the traditional blood vascular EC classification (artery, capillary, vein). For instance, twenty-four renal endothelial populations were identified across the glomerular, cortical and medullary compartments[37], whilst studies on pulmonary ECs highlighted extensive heterogeneity within the capillary endothelium[68,69]. The murine alveolar microvasculature was reported to consist of two cellular subtypes, aerocytes and general capillary ECs (gCap), both of which were morphologically distinct from other capillary cells in the bronchial circulation and other organs[68]. Aerocytes with large, thin and expansive morphology, are anatomically localised with alveolar type I (AT1) cells and enriched with adhesion and leukocyte-sequestration genes, suggesting that these capillary ECs are unique to the lungs and are specialised for optimal gas exchange and leukocyte trafficking (Figure 2). In contrast, gCap cells are positioned in thick regions of the pulmonary stroma, regulate vasomotor tone, and function as specialised stem/progenitor cells in alveolar capillary homeostasis and repair. Both these alveolar capillary EC subtypes and their subtype-specific functions are conserved in humans, although human aerocytes express MHC class II genes whilst in mice, these genes are preferentially expressed by gCap cells[68]. scRNA-seq of human pulmonary cells also identified nine subpopulations of ECs, including two bronchial endothelial groups that were distinctly enriched in matrix, fenestration and cell cycle-related genes, compared to ECs that make up the pulmonary circulation[69] (Figure 2). In addition, there were also two rare capillary subpopulations with features of both aerocytes and gCap cells.

One of the first studies to use scRNA-seq to systematically investigate the molecular profiles of vascular cells in the adult mouse brain identified gradual changes in endothelial

transcriptional profiles along the arteriovenous axis, known as zonation[70]. While clusters of cells corresponding to arterial, microvascular and vein ECs could be identified, these cells could be ordered into a single one-dimensional range with markers of the different clusters displaying a gradual change across this axis. Arterial ECs were enriched in transcription factors, whilst transporter transcripts were dominantly expressed in capillary and vein ECs, suggesting that trans-endothelial transport of molecules across the blood-brain barrier (BBB) are concentrated in the latter regions. Similar zonation was observed in liver sinusoids, with 67% of liver sinusoidal ECs asymmetrically distributed along the portal vein-central vein axis, though there was limited conservation of zonation profiles between human and mouse scRNA-seq data[38-40]. These studies provided insights into how zonation influences endothelial function and have implications for improving central nervous system (CNS) drug delivery in treating brain diseases, as the BBB remains a significant physiological hurdle for drug design and development[71], and for understanding the relevance of EC zonation in disease pathogenesis.

Non-organotypic/vascular bed heterogeneity

Apart from organotypic and vascular heterogeneity, endothelial phenotypes were also found to differ between gender and ages in normal health.

Gender: Since the Tabula Muris consortium used both male and female mice, their single cell studies allow for the assessment of gender as a potential factor contributing to transcriptome diversity among ECs from the same organ. Indeed, adult male and female mice showcase different endothelial gene expression signatures and subpopulations in the brain, heart and lung[42]. For instance, the gene encoding mitochondrial leucyl-tRNA synthetase (*Lars2*) is enriched in male *versus* female ECs (Figure 2). Another study using the endothelial

compartment from the same Tabula Muris dataset did not find any differences in EC subtype abundance between male and female mice, though this particular study had used another EC annotation method (scmap and top 10 marker genes of each EC phenotype) to map the Tabula Muris-derived ECs onto the subpopulations identified in their EC Atlas[41]. However, this study did not further examine EC sex differences beyond the comparison of subtype proportion. A third study, using an independent Tabula Muris dataset generated from young and aged mice (3 months and 18 months)[43,44], found similar upregulation of *Lars2* in the young male mice in addition to the upregulation of S100a8 and S100a9 in the older male mice, when compared to the female. However, they concluded that EC gene expression was largely similar between the sexes when taking age into consideration. Further investigation is warranted to reveal how gender influences EC heterogeneity, and may explain gender differences in cardiovascular risks.

Aging: Natural aging influences changes in endothelial phenotypes and may explain age-related susceptibility to diseases[72]. In an attempt to uncover the impact of aging on the mammalian heart, one study compared the single-cell transcriptomes of cardiac cells from 12-week-old and 18-month-old mice[73]. Findings from this study suggest that the paracrine crosstalk between cardiac fibroblasts and cardiac ECs is impaired during aging. Blunted angiogenesis and autophagy, as well as proinflammatory activation in aged cardiac ECs were attributed to aged fibroblasts, which had the most significant differential gene expression. Increased expression of serpins in aged fibroblasts was found to mediate the anti-angiogenic effects on cardiac ECs. A separate study investigating how aging affects neurovascular dysfunction compared single-endothelial transcriptomes from young (2-3 months old) and aged (18-20 months old) mouse brains[74]. The age-associated transcriptional changes were involved in immune/cytokine signalling (*Arhgap5*, *Pak2*, *Rdx*, *Gng5*, *Cdkn1a*, *Hnrnpk*), blood

brain barrier (BBB) integrity (*Afdn*, *Ctnna1*, *Iqgap1*, *Cgnl1*, *Nedd4*, *Ocln*), and energy metabolism (*Cox6c*, *Cox7b*, *Ucp2*, *Hmgcs2*, *Pea15a*), most prominently in capillary ECs. Another study observed up-regulation of von Willebrand factor, a marker of endothelial dysfunction, in gCap cells but not aerocytes, in the lungs of aged mice[68]. ECs across five different organs in aged mice (18 months) have higher expression of immune and inflammation-related genes, compared to their younger counterparts[43]. Taken together, these findings suggest heterogeneous regulation of the different EC populations during aging that may contribute to the development of chronic diseases such as atherosclerosis, hypertension and Alzheimer's disease (Figure 2).

Endothelial heterogeneity in development

EC functional heterogeneity during development is evident in the developing heart[75], where the endocardium, a specialised endothelium lining the inner heart walls, acts not only as a physical barrier protecting the cardiac tissue from the chamber circulation but also as an essential source of different cardiac cell types[76]. Heart valve formation begins with the development of endocardial cushions at the atrioventricular canal and outflow tract, and at E8.5 to 9.0, a subset of these cushion endocardial cells undergoes endothelial-to-mesenchymal transition (EndMT) to give rise to the precursor cells that will eventually go on to form the mature heart valves[77]. Previously, it was unknown if this endocardial subset was predetermined to undergo EndMT or if the surrounding myocardium and haemodynamic circulation push this subset towards such a fate, since the trabeculae endocardium does not undergo EndMT. Endocardial heterogeneity was confirmed by a recent scRNA-seq study, which sequenced 36,000 cardiac cells from three distinct developmental stages at E7.75 when cardiac progenitor cells begin to differentiate, during heart tube formation at E8.25 and at

E9.25 when the heart tube loops[75]. This study identified three endocardial subpopulations: haematoendothelial progenitors, ECs and endocardial cells initiating EndMT. However, this study did not further examine these subpopulations beyond their identification and assignment in the single cell dataset. As such, important questions remain about the origin(s) of endocardial subpopulations and the wider endothelial heterogeneity in vascular development: (i) Are all ECs different from the initial point of their formation; and (ii) If not, when do they start becoming different and what drives this differentiation during development?

Since a functioning circulatory system is vital for embryonic growth, formation of the vascular network precedes the formation of all other organ systems. ECs originate *de novo* by vasculogenesis from mesodermal precursors in at least three sites: the yolk sac, allantois and embryo proper. Primitive ECs at this stage are highly plastic and were presumed to be non-specialised as they undergo rapid expansion and coalesce to form the primary vascular plexus, before acquiring arterial, venous and lymphatic identities. A scRNA-seq of whole mouse embryos at E8.25 reported that subsets of these primitive ECs show unique identities that could be demarcated by their maturity and anatomical origins[78]. Allantoic ECs express distinct transcriptional signatures, characterised by *Tbx4*, *Hoxa10* and *Hoxa11* expression, while non-allantoic ECs could be subdivided by their maturity based on their expression levels of *Etv2*, *Cdh5* and *Pecam1*. These findings, alongside scRNA-seq profiling of early Xenopus embryos[79], suggest that EC diversity begins much earlier in development than previously thought. It remains to be seen if and how this early diversification of EC identity influences their heterogeneous function and phenotypes later in life, and in the pathophysiology of diseases.

As the vascular plexus continues to remodel into distinct vasculatures, developing ECs continue to differentiate into the different vessel types and subsequently specialise to meet

the needs of their resident organs during organ vascularisation. Bipotentiality has been reported in pulmonary plexus cells, as they give rise to both subsets of alveolar capillary ECs (aerocytes and gCap cells) during development[68]. Aerocyte development has also been reported to depend on AT1-derived VEGFA, as this population of ECs is specifically and completely lost in AT1-specific *Vegfa* mutant lungs[80]. These findings again suggest early specification of EC phenotype during development that continues to persist in the adult.

Lineage-tracing and time-lapse imaging studies provided evidence that a subset of primitive ECs, termed hemogenic ECs, give rise to haematopoietic stem and progenitor cells (HSPCs) and intra-aortic haematopoietic clusters (IAHCs) in the later (definitive) wave of haematopoiesis[81,82]. It is less well-defined if hemogenic ECs are responsible for the primitive wave, where blood cell production occurs in blood islands in the yolk sac, prior to initial vascular formation. This is largely due to the overlap in their cell surface marker expression with haematopoietic cells, though previous studies have shown that the primitive wave can arise from cells expressing endothelial markers *Tie2*, *VE-cadherin* and *Pecam1*[83].

A pseudotemporal dataset of the developing mouse embryo was generated through scRNA-seq from nine sequential timepoints, E6.0 to E8.5[50]. This study identified two discrete subsets of hemogenic ECs, expressing both endothelial and haematopoietic markers. One of the subpopulations showed a more mature EC phenotype, with a high expression of classical markers of mature ECs such as *Cdh5* and *Pecam1*. By incorporating temporal information of each individual cell, this group was identified as the hemogenic ECs involved in the definitive wave, suggesting that EC maturity is essential to give rise to HSPCs. In addition, they also observed that these second wave ECs were transcriptionally heterogeneous, and through clustering analysis, this heterogeneity was associated with their anatomical origins. This study also reported TAL1 as a transcriptional regulator of the two haematopoietic waves, and

documented that *Tal1*$^{-/-}$ ECs deviate into an aberrant mesodermal phenotype. An additional study using ATAC-seq on single nuclei from 10 mouse embryos at E8.25 identified EC-specific regions of open chromatin[84]. Integrative analysis with TAL1 ChIP-seq data from past studies and validation in transgenic mouse assays revealed that TAL1 binds to both known (*Fli* -15 kb and *Erg* +86 kb) and novel (*Flt1* +67 kb and *Malm3* +360 kb) endothelial enhancers. Altogether, important transcriptomic and epigenetic mechanisms direct ECs towards a hemogenic fate during development.

ENDOTHELIAL HETEROGENEITY IN DISEASE

Endothelial cell population shifts in disease

Dimensionality reduction and clustering analysis allowed the comparison of EC populations in disease samples. First, a change of the relative proportion of ECs compared to other cell types has been noted in some diseases (Figure 3), with for example fewer ECs detected in metastasis compared to primary tumours[85], while more ECs have been observed in Alzheimer's disease *versus* control samples[64] .

Within the EC population, a change in the proportion of EC subtypes corresponds to a second level of heterogeneity observed in disease (Figure 3). Expansion of one of the three EC subtypes, probably corresponding to postcapillary venular cells, was observed in human skin samples from patients with atopic dermatitis or psoriasis[86]. In idiopathic pulmonary fibrosis (IPF), the peribronchial EC population was increased compared to control or obstructive pulmonary disease conditions and associated to areas of bronchiolization and fibrosis, showing the distinct response of this population between two diseases[52]. In mouse lungs exposed to hyperoxic conditions, an increase of the aerocytes/*Car4*$^{+}$ ECs population was observed[87].

An increase in EC proliferation was previously associated with several diseases[88] and scRNA-seq showed evidence of such an increase after myocardial infarction (MI)[89] or H1N1 influenza lung injury[90] in mice. In the lungs, most vessel-type ECs contribute to the proliferating response[90], while, in the myocardial infarction (MI) study, the use of a PDGFB-driven multispectral (Confetti reporter) EC tracing mouse model confirmed that proliferating ECs originated from resident cells via clonal expansion[89]. This Confetti reporter mouse line system was previously used to show EC clonal expansion after ischemia-induced neovascularization, and clonally expanded ECs selected by laser capture microscopy were analysed by bulk transcriptomics without single-cell resolution[91]. scRNA-seq was also used to study EC populations contributing to liver[92] and aorta[93] regeneration after injury in mice. In liver injury, a tissue-resident *Cd157*$^{+}$ population contributes to the regeneration of large vessels expressing only EC-specific genes[92]. In the aorta, regeneration originates from local adjacent ECs; both bulk and scRNA-seq studies revealed transcriptomic changes, including an increase of the progenitor marker *Ly6a*/*Sca1* and the transcription factor *Aft3*[93].

Disease can lead to a third level of heterogeneity in the endothelium, with the presence of EC subpopulations being almost exclusively restricted to control or disease conditions (Figure 3). After MI in mice, several clusters were predominantly composed of cells from disease samples and were characterised by a higher expression of the plasmalemma vesicle-associated protein gene *Plvap*[89], shown to regulate EC proliferation *in vitro*[89] and previously involved in EC permeability and angiogenesis[94]. In human liver cirrhosis, two disease-specific EC populations restricted to the fibrotic niche were identified and annotated as scar-associated ECs, in which marker gene analysis revealed the expression of pre-fibrotic and immune response genes[59]. Furthermore, pro-inflammatory and pro-atherogenic genes characterised EC clusters from the mouse aorta exposed to disturbed flow[48]. Similar pathways

seem to be identified in ECs from human atherosclerotic plaques, in which atherosclerosis-specific EC populations were described as activated ECs[49]. The term "activated ECs" was also used to describe EC populations identified in prostate cancer, which express cancer-associated fibroblast markers and extracellular matrix (ECM) genes but show a down-regulation of genes related to immunoregulatory pathways[95].

Tip ECs are critical for vessel sprouting, by leading the sprout at the forefront[96]. In both human and mouse lung tumours, tip EC populations have been detected in scRNA-seq studies, in agreement with the role of angiogenesis in tumour growth and proliferation[53,65]. Proliferating cells were detected, at substantial rates in mouse tumours, but at negligible rates in human (lung) tumours[53]. Tip cells were also found in scRNA-seq studies of mouse choroidal neovascularization[65]. Common/congruent tip cell markers, conserved across species (mouse /human), diseases and tissues (cancer/choroidal neovascularization), and experimental conditions (freshly isolated/cultured) were identified, allowing a better understanding of angiogenesis across disease conditions[53,65]. Congruent tip cell markers included genes previously detected in tip cells, such as *APLN,* but also novel tip cell transcription factors *TCF4*, *SOX4* and *SMAD1*, and novel genes relevant to the migratory tip EC phenotype[53]. Silencing of two novel markers, LXN (latexin) and FSCN1 (Fascin), in human umbilical vein ECs furthermore affected tip cell competitivity in a mosaic spheroid assay, confirming the tip cell role of these markers[53]. In addition to tip cells, another population of so-called "breach" cells has recently been identified in murine lung tumors by scRNA-seq. Based on their transcriptional profile breach cells are hypothesized to assist tip cells to lead the vessel sprout[53].

In addition, transitioning populations and pseudotime trajectories leading to these tip cells were characterised, revealing a change in the expression of genes related to metabolic

pathways[65]. Such metabolic changes in ECs, key to angiogenesis, were previously reported in scRNA-seq of all cells from lung cancer[55]. Moreover, in mouse cerebral cavernous malformations, based on a *Pcd10* deletion model, ECs with tip cell traits have been reported[97] but further characterisation is required to confirm if they indeed represent genuine tip cells.

EndMT occurs in many cardiovascular diseases[98], yet with some controversies due to the lack of standard in diagnosing the transition, and difficulties comparing different time points and/or models[99]. Using scRNA-seq of EC reporter mice, no evidence of EndMT was found in liver cirrhosis[100]. In contrast, EndMT was reported in human calcific aortic valve disease[101], in human atherosclerosis[49] and in mouse atherosclerosis induced by disturbed flow[48] or the high-cholesterol high fat diet in Apoe$^{-/-}$ mouse[102]. However, these scRNA-seq studies reporting EndMT did not use an EC tracing system, not allowing the full confirmation of the transition, and relied essentially on trajectory analysis. Additional analysis, such as RNA velocity might help to define the directionality of the observed trajectories and the cell population origins. Recently, activation of ECM genes was observed 7 days after MI in the mouse, and confirmed in scRNA-seq analysis of an EC lineage tracing model[103]. This study, based on a time course experiment, showed that EndMT is transient and reversible in MI[103], in contrast to the sustained EndMT observed in atherosclerosis and likely due to the chronic nature of the stimuli[48,49,102]. The potential transient nature of EndMT might explain why EndMT was not detected in another MI mouse study[89] and highlights the need to study different stages of disease development in association with a better EndMT diagnosis[98].

Transcriptomics changes leading to EC heterogeneity

In addition to a change of the population landscape, scRNA-seq also revealed EC global and subtype-specific transcriptomics changes in disease, highlighting a heterogeneity of phenotypes (Figure 3).

Changes in genes related to inflammation have been observed in ECs in several contexts. In adult mouse peripheral lymph nodes, antigenic stimulation by oxazolone led to an up-regulation of inflammatory genes such as *Sele* and *Cxcl9* in HEVs[104]. In mouse hyperoxic lung, genes known to be regulated by inflammation (*Ctgf*, *Fxyd5*) were up-regulated in the aerocyte EC populations[87]. In Alzheimer's diseases, up-regulation of genes from the major histocompatibility complex (MHC) class I were observed in ECs[64], while the expression of the MHC class II genes, part of the capillary gene signature, are up-regulated in pulmonary arterial hypertension (PAH)[105] and down-regulated in ECs from murine and human lung tumours[53]. Changes in inflammation-related genes were also reported in atherosclerotic Apoe$^{-/-}$ mice[102], and a recent study of the mouse aorta during disturbed flow suggested a potential transition of ECs towards an immune-like phenotype as an additional type of EC reprogramming[48]. All these studies confirm that the endothelium is a target of the inflammatory process, but likely also acts as an immuno-regulator, in part by working as semi-professional antigen-presenting cells. Indeed, the term "immunomodulatory ECs" (IMECs) was recently coined to describe the immunoregulatory EC phenotype[106].

Vessel growth dysregulation contributes to the pathogenesis of many diseases such as cancer and pulmonary arterial hypertension (PAH). In addition to the identification of angiogenic tip cells, angiogenesis pathway regulation has also been documented in several studies. Indeed, down-regulation of genes relevant to capillarization were observed in ECs in human systemic sclerosis[107], while anti-angiogenic genes were up-regulated in ECs from hyperoxic lungs[87]. In contrast, pro-angiogenic/ capillarization genes were activated in ECs in

Alzheimer's disease[64] and cirrhotic mouse liver[100] and in one capillary EC subtype in PAH[105]. Interestingly, in cirrhotic liver, the activation was zonation-dependent and restricted to a specific region of the liver sinusoidal ECs[100]. As most changes of angiogenesis pathway did not seem to be associated with the detection of a tip cell population, these regulations might not be linked to sprouting angiogenesis but might possibly reflect other vessel formation modes such as splitting angiogenesis, not characterised so far by any standard marker expression, or EC migration. Further studies are needed to understand the contribution of these different processes to vessel growth or regression.

Several studies reported the up-regulation of the ECM genes in ECs in disease conditions, probably reflecting structural EC changes. In prostate cancer, activated ECs were characterised by an up-regulation of ECM genes[95], while the transient mesenchymal gene activation in MI also included ECM gene changes. In addition, ECM gene up-regulation was observed in liver cirrhosis[100], lung cancer[53] and in systemic sclerosis[107]. Additional transcriptome regulations in ECs have also been described. Down-regulation of several members of the Notch signalling pathway occurs in ECs in pulmonary fibrosis[57]. In atopic dermatitis and psoriasis, ECs activate fetal genes[86], while in oxygen-induced retinopathy, the peak of neovascularization was associated with expression of senescence genes[108]. Further investigation is required to define the functional effect of these changes and their relevance across diseases.

To understand the regulation leading to these transcriptomics changes, scRNA-seq studies were performed together with single-cell ATAC-seq, confirming chromatin accessibility changes in correlation with the transcriptomics changes and reporting disease-induced peaks such as in mouse MI[47]. As transcription factors (TFs) play a key role in shaping the transcriptome, motif enrichment analysis of scATAC-seq of mouse carotid artery in

different flow conditions identified KLF2/KLF4 motifs in stable flow, while motifs for RELA, AP1, STAT1 and TEAD1 were enriched in accessible regions from disturbed flow conditions[48]. Approaches developed for TF target and/or regulon-based analysis of scRNA-seq data[109,110] revealed the possible role of FLI1 and TEAD1 in tumour ECs[55], and of SOX18 in human PAH[51].

Contribution of the microenvironment to EC heterogeneity in disease

ECs plastically adapt to the physiological needs of different tissues. Unsurprisingly therefore, signals in the microenvironment shape the EC subtype landscape[111]. ECs acquire a specialized role depending on their location and status in physiological conditions that can make them more or less responsive to certain stimuli in disease. For instance, in cerebral cavernous malformation, venous capillary ECs are the main contributor of the lesion, as arterial ECs remain non-responsive to the transformation[97]. Furthermore, HEVs in lymph nodes possess an activated phenotype that is lost upon changes to the microenvironment such as inhibition of lymphotoxin-b receptor signalling[104].

Complex communicative circuits between ECs and other cell types play a key role in disease pathogenesis (Figure 3). For example, tumour aggressiveness is regulated through a crosstalk of ECs with cancer cells or tumour-associated macrophages in the microenvironment, regulating (among others) induction of metastasis and tumour angiogenesis[112,113]. Moreover, interactions between ECs and cardiomyocytes are key during development and cardiac homeostasis, and become dysregulated in cardiovascular disease[114,115].

Cell-cell communication and interaction can be assessed in scRNA-seq data by an unbiased analysis of receptor-ligand interaction (RLI) pairs using popular tools such as CellPhoneDB[116] or more recent and comprehensive tools including CellChat[117] and

NicheNet[118], detailed hereafter in the "Recent Advances & Future Perspectives" section of this review. Increased interactions of ECs with other cells were detected in the heart of postnatal day 8 mice 3 days after MI[47] but also in human atherosclerotic plaque[49]. In the murine regenerative heart, R-Spondin was identified as an EC ligand expressed by epithelial cells with pro-angiogenic effect to EC *in vitro*[47]. ECs appear to receive communication from fibroblasts in the murine hyperoxic lung, with the ligand and receptor *Bmp5* and *Bmpr2* expressed by fibroblast and ECs respectively[87]. In atherosclerosis, the PDGF/PDGFRB interaction between myeloid cells and ECs led to the hypothesis of a myeloid-driven angiogenic contribution to plaque destabilization[49]. In the heart, evidence of communication between fibroblasts and ECs was detected in both healthy and injured conditions using scRNA-seq and the proximity between fibroblasts and ECs was confirmed by immunofluorescence[119]. As mentioned previously, a study of the murine aging heart revealed the deterioration of this paracrine crosstalk, with *in-vitro* experiments showing a reduced angiogenic property of the conditioned medium from heart-derived aged fibroblasts[73]. In contrast, ECs might communicate with mesenchymal cells in human cirrhotic liver, where the scar-associated ECs express the non-canonical Notch ligand *JAG1*, *JAG2* and *DLL4,* whereas the *NOTCH3* receptor is expressed by scar-associated mesenchymal cells[59]. Co-culture experiments, using primary human hepatic stellate cells (HPCs) and ECs from cirrhotic livers, validated that this interaction promotes fibrillar collagen production by HPCs, which could be inhibited by perturbation of *NOTCH3* expression[59], highlighting the translational potential of findings identified through scRNA-seq and interactome analyses.

RLI analysis also highlighted cell-cell interactions in physiological conditions, with potential implication for development and disease. In the lung, the epithelium was identified as a key hub for spatially-restricted regulation of EC morphogenesis, by means of their

preferential expression of semaphorins and VEGF family members, a phenomenon that is conserved across multiple species[66]. Lastly, and in line with their well-appreciated immunoregulatory role, interactome analyses revealed novel interactions between pulmonary ECs and immune cells, including possible recruitment of *CX3CR1+* non-classical monocytes to ECs (*CX3CL1+*), and attraction of *CCR1+* dendritic cells to veins (*CCL23+*), bronchial vessels (*CCL14+*) and lymphocytes (*CCL5+*)[69], highlighting interesting avenues for future research in light of lung cancer and/or inflammatory disease.

Overall, a high level of EC heterogeneity has been observed across developmental, physiological and pathological conditions. Further investigation into this heterogeneity may help understand therapy resistance mechanisms, and should be factored into future EC-focused therapeutic development.

THERAPEUTIC IMPLICATIONS

Anti-angiogenic therapies in cancer – targets and resistance

As angiogenesis is critical for a variety of diseases, therapies have been devised to either promote or inhibit angiogenesis[120]. While pro-angiogenic efforts promise to offer novel therapeutic opportunities for cardiovascular disease and diabetes, here we focus on anti-angiogenic therapies (AATs). Cancer presents one of the main pathologies for which AAT is used, due to the critical role of angiogenesis in cancer progression and metastasis[121]. Currently approved AATs center around blocking the key pro-angiogenic target VEGF, though other targets are emerging (Figure 4). While initially designed to prune the tumour vasculature[122-124], current clinical trials explore whether VEGF-blockade can improve immunotherapy by normalizing the tumour vasculature[125]. The success of VEGF-blockade therapy is however tampered by insufficient efficacy and resistance[126,127]. Several resistance

mechanisms have been proposed, ranging from alternative growth factor signalling to other modes of tumour vascularization, such as vessel co-option[128,129], but only recent studies explored additional mechanisms at the single EC level[53,130].

In a mouse lung cancer model, tip cells and breach cells (putatively assisting tip cells to lead the vessel sprout[53]) represent the EC subtypes most sensitive to VEGF blockade[53], whereas other EC subtypes were less or differentially sensitive. In fact, postcapillary vein ECs increased in abundance upon anti-VEGF treatment[53]. Whether the increases in capillary and postcapillary vein ECs is a consequence of switching from sprouting angiogenesis (SA) to vessel co-option (a known escape mechanism to AAT therapy[131]), remains to be determined. This may explain – at least in part – the limited success and therapeutic immunity towards AAT.

In addition, the various distinct EC types identified by single-cell transcriptomic studies might also contribute to a better understanding of AAT resistance[53,132]. Tip cells, which are the presumed key targets of AAT, amount to fewer than 10% of all ECs within lung tumours[53], thus the majority of ECs is in fact not targeted by AAT (Figure 4). Differences in the composition of different EC subtypes in tumours from distinct patients[53] might furthermore explain why some patients respond better than others to AAT. Moreover, venous ECs in tumours contain a subset of so-called resident endothelial stem cells (rESCs)[53]. rESCs were also identified in large vessels of multiple murine organs and showed self-renewal capacity as well as contributed to vessel regeneration in different models of vessel injury[65,92,93]. As venous ECs expand upon AAT[53], it raises the question whether these rESCs might reconstitute vessels upon AAT, thereby contributing to therapy resistance. Endothelial progenitor cells were identified in human metastatic lung adenocarcinoma[133]. Moreover, aldehyde dehydrogenase (ALDH)-positive ECs with stem-like properties were found in melanoma (xenograft models)

524 and human renal cell carcinomas. These ALDH-positive stem-like ECs display pro-angiogenic
525 properties, and resisted to chemotherapy treatment[134,135]. How such progenitor-like ECs are
526 impacted by AAT remains to be determined. Future studies will determine whether such cells
527 are present in other tumour types, and contribute to AAT resistance by induction of
528 neoangiogenesis upon treatment. Interestingly, "Myc targets" was amongst the top up-
529 regulated pathways in tumour ECs in a single-cell analysis of human NSCLC[55]. Myc has been
530 identified as a driver of the endothelial regeneration process[93], thereby raising the question
531 whether progenitor-like ECs might arise in tumours, and if so, whether they harbour
532 additional heterogeneity in terms of their transcriptome or their response to anti-cancer
533 therapy/AAT. Of note, while several scRNA-seq studies identified EC populations with stem-
534 or progenitor-like potential, future studies are needed to carefully assess potentially distinct
535 vascular progenitors, that might be tissue and/or disease specific. Thus far, there is not yet a
536 consensus definition of EC stem- and/or progenitor cells available based on scRNA-seq.

537 Alternative mechanisms of blood vessel growth, in addition to sprouting angiogenesis
538 (SA), which is the most studied form of angiogenesis, also need to be considered in the
539 context of EC heterogeneity and its impact on cancer progression and therapy response. In
540 fact, VEGF inhibition can induce substitute mechanisms of vessel growth, such as
541 intussusceptive angiogenesis (IA)[136] and vessel co-option[131]. Also, vascular mimicry and
542 vasculogenesis were identified as potential alternate processes that promote AAT
543 resistance[137,138]. However, single-cell studies investigating phenotypical and functional EC
544 heterogeneity in these processes remain elusive. Such studies would be critical to identify
545 novel targets to enable the control of pathologic angiogenesis by simultaneously attacking
546 several aspects of vessel growth.

Importantly, combination of AAT with other anti-cancer therapies, such as chemotherapy or immunotherapy has shown promising results not only in pre-clinical models, but also in the clinic. In fact, several AAT agents (e.g. bevacizumab, aflibercept, sorafenib, sunitinib), apart from being approved as single-agent therapy, have reached approval in combination with chemotherapy, or as second-line therapy after patients progressed on chemotherapy[139]. Moreover, the combination of IFN-α treatment with anti-VEGF therapy has been approved by the FDA for treatment of metastatic renal cell carcinoma[140]. With the advent of novel immunotherapies, such as immune checkpoint blockade, there are many new promising anti-cancer therapeutic opportunities[139]. New insights into distinct EC phenotypes could help to develop more precise treatments tailored to target specific EC populations, which might create a favourable environment, in particular for immunotherapy to work. IMECs or other specialized EC phenotypes might offer such opportunities. For instance, HEVs are involved in the recruitment of different immune cells[141], thus promoting HEV growth is expected to be beneficial for enhancing the anti-cancer effect of immunotherapy. This concept to "tune rather than only prune" is a novel strategy for future AAT.

EC metabolism as alternative target to modulate angiogenesis

More than a decade ago, ECs were shown to undergo metabolic changes to execute their various functions. This metabolic reprogramming is driven in part by different signalling cascades, for instance growth factor signalling (e.g. VEGF can induce glycolysis) or Notch signalling (Notch suppresses glycolysis in stalk cells)[142]. However, it is now clear that EC metabolism is not only necessary, but also sufficient (independent of growth factors or other stimuli) to control EC function[143]. Several metabolic pathways have been implicated in distinct

functions. Single-cell studies alongside metabolomic investigations have uncovered several metabolically distinct EC subtypes. For instance, during sprouting angiogenesis, tip cells up-regulate glycolysis and amino acid (AA) metabolism to support migration[142-144]. These metabolic pathways are also used by stalk cells (however, at lower levels), where they support proliferation and biomass production[143]. Stalk cells as well as phalanx cells also rely on fatty acid oxidation (FAO)[145]. In quiescent phalanx ECs, FAO contributes to maintainence of their quiescent phenotype[146] (Figure 4). It has also been recognized that different EC subsets display distinct metabolic signatures, in a tissue-specific manner[41]. For instance, different metabolic transporters are most highly expressed in brain ECs, spleen ECs are enriched in cholesterol metabolism, while cardiac and muscle ECs show elevated fatty acid metabolism[41]. For a detailed review of EC metabolism, and metabolic heterogeneity in different EC types, we refer to recent excellent reviews[143,147,148].

When comparing ECs from healthy tissues to those in disease, different metabolic gene signatures were observed as well. For instance, compared to their respective controls, ECs from choroidal neovascularization or murine lung tumour models displayed an increase in gene expression related to several metabolic pathways, such as glycolysis, tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), one-carbon metabolism and nucleotide synthesis[65]. In line with these findings, single-cell analysis of colorectal, lung and ovarian cancer revealed that tip ECs in all three cancer types up-regulate glycolysis and OXPHOS gene signatures[56]. Moreover, EC subtypes in human lung cancer also presented with metabolic gene adaptations compared to their healthy counterparts, with an up-regulation of genes involved in lipid metabolism in capillary tumour ECs, and increased prostaglandin metabolism in venous tumour ECs[53]. Compared to ECs from early stage ground glass nodules adenocarcinoma, ECs from late stage solid lung adenocarcinoma were also enriched in

595 metabolic gene processes[149], and circulating ECs from metastatic prostate cancer patients
596 showed enriched metabolic gene expression compared to circulating ECs from healthy
597 controls[150].

598 The findings of EC metabolism as critical propeller to EC function, along with the
599 observed metabolic changes in tumour ECs, led to the hypothesis that metabolic targeting of
600 ECs might offer new therapeutic opportunities to keep tumour angiogenesis at bay (Figure 4).
601 The glycolytic enzyme PFKFB3 regulates tip and stalk cell phenotypes, and associates with
602 actin remodelling[142]. Genetic silencing of PFKFB3 inhibited tip cell function and resulted in
603 acquisition of a quiescent phenotype[142]. Pharmacological inhibition of PFKFB3 with the
604 inhibitor 3PO (3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one) impeded vessel sprouting in
605 models of retinal angiogenesis and vascular development in zebrafish[144]. Notably,
606 pathological angiogenesis in different disease models (age-related macular
607 degeneration, retinopathy of prematurity, skin psoriasis, inflammatory bowel disease and
608 cancer) was also suppressed by 3PO treatment[144,151] (Figure 4). Importantly, while
609 pharmacological PFKFB3 inhibition impedes angiogenesis in pre-clinical models, the efficacy
610 of the treatment in clinical settings remains to be tested (Figure 4). Moreover, blocking of
611 FAO hampers pathological angiogenesis. Etomoxir, which inhibits the FAO enzyme Carnitine
612 Palmitoyltransferase 1A (CPT1A) reduces pathological angiogenesis in a model of retinopathy
613 of prematurity[145]. Tip and stalk cells also rely on fatty acid synthesis[143]. In fact,
614 pharmacological inhibition of the fatty acid synthase (*FASN*) using Orlistat, reduces EC
615 proliferation and angiogenesis in pathological ocular neovascularization and melanoma
616 animal models[152,153]. Thus far, no apparent off-target effects were discovered in preclinical
617 models, however it is critical to note that targeting metabolic pathways affects not specifically
618 ECs, but all cell types. Therefore, the suitability of metabolic targets to specifically inhibit EC

functions in patients remains to be investigated. However, as discussed in the following paragraph, recent developments in precision medicine might allow targeting of EC-specific metabolic pathways. In summary, these promising results demonstrate the need for future studies on the metabolic heterogeneity of ECs to identify additional metabolic targets.

Novel targets from single cell studies - prioritization & targeting

Whilst the unravelling of EC heterogeneity at single cell resolution has led to the discovery of exciting novel and specialized EC subtypes with a presumable key role in disease, the prioritization of functionally important candidate (metabolic) genes that are most reflective of these EC subtypes remains a formidable challenge. It demands the development of efficient means to transcend the atlas-like descriptive listing of EC-subtype specific marker genes into the most promising functionally relevant and therapeutically targetable candidates, and various *in silico* methods have been developed and reported in the recent years to aid in this challenge. For instance, use of an integrated (meta-)analysis of candidate gene expression across species, diseases and models identified *PLOD1* and *PLOD2* as novel angiogenic candidates[53]. Silencing or inhibition of both genes furthermore impaired *in vitro* and *in vivo* vessel sprouting, validating the therapeutic potential of these genes[53]. Moreover, a similar meta-analysis approach, yet combined with scRNA-seq data-tailored genome-scale metabolic models (GEMs), proved an efficient method for prioritization of *SQLE* and *ALDH18* as promising new metabolic targets for AAT[65] (Figure 5). Again, *in vitro* and *in vivo* perturbation experiments confirmed the functional relevance of both genes for angiogenesis, stressing their translational potential[65].

Querying of cell types enriched for trait-relevant genes based on genome-wide association studies (GWAS)[154,155] represents another intriguing strategy for the identification

643 of EC-specific genes associated with a particular disease or condition. For instance, a GWAS-
644 based analysis of genes associated with cardiovascular disease was performed in a scRNA-seq
645 study of human atherosclerotic plaques[49]. Eight of such genes (*SHE*, *KCNN3*, *VAMP5*, *SEMA3F*,
646 *HDAC9*, *GIMAP1*, *NOS3*, and *DOCK6*) showed an EC-enriched expression pattern, supporting
647 EC contribution to the disease and providing crucial information for future functional
648 characterisations[49]. Furthermore, in scRNA-seq data of two rat models of pulmonary arterial
649 hypertension (PAH), relevance to the human disease was investigated by analysing the
650 expression of genes implicated in PAH based on DisGeNET and the Comparative
651 Toxicogenomics Database[156], and in a human PAH scRNA-seq study, differential expression of
652 genes associated with hereditary PAH (e.g. *BMPR2, ENG, SMAD9*) was confirmed in several
653 cell types, including ECs[51]. Interestingly, the rat PAH scRNA-seq study also assessed the
654 therapeutic potential of existing drugs in PAH, by means of *in silico* drug screening[156]. This
655 screening relied on the "Connectivity Map" resource, that allows the comparison of scRNA-
656 seq transcriptional signatures with a reference collection of drug-induced gene expression
657 profiles from cultured human cells[157] (Figure 5). Another recent method, Augur, allows
658 prioritization of cellular subtypes most responsive to a biological perturbation[158], in lieu of
659 the traditional prioritization based on differential gene expression. This enables the
660 identification of the individual contributions of distinct cell types to a condition or their
661 discrete responses to different treatments, thereby deciphering the roles of distinct cell
662 subtypes on a broader scale[158]. The *in silico* construction of multicellular disease models
663 (MCDMs)[159] is yet an additional method for target prioritization. This systems-level approach
664 uses scRNA-seq data to construct models of disease-associated cell types, their expression
665 profiles, and predicted cell-cell interactions. By integrating this method with disease context-
666 specific genetic and epigenetic data, the possibility of identifying the most (therapeutically)

667 relevant cell types was showcased in single cell datasets of human and mouse rheumatoid
668 arthritis[159]. These novel approaches all showcased the ability of cell type and target
669 prioritization from complex scRNA-seq datasets, and their application to EC-specific OMICs
670 data promises to unveil important insights into vascular subtypes and marker genes most
671 relevant for follow-up in a disease or condition-specific context (Figure 5).

672 While identifying the EC subtype and associated marker(s) most likely to be of
673 therapeutic interest already poses a challenge, subsequent specific targeting of the prioritized
674 vascular subset may present an even bigger hurdle. Developments in the selective targeting
675 of an EC subtype, recently coined "precision angioscience"[160], will therefore be instrumental
676 in translating EC-derived scRNA-seq data into clinically interesting and feasible follow-up
677 studies. Selective delivery of small interfering RNAs (siRNAs), single-guide RNAs (sgRNAs),
678 messenger RNAs (mRNAs), small molecules, and therapeutic proteins represents another
679 strategy for specific targeting of the endothelium, and has thus far been experimentally
680 achieved through the use of targeting ligands (for instance monoclonal antibodies), directed
681 against EC-specific adhesion molecules or other surface markers. Vascular cell adhesion
682 molecule-1 (VCAM1)-targeted nanoparticles have shown promising results in light of imaging
683 inflamed or ischemic tissues in the mouse[161-164]. Further, enzyme-antibody conjugates and
684 nanoparticle formulation aimed at specific targeting of the pulmonary[165,166] or splenic[167]
685 murine vasculature have been reported so far, often with negligible alterations in non-
686 vascular cell types or other tissues.

687 Although promising, *in vivo* gene delivery to a particular EC subtype identified by
688 scRNA-seq has thus far not been achieved but may harbour benefits over pan-EC targeted
689 strategies in terms of toxicity to other parts of the vascular bed within and outside of the
690 tissue of interest. One major reason why targeting of specific EC subtypes identified by scRNA-

seq studies has not yet been achieved, is the lack of consensus marker genes for distinct EC subpopulations. Future scRNA-seq analyses might provide further insights into construction of specific promoters for inclusion into gene therapy vectors in order to selectively target specific EC populations. This strategy however depends on the mutual exclusivity of EC subtype specific marker genes, and may be more challenging in case of tissues, where EC expression signatures exhibit spatial zonation, as for instance shown in the hepatic vasculature[168].

RECENT ADVANCES & FUTURE PERSPECTIVES

A compendium of all publicly available single ECs

Despite the vast amount of scRNA-seq studies published to date, the abundance of the vascular compartment within individual studies is often relatively low, precluding a detailed and all-encompassing interrogation of its heterogeneity. Increasing the magnitude of EC-derived single cell datasets, by performing a joint analysis across all publicly available studies, could offer a solution to this problem. Although seemingly straightforward, this strategy nevertheless faces multiple challenges, including the need for effective batch effect correction, lack of standardization in EC isolation protocols, and variation in single cell data analysis, subclustering and annotation strategies (see Box 3).

The latter issue is expected to improve in the coming years with the advancement of automated cell type annotation tools, which are rising in number and user-friendliness[169-172], but even more so with the development of tools like Azimuth[173], providing rapid and automated mapping, visualization and annotation of single cell datasets through an online web application. Yet, these tools often provide reference datasets representing major cellular lineages in various tissues/organs but preclude annotation of different EC subtypes within a

particular tissue or vascular bed. There is thus a need for the generation of tissue-specific "gold standard" vascular atlases, to both improve and progress standardization of EC OMICs annotations. A recent integration of six lung scRNA-seq datasets resulted in joint profiling of over 15,000 ECs from 73 individuals[174], and although not covering the full spectrum of published (healthy/normal) lung single EC RNA-seq data, this study provided one of the first in-depth reference atlases of healthy/normal lung ECs and is likely to aid annotation of future pulmonary EC studies in health and disease. When such efforts will be combined with automated cell type mapping tools and standardized whole tissue/EC isolation protocols, harmonized EC annotation across laboratories, tissues and experimental setups should be feasible in the foreseeable future.

Another obstacle in integrated analysis of EC OMICs data is represented by the inconsistent formats in which raw data is deposited, and the (sometimes) severe lack of detail regarding sample origin information and data processing parameters. The availability of processed counts and annotated metadata is furthermore limited, yet inevitable to ensure reproducibility of the data across labs of different expertise. Data-sharing methods also become increasingly variable, complicating uniform methods of dataset curation. While lab-hosted servers, offering virtual exploration and downloading of data, are rising in popularity and enable non-bioinformatics focused labs an affordable and reliable method of data exploration, a more centralized storage platform would greatly enhance our ability to study vascular OMICs in a streamlined and comprehensive manner. Various recent efforts aimed at offering solace, either by generation of free-of-charge portals harbouring curated and harmonized processed datasets, or frequently updated overviews of published scRNA-seq datasets[175-178]. Specialized databases, like *JingleBells*[179] for immune cells, *cancerSEA*[180] for cancer cell states, *The Human Cell Atlas* portal for all tissues and cell types of the human

739 body[181], or the *NIH Human Biomolecular Atlas Program (HuBMAP)*[182], furthermore provide
740 tempting field-specific opportunities in terms of scRNA-seq data exploration and analysis. Yet,
741 none of these portals/efforts capture the complete spectrum of published datasets, and their
742 usefulness relies on continuous data curation and updates.

743 If we are to make progress in deciphering vascular heterogeneity across species,
744 tissues and conditions, a dedicated portal housing all publicly available vascular-centered
745 single OMICs data appears to become a key milestone waiting to be accomplished. However,
746 as the ever-increasing number of single cell datasets published is becoming difficult to curate,
747 a demand for artificial intelligence (AI)-based data-mining approaches is likely to arise in
748 parallel to realize such an effort in an all-encompassing manner. Implementation of natural
749 language processing (NLP) strategies and recent developments in their specific moulding
750 towards biomedical sciences appear promising[183,184]. Amidst the current single cell OMICs
751 "tsunami" of data, tailoring of text-mining tools toward identifying OMICs publications
752 harbouring a particular cell type of interest (in this case, ECs) has the potential to greatly
753 enhance their identification and prioritization, accelerating the generation of comprehensive
754 single EC OMICs repositories and furthering data-driven research in the (vascular) biology
755 field (Figure 6).

756

757 ***ECs never work alone – interactomes and spatial resolution***

758 As described above, intricate cellular communication between ECs and their neighbouring
759 cells are of vital importance for maintaining vascular homeostasis and remodelling, and
760 recent advances in the development of interactome prediction tools for single cell data
761 revealed intriguing findings regarding the interplay of ECs and other cell types[47,49,59,87].
762 Although fascinating, it must be noted that the findings and interactions resulting from RLI

analysis represent predictions, requiring functional validation. Recently developed tools provide more comprehensive solutions, including CellChat[117], taking into account interactions between ligands, receptors and their co-factors, or NicheNet[118], aimed at diving deeper into the intracellular response of cell types on the "receiving end" of these predicted interactions (Figure 7A). Ultimately, however, RLIs can be more accurately investigated when positional information is preserved. Advances in spatial transcriptomics, crowned as "Method of the year 2020" by Nature Methods[185], presumably hold great promise for future enhancements in studying the interplay between ECs and their environment in a tissue architecture-dependent context (Figure 7B). Interestingly, several computational tools have been recently developed with the aim to provide a more cost-effective alternative to spatial transcriptomics, either by integration of scRNA-seq data with reference *in situ* hybridization (ISH) data (Perler[186]), prediction of cellular coordinates in a three-dimensional pseudo-space based on input scRNA-seq data and known ligand-receptor interactions (CSOmap[187]), *de novo* spatial reconstruction of single-cell gene expression (novoSpaRc[188]), or prediction of whole-transcriptome expressions in their spatial configuration by mapping of untargeted scRNA-seq data to smaller, targeted spatial transcriptomics datasets (SpaGE[189], SpaOTsc[190]) (Figure 7B).

Finally, translation of cellular cross-talk predictions to the protein level, for instance by applying established methods including cite-SEQ[191], REAP-seq[192], or cytometry by time of flight (CyTOF)[193], or the more newly developed Nativeomics[194] (allowing detection of intact ligand–receptor assemblies using mass spectrometry), INs-seq[195] (allowing more accurate exploration of transcription factors, active signalling networks and metabolic activity by parallel transcriptome and intracellular proteomic profiling at single cell resolution), or single cell proteomics[196] will be essential to complement and finetune EC-interactomes predicted from scRNA-seq data (Figure 7C). Lastly, as spatial juxtaposition of an EC and another cell type

787 does not automatically imply their active communication, the abovementioned tools will
788 undoubtedly help prioritize the interactions that are most promising for further functional
789 validation.
790

791 CONCLUSION

792 Collectively, EC OMICs studies have opened up a staggering amount of data readily available
793 for analysis, of which we have currently only scratched the surface. Nevertheless, the single
794 endothelial landscape uncovered thus far has revealed an intriguing degree of transcriptional
795 heterogeneity, and has already propelled the vascular biology field at unprecedented speed.
796 Further efforts aimed at unravelling the associated biological and functional relevance of this
797 heterogeneity will undoubtedly help forward our understanding of the molecular drivers by
798 leaps and bounds, and reveal the translational potential of exploiting EC heterogeneity for
799 the development of novel AAT or endothelial-targeted therapies.
800

801 BOXES

802 BOX 1: WHAT IS HETEROGENEITY AND HOW CAN IT BE QUANTIFIED?

803 Heterogeneity is an immanent trait of living systems that is omnipresent across all biological
804 levels. It can manifest in different scale, ranging from different species arising from evolution
805 to genetic differences within a population of seemingly identical cells. Although biological
806 diversity is vital for the survival of organisms in a changing environment, it presents a
807 formidable challenge for biologists to determine which of the observed heterogeneity have a
808 biologically meaningful function. Heterogeneity can be summised as a statistical
809 characteristic of a cell population. It is most commonly quantified through epigenomic,

genomic, transcriptomic and proteomic studies, though the extent of heterogeneity at one level of regulation is not indicative of the heterogeneity at another level of expression. Conceptually, heterogeneity within a cell population can be probed by first collecting single-cell measurements from the population. Next, patterns of diversity can be identified by distilling distinct cellular behaviours into defined categories. Finally, functional significance of the patterns observed can be tested by measuring whether one subpopulation significantly differs from another or if the heterogeneity is informative as a predictor of responses to certain stimuli. We recommend the following commentaries for further conceptual exploration of heterogeneity in biology and single-cell[5,9].

BOX 2: STUDY DESIGN AND BIOINFORMATICS CONSIDERATION FOR SCRNA-SEQ STUDIES TO IDENTIFY AND CHARACTERISE EC POPULATIONS ON THE TRANSCRIPTOME LEVEL

Whole tissues vs EC enrichment vs EC isolated from reporter mice: Whole tissue/organ analysis potentially lowers the power and resolution of EC analysis, yet allows their analysis amidst other cell types and querying of cell-cell interactions. To obtain a better resolution of the EC transcriptomic landscape, enrichment strategies based on CD31 expression can be performed prior to sequencing[41,65,97,102]. scRNA-seq of ECs isolated from reporter mice have also been implemented in liver cirrhosis[100], after myocardial infarction[89,103] and in atherosclerosis[197]. Such designs allow us to track the changes ECs undergo in diseases and reveal the presence and/or absence of cell transitions, such as endothelial-to-mesenchymal transition (EndMT)[103].

Cell number: Low number of sequenced cells could limit the identification of minor EC populations.

Inclusion of technical and biological replicates: As expected for novel technologies and in part due to their costs, study designs vary considerably in terms of biological/technical replicates. In some studies, lack of or low number of replicates prevent an analysis of variability and reproducibility and will require further studies and additional validations.

Depth of EC downstream analysis: The depth of downstream EC analyses also varies across different studies, sometimes due to the study design (e.g. limited number of isolated ECs), or to incomplete characterization of EC clusters. Especially in cases of whole tissue scRNA-seq, EC analysis has often been performed alongside the analysis of other more abundant cell types, and lacks in-depth investigation and/or detailed subclustering of ECs. For instance, in studies of abdominal aortic aneurysm[198], Alzheimer disease[199], cancer[56], cirrhosis/fibrosis[52,59] and atherosclerosis[200], ECs were present but their EC subsets were not studied.

scRNA-seq analysis: general caveats: Besides EC-specific considerations in terms of study design and analysis, hurdles in quality control (QC) of the data remain an ongoing challenge in the field of single-cell OMICs. For instance, during library preparation using droplet-based methods, multiple cells may have been captured together (doublets), non-viable cells may have been captured, or, droplets may have been sequenced that harbored no cells (empty droplets). Differences in library preparation might also stem from variability in cell recovery and quality, which results from different isolation protocols. After sequencing, it is thus imperative to implement a series of QC steps to ensure the analysis will be performed on high-quality cells only. Generally, QC of scRNA-seq data is based on three variables: (i) the number of counts per cell, (ii) the number of genes per cell, and (iii) the fraction of counts

858 from mitochondrial genes per cell. Filtering of outliers, based in examination of the
859 distributions of these QC variables, can be applied to eliminate unwanted cells. For example,
860 low-quality cells can be identified by a low number of detected genes, non-viable cells are
861 characterized by a high fraction of mitochondrial counts, and cells with an unexpectedly large
862 number of detected genes may represent doublets. Specifically for doublet removal, several
863 computational tools can additionally be used to further optimize their detection beyond
864 manual inspection of gene counts (DoubletDecon[201], Solo[202], scds[203], Scrublet[204], Doublet
865 Finder[205]). Additionally, cell hashing strategies can be implemented to enhance the detection
866 of doublets[206].

867 scRNA-seq results typically also suffer from sparsity, as the data often only captures a small
868 fraction of the transcriptome, and genes can be detected at a low or moderate expression
869 level in one cell, yet go undetected in another cell of the same cell type (zeros). Several
870 computational approaches can be implemented to tackle this problem. Selecting only the
871 most highly variable genes in the data, and applying several dimensionality reduction
872 strategies represent common methods of handling data sparsity[207]. Moreover, various
873 methods have been developed to "impute" values for observed zeros, including SAVER[208] and
874 MAGIC[209].

875 Furthermore, to accurately decipher findings from scRNA-seq data, normalization is an
876 essential step to adjust for unwanted biases resulting from sequencing depth, sparsity, and
877 other potential technical artefacts. Numerous normalization methods have been developed
878 specifically for scRNA-seq data. One of the most general methods of normalization is the
879 NormalizeData function, implemented within the Seurat R package. With this method, gene
880 counts for each cell are normalized by the total expression, before multiplying by the scale

881 factor (10,000 by default) and natural log transforming the result. Various alternative
882 normalization methods have been described and tested, but these appear highly comparable
883 to the method built in to Seurat[210].

884

885 **BOX 3: CHALLENGES OF INTEGRATING MULTIPLE SINGLE EC DATASETS**

886 An integrated analysis of ECs extracted from multiple, publicly available single cell datasets
887 would provide a solution to the problem of overall low numbers of high-quality ECs in most
888 individual (whole tissue) studies. However, this strategy faces multiple challenges:

889 • Unavoidable "batches" across single cell datasets arise when they are generated in
890 different labs, and/or comprise different experimental models, sample cohorts,
891 library preparation methods or sequencing platforms. If not properly accounted for,
892 these batch effects could severely bias conclusions drawn from comparative and/or
893 integrated analyses. Despite the rapid development, optimization and benchmarking
894 of user-friendly data integration or batch correction methods for single cell
895 datasets[211-214], their use is limited to only certain aspects of downstream data
896 analyses, and finding a proper balance between aligning multiple datasets while
897 preserving key biological variation remains challenging. Not surprisingly, batch
898 correction is recognized as one of the major challenges in the single cell OMICs
899 community[215].

900 • With the increasing number of published single cell studies, insufficient
901 standardization of tissue isolation, as well as inconsistencies in annotation of EC
902 subtypes are arising as a major hurdle in the vascular single cell field. Usage of
903 different isolation protocols inevitably leads to variation in the overall yield of cellular

lineages, and the vascular compartment is no exception[216]. Standardized protocols for EC isolation from various mouse tissues are rising[217-219], and optimized pipelines for pan-cell type isolation of single cells or nuclei from human tumour samples are also being developed[216]. The continuation of such developments in additional tissues, conditions and species are expected to reduce discrepancies in overall EC/EC subtype yields across studies.

- The categorization of ECs into transcriptomically distinct phenotypes or subgroups within the identified vascular compartments, which by itself is not a trivial pursuit, varies substantially across studies. Whereas this variability can likely be attributed to differences in the overall EC yield across these studies (indeed, studies analysing enriched EC populations generally report a higher number of transcriptomically distinct EC subtypes as compared to whole-tissue analyses[41,53,55,65,133]), differences in the applied subclustering parameters and annotation strategies may also play a role.

FIGURE/TABLE LEGENDS

FIGURE 1: OVERVIEW OF SINGLE CELL OMICS TECHONOLOGIES AND CHARACTERISTICS OF THE TWO MAIN SCRNA-SEQ APPROACHES.

Single cell OMICs technologies are diverse, profiling different molecules at the single cell level. scATAc-seq analyses chromatin accessibility while scRNA-seq defines gene expression by measuring RNA steady state level. Other OMICs technologies such as proteomics and metabolomics are less commonly used at the single-cell level. B. Comparison of the two main scRNA-seq technologies in terms of cell isolation, recovered cell number, sequencing depth and sequencing type. 10X Genomics with its droplet based microfluidics technology allows

the sequenting of thousands of cells providing a high resolution of cell populations but without a full coverage of the transcriptome and no information on gene structure. In contrast, Smart-seq, with its higher sequencing depth and full length sequencing provides a better transcriptomics coverage but for a lower number of cells.

FIGURE 2: ENDOTHELIAL HETEROGENEITY IN HEALTH

EC phenotypes in health differ across organs, vascular beds and non-organotypic/vascular bed factors including sex and aging. ***Organotypic heterogeneity:*** ECs from different organs highly express genes involved in different biological processes. Liver and spleen ECs have a shared high expression of gene sets involved in immunoregulation, whilst heart and skeletal muscle ECs have upregulated expression of genes associated with membrane transporter and redox homeostasis. ***Vascular bed heterogeneity:*** Within each vascular bed, ECs from different segments of the same vessel type are diverse with several different EC subtypes. Two different subtypes of murine lung capillary ECs have been identified, aerocytes and general capillary ECs (gCap). Modified illustration from Gillich et al.[68]. ***Non-organotypic/vascular bed heterogeneity:*** EC phenotypes also vary across sex and age. Male ECs have enriched *Lars2* expression, compared to female ECs. Aged ECs are phenotypically different from younger ECs, such as brain capillary ECs expressing more pro-inflammatory and senescence-associated genes, resulting in dysregulated tight junctions in the blood-brain barrier.

FIGURE 3: ENDOTHELIAL HETEROGENEITY IN DISEASE.

ECs in a pathological context can differ from those in healthy organs on several levels. Each level of heterogeneity highlighted in this figure has been illustrated by a representative example. 1. The relative proportion of ECs (out of all cell types) can change in disease with,

for example, more ECs observed in Alzheimer brain compared to other cell types. 2. Disease can trigger a change in the relative proportion of EC subtypes such as an increased of peribronchial Ecs (pEC) but not arterial, vein and capillary Ecs (aEC, vEC and cEC) in idiopathic pulmonary fibrosis (IPF). 3. Specific EC subpopulation can be specifically observed in control or disease condition. For instance, “activated” ECs expressing pro-inflammatory and pro-atherogenic genes were observed in human atherosclerotic plaque. 4. Disease-mediated transcriptional changes constitute an additional level of heterogeneity. Genes involved in the major histocompatibility complex of class II (MHC-II) are down-regulated in lung tumour Ecs. 5. In diseases, ECs can change their interactions with neighbouring cell types. In atherosclerotic plaques, an increased interaction was observed between myeloid cells and Ecs mediated by PDGF/PDGFRB and leading to angiogenesis. 6. Ec can transition to another cell type by loosing their EC markers and gain other cell type identity markers. In mouse, the transient activation of mesenchymal genes has been observed 7 days after myocardial infarction (MI).

FIGURE 4: ANTI-ANGIOGENIC THERAPIES IN TUMOURS

Traditional AAT: Traditional AAT therapies target angiogenic growth factors, such as VEGF. VEGF-inhibition leads to inhibition of <10% of all ECs (including tip cells). ***Targeting EC metabolism:*** A potential alternative approach to inhibit angiogenesis in tumorigenesis presents targeting EC metabolism. Here inhibition of the glycolytic activator PFKFB3 has led to decreased tumor angiogenesis and impaired tumor growth in animal models. However, unlike traditional AAT, this approach has not yet been established in the clinical setting.

FIGURE 5: EC-SPECIFIC TARGET DISCOVERY AND PRIORITISATION FOR THERAPY

975 Meta-analyses using different platforms (for instance scRNAseq, Cytof, Bulk
976 proteomics/transcriptomics, epigenetic analyses etc.) and comparing data between different
977 species (mouse, rat, human…) can narrow down candidate cell types and genes with
978 important biological functions in a pathological setting. This approach focuses on
979 genes/proteins repetitively up- or downregulated in the pathological setting independent of
980 the method used and congruently changed between different species. Drug and toxicity
981 databases can then be exploited to identify potential drugs/drug classes to reverse the
982 determined genes/gene signatures. The availability of FDA/EMA-approved drugs potentially
983 capable of targeting certain genes can also help in target prioritisation.
984
985 **FIGURE 6: A SINGLE CELL VASCULAR DATABASE**
986 Single cell OMICs studies generate vast amounts of data. The challenge is to identify
987 biologically relevant EC phenotypes and disease-specific changes in ECs. Here, text-mining
988 tools can be tailored to identify OMICs publications including ECs, to aid in the generation of
989 an all-encompassing repository of EC OMICs data. Such a database will facilitate automated
990 and consistent EC annotation, as well as the comparison of ECs between different tissues,
991 species and conditions, advancing and harmonizing data-driven research in vascular biology.
992
993 **FIGURE 7: EXTENSION AND VALIDATION OF RLIS PREDICTED FROM SINGLE CELL DATA.**
994 An overview of methods for further exploration and validation of predicted RLIs from scRNA-
995 seq data, either on the transcriptome level (upper panel) or protein level (lower panel). (A)
996 Computational tools can be used to retrieve information regarding interactions between
997 ligands, receptors and their co-factors (CellChat), or the intracellular response of cell types on

998 the “receiving end” of predicted RLIs (NicheNet). (B) RLIs can also be placed in a spatial
999 context by implementation of computational tools allowing the integration of (i) scRNA-seq
1000 data with reference *in situ* hybridization data (Perler), or (ii) scRNA-seq data and its predicted
1001 RLI landscape (CSOmap). Spatial information can also be reconstructed *de novo* using scRNA-
1002 seq data (novoSpaRc), or by means of mapping untargeted scRNA-seq data to smaller,
1003 targeted spatial transcriptomics datasets (SpaGE, SpaOTsc). (C) Protein level exploration of
1004 RLIs can be achieved by applying established methods aimed at generating a dual
1005 transcriptome and protein read-out in scRNA-seq experiments (cite-SEQ, REAP-seq, INs-seq),
1006 or by using mass-spectometry based methods (cytometry by time of flight (CyTOF),
1007 Nativeomics).
1008
1009

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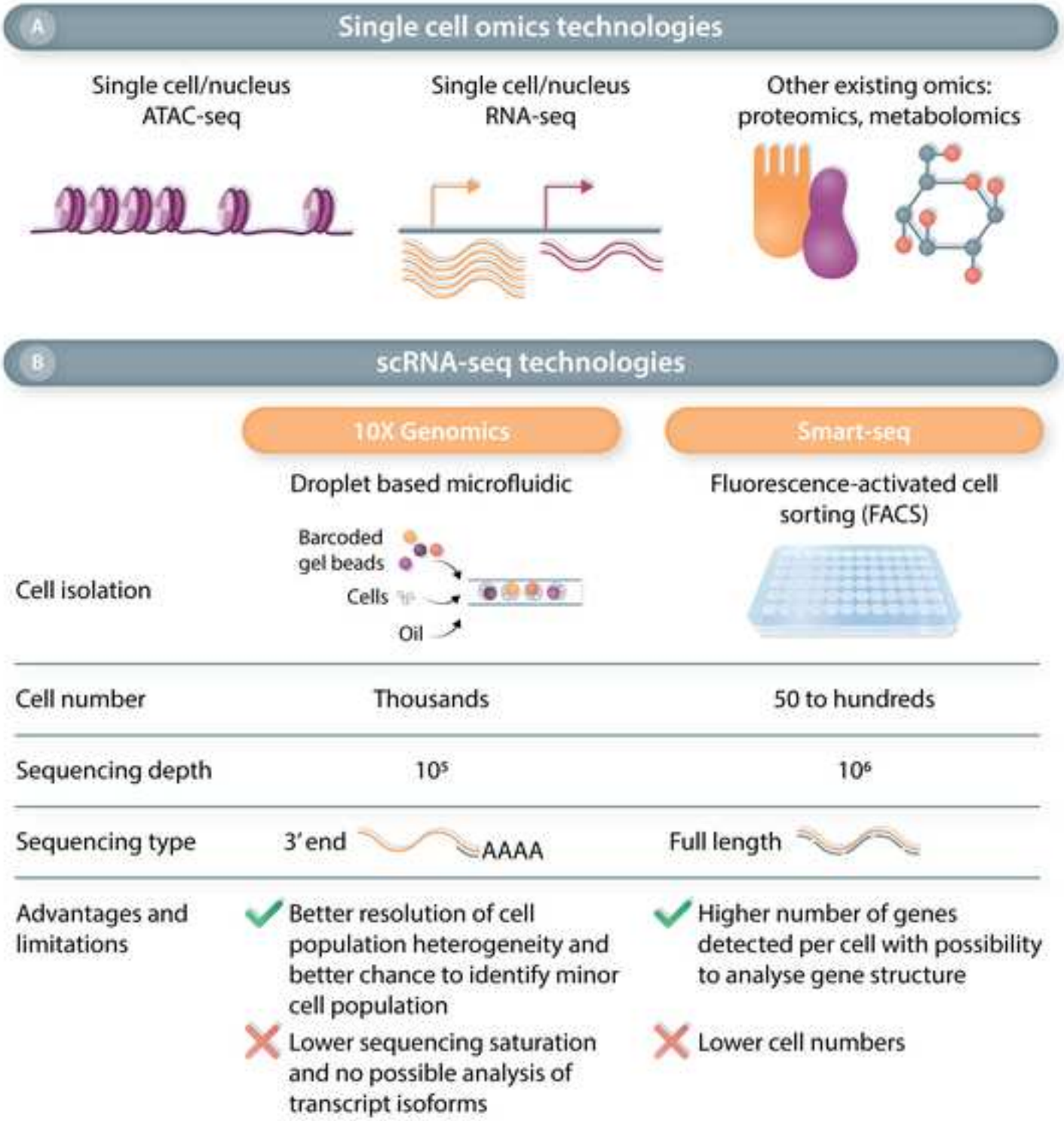


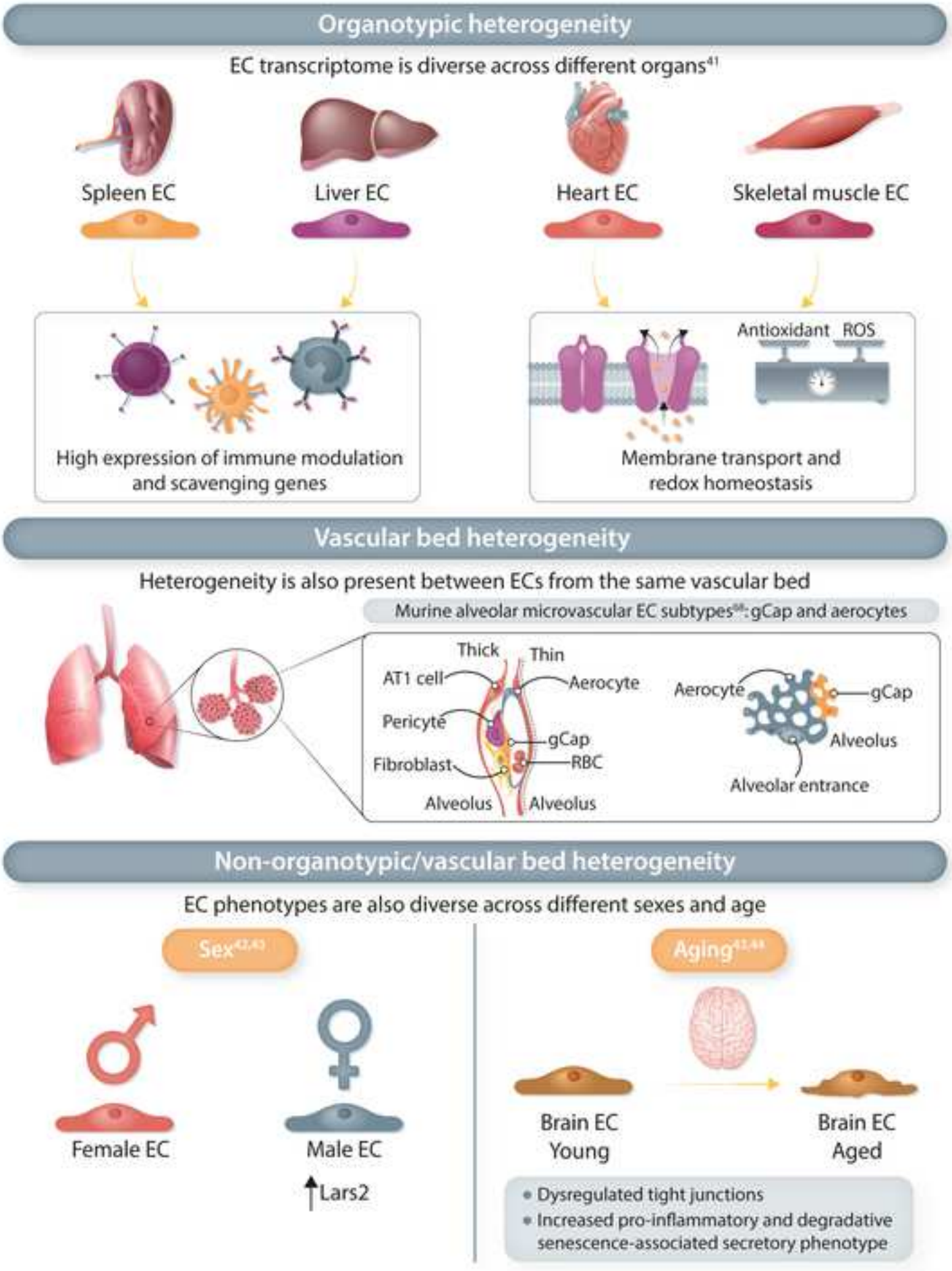
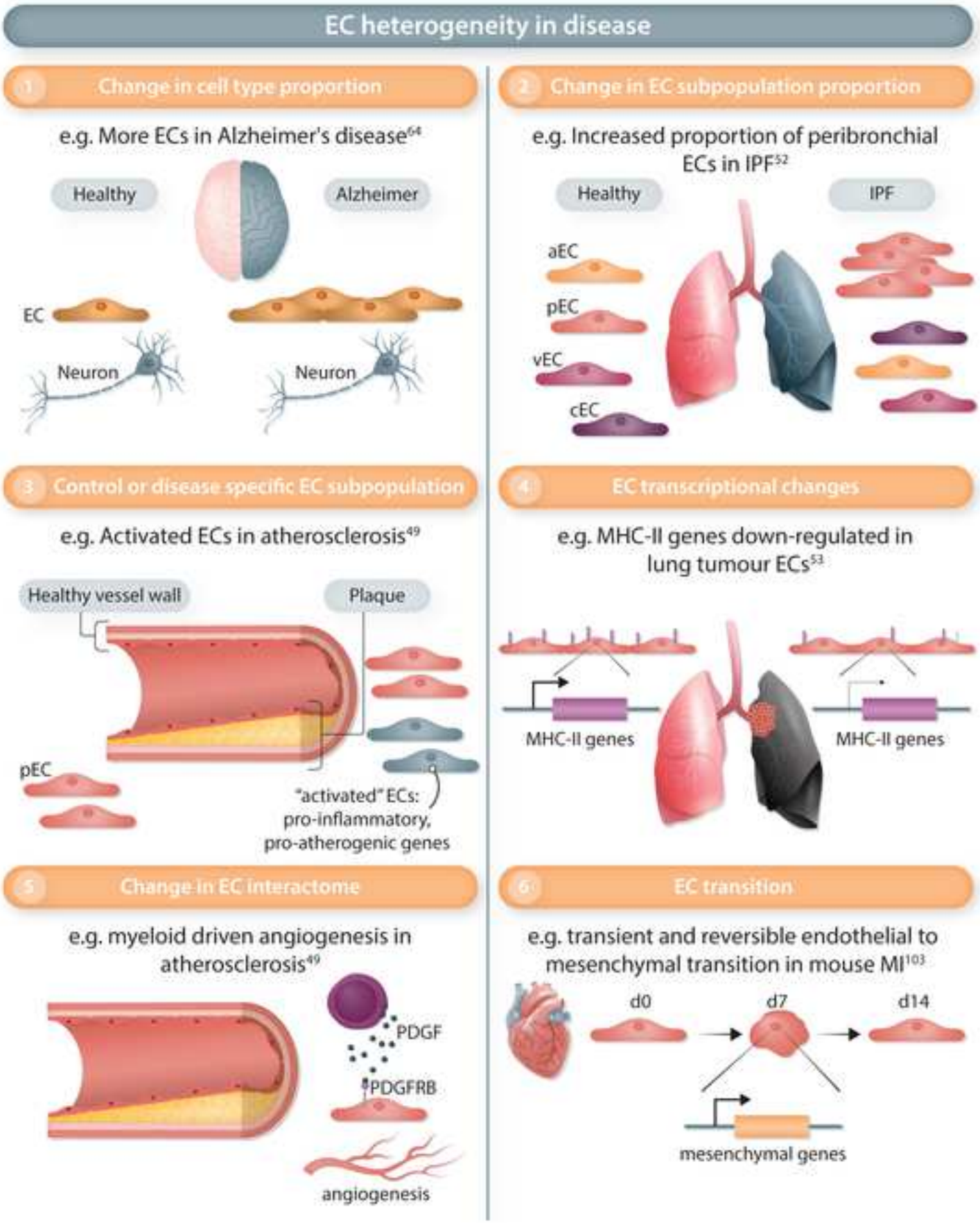


Figure 3



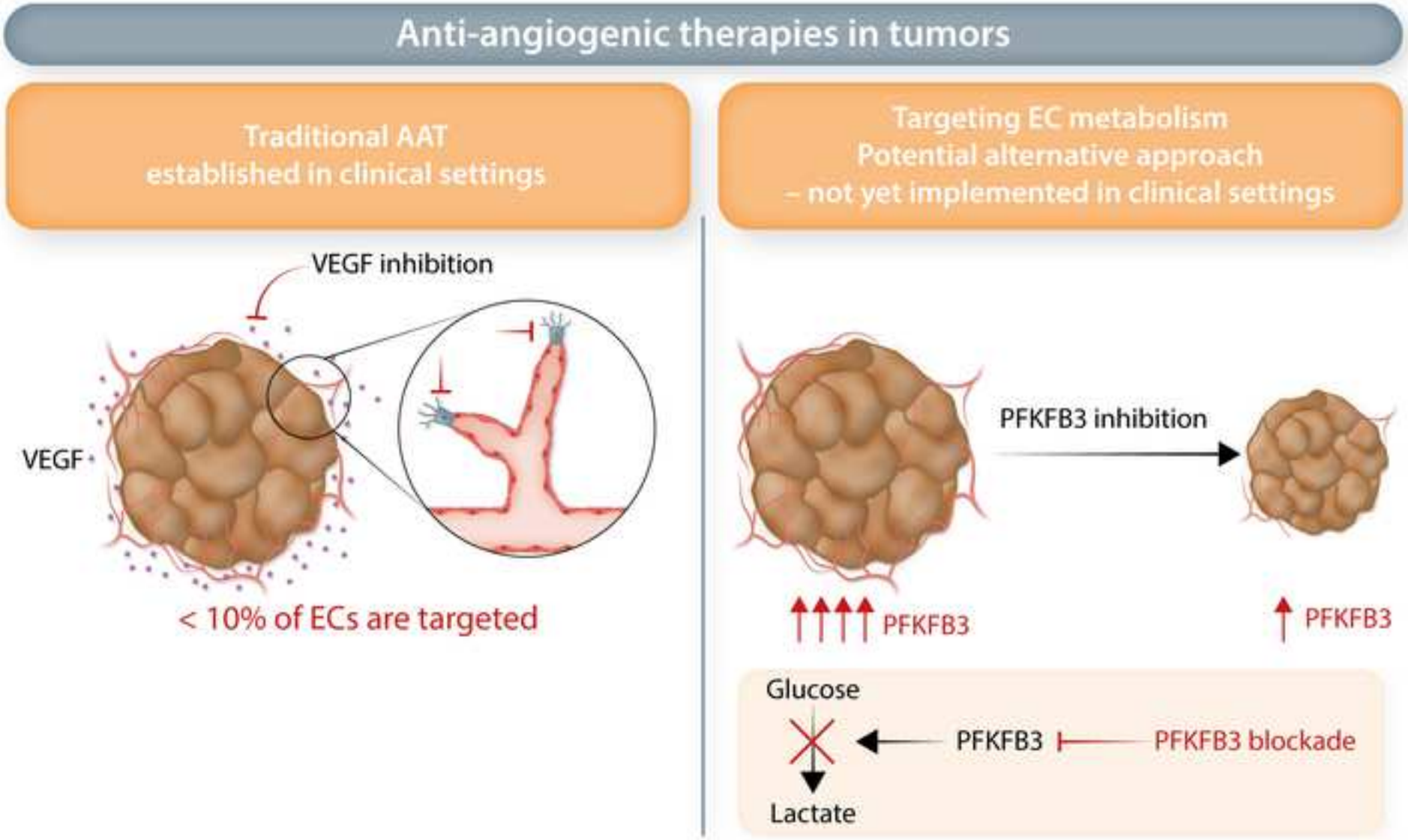


Figure 5