



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

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

Citation for published version:

Becker, LM, Chen, S-H, Rodor, J, de Rooij, LPMH, Baker, AH & Carmeliet, P 2022, 'Deciphering endothelial heterogeneity in health and disease at single cell resolution: progress and perspectives', *Cardiovascular Research*. <https://doi.org/10.1093/cvr/cvac018>

Digital Object Identifier (DOI):

[10.1093/cvr/cvac018](https://doi.org/10.1093/cvr/cvac018)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Cardiovascular Research

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



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

Lisa M. Becker^{1*}, Shiau-Haln Chen^{2*}, Julie Rodor^{2*}, Laura P.M.H. de Rooij^{1*}, Andrew H. Baker^{2,3&} & Peter Carmeliet^{1,4,5&}

Affiliations

- 1) Laboratory of Angiogenesis and Vascular Metabolism, Center for Cancer Biology, VIB & Department of Oncology, Leuven Cancer Institute, KU Leuven, Leuven 3000, Belgium;
- 2) The Queens Medical Research Institute, Centre for Cardiovascular Science, University of Edinburgh, Edinburgh EH16 4TJ, UK;
- 3) CARIM Institute, University of Maastricht, Universiteitssingel 50, 6200 MD Maastricht, The Netherlands.
- 4) State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-Sen University, Guangzhou 510060, Guangdong, China;
- 5) Laboratory of Angiogenesis and Vascular Heterogeneity, Department of Biomedicine, Aarhus University, Aarhus 8000, Denmark.

*equal contribution as first authors

&co-corresponding authors

Corresponding authors

Peter Carmeliet (peter.carmeliet@kuleuven.be)

Andrew H. Baker (andy.baker@ed.ac.uk)

Short title: EC heterogeneity at single cell resolution

Category of manuscript: Invited Review

Total word count: 17003

1 ABSTRACT

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

16

17

18

19

20

21

22

23 INTRODUCTION

24 Endothelial cells (ECs) line the interior surface of blood and lymph vessels. The endothelium
25 plays a crucial role in maintaining tissue homeostasis in normal health¹, but also contributes
26 to the progression of many diseases². ECs respond to various physical and chemical stimuli
27 and interact with other cells in the vessel wall, such as smooth muscle cells or pericytes, to
28 regulate vascular tone, blood flow, inflammation, permeability of solutes, and cellular
29 adhesion¹. Blood vessel overgrowth promotes diseases like cancer³, while EC dysfunction
30 contributes to vascular complications in diabetes, cardiovascular disease, and ageing-
31 associated pathologies (including neurological diseases with a vascular component, such as
32 Alzheimer's disease⁴). Hence, understanding the basic function and dysfunction of the
33 endothelium in health and disease has broad reaching implications.

34 Despite their common characteristics⁵, ECs are heterogeneous under physiological
35 and disease conditions⁶⁻⁸ (see Box 1 for definition of heterogeneity). Whilst they are present
36 throughout the whole body, ECs are highly specialised to meet the distinct needs of the
37 organs and sites they reside in. Within each organ, this heterogeneity is evident between
38 different vascular beds (arteries, veins, capillaries, lymphatics), between different segments
39 of the same vessel type, and even between neighbouring ECs⁸. EC phenotypes in disease are
40 equally diverse, exemplified by their ability to activate or inhibit angiogenesis, metabolic
41 switching or the release of vasodilators, reflecting varying responses to different stimuli and
42 changes in the pathological microenvironment³. While EC heterogeneity was highlighted in
43 other reviews⁶⁻⁸, recent advances in single cell technologies brought new resolution and new
44 insights into this heterogeneity. Characterising the different heterogeneity levels and their
45 functional relevance is crucial⁵, albeit dependent on the technologies used to measure and
46 quantify heterogeneity⁹.

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

52

53 **HISTORICAL PERSPECTIVE ON METHODS TO UNRAVEL EC HETEROGENEITY**

54 Prior to the advent of single-cell technologies, various *in situ* and *in vivo* methods were
55 developed to identify organ- and vessel type-specific endothelial markers whilst
56 circumventing difficulties faced in isolating pure populations of ECs from tissues and the loss
57 of *in vivo* phenotype of ECs cultured *in vitro* (reviewed in¹⁰⁻¹²). The concept that ECs from
58 different organs and vascular beds express different molecular markers was fueled by early
59 evidence of cancer and immune cells, preferentially migrating to specific organs – likewise,
60 peptides with a particular sequence homed to specific vascular beds¹³⁻¹⁶. For instance, the
61 Stamper-Woodruff assay was designed to study lymphocyte-endothelial binding in lymph
62 nodes¹³, later modified for use in other tissues^{14,15}, and alongside an emerging monoclonal
63 antibody technology, led to the identification of L-selectin as the receptor responsible for
64 selective homing of lymphocytes to high endothelial venules (HEVs) in lymph nodes¹⁶.

65 Phage display peptide libraries were used to unbiasedly screen peptide sequences
66 that home to particular organs¹⁷ or vascular beds *in vivo*¹⁸. These approaches, as well as SAGE
67 analysis^{19,20} and microarrays²¹⁻²³, contributed to the mapping of endothelial markers across
68 different organs and vascular beds within organs²⁴⁻³⁰, the development of tissue-targeted
69 pharmacodelivery^{18,31,32}, and to increasing our understanding of baseline EC phenotypes in
70 different organs³³. However, these methods suffer from relatively low throughput and

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

76

77 **SINGLE-CELL STUDIES IN ENDOTHELIAL CELLS**

78 Our characterisation and understanding of EC heterogeneity has advanced considerably in
79 the past years, due to the development of single-cell OMICs approaches (Figure 1A). These
80 techniques offer simultaneous analysis of hundreds to thousands of cells from complex
81 samples, such as tissues and heterogeneous cell populations, often without any prior
82 knowledge of cell markers³⁴. In particular, single-cell transcriptomics have been used to
83 identify and study EC populations in health and disease, across virtually all stages of life (e.g.
84 development, adulthood, aging), as detailed hereafter. While initial studies often described a
85 single organ in healthy condition³⁵⁻⁴⁰, recent studies now provide multi-organ analysis⁴¹⁻⁴⁴ or
86 focus on a specific disease, allowing to study ECs in physiological and pathological conditions.
87 Most single-cell RNA-sequencing (scRNA-seq) experiments relied on a droplet-based
88 approach, with the majority using the 3' end sequencing Chromium 10X technology. The main
89 characteristics and advantages of the two major scRNA-seq platforms (10X Genomics and
90 Smart-Seq) are described in Figure 1B, and more details on these technologies and
91 applications can be found in several reviews^{45,46}. Some scRNA-seq studies were also
92 accompanied by single-cell ATAC-seq (Assay for Transposase-Accessible Chromatin
93 sequencing), revealing the (epigenetic) chromatin accessibility landscape in ECs⁴⁷⁻⁵⁰. scRNA-
94 seq studies of ECs using mouse tissues/models took advantage of tissue availability, allowing

95 a more in-depth study of development and/or early disease stages, for which only late-stage
96 human disease samples are available, such as pulmonary arterial hypertension⁵¹. scRNA-seq
97 was also performed in healthy and diseased human tissues, such as types of lung⁵²⁻⁵⁸, liver⁵⁹,
98 heart⁶⁰⁻⁶³ or brain⁶⁴ diseases. Only a few studies combined both human and mouse
99 analysis^{53,65,66}, allowing a cross-species comparison. Of note, the interpretation and findings
100 of the EC scRNA-seq studies described below are also limited by the study design and chosen
101 data analysis pipelines. Some of these limitations, as well as the general caveats of scRNA-seq
102 analyses, have been highlighted in Box 2.

103

104 **ENDOTHELIAL HETEROGENEITY IN HEALTH**

105 In the healthy adult, phenotypic and structural diversity of ECs are a reflection of the breadth
106 of functions they perform to maintain tissue homeostasis, and are highly dependent on the
107 organs and microenvironment in which they reside. Given that different organs have different
108 needs⁸, dissecting the functional specialisation of ECs in healthy organs is key to
109 understanding EC health and behaviour, and essential for identifying how and why they
110 become dysfunctional in disease.

111

112 ***Organotypic heterogeneity***

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

118 comprehensive single-EC transcriptome atlas (EC atlas)⁴¹. Across different organs, inter-tissue
119 heterogeneity in EC transcriptional states was detected. ECs from different organs expressed
120 distinct transcriptional signatures, although ECs from certain organs had overlapping gene
121 signatures, suggesting shared biological processes⁴¹. Higher expression of gene sets involved
122 in immune modulation and scavenging were for example shared by liver and spleen ECs, while
123 an enrichment of genes involved in membrane transport was detected in heart and skeletal
124 muscle ECs⁴¹ (Figure 2). Different transcription factor networks were moreover up-regulated
125 in ECs from different tissues, which may drive organotypic diversity of ECs. Regulons of the
126 *Gata* family were for example enriched in liver and spleen ECs, while spleen ECs additionally
127 showed up-regulation of *Nr5a1* (Figure 2). Skeletal muscle ECs displayed enriched expression
128 of the *Pparg* network, while pulmonary ECs showed higher expression of the *Foxf1* network.
129 Another multi-organ study extracted mouse EC transcriptomes across 12 different organs
130 from the single-cell dataset generated by the Tabula Muris consortium⁴². Largely similar
131 overall findings were reported in both studies regarding unique EC molecular profiles across
132 different organs and overlapping gene expression between certain organs, as well as
133 enrichment of similar gene sets in the same organs (e.g. up-regulation of transporter-related
134 genes in brain ECs).

135

136 *Vascular bed heterogeneity*

137 Apart from organotypic heterogeneity of ECs, endothelial diversity also exists within the
138 vascular bed (artery, vein, capillary, lymphatic). In the aforementioned multi-organ study⁴¹,
139 conservation of EC vascular diversity across different organs was reported, as arterial, venous,
140 capillary and lymphatic ECs clustered together, regardless of the organ they originated from.
141 The vasculature in all organs displayed an arteriovenous hierarchy and the topography of

142 various endothelial subclusters along the vascular tree paralleled differences in blood flow,
143 pressure, and chemical composition in the circulation⁴¹.

144 scRNA-seq allowed finetuning of the traditional blood vascular EC classification
145 (artery, capillary, vein). For instance, twenty-four renal endothelial populations were
146 identified across the glomerular, cortical and medullary compartments³⁷, whilst studies on
147 pulmonary ECs highlighted extensive heterogeneity within the capillary endothelium^{68,69}. The
148 murine alveolar microvasculature was reported to consist of two cellular subtypes, aerocytes
149 and general capillary ECs (gCap), both of which were morphologically distinct from other
150 capillary cells in the bronchial circulation and other organs⁶⁸. Aerocytes with large, thin and
151 expansive morphology, are anatomically localised with alveolar type I (AT1) cells and enriched
152 with adhesion and leukocyte-sequestration genes, suggesting that these capillary ECs are
153 unique to the lungs and are specialised for optimal gas exchange and leukocyte trafficking
154 (Figure 2). In contrast, gCap cells are positioned in thick regions of the pulmonary stroma,
155 regulate vasomotor tone, and function as specialised stem/progenitor cells in alveolar
156 capillary homeostasis and repair. Both these alveolar capillary EC subtypes and their subtype-
157 specific functions are conserved in humans, although human aerocytes express MHC class II
158 genes whilst in mice, these genes are preferentially expressed by gCap cells⁶⁸. scRNA-seq of
159 human pulmonary cells also identified nine subpopulations of ECs, including two bronchial
160 endothelial groups that were distinctly enriched in matrix, fenestration and cell cycle-related
161 genes, compared to ECs that make up the pulmonary circulation⁶⁹ (Figure 2). In addition,
162 there were also two rare capillary subpopulations with features of both aerocytes and gCap
163 cells.

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

166 transcriptional profiles along the arteriovenous axis, known as zonation⁷⁰. While clusters of
167 cells corresponding to arterial, microvascular and vein ECs could be identified, these cells
168 could be ordered into a single one-dimensional range with markers of the different clusters
169 displaying a gradual change across this axis. Arterial ECs were enriched in transcription
170 factors, whilst transporter transcripts were dominantly expressed in capillary and vein ECs,
171 suggesting that trans-endothelial transport of molecules across the blood-brain barrier (BBB)
172 are concentrated in the latter regions. Similar zonation was observed in liver sinusoids, with
173 67% of liver sinusoidal ECs asymmetrically distributed along the portal vein-central vein axis,
174 though there was limited conservation of zonation profiles between human and mouse
175 scRNA-seq data³⁸⁻⁴⁰. These studies provided insights into how zonation influences endothelial
176 function and have implications for improving central nervous system (CNS) drug delivery in
177 treating brain diseases, as the BBB remains a significant physiological hurdle for drug design
178 and development⁷¹, and for understanding the relevance of EC zonation in disease
179 pathogenesis.

180

181 *Non-organotypic/vascular bed heterogeneity*

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

184 **Gender:** Since the Tabula Muris consortium used both male and female mice, their single cell
185 studies allow for the assessment of gender as a potential factor contributing to transcriptome
186 diversity among ECs from the same organ. Indeed, adult male and female mice showcase
187 different endothelial gene expression signatures and subpopulations in the brain, heart and
188 lung⁴². For instance, the gene encoding mitochondrial leucyl-tRNA synthetase (*Lars2*) is
189 enriched in male *versus* female ECs (Figure 2). Another study using the endothelial

190 compartment from the same Tabula Muris dataset did not find any differences in EC subtype
191 abundance between male and female mice, though this particular study had used another EC
192 annotation method (scmap and top 10 marker genes of each EC phenotype) to map the
193 Tabula Muris-derived ECs onto the subpopulations identified in their EC Atlas⁴¹. However, this
194 study did not further examine EC sex differences beyond the comparison of subtype
195 proportion. A third study, using an independent Tabula Muris dataset generated from young
196 and aged mice (3 months and 18 months)^{43,44}, found similar upregulation of *Lars2* in the young
197 male mice in addition to the upregulation of S100a8 and S100a9 in the older male mice, when
198 compared to the female. However, they concluded that EC gene expression was largely
199 similar between the sexes when taking age into consideration. Further investigation is
200 warranted to reveal how gender influences EC heterogeneity, and may explain gender
201 differences in cardiovascular risks.

202 **Aging:** Natural aging influences changes in endothelial phenotypes and may explain age-
203 related susceptibility to diseases⁷². In an attempt to uncover the impact of aging on the
204 mammalian heart, one study compared the single-cell transcriptomes of cardiac cells from
205 12-week-old and 18-month-old mice⁷³. Findings from this study suggest that the paracrine
206 crosstalk between cardiac fibroblasts and cardiac ECs is impaired during aging. Blunted
207 angiogenesis and autophagy, as well as proinflammatory activation in aged cardiac ECs were
208 attributed to aged fibroblasts, which had the most significant differential gene expression.
209 Increased expression of serpins in aged fibroblasts was found to mediate the anti-angiogenic
210 effects on cardiac ECs. A separate study investigating how aging affects neurovascular
211 dysfunction compared single-endothelial transcriptomes from young (2-3 months old) and
212 aged (18-20 months old) mouse brains⁷⁴. The age-associated transcriptional changes were
213 involved in immune/cytokine signalling (*Arhgap5*, *Pak2*, *Rdx*, *Gng5*, *Cdkn1a*, *Hnrnpk*), blood

214 brain barrier (BBB) integrity (*Afdn, Ctnna1, Iqgap1, Cgnl1, Nedd4, Ocln*), and energy
215 metabolism (*Cox6c, Cox7b, Ucp2, Hmgcs2, Pea15a*), most prominently in capillary ECs.
216 Another study observed up-regulation of von Willebrand factor, a marker of endothelial
217 dysfunction, in gCap cells but not aerocytes, in the lungs of aged mice⁶⁸. ECs across five
218 different organs in aged mice (18 months) have higher expression of immune and
219 inflammation-related genes, compared to their younger counterparts⁴³. Taken together,
220 these findings suggest heterogeneous regulation of the different EC populations during aging
221 that may contribute to the development of chronic diseases such as atherosclerosis,
222 hypertension and Alzheimer's disease (Figure 2).

223

224 ***Endothelial heterogeneity in development***

225 EC functional heterogeneity during development is evident in the developing heart⁷⁵, where
226 the endocardium, a specialised endothelium lining the inner heart walls, acts not only as a
227 physical barrier protecting the cardiac tissue from the chamber circulation but also as an
228 essential source of different cardiac cell types⁷⁶. Heart valve formation begins with the
229 development of endocardial cushions at the atrioventricular canal and outflow tract, and at
230 E8.5 to 9.0, a subset of these cushion endocardial cells undergoes endothelial-to-
231 mesenchymal transition (EndMT) to give rise to the precursor cells that will eventually go on
232 to form the mature heart valves⁷⁷. Previously, it was unknown if this endocardial subset was
233 predetermined to undergo EndMT or if the surrounding myocardium and haemodynamic
234 circulation push this subset towards such a fate, since the trabeculae endocardium does not
235 undergo EndMT. Endocardial heterogeneity was confirmed by a recent scRNA-seq study,
236 which sequenced 36,000 cardiac cells from three distinct developmental stages at E7.75 when
237 cardiac progenitor cells begin to differentiate, during heart tube formation at E8.25 and at

238 E9.25 when the heart tube loops⁷⁵. This study identified three endocardial subpopulations:
239 haematoendothelial progenitors, ECs and endocardial cells initiating EndMT. However, this
240 study did not further examine these subpopulations beyond their identification and
241 assignment in the single cell dataset. As such, important questions remain about the origin(s)
242 of endocardial subpopulations and the wider endothelial heterogeneity in vascular
243 development: (i) Are all ECs different from the initial point of their formation; and (ii) If not,
244 when do they start becoming different and what drives this differentiation during
245 development?

246 Since a functioning circulatory system is vital for embryonic growth, formation of the
247 vascular network precedes the formation of all other organ systems. ECs originate *de novo* by
248 vasculogenesis from mesodermal precursors in at least three sites: the yolk sac, allantois and
249 embryo proper. Primitive ECs at this stage are highly plastic and were presumed to be non-
250 specialised as they undergo rapid expansion and coalesce to form the primary vascular plexus,
251 before acquiring arterial, venous and lymphatic identities. A scRNA-seq of whole mouse
252 embryos at E8.25 reported that subsets of these primitive ECs show unique identities that
253 could be demarcated by their maturity and anatomical origins⁷⁸. Allantoic ECs express distinct
254 transcriptional signatures, characterised by *Tbx4*, *Hoxa10* and *Hoxa11* expression, while non-
255 allantoic ECs could be subdivided by their maturity based on their expression levels of *Etv2*,
256 *Cdh5* and *Pecam1*. These findings, alongside scRNA-seq profiling of early *Xenopus* embryos⁷⁹,
257 suggest that EC diversity begins much earlier in development than previously thought. It
258 remains to be seen if and how this early diversification of EC identity influences their
259 heterogeneous function and phenotypes later in life, and in the pathophysiology of diseases.

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

262 the needs of their resident organs during organ vascularisation. Bipotentiality has been
263 reported in pulmonary plexus cells, as they give rise to both subsets of alveolar capillary ECs
264 (aerocytes and gCap cells) during development⁶⁸. Aerocyte development has also been
265 reported to depend on AT1-derived VEGFA, as this population of ECs is specifically and
266 completely lost in AT1-specific *Vegfa* mutant lungs⁸⁰. These findings again suggest early
267 specification of EC phenotype during development that continues to persist in the adult.

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

276 A pseudotemporal dataset of the developing mouse embryo was generated through
277 scRNA-seq from nine sequential timepoints, E6.0 to E8.5⁵⁰. This study identified two discrete
278 subsets of hemogenic ECs, expressing both endothelial and haematopoietic markers. One of
279 the subpopulations showed a more mature EC phenotype, with a high expression of classical
280 markers of mature ECs such as *Cdh5* and *Pecam1*. By incorporating temporal information of
281 each individual cell, this group was identified as the hemogenic ECs involved in the definitive
282 wave, suggesting that EC maturity is essential to give rise to HSPCs. In addition, they also
283 observed that these second wave ECs were transcriptionally heterogeneous, and through
284 clustering analysis, this heterogeneity was associated with their anatomical origins. This study
285 also reported TAL1 as a transcriptional regulator of the two haematopoietic waves, and

286 documented that *Tal1*^{-/-} ECs deviate into an aberrant mesodermal phenotype. An additional
287 study using ATAC-seq on single nuclei from 10 mouse embryos at E8.25 identified EC-specific
288 regions of open chromatin⁸⁴. Integrative analysis with TAL1 ChIP-seq data from past studies
289 and validation in transgenic mouse assays revealed that TAL1 binds to both known (*Fli* -15 kb
290 and *Erg* +86 kb) and novel (*Flt1* +67 kb and *Malm3* +360 kb) endothelial enhancers.
291 Altogether, important transcriptomic and epigenetic mechanisms direct ECs towards a
292 hemogenic fate during development.

293

294 **ENDOTHELIAL HETEROGENEITY IN DISEASE**

295 *Endothelial cell population shifts in disease*

296 Dimensionality reduction and clustering analysis allowed the comparison of EC populations
297 in disease samples. First, a change of the relative proportion of ECs compared to other cell
298 types has been noted in some diseases (Figure 3), with for example fewer ECs detected in
299 metastasis compared to primary tumours⁸⁵, while more ECs have been observed in
300 Alzheimer's disease *versus* control samples⁶⁴.

301 Within the EC population, a change in the proportion of EC subtypes corresponds to a
302 second level of heterogeneity observed in disease (Figure 3). Expansion of one of the three
303 EC subtypes, probably corresponding to postcapillary venular cells, was observed in human
304 skin samples from patients with atopic dermatitis or psoriasis⁸⁶. In idiopathic pulmonary
305 fibrosis (IPF), the peribronchial EC population was increased compared to control or
306 obstructive pulmonary disease conditions and associated to areas of bronchiolization and
307 fibrosis, showing the distinct response of this population between two diseases⁵². In mouse
308 lungs exposed to hyperoxic conditions, an increase of the aerocytes/*Car4*⁺ ECs population was
309 observed⁸⁷.

310 An increase in EC proliferation was previously associated with several diseases⁸⁸ and
311 scRNA-seq showed evidence of such an increase after myocardial infarction (MI)⁸⁹ or H1N1
312 influenza lung injury⁹⁰ in mice. In the lungs, most vessel-type ECs contribute to the
313 proliferating response⁹⁰, while, in the myocardial infarction (MI) study, the use of a PDGFB-
314 driven multispectral (Confetti reporter) EC tracing mouse model confirmed that proliferating
315 ECs originated from resident cells via clonal expansion⁸⁹. This Confetti reporter mouse line
316 system was previously used to show EC clonal expansion after ischemia-induced
317 neovascularization, and clonally expanded ECs selected by laser capture microscopy were
318 analysed by bulk transcriptomics without single-cell resolution⁹¹. scRNA-seq was also used to
319 study EC populations contributing to liver⁹² and aorta⁹³ regeneration after injury in mice. In
320 liver injury, a tissue-resident *Cd157⁺* population contributes to the regeneration of large
321 vessels expressing only EC-specific genes⁹². In the aorta, regeneration originates from local
322 adjacent ECs; both bulk and scRNA-seq studies revealed transcriptomic changes, including an
323 increase of the progenitor marker *Ly6a/Sca1* and the transcription factor *Aft3*⁹³.

324 Disease can lead to a third level of heterogeneity in the endothelium, with the
325 presence of EC subpopulations being almost exclusively restricted to control or disease
326 conditions (Figure 3). After MI in mice, several clusters were predominantly composed of cells
327 from disease samples and were characterised by a higher expression of the plasmalemma
328 vesicle-associated protein gene *Plvap*⁸⁹, shown to regulate EC proliferation *in vitro*⁸⁹ and
329 previously involved in EC permeability and angiogenesis⁹⁴. In human liver cirrhosis, two
330 disease-specific EC populations restricted to the fibrotic niche were identified and annotated
331 as scar-associated ECs, in which marker gene analysis revealed the expression of pre-fibrotic
332 and immune response genes⁵⁹. Furthermore, pro-inflammatory and pro-atherogenic genes
333 characterised EC clusters from the mouse aorta exposed to disturbed flow⁴⁸. Similar pathways

334 seem to be identified in ECs from human atherosclerotic plaques, in which atherosclerosis-
335 specific EC populations were described as activated ECs⁴⁹. The term “activated ECs” was also
336 used to describe EC populations identified in prostate cancer, which express cancer-
337 associated fibroblast markers and extracellular matrix (ECM) genes but show a down-
338 regulation of genes related to immunoregulatory pathways⁹⁵.

339 Tip ECs are critical for vessel sprouting, by leading the sprout at the forefront⁹⁶. In both
340 human and mouse lung tumours, tip EC populations have been detected in scRNA-seq studies,
341 in agreement with the role of angiogenesis in tumour growth and proliferation^{53,65}.
342 Proliferating cells were detected, at substantial rates in mouse tumours, but at negligible
343 rates in human (lung) tumours⁵³. Tip cells were also found in scRNA-seq studies of mouse
344 choroidal neovascularization⁶⁵. Common/congruent tip cell markers, conserved across
345 species (mouse /human), diseases and tissues (cancer/choroidal neovascularization), and
346 experimental conditions (freshly isolated/cultured) were identified, allowing a better
347 understanding of angiogenesis across disease conditions^{53,65}. Congruent tip cell markers
348 included genes previously detected in tip cells, such as *APLN*, but also novel tip cell
349 transcription factors *TCF4*, *SOX4* and *SMAD1*, and novel genes relevant to the migratory tip
350 EC phenotype⁵³. Silencing of two novel markers, *LXN* (latexin) and *FSCN1* (Fascin), in human
351 umbilical vein ECs furthermore affected tip cell competitiveness in a mosaic spheroid assay,
352 confirming the tip cell role of these markers⁵³. In addition to tip cells, another population of
353 so-called “breach” cells has recently been identified in murine lung tumors by scRNA-seq.
354 Based on their transcriptional profile breach cells are hypothesized to assist tip cells to lead
355 the vessel sprout⁵³.

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

358 pathways⁶⁵. Such metabolic changes in ECs, key to angiogenesis, were previously reported in
359 scRNA-seq of all cells from lung cancer⁵⁵. Moreover, in mouse cerebral cavernous
360 malformations, based on a *Pcd10* deletion model, ECs with tip cell traits have been reported⁹⁷
361 but further characterisation is required to confirm if they indeed represent genuine tip cells.

362 EndMT occurs in many cardiovascular diseases⁹⁸, yet with some controversies due to
363 the lack of standard in diagnosing the transition, and difficulties comparing different time
364 points and/or models⁹⁹. Using scRNA-seq of EC reporter mice, no evidence of EndMT was
365 found in liver cirrhosis¹⁰⁰. In contrast, EndMT was reported in human calcific aortic valve
366 disease¹⁰¹, in human atherosclerosis⁴⁹ and in mouse atherosclerosis induced by disturbed
367 flow⁴⁸ or the high-cholesterol high fat diet in *Apoe*^{-/-} mouse¹⁰². However, these scRNA-seq
368 studies reporting EndMT did not use an EC tracing system, not allowing the full confirmation
369 of the transition, and relied essentially on trajectory analysis. Additional analysis, such as RNA
370 velocity might help to define the directionality of the observed trajectories and the cell
371 population origins. Recently, activation of ECM genes was observed 7 days after MI in the
372 mouse, and confirmed in scRNA-seq analysis of an EC lineage tracing model¹⁰³. This study,
373 based on a time course experiment, showed that EndMT is transient and reversible in MI¹⁰³,
374 in contrast to the sustained EndMT observed in atherosclerosis and likely due to the chronic
375 nature of the stimuli^{48,49,102}. The potential transient nature of EndMT might explain why
376 EndMT was not detected in another MI mouse study⁸⁹ and highlights the need to study
377 different stages of disease development in association with a better EndMT diagnosis⁹⁸.

378

379 *Transcriptomics changes leading to EC heterogeneity*

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

383 Changes in genes related to inflammation have been observed in ECs in several
384 contexts. In adult mouse peripheral lymph nodes, antigenic stimulation by oxazolone led to
385 an up-regulation of inflammatory genes such as *Sele* and *Cxcl9* in HEVs¹⁰⁴. In mouse hyperoxic
386 lung, genes known to be regulated by inflammation (*Ctgf*, *Fxyd5*) were up-regulated in the
387 aeryocyte EC populations⁸⁷. In Alzheimer's diseases, up-regulation of genes from the major
388 histocompatibility complex (MHC) class I were observed in ECs⁶⁴, while the expression of the
389 MHC class II genes, part of the capillary gene signature, are up-regulated in pulmonary arterial
390 hypertension (PAH)¹⁰⁵ and down-regulated in ECs from murine and human lung tumours⁵³.
391 Changes in inflammation-related genes were also reported in atherosclerotic *Apoe*^{-/-} mice¹⁰²,
392 and a recent study of the mouse aorta during disturbed flow suggested a potential transition
393 of ECs towards an immune-like phenotype as an additional type of EC reprogramming⁴⁸. All
394 these studies confirm that the endothelium is a target of the inflammatory process, but likely
395 also acts as an immuno-regulator, in part by working as semi-professional antigen-presenting
396 cells. Indeed, the term "immunomodulatory ECs" (IMECs) was recently coined to describe the
397 immunoregulatory EC phenotype¹⁰⁶.

398 Vessel growth dysregulation contributes to the pathogenesis of many diseases such
399 as cancer and pulmonary arterial hypertension (PAH). In addition to the identification of
400 angiogenic tip cells, angiogenesis pathway regulation has also been documented in several
401 studies. Indeed, down-regulation of genes relevant to capillarization were observed in ECs in
402 human systemic sclerosis¹⁰⁷, while anti-angiogenic genes were up-regulated in ECs from
403 hyperoxic lungs⁸⁷. In contrast, pro-angiogenic/ capillarization genes were activated in ECs in

404 Alzheimer's disease⁶⁴ and cirrhotic mouse liver¹⁰⁰ and in one capillary EC subtype in PAH¹⁰⁵.
405 Interestingly, in cirrhotic liver, the activation was zonation-dependent and restricted to a
406 specific region of the liver sinusoidal ECs¹⁰⁰. As most changes of angiogenesis pathway did not
407 seem to be associated with the detection of a tip cell population, these regulations might not
408 be linked to sprouting angiogenesis but might possibly reflect other vessel formation modes
409 such as splitting angiogenesis, not characterised so far by any standard marker expression, or
410 EC migration. Further studies are needed to understand the contribution of these different
411 processes to vessel growth or regression.

412 Several studies reported the up-regulation of the ECM genes in ECs in disease
413 conditions, probably reflecting structural EC changes. In prostate cancer, activated ECs were
414 characterised by an up-regulation of ECM genes⁹⁵, while the transient mesenchymal gene
415 activation in MI also included ECM gene changes. In addition, ECM gene up-regulation was
416 observed in liver cirrhosis¹⁰⁰, lung cancer⁵³ and in systemic sclerosis¹⁰⁷. Additional
417 transcriptome regulations in ECs have also been described. Down-regulation of several
418 members of the Notch signalling pathway occurs in ECs in pulmonary fibrosis⁵⁷. In atopic
419 dermatitis and psoriasis, ECs activate fetal genes⁸⁶, while in oxygen-induced retinopathy, the
420 peak of neovascularization was associated with expression of senescence genes¹⁰⁸. Further
421 investigation is required to define the functional effect of these changes and their relevance
422 across diseases.

423 To understand the regulation leading to these transcriptomics changes, scRNA-seq
424 studies were performed together with single-cell ATAC-seq, confirming chromatin
425 accessibility changes in correlation with the transcriptomics changes and reporting disease-
426 induced peaks such as in mouse MI⁴⁷. As transcription factors (TFs) play a key role in shaping
427 the transcriptome, motif enrichment analysis of scATAC-seq of mouse carotid artery in

428 different flow conditions identified KLF2/KLF4 motifs in stable flow, while motifs for RELA,
429 AP1, STAT1 and TEAD1 were enriched in accessible regions from disturbed flow conditions⁴⁸.
430 Approaches developed for TF target and/or regulon-based analysis of scRNA-seq data^{109,110}
431 revealed the possible role of FLI1 and TEAD1 in tumour ECs⁵⁵, and of SOX18 in human PAH⁵¹.

432

433 *Contribution of the microenvironment to EC heterogeneity in disease*

434 ECs plastically adapt to the physiological needs of different tissues. Unsurprisingly therefore,
435 signals in the microenvironment shape the EC subtype landscape¹¹¹. ECs acquire a specialized
436 role depending on their location and status in physiological conditions that can make them
437 more or less responsive to certain stimuli in disease. For instance, in cerebral cavernous
438 malformation, venous capillary ECs are the main contributor of the lesion, as arterial ECs
439 remain non-responsive to the transformation⁹⁷. Furthermore, HEVs in lymph nodes possess
440 an activated phenotype that is lost upon changes to the microenvironment such as inhibition
441 of lymphotoxin-b receptor signalling¹⁰⁴.

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

449 Cell-cell communication and interaction can be assessed in scRNA-seq data by an
450 unbiased analysis of receptor-ligand interaction (RLI) pairs using popular tools such as
451 CellPhoneDB¹¹⁶ or more recent and comprehensive tools including CellChat¹¹⁷ and

452 NicheNet¹¹⁸, detailed hereafter in the “Recent Advances & Future Perspectives” section of
453 this review. Increased interactions of ECs with other cells were detected in the heart of
454 postnatal day 8 mice 3 days after MI⁴⁷ but also in human atherosclerotic plaque⁴⁹. In the
455 murine regenerative heart, R-Spondin was identified as an EC ligand expressed by epithelial
456 cells with pro-angiogenic effect to EC *in vitro*⁴⁷. ECs appear to receive communication from
457 fibroblasts in the murine hyperoxic lung, with the ligand and receptor *Bmp5* and *Bmpr2*
458 expressed by fibroblast and ECs respectively⁸⁷. In atherosclerosis, the PDGF/PDGFRB
459 interaction between myeloid cells and ECs led to the hypothesis of a myeloid-driven
460 angiogenic contribution to plaque destabilization⁴⁹. In the heart, evidence of communication
461 between fibroblasts and ECs was detected in both healthy and injured conditions using
462 scRNA-seq and the proximity between fibroblasts and ECs was confirmed by
463 immunofluorescence¹¹⁹. As mentioned previously, a study of the murine aging heart revealed
464 the deterioration of this paracrine crosstalk, with *in-vitro* experiments showing a reduced
465 angiogenic property of the conditioned medium from heart-derived aged fibroblasts⁷³. In
466 contrast, ECs might communicate with mesenchymal cells in human cirrhotic liver, where the
467 scar-associated ECs express the non-canonical Notch ligand *JAG1*, *JAG2* and *DLL4*, whereas
468 the *NOTCH3* receptor is expressed by scar-associated mesenchymal cells⁵⁹. Co-culture
469 experiments, using primary human hepatic stellate cells (HPCs) and ECs from cirrhotic livers,
470 validated that this interaction promotes fibrillar collagen production by HPCs, which could be
471 inhibited by perturbation of *NOTCH3* expression⁵⁹, highlighting the translational potential of
472 findings identified through scRNA-seq and interactome analyses.

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

476 preferential expression of semaphorins and VEGF family members, a phenomenon that is
477 conserved across multiple species⁶⁶. Lastly, and in line with their well-appreciated
478 immunoregulatory role, interactome analyses revealed novel interactions between
479 pulmonary ECs and immune cells, including possible recruitment of *CX3CR1+* non-classical
480 monocytes to ECs (*CX3CL1+*), and attraction of *CCR1+* dendritic cells to veins (*CCL23+*),
481 bronchial vessels (*CCL14+*) and lymphocytes (*CCL5+*)⁶⁹, highlighting interesting avenues for
482 future research in light of lung cancer and/or inflammatory disease.

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

487

488 THERAPEUTIC IMPLICATIONS

489 *Anti-angiogenic therapies in cancer – targets and resistance*

490 As angiogenesis is critical for a variety of diseases, therapies have been devised to either
491 promote or inhibit angiogenesis¹²⁰. While pro-angiogenic efforts promise to offer novel
492 therapeutic opportunities for cardiovascular disease and diabetes, here we focus on anti-
493 angiogenic therapies (AATs). Cancer presents one of the main pathologies for which AAT is
494 used, due to the critical role of angiogenesis in cancer progression and metastasis¹²¹.
495 Currently approved AATs center around blocking the key pro-angiogenic target VEGF, though
496 other targets are emerging (Figure 4). While initially designed to prune the tumour
497 vasculature¹²²⁻¹²⁴, current clinical trials explore whether VEGF-blockade can improve
498 immunotherapy by normalizing the tumour vasculature¹²⁵. The success of VEGF-blockade
499 therapy is however tampered by insufficient efficacy and resistance^{126,127}. Several resistance

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

503 In a mouse lung cancer model, tip cells and breach cells (putatively assisting tip cells
504 to lead the vessel sprout⁵³) represent the EC subtypes most sensitive to VEGF blockade⁵³,
505 whereas other EC subtypes were less or differentially sensitive. In fact, postcapillary vein ECs
506 increased in abundance upon anti-VEGF treatment⁵³. Whether the increases in capillary and
507 postcapillary vein ECs is a consequence of switching from sprouting angiogenesis (SA) to
508 vessel co-option (a known escape mechanism to AAT therapy¹³¹), remains to be determined.
509 This may explain – at least in part – the limited success and therapeutic immunity towards
510 AAT.

511 In addition, the various distinct EC types identified by single-cell transcriptomic studies
512 might also contribute to a better understanding of AAT resistance^{53,132}. Tip cells, which are
513 the presumed key targets of AAT, amount to fewer than 10% of all ECs within lung tumours⁵³,
514 thus the majority of ECs is in fact not targeted by AAT (Figure 4). Differences in the
515 composition of different EC subtypes in tumours from distinct patients⁵³ might furthermore
516 explain why some patients respond better than others to AAT. Moreover, venous ECs in
517 tumours contain a subset of so-called resident endothelial stem cells (rESCs)⁵³. rESCs were
518 also identified in large vessels of multiple murine organs and showed self-renewal capacity as
519 well as contributed to vessel regeneration in different models of vessel injury^{65,92,93}. As venous
520 ECs expand upon AAT⁵³, it raises the question whether these rESCs might reconstitute vessels
521 upon AAT, thereby contributing to therapy resistance. Endothelial progenitor cells were
522 identified in human metastatic lung adenocarcinoma¹³³. Moreover, aldehyde dehydrogenase
523 (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 NSCLC⁵⁵. Myc has been
530 identified as a driver of the endothelial regeneration process⁹³, 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-option¹³¹. 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.

547 Importantly, combination of AAT with other anti-cancer therapies, such as
548 chemotherapy or immunotherapy has shown promising results not only in pre-clinical
549 models, but also in the clinic. In fact, several AAT agents (e.g. bevacizumab, aflibercept,
550 sorafenib, sunitinib), apart from being approved as single-agent therapy, have reached
551 approval in combination with chemotherapy, or as second-line therapy after patients
552 progressed on chemotherapy¹³⁹. Moreover, the combination of IFN- α treatment with anti-
553 VEGF therapy has been approved by the FDA for treatment of metastatic renal cell
554 carcinoma¹⁴⁰. With the advent of novel immunotherapies, such as immune checkpoint
555 blockade, there are many new promising anti-cancer therapeutic opportunities¹³⁹. New
556 insights into distinct EC phenotypes could help to develop more precise treatments tailored
557 to target specific EC populations, which might create a favourable environment, in particular
558 for immunotherapy to work. IMECs or other specialized EC phenotypes might offer such
559 opportunities. For instance, HEVs are involved in the recruitment of different immune cells¹⁴¹,
560 thus promoting HEV growth is expected to be beneficial for enhancing the anti-cancer effect
561 of immunotherapy. This concept to “tune rather than only prune” is a novel strategy for
562 future AAT.

563

564 *EC metabolism as alternative target to modulate angiogenesis*

565 More than a decade ago, ECs were shown to undergo metabolic changes to execute their
566 various functions. This metabolic reprogramming is driven in part by different signalling
567 cascades, for instance growth factor signalling (e.g. VEGF can induce glycolysis) or Notch
568 signalling (Notch suppresses glycolysis in stalk cells)¹⁴². However, it is now clear that EC
569 metabolism is not only necessary, but also sufficient (independent of growth factors or other
570 stimuli) to control EC function¹⁴³. Several metabolic pathways have been implicated in distinct

571 functions. Single-cell studies alongside metabolomic investigations have uncovered several
572 metabolically distinct EC subtypes. For instance, during sprouting angiogenesis, tip cells up-
573 regulate glycolysis and amino acid (AA) metabolism to support migration¹⁴²⁻¹⁴⁴. These
574 metabolic pathways are also used by stalk cells (however, at lower levels), where they support
575 proliferation and biomass production¹⁴³. Stalk cells as well as phalanx cells also rely on fatty
576 acid oxidation (FAO)¹⁴⁵. In quiescent phalanx ECs, FAO contributes to maintenance of their
577 quiescent phenotype¹⁴⁶ (Figure 4). It has also been recognized that different EC subsets
578 display distinct metabolic signatures, in a tissue-specific manner⁴¹. For instance, different
579 metabolic transporters are most highly expressed in brain ECs, spleen ECs are enriched in
580 cholesterol metabolism, while cardiac and muscle ECs show elevated fatty acid metabolism⁴¹.
581 For a detailed review of EC metabolism, and metabolic heterogeneity in different EC types,
582 we refer to recent excellent reviews^{143,147,148}.

583 When comparing ECs from healthy tissues to those in disease, different metabolic
584 gene signatures were observed as well. For instance, compared to their respective controls,
585 ECs from choroidal neovascularization or murine lung tumour models displayed an increase
586 in gene expression related to several metabolic pathways, such as glycolysis, tricarboxylic acid
587 (TCA) cycle, oxidative phosphorylation (OXPHOS), one-carbon metabolism and nucleotide
588 synthesis⁶⁵. In line with these findings, single-cell analysis of colorectal, lung and ovarian
589 cancer revealed that tip ECs in all three cancer types up-regulate glycolysis and OXPHOS gene
590 signatures⁵⁶. Moreover, EC subtypes in human lung cancer also presented with metabolic
591 gene adaptations compared to their healthy counterparts, with an up-regulation of genes
592 involved in lipid metabolism in capillary tumour ECs, and increased prostaglandin metabolism
593 in venous tumour ECs⁵³. Compared to ECs from early stage ground glass nodules
594 adenocarcinoma, ECs from late stage solid lung adenocarcinoma were also enriched in

595 metabolic gene processes¹⁴⁹, and circulating ECs from metastatic prostate cancer patients
596 showed enriched metabolic gene expression compared to circulating ECs from healthy
597 controls¹⁵⁰.

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 remodelling¹⁴². Genetic silencing of PFKFB3 inhibited tip cell function and resulted in
603 acquisition of a quiescent phenotype¹⁴². 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 zebrafish¹⁴⁴. 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 prematurity¹⁴⁵. Tip and stalk cells also rely on fatty acid synthesis¹⁴³. 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

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

623

624 ***Novel targets from single cell studies - prioritization & targeting***

625 Whilst the unravelling of EC heterogeneity at single cell resolution has led to the discovery of
626 exciting novel and specialized EC subtypes with a presumable key role in disease, the
627 prioritization of functionally important candidate (metabolic) genes that are most reflective
628 of these EC subtypes remains a formidable challenge. It demands the development of efficient
629 means to transcend the atlas-like descriptive listing of EC-subtype specific marker genes into
630 the most promising functionally relevant and therapeutically targetable candidates, and
631 various *in silico* methods have been developed and reported in the recent years to aid in this
632 challenge. For instance, use of an integrated (meta-)analysis of candidate gene expression
633 across species, diseases and models identified *PLOD1* and *PLOD2* as novel angiogenic
634 candidates⁵³. Silencing or inhibition of both genes furthermore impaired *in vitro* and *in vivo*
635 vessel sprouting, validating the therapeutic potential of these genes⁵³. Moreover, a similar
636 meta-analysis approach, yet combined with scRNA-seq data-tailored genome-scale metabolic
637 models (GEMs), proved an efficient method for prioritization of *SQLE* and *ALDH18* as
638 promising new metabolic targets for AAT⁶⁵ (Figure 5). Again, *in vitro* and *in vivo* perturbation
639 experiments confirmed the functional relevance of both genes for angiogenesis, stressing
640 their translational potential⁶⁵.

641 Querying of cell types enriched for trait-relevant genes based on genome-wide
642 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 plaques⁴⁹. 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 characterisations⁴⁹. 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 Database¹⁵⁶, 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 ECs⁵¹. Interestingly, the rat PAH scRNA-seq study also assessed the
654 therapeutic potential of existing drugs in PAH, by means of *in silico* drug screening¹⁵⁶. 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 cells¹⁵⁷ (Figure 5). Another recent method, Augur, allows
658 prioritization of cellular subtypes most responsive to a biological perturbation¹⁵⁸, 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 scale¹⁵⁸. 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 arthritis¹⁵⁹. 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 mouse¹⁶¹⁻¹⁶⁴. Further, enzyme-antibody conjugates and
684 nanoparticle formulation aimed at specific targeting of the pulmonary^{165,166} or splenic¹⁶⁷
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-

691 seq studies has not yet been achieved, is the lack of consensus marker genes for distinct EC
692 subpopulations. Future scRNA-seq analyses might provide further insights into construction
693 of specific promoters for inclusion into gene therapy vectors in order to selectively target
694 specific EC populations. This strategy however depends on the mutual exclusivity of EC
695 subtype specific marker genes, and may be more challenging in case of tissues, where EC
696 expression signatures exhibit spatial zonation, as for instance shown in the hepatic
697 vasculature¹⁶⁸.

698

699 **RECENT ADVANCES & FUTURE PERSPECTIVES**

700 *A compendium of all publicly available single ECs*

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

709 The latter issue is expected to improve in the coming years with the advancement of
710 automated cell type annotation tools, which are rising in number and user-friendliness¹⁶⁹⁻¹⁷²,
711 but even more so with the development of tools like Azimuth¹⁷³, providing rapid and
712 automated mapping, visualization and annotation of single cell datasets through an online
713 web application. Yet, these tools often provide reference datasets representing major cellular
714 lineages in various tissues/organs but preclude annotation of different EC subtypes within a

715 particular tissue or vascular bed. There is thus a need for the generation of tissue-specific
716 “gold standard” vascular atlases, to both improve and progress standardization of EC OMICs
717 annotations. A recent integration of six lung scRNA-seq datasets resulted in joint profiling of
718 over 15,000 ECs from 73 individuals¹⁷⁴, and although not covering the full spectrum of
719 published (healthy/normal) lung single EC RNA-seq data, this study provided one of the first
720 in-depth reference atlases of healthy/normal lung ECs and is likely to aid annotation of future
721 pulmonary EC studies in health and disease. When such efforts will be combined with
722 automated cell type mapping tools and standardized whole tissue/EC isolation protocols,
723 harmonized EC annotation across laboratories, tissues and experimental setups should be
724 feasible in the foreseeable future.

725 Another obstacle in integrated analysis of EC OMICs data is represented by the
726 inconsistent formats in which raw data is deposited, and the (sometimes) severe lack of detail
727 regarding sample origin information and data processing parameters. The availability of
728 processed counts and annotated metadata is furthermore limited, yet inevitable to ensure
729 reproducibility of the data across labs of different expertise. Data-sharing methods also
730 become increasingly variable, complicating uniform methods of dataset curation. While lab-
731 hosted servers, offering virtual exploration and downloading of data, are rising in popularity
732 and enable non-bioinformatics focused labs an affordable and reliable method of data
733 exploration, a more centralized storage platform would greatly enhance our ability to study
734 vascular OMICs in a streamlined and comprehensive manner. Various recent efforts aimed at
735 offering solace, either by generation of free-of-charge portals harbouring curated and
736 harmonized processed datasets, or frequently updated overviews of published scRNA-seq
737 datasets¹⁷⁵⁻¹⁷⁸. Specialized databases, like *JingleBells*¹⁷⁹ for immune cells, *cancerSEA*¹⁸⁰ for
738 cancer cell states, *The Human Cell Atlas* portal for all tissues and cell types of the human

739 body¹⁸¹, 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

763 analysis represent predictions, requiring functional validation. Recently developed tools
764 provide more comprehensive solutions, including CellChat¹¹⁷, taking into account interactions
765 between ligands, receptors and their co-factors, or NicheNet¹¹⁸, aimed at diving deeper into
766 the intracellular response of cell types on the “receiving end” of these predicted interactions
767 (Figure 7A). Ultimately, however, RLIs can be more accurately investigated when positional
768 information is preserved. Advances in spatial transcriptomics, crowned as “Method of the
769 year 2020” by Nature Methods¹⁸⁵, presumably hold great promise for future enhancements
770 in studying the interplay between ECs and their environment in a tissue architecture-
771 dependent context (Figure 7B). Interestingly, several computational tools have been recently
772 developed with the aim to provide a more cost-effective alternative to spatial
773 transcriptomics, either by integration of scRNA-seq data with reference *in situ* hybridization
774 (ISH) data (Perler¹⁸⁶), prediction of cellular coordinates in a three-dimensional pseudo-space
775 based on input scRNA-seq data and known ligand-receptor interactions (CSOmap¹⁸⁷), *de novo*
776 spatial reconstruction of single-cell gene expression (novoSpaRc¹⁸⁸), or prediction of whole-
777 transcriptome expressions in their spatial configuration by mapping of untargeted scRNA-seq
778 data to smaller, targeted spatial transcriptomics datasets (SpaGE¹⁸⁹, SpaOTsc¹⁹⁰) (Figure 7B).

779 Finally, translation of cellular cross-talk predictions to the protein level, for instance
780 by applying established methods including cite-SEQ¹⁹¹, REAP-seq¹⁹², or cytometry by time of
781 flight (CyTOF)¹⁹³, or the more newly developed Nativeomics¹⁹⁴ (allowing detection of intact
782 ligand–receptor assemblies using mass spectrometry), INs-seq¹⁹⁵ (allowing more accurate
783 exploration of transcription factors, active signalling networks and metabolic activity by
784 parallel transcriptome and intracellular proteomic profiling at single cell resolution), or single
785 cell proteomics¹⁹⁶ will be essential to complement and finetune EC-interactomes predicted
786 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 summarised as a statistical
809 characteristic of a cell population. It is most commonly quantified through epigenomic,

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

819

820 **BOX 2: STUDY DESIGN AND BIOINFORMATICS CONSIDERATION FOR scRNA-SEQ STUDIES TO**

821 **IDENTIFY AND CHARACTERISE EC POPULATIONS ON THE TRANSCRIPTOME LEVEL**

822 ***Whole tissues vs EC enrichment vs EC isolated from reporter mice:*** Whole tissue/organ
823 analysis potentially lowers the power and resolution of EC analysis, yet allows their analysis
824 amidst other cell types and querying of cell-cell interactions. To obtain a better resolution of
825 the EC transcriptomic landscape, enrichment strategies based on CD31 expression can be
826 performed prior to sequencing^{41,65,97,102}. scRNA-seq of ECs isolated from reporter mice have
827 also been implemented in liver cirrhosis¹⁰⁰, after myocardial infarction^{89,103} and in
828 atherosclerosis¹⁹⁷. Such designs allow us to track the changes ECs undergo in diseases and
829 reveal the presence and/or absence of cell transitions, such as endothelial-to-mesenchymal
830 transition (EndMT)¹⁰³.

831

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

834

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

839

840 ***Depth of EC downstream analysis:*** The depth of downstream EC analyses also varies across
841 different studies, sometimes due to the study design (e.g. limited number of isolated ECs), or
842 to incomplete characterization of EC clusters. Especially in cases of whole tissue scRNA-seq,
843 EC analysis has often been performed alongside the analysis of other more abundant cell
844 types, and lacks in-depth investigation and/or detailed subclustering of ECs. For instance, in
845 studies of abdominal aortic aneurysm¹⁹⁸, Alzheimer disease¹⁹⁹, cancer⁵⁶, cirrhosis/fibrosis^{52,59}
846 and atherosclerosis²⁰⁰, ECs were present but their EC subsets were not studied.

847

848 ***scRNA-seq analysis: general caveats:*** Besides EC-specific considerations in terms of study
849 design and analysis, hurdles in quality control (QC) of the data remain an ongoing challenge
850 in the field of single-cell OMICs. For instance, during library preparation using droplet-based
851 methods, multiple cells may have been captured together (doublets), non-viable cells may
852 have been captured, or, droplets may have been sequenced that harbored no cells (empty
853 droplets). Differences in library preparation might also stem from variability in cell recovery
854 and quality, which results from different isolation protocols. After sequencing, it is thus
855 imperative to implement a series of QC steps to ensure the analysis will be performed on
856 high-quality cells only. Generally, QC of scRNA-seq data is based on three variables: (i) the
857 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 (DoubletDecon²⁰¹, Solo²⁰², scds²⁰³, Scrublet²⁰⁴, Doublet
865 Finder²⁰⁵). Additionally, cell hashing strategies can be implemented to enhance the detection
866 of doublets²⁰⁶.

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 sparsity²⁰⁷. Moreover, various
873 methods have been developed to “impute” values for observed zeros, including SAVER²⁰⁸ and
874 MAGIC²⁰⁹.

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 Seurat²¹⁰.

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 datasets²¹¹⁻²¹⁴, 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 community²¹⁵.

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

904 lineages, and the vascular compartment is no exception²¹⁶. Standardized protocols
905 for EC isolation from various mouse tissues are rising²¹⁷⁻²¹⁹, and optimized pipelines
906 for pan-cell type isolation of single cells or nuclei from human tumour samples are
907 also being developed²¹⁶. The continuation of such developments in additional tissues,
908 conditions and species are expected to reduce discrepancies in overall EC/EC subtype
909 yields across studies.

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

917

918 **FIGURE/TABLE LEGENDS**

919 **FIGURE 1: OVERVIEW OF SINGLE CELL OMICS TECHNOLOGIES AND CHARACTERISTICS OF THE TWO** 920 **MAIN SCRNA-SEQ APPROACHES.**

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

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

931

932 **FIGURE 2: ENDOTHELIAL HETEROGENEITY IN HEALTH**

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

946

947 **FIGURE 3: ENDOTHELIAL HETEROGENEITY IN DISEASE.**

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

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

965

966 **FIGURE 4: ANTI-ANGIOGENIC THERAPIES IN TUMOURS**

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

973

974 **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-spectrometry based methods (cytometry by time of flight (CyTOF),
1007 Nativeomics).

1008

1009

1010 **FUNDING**

1011 L.D.R. and L.M.B. are supported by the “Fonds Wetenschappelijk Onderzoek” (FWO). L.M.B.
1012 is supported by a Marie Skłodowska-Curie-individual fellowship. S.H.C. is supported by the
1013 Wellcome Trust Tissue Repair PhD (108906/B/15/Z); and the University of Edinburgh Global
1014 Award. J.R is supported by the British Heart Foundation (BHF). A.H.B is supported by VASCMIR
1015 ERC Advanced Grant, BHF Chair of Translational Cardiovascular Sciences (CH/11/2/28733),
1016 BHF programme grants (RG/14/3/30706, RG/20/5/34796 and RG/19/3/34265), BHF Centre
1017 for Vascular Regeneration and BHF Research Excellence Award RE/18/5/34216. P.C. is
1018 supported by grants from Methusalem funding (Flemish government); the Fund for Scientific
1019 Research-Flanders (FWO-Vlaanderen); the European Research Council ERC Proof of Concept
1020 - ERC-713758; the European Research Council ERC Advanced Research Grant EU- ERC74307;
1021 and the NNF Laureate Research Grant from Novo Nordisk Foundation (Denmark).

1022

1023

1024 **AUTHOR INFORMATION**

1025 **Contributions:** L.M.B., S.H.C., J.R., and L.D.R. wrote the manuscript, and designed the figures.

1026 A.H.B. and P.C. conceptualized the manuscript. All authors approved the manuscript.

1027 **Author notes:** These authors contributed equally: Lisa M. Becker, Shiau-Haln Chen, Julie

1028 Rodor, Laura P.M.H. de Rooij.

1029 **Corresponding author:** Andrew H. Baker and Peter Carmeliet are co-corresponding authors.

1030 Correspondence should be addressed to A.H.B.

1031

1032

1033 **REFERENCES**

- 1034 1 Deanfield, J. E., Halcox, J. P. & Rabelink, T. J. Endothelial function and dysfunction:
1035 testing and clinical relevance. *Circulation* **115**, 1285-1295,
1036 doi:10.1161/CIRCULATIONAHA.106.652859 (2007).
- 1037 2 Tesfamariam, B. & DeFelice, A. F. Endothelial injury in the initiation and progression
1038 of vascular disorders. *Vascul Pharmacol* **46**, 229-237, doi:10.1016/j.vph.2006.11.005
1039 (2007).
- 1040 3 Rajendran, P. *et al.* The vascular endothelium and human diseases. *Int J Biol Sci* **9**,
1041 1057-1069, doi:10.7150/ijbs.7502 (2013).
- 1042 4 Zenaro, E., Piacentino, G. & Constantin, G. The blood-brain barrier in Alzheimer's
1043 disease. *Neurobiol Dis* **107**, 41-56, doi:10.1016/j.nbd.2016.07.007 (2017).
- 1044 5 Altschuler, S. J. & Wu, L. F. Cellular heterogeneity: do differences make a difference?
1045 *Cell* **141**, 559-563, doi:10.1016/j.cell.2010.04.033 (2010).
- 1046 6 Aird, W. C. Endothelial cell heterogeneity. *Cold Spring Harb Perspect Med* **2**,
1047 a006429, doi:10.1101/cshperspect.a006429 (2012).
- 1048 7 Aird, W. C. Phenotypic heterogeneity of the endothelium: I. Structure, function, and
1049 mechanisms. *Circ Res* **100**, 158-173, doi:10.1161/01.RES.0000255691.76142.4a
1050 (2007).
- 1051 8 Aird, W. C. Phenotypic heterogeneity of the endothelium: II. Representative vascular
1052 beds. *Circ Res* **100**, 174-190, doi:10.1161/01.RES.0000255690.03436.ae (2007).
- 1053 9 Goldman, S. L. *et al.* The Impact of Heterogeneity on Single-Cell Sequencing.
1054 *Frontiers in Genetics* **10**, 8 (2019).

1055 10 Ruoslahti, E. & Rajotte, D. An address system in the vasculature of normal tissues
1056 and tumors. *Annu Rev Immunol* **18**, 813-827,
1057 doi:10.1146/annurev.immunol.18.1.813 (2000).

1058 11 Simonson, A. B. & Schnitzer, J. E. Vascular proteomic mapping in vivo. *J Thromb*
1059 *Haemost* **5 Suppl 1**, 183-187, doi:10.1111/j.1538-7836.2007.02551.x (2007).

1060 12 Griffin, N. M. & Schnitzer, J. E. Chapter 8. Proteomic mapping of the vascular
1061 endothelium in vivo for vascular targeting. *Methods Enzymol* **445**, 177-208,
1062 doi:10.1016/S0076-6879(08)03008-5 (2008).

1063 13 Stamper, H. B., Jr. & Woodruff, J. J. Lymphocyte homing into lymph nodes: in vitro
1064 demonstration of the selective affinity of recirculating lymphocytes for high-
1065 endothelial venules. *J Exp Med* **144**, 828-833, doi:10.1084/jem.144.3.828 (1976).

1066 14 Hill, T. A., Stanford, M. R., Graham, E. M., Dumonde, D. C. & Brown, K. A. A new
1067 method for studying the selective adherence of blood lymphocytes to the
1068 microvasculature of human retina. *Invest Ophthalmol Vis Sci* **38**, 2608-2618 (1997).

1069 15 Grober, J. S. *et al.* Monocyte-endothelial adhesion in chronic rheumatoid arthritis. In
1070 situ detection of selectin and integrin-dependent interactions. *J Clin Invest* **91**, 2609-
1071 2619, doi:10.1172/JCI116500 (1993).

1072 16 Gallatin, W. M., Weissman, I. L. & Butcher, E. C. A cell-surface molecule involved in
1073 organ-specific homing of lymphocytes. *Nature* **304**, 30-34, doi:10.1038/304030a0
1074 (1983).

1075 17 Pasqualini, R. & Ruoslahti, E. Organ targeting in vivo using phage display peptide
1076 libraries. *Nature* **380**, 364-366, doi:10.1038/380364a0 (1996).

1077 18 Arap, W. *et al.* Targeting the prostate for destruction through a vascular address.
1078 *Proc Natl Acad Sci U S A* **99**, 1527-1531, doi:10.1073/pnas.241655998 (2002).

1079 19 Sengoelge, G. *et al.* A SAGE-based comparison between glomerular and aortic
1080 endothelial cells. *Am J Physiol Renal Physiol* **288**, F1290-1300,
1081 doi:10.1152/ajprenal.00076.2004 (2005).

1082 20 Sengoelge, G. *et al.* A SAGE based approach to human glomerular endothelium:
1083 defining the transcriptome, finding a novel molecule and highlighting endothelial
1084 diversity. *BMC Genomics* **15**, 725, doi:10.1186/1471-2164-15-725 (2014).

1085 21 Chi, J. T. *et al.* Endothelial cell diversity revealed by global expression profiling. *Proc*
1086 *Natl Acad Sci U S A* **100**, 10623-10628, doi:10.1073/pnas.1434429100 (2003).

1087 22 Schweighofer, B., Rohringer, S., Proll, J. & Holnthoner, W. A microarray analysis of
1088 two distinct lymphatic endothelial cell populations. *Genom Data* **4**, 115-118,
1089 doi:10.1016/j.gdata.2015.04.005 (2015).

1090 23 Chen, B. P. *et al.* DNA microarray analysis of gene expression in endothelial cells in
1091 response to 24-h shear stress. *Physiol Genomics* **7**, 55-63,
1092 doi:10.1152/physiolgenomics.2001.7.1.55 (2001).

1093 24 Voyta, J. C., Via, D. P., Butterfield, C. E. & Zetter, B. R. Identification and isolation of
1094 endothelial cells based on their increased uptake of acetylated-low density
1095 lipoprotein. *J Cell Biol* **99**, 2034-2040, doi:10.1083/jcb.99.6.2034 (1984).

1096 25 Nolan, D. J. *et al.* Molecular signatures of tissue-specific microvascular endothelial
1097 cell heterogeneity in organ maintenance and regeneration. *Dev Cell* **26**, 204-219,
1098 doi:10.1016/j.devcel.2013.06.017 (2013).

1099 26 Fina, L. *et al.* Expression of the CD34 gene in vascular endothelial cells. *Blood* **75**,
1100 2417-2426 (1990).

- 1101 27 Ghitescu, L. D., Crine, P. & Jacobson, B. S. Antibodies specific to the plasma
1102 membrane of rat lung microvascular endothelium. *Exp Cell Res* **232**, 47-55,
1103 doi:10.1006/excr.1997.3490 (1997).
- 1104 28 Pusztaszeri, M. P., Seelentag, W. & Bosman, F. T. Immunohistochemical expression
1105 of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal
1106 human tissues. *J Histochem Cytochem* **54**, 385-395, doi:10.1369/jhc.4A6514.2005
1107 (2006).
- 1108 29 Ponder, B. A. & Wilkinson, M. M. Organ-related differences in binding of Dolichos
1109 biflorus agglutinin to vascular endothelium. *Dev Biol* **96**, 535-541, doi:10.1016/0012-
1110 1606(83)90191-4 (1983).
- 1111 30 Belloni, P. N. & Nicolson, G. L. Differential expression of cell surface glycoproteins on
1112 various organ-derived microvascular endothelia and endothelial cell cultures. *J Cell*
1113 *Physiol* **136**, 398-410, doi:10.1002/jcp.1041360303 (1988).
- 1114 31 McIntosh, D. P., Tan, X. Y., Oh, P. & Schnitzer, J. E. Targeting endothelium and its
1115 dynamic caveolae for tissue-specific transcytosis in vivo: a pathway to overcome cell
1116 barriers to drug and gene delivery. *Proc Natl Acad Sci U S A* **99**, 1996-2001,
1117 doi:10.1073/pnas.251662398 (2002).
- 1118 32 Arap, W., Pasqualini, R. & Ruoslahti, E. Cancer treatment by targeted drug delivery to
1119 tumor vasculature in a mouse model. *Science* **279**, 377-380,
1120 doi:10.1126/science.279.5349.377 (1998).
- 1121 33 Gunawardana, H. *et al.* Tissue-specific endothelial cell heterogeneity contributes to
1122 unequal inflammatory responses. *Sci Rep* **11**, 1949, doi:10.1038/s41598-020-80102-
1123 w (2021).
- 1124 34 Mincarelli, L., Lister, A., Lipscombe, J. & Macaulay, I. C. Defining Cell Identity with
1125 Single-Cell Omics. *Proteomics* **18**, e1700312, doi:10.1002/pmic.201700312 (2018).
- 1126 35 Vanlandewijck, M. *et al.* A molecular atlas of cell types and zonation in the brain
1127 vasculature. *Nature* **554**, 475-480, doi:10.1038/nature25739 (2018).
- 1128 36 Karaiskos, N. *et al.* A Single-Cell Transcriptome Atlas of the Mouse Glomerulus. *J Am*
1129 *Soc Nephrol* **29**, 2060-2068, doi:10.1681/ASN.2018030238 (2018).
- 1130 37 Dumas, S. J. *et al.* Single-Cell RNA Sequencing Reveals Renal Endothelium
1131 Heterogeneity and Metabolic Adaptation to Water Deprivation. *J Am Soc Nephrol* **31**,
1132 118-138, doi:10.1681/ASN.2019080832 (2020).
- 1133 38 MacParland, S. A. *et al.* Single cell RNA sequencing of human liver reveals distinct
1134 intrahepatic macrophage populations. *Nat Commun* **9**, 4383, doi:10.1038/s41467-
1135 018-06318-7 (2018).
- 1136 39 Aizarani, N. *et al.* A human liver cell atlas reveals heterogeneity and epithelial
1137 progenitors. *Nature* **572**, 199-204, doi:10.1038/s41586-019-1373-2 (2019).
- 1138 40 Halpern, K. B. *et al.* Single-cell spatial reconstruction reveals global division of labour
1139 in the mammalian liver. *Nature* **542**, 352-356, doi:10.1038/nature21065 (2017).
- 1140 41 Kalucka, J. *et al.* Single-Cell Transcriptome Atlas of Murine Endothelial Cells. *Cell* **180**,
1141 764-779 e720, doi:10.1016/j.cell.2020.01.015 (2020).
- 1142 42 Paik, D. T. *et al.* Single-Cell RNA Sequencing Unveils Unique Transcriptomic
1143 Signatures of Organ-Specific Endothelial Cells. *Circulation* **142**, 1848-1862,
1144 doi:10.1161/CIRCULATIONAHA.119.041433 (2020).
- 1145 43 Huang, X. *et al.* Single-Cell Transcriptional Profiling Reveals Sex and Age Diversity of
1146 Gene Expression in Mouse Endothelial Cells. *Front Genet* **12**, 590377,
1147 doi:10.3389/fgene.2021.590377 (2021).

1148 44 Almanzar, N. *et al.* A single-cell transcriptomic atlas characterizes ageing tissues in
1149 the mouse. *Nature* **583**, 590-595, doi:10.1038/s41586-020-2496-1 (2020).

1150 45 Kashima, Y. *et al.* Single-cell sequencing techniques from individual to multiomics
1151 analyses. *Exp Mol Med* **52**, 1419-1427, doi:10.1038/s12276-020-00499-2 (2020).

1152 46 Baran-Gale, J., Chandra, T. & Kirschner, K. Experimental design for single-cell RNA
1153 sequencing. *Brief Funct Genomics* **17**, 233-239, doi:10.1093/bfgp/elx035 (2018).

1154 47 Wang, Z. *et al.* Cell-Type-Specific Gene Regulatory Networks Underlying Murine
1155 Neonatal Heart Regeneration at Single-Cell Resolution. *Cell Rep* **33**, 108472,
1156 doi:10.1016/j.celrep.2020.108472 (2020).

1157 48 Andueza, A. *et al.* Endothelial Reprogramming by Disturbed Flow Revealed by Single-
1158 Cell RNA and Chromatin Accessibility Study. *Cell Rep* **33**, 108491,
1159 doi:10.1016/j.celrep.2020.108491 (2020).

1160 49 Depuydt, M. A. C. *et al.* Microanatomy of the Human Atherosclerotic Plaque by
1161 Single-Cell Transcriptomics. *Circ Res* **127**, 1437-1455,
1162 doi:10.1161/CIRCRESAHA.120.316770 (2020).

1163 50 Pijuan-Sala, B. *et al.* A single-cell molecular map of mouse gastrulation and early
1164 organogenesis. *Nature* **566**, 490-495, doi:10.1038/s41586-019-0933-9 (2019).

1165 51 Saygin, D. *et al.* Transcriptional profiling of lung cell populations in idiopathic
1166 pulmonary arterial hypertension. *Pulm Circ* **10**, doi:10.1177/2045894020908782
1167 (2020).

1168 52 Adams, T. S. *et al.* Single-cell RNA-seq reveals ectopic and aberrant lung-resident cell
1169 populations in idiopathic pulmonary fibrosis. *Science Advances* **6**, eaba1983,
1170 doi:10.1126/sciadv.aba1983 (2020).

1171 53 Goveia, J. *et al.* An Integrated Gene Expression Landscape Profiling Approach to
1172 Identify Lung Tumor Endothelial Cell Heterogeneity and Angiogenic Candidates.
1173 *Cancer Cell* **37**, 21-36.e13, doi:<https://doi.org/10.1016/j.ccell.2019.12.001> (2020).

1174 54 Habermann, A. C. *et al.* Single-cell RNA sequencing reveals profibrotic roles of
1175 distinct epithelial and mesenchymal lineages in pulmonary fibrosis. *Science Advances*
1176 **6**, eaba1972, doi:10.1126/sciadv.aba1972 (2020).

1177 55 Lambrechts, D. *et al.* Phenotype molding of stromal cells in the lung tumor
1178 microenvironment. *Nature Medicine* **24**, 1277-1289, doi:10.1038/s41591-018-0096-5
1179 (2018).

1180 56 Qian, J. *et al.* A pan-cancer blueprint of the heterogeneous tumor microenvironment
1181 revealed by single-cell profiling. *Cell Research* **30**, 745-762, doi:10.1038/s41422-020-
1182 0355-0 (2020).

1183 57 Reyfman, P. A. *et al.* Single-Cell Transcriptomic Analysis of Human Lung Provides
1184 Insights into the Pathobiology of Pulmonary Fibrosis. *Am J Respir Crit Care Med* **199**,
1185 1517-1536, doi:10.1164/rccm.201712-2410OC (2019).

1186 58 Melms, J. C. *et al.* A molecular single-cell lung atlas of lethal COVID-19. *Nature*,
1187 doi:10.1038/s41586-021-03569-1 (2021).

1188 59 Ramachandran, P. *et al.* Resolving the fibrotic niche of human liver cirrhosis at single-
1189 cell level. *Nature* **575**, 512-518, doi:10.1038/s41586-019-1631-3 (2019).

1190 60 Nicin, L. *et al.* Cell type-specific expression of the putative SARS-CoV-2 receptor ACE2
1191 in human hearts. *Eur Heart J* **41**, 1804-1806, doi:10.1093/eurheartj/ehaa311 (2020).

1192 61 Wang, L. *et al.* Single-cell reconstruction of the adult human heart during heart
1193 failure and recovery reveals the cellular landscape underlying cardiac function. *Nat*
1194 *Cell Biol* **22**, 108-119, doi:10.1038/s41556-019-0446-7 (2020).

1195 62 Suryawanshi, H. *et al.* Cell atlas of the foetal human heart and implications for
1196 autoimmune-mediated congenital heart block. *Cardiovasc Res* **116**, 1446-1457,
1197 doi:10.1093/cvr/cvz257 (2020).

1198 63 Nicin, L. *et al.* Single Nuclei Sequencing Reveals Novel Insights Into the Regulation of
1199 Cellular Signatures in Children With Dilated Cardiomyopathy. *Circulation* **143**, 1704-
1200 1719, doi:10.1161/CIRCULATIONAHA.120.051391 (2021).

1201 64 Lau, S.-F., Cao, H., Fu, A. K. Y. & Ip, N. Y. Single-nucleus transcriptome analysis reveals
1202 dysregulation of angiogenic endothelial cells and neuroprotective glia in Alzheimer's
1203 disease. *Proceedings of the National Academy of Sciences* **117**, 25800,
1204 doi:10.1073/pnas.2008762117 (2020).

1205 65 Rohlenova, K. *et al.* Single-Cell RNA Sequencing Maps Endothelial Metabolic
1206 Plasticity in Pathological Angiogenesis. *Cell Metab* **31**, 862-877 e814,
1207 doi:10.1016/j.cmet.2020.03.009 (2020).

1208 66 Raredon, M. S. B. *et al.* Single-cell connectomic analysis of adult mammalian lungs.
1209 *Science Advances* **5**, eaaw3851, doi:10.1126/sciadv.aaw3851 (2019).

1210 67 Baryawno, N. *et al.* A Cellular Taxonomy of the Bone Marrow Stroma in Homeostasis
1211 and Leukemia. *Cell* **177**, 1915-1932 e1916, doi:10.1016/j.cell.2019.04.040 (2019).

1212 68 Gillich, A. *et al.* Capillary cell-type specialization in the alveolus. *Nature* **586**, 785-789,
1213 doi:10.1038/s41586-020-2822-7 (2020).

1214 69 Travaglini, K. J. *et al.* A molecular cell atlas of the human lung from single-cell RNA
1215 sequencing. *Nature* **587**, 619-625, doi:10.1038/s41586-020-2922-4 (2020).

1216 70 Vanlandewijck, M. & Betsholtz, C. Single-Cell mRNA Sequencing of the Mouse Brain
1217 Vasculature. *Methods Mol Biol* **1846**, 309-324, doi:10.1007/978-1-4939-8712-2_21
1218 (2018).

1219 71 Banks, W. A. From blood-brain barrier to blood-brain interface: new opportunities
1220 for CNS drug delivery. *Nat Rev Drug Discov* **15**, 275-292, doi:10.1038/nrd.2015.21
1221 (2016).

1222 72 Brandes, R. P., Fleming, I. & Busse, R. Endothelial aging. *Cardiovasc Res* **66**, 286-294,
1223 doi:10.1016/j.cardiores.2004.12.027 (2005).

1224 73 Vidal, R. *et al.* Transcriptional heterogeneity of fibroblasts is a hallmark of the aging
1225 heart. *JCI Insight* **4**, doi:10.1172/jci.insight.131092 (2019).

1226 74 Zhao, L. *et al.* Pharmacologically reversible zonation-dependent endothelial cell
1227 transcriptomic changes with neurodegenerative disease associations in the aged
1228 brain. *Nat Commun* **11**, 4413, doi:10.1038/s41467-020-18249-3 (2020).

1229 75 de Soysa, T. Y. *et al.* Single-cell analysis of cardiogenesis reveals basis for organ-level
1230 developmental defects. *Nature* **572**, 120-124, doi:10.1038/s41586-019-1414-x
1231 (2019).

1232 76 Zhang, H., Lui, K. O. & Zhou, B. Endocardial Cell Plasticity in Cardiac Development,
1233 Diseases and Regeneration. *Circ Res* **122**, 774-789,
1234 doi:10.1161/CIRCRESAHA.117.312136 (2018).

1235 77 de Lange, F. J. *et al.* Lineage and morphogenetic analysis of the cardiac valves. *Circ*
1236 *Res* **95**, 645-654, doi:10.1161/01.RES.0000141429.13560.cb (2004).

1237 78 Ibarra-Soria, X. *et al.* Defining murine organogenesis at single-cell resolution reveals
1238 a role for the leukotriene pathway in regulating blood progenitor formation. *Nat Cell*
1239 *Biol* **20**, 127-134, doi:10.1038/s41556-017-0013-z (2018).

1240 79 Briggs, J. A. *et al.* The dynamics of gene expression in vertebrate embryogenesis at
1241 single-cell resolution. *Science* **360**, doi:10.1126/science.aar5780 (2018).

1242 80 Vila Ellis, L. *et al.* Epithelial Vegfa Specifies a Distinct Endothelial Population in the
1243 Mouse Lung. *Dev Cell* **52**, 617-630 e616, doi:10.1016/j.devcel.2020.01.009 (2020).

1244 81 Boisset, J. C. *et al.* In vivo imaging of haematopoietic cells emerging from the mouse
1245 aortic endothelium. *Nature* **464**, 116-120, doi:10.1038/nature08764 (2010).

1246 82 Zovein, A. C. *et al.* Fate tracing reveals the endothelial origin of hematopoietic stem
1247 cells. *Cell Stem Cell* **3**, 625-636, doi:10.1016/j.stem.2008.09.018 (2008).

1248 83 Ema, M. *et al.* Primitive erythropoiesis from mesodermal precursors expressing VE-
1249 cadherin, PECAM-1, Tie2, endoglin, and CD34 in the mouse embryo. *Blood* **108**,
1250 4018-4024, doi:10.1182/blood-2006-03-012872 (2006).

1251 84 Pijuan-Sala, B. *et al.* Single-cell chromatin accessibility maps reveal regulatory
1252 programs driving early mouse organogenesis. *Nat Cell Biol* **22**, 487-497,
1253 doi:10.1038/s41556-020-0489-9 (2020).

1254 85 Rao, M. *et al.* Comparative single-cell RNA sequencing (scRNA-seq) reveals liver
1255 metastasis-specific targets in a patient with small intestinal neuroendocrine cancer.
1256 *Cold Spring Harb Mol Case Stud* **6**, doi:10.1101/mcs.a004978 (2020).

1257 86 Reynolds, G. *et al.* Developmental cell programs are co-opted in inflammatory skin
1258 disease. *Science* **371**, doi:10.1126/science.aba6500 (2021).

1259 87 Hurskainen, M. *et al.* Single cell transcriptomic analysis of murine lung development
1260 on hyperoxia-induced damage. *Nat Commun* **12**, 1565, doi:10.1038/s41467-021-
1261 21865-2 (2021).

1262 88 Muhleder, S., Fernandez-Chacon, M., Garcia-Gonzalez, I. & Benedito, R. Endothelial
1263 sprouting, proliferation, or senescence: tipping the balance from physiology to
1264 pathology. *Cell Mol Life Sci* **78**, 1329-1354, doi:10.1007/s00018-020-03664-y (2021).

1265 89 Li, Z. *et al.* Single-cell transcriptome analyses reveal novel targets modulating cardiac
1266 neovascularization by resident endothelial cells following myocardial infarction. *Eur*
1267 *Heart J* **40**, 2507-2520, doi:10.1093/eurheartj/ehz305 (2019).

1268 90 Niethamer, T. K. *et al.* Defining the role of pulmonary endothelial cell heterogeneity
1269 in the response to acute lung injury. *Elife* **9**, doi:10.7554/eLife.53072 (2020).

1270 91 Manavski, Y. *et al.* Clonal Expansion of Endothelial Cells Contributes to Ischemia-
1271 Induced Neovascularization. *Circulation Research* **122**, 670-677,
1272 doi:10.1161/CIRCRESAHA.117.312310 (2018).

1273 92 Wakabayashi, T. *et al.* CD157 Marks Tissue-Resident Endothelial Stem Cells with
1274 Homeostatic and Regenerative Properties. *Cell Stem Cell* **22**, 384-397 e386,
1275 doi:10.1016/j.stem.2018.01.010 (2018).

1276 93 McDonald, A. I. *et al.* Endothelial Regeneration of Large Vessels Is a Biphasic Process
1277 Driven by Local Cells with Distinct Proliferative Capacities. *Cell Stem Cell* **23**, 210-225
1278 e216, doi:10.1016/j.stem.2018.07.011 (2018).

1279 94 Guo, L., Zhang, H., Hou, Y., Wei, T. & Liu, J. Plasmalemma vesicle-associated protein:
1280 A crucial component of vascular homeostasis. *Exp Ther Med* **12**, 1639-1644,
1281 doi:10.3892/etm.2016.3557 (2016).

1282 95 Chen, S. *et al.* Single-cell analysis reveals transcriptomic remodellings in distinct cell
1283 types that contribute to human prostate cancer progression. *Nat Cell Biol* **23**, 87-98,
1284 doi:10.1038/s41556-020-00613-6 (2021).

1285 96 Carmeliet, P., De Smet, F., Loges, S. & Mazzone, M. Branching morphogenesis and
1286 antiangiogenesis candidates: tip cells lead the way. *Nat Rev Clin Oncol* **6**, 315-326,
1287 doi:10.1038/nrclinonc.2009.64 (2009).

- 1288 97 Orsenigo, F. *et al.* Mapping endothelial-cell diversity in cerebral cavernous
1289 malformations at single-cell resolution. *Elife* **9**, doi:10.7554/eLife.61413 (2020).
- 1290 98 Kovacic, J. C. *et al.* Endothelial to Mesenchymal Transition in Cardiovascular Disease:
1291 JACC State-of-the-Art Review. *J Am Coll Cardiol* **73**, 190-209,
1292 doi:10.1016/j.jacc.2018.09.089 (2019).
- 1293 99 Hong, L. *et al.* EndMT: A promising and controversial field. *Eur J Cell Biol* **97**, 493-500,
1294 doi:10.1016/j.ejcb.2018.07.005 (2018).
- 1295 100 Su, T. *et al.* Single-Cell Transcriptomics Reveals Zone-Specific Alterations of Liver
1296 Sinusoidal Endothelial Cells in Cirrhosis. *Cell Mol Gastroenterol Hepatol* **11**, 1139-
1297 1161, doi:10.1016/j.jcmgh.2020.12.007 (2021).
- 1298 101 Xu, K. *et al.* Cell-Type Transcriptome Atlas of Human Aortic Valves Reveal Cell
1299 Heterogeneity and Endothelial to Mesenchymal Transition Involved in Calcific Aortic
1300 Valve Disease. *Arterioscler Thromb Vasc Biol* **40**, 2910-2921,
1301 doi:10.1161/ATVBAHA.120.314789 (2020).
- 1302 102 Chen, P. Y. *et al.* Endothelial TGF-beta signalling drives vascular inflammation and
1303 atherosclerosis. *Nat Metab* **1**, 912-926, doi:10.1038/s42255-019-0102-3 (2019).
- 1304 103 Tombor, L. S. *et al.* Single cell sequencing reveals endothelial plasticity with transient
1305 mesenchymal activation after myocardial infarction. *Nat Commun* **12**, 681,
1306 doi:10.1038/s41467-021-20905-1 (2021).
- 1307 104 Veerman, K., Tardiveau, C., Martins, F., Coudert, J. & Girard, J. P. Single-Cell Analysis
1308 Reveals Heterogeneity of High Endothelial Venules and Different Regulation of
1309 Genes Controlling Lymphocyte Entry to Lymph Nodes. *Cell Rep* **26**, 3116-3131 e3115,
1310 doi:10.1016/j.celrep.2019.02.042 (2019).
- 1311 105 Rodor, J. *et al.* Single-cell RNA-seq profiling of mouse endothelial cells in response to
1312 pulmonary arterial hypertension. *Cardiovasc Res*, doi:10.1093/cvr/cvab296 (2021).
- 1313 106 Pasut, A., Becker, L. M., Cuypers, A. & Carmeliet, P. Endothelial cell plasticity at the
1314 single-cell level. *Angiogenesis* **24**, 311-326, doi:10.1007/s10456-021-09797-3 (2021).
- 1315 107 Apostolidis, S. A. *et al.* Single Cell RNA Sequencing Identifies HSPG2 and APLNR as
1316 Markers of Endothelial Cell Injury in Systemic Sclerosis Skin. *Front Immunol* **9**, 2191,
1317 doi:10.3389/fimmu.2018.02191 (2018).
- 1318 108 Crespo-Garcia, S. *et al.* Pathological angiogenesis in retinopathy engages cellular
1319 senescence and is amenable to therapeutic elimination via BCL-xL inhibition. *Cell*
1320 *Metab* **33**, 818-832 e817, doi:10.1016/j.cmet.2021.01.011 (2021).
- 1321 109 Aibar, S. *et al.* SCENIC: single-cell regulatory network inference and clustering. *Nat*
1322 *Methods* **14**, 1083-1086, doi:10.1038/nmeth.4463 (2017).
- 1323 110 Holland, C. H. *et al.* Robustness and applicability of transcription factor and pathway
1324 analysis tools on single-cell RNA-seq data. *Genome Biol* **21**, 36, doi:10.1186/s13059-
1325 020-1949-z (2020).
- 1326 111 Minami, T., Muramatsu, M. & Kume, T. Organ/Tissue-Specific Vascular Endothelial
1327 Cell Heterogeneity in Health and Disease. *Biol Pharm Bull* **42**, 1609-1619,
1328 doi:10.1248/bpb.b19-00531 (2019).
- 1329 112 Yang, Y. *et al.* M2 Macrophage-Derived Exosomes Promote Angiogenesis and Growth
1330 of Pancreatic Ductal Adenocarcinoma by Targeting E2F2. *Molecular Therapy* **29**,
1331 1226-1238, doi:10.1016/j.ymthe.2020.11.024 (2021).
- 1332 113 Choi, H. & Moon, A. Crosstalk between cancer cells and endothelial cells:
1333 implications for tumor progression and intervention. *Archives of Pharmacal Research*
1334 **41**, 711-724, doi:10.1007/s12272-018-1051-1 (2018).

1335 114 Colliva, A., Braga, L., Giacca, M. & Zacchigna, S. Endothelial cell-cardiomyocyte
1336 crosstalk in heart development and disease. *J Physiol* **598**, 2923-2939,
1337 doi:10.1113/JP276758 (2020).

1338 115 Wan, A. & Rodrigues, B. Endothelial cell–cardiomyocyte crosstalk in diabetic
1339 cardiomyopathy. *Cardiovascular Research* **111**, 172-183, doi:10.1093/cvr/cvw159
1340 (2016).

1341 116 Efremova, M., Vento-Tormo, M., Teichmann, S. A. & Vento-Tormo, R. CellPhoneDB:
1342 inferring cell-cell communication from combined expression of multi-subunit ligand-
1343 receptor complexes. *Nat Protoc* **15**, 1484-1506, doi:10.1038/s41596-020-0292-x
1344 (2020).

1345 117 Jin, S. *et al.* Inference and analysis of cell-cell communication using CellChat. *Nature*
1346 *Communications* **12**, 1088, doi:10.1038/s41467-021-21246-9 (2021).

1347 118 Browaeys, R., Saelens, W. & Saeys, Y. NicheNet: modeling intercellular
1348 communication by linking ligands to target genes. *Nature Methods* **17**, 159-162,
1349 doi:10.1038/s41592-019-0667-5 (2020).

1350 119 Farbehi, N. *et al.* Single-cell expression profiling reveals dynamic flux of cardiac
1351 stromal, vascular and immune cells in health and injury. *Elife* **8**,
1352 doi:10.7554/eLife.43882 (2019).

1353 120 Cook, K. M. & Figg, W. D. Angiogenesis inhibitors: current strategies and future
1354 prospects. *CA Cancer J Clin* **60**, 222-243, doi:10.3322/caac.20075 (2010).

1355 121 Potente, M., Gerhardt, H. & Carmeliet, P. Basic and Therapeutic Aspects of
1356 Angiogenesis. *Cell* **146**, 873-887, doi:10.1016/j.cell.2011.08.039 (2011).

1357 122 Folkman, J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* **285**, 1182-
1358 1186, doi:10.1056/NEJM197111182852108 (1971).

1359 123 Yuan, F. *et al.* Time-dependent vascular regression and permeability changes in
1360 established human tumor xenografts induced by an anti-vascular endothelial growth
1361 factor/vascular permeability factor antibody. *Proc Natl Acad Sci U S A* **93**, 14765-
1362 14770, doi:10.1073/pnas.93.25.14765 (1996).

1363 124 Kim, K. J. *et al.* Inhibition of vascular endothelial growth factor-induced angiogenesis
1364 suppresses tumour growth in vivo. *Nature* **362**, 841-844, doi:10.1038/362841a0
1365 (1993).

1366 125 Eelen, G., Treppe, L., Li, X. & Carmeliet, P. Basic and Therapeutic Aspects of
1367 Angiogenesis Updated. *Circ Res* **127**, 310-329, doi:10.1161/CIRCRESAHA.120.316851
1368 (2020).

1369 126 De Bock, K., Mazzone, M. & Carmeliet, P. Antiangiogenic therapy, hypoxia, and
1370 metastasis: risky liaisons, or not? *Nat Rev Clin Oncol* **8**, 393-404,
1371 doi:10.1038/nrclinonc.2011.83 (2011).

1372 127 Loges, S., Mazzone, M., Hohensinner, P. & Carmeliet, P. Silencing or fueling
1373 metastasis with VEGF inhibitors: antiangiogenesis revisited. *Cancer cell* **15**, 167-170,
1374 doi:10.1016/j.ccr.2009.02.007 (2009).

1375 128 Bottsford-Miller, J. N., Coleman, R. L. & Sood, A. K. Resistance and escape from
1376 antiangiogenesis therapy: clinical implications and future strategies. *J Clin Oncol* **30**,
1377 4026-4034, doi:10.1200/JCO.2012.41.9242 (2012).

1378 129 Sennino, B. & McDonald, D. M. Controlling escape from angiogenesis inhibitors. *Nat*
1379 *Rev Cancer* **12**, 699-709, doi:10.1038/nrc3366 (2012).

1380 130 Teuwen, L. A. *et al.* Tumor vessel co-option probed by single-cell analysis. *Cell Rep*
1381 **35**, 109253, doi:10.1016/j.celrep.2021.109253 (2021).

1382 131 Bridgeman, V. L. *et al.* Vessel co-option is common in human lung metastases and
1383 mediates resistance to anti-angiogenic therapy in preclinical lung metastasis models.
1384 *J Pathol* **241**, 362-374, doi:10.1002/path.4845 (2017).

1385 132 Zhao, Q. *et al.* Single-Cell Transcriptome Analyses Reveal Endothelial Cell
1386 Heterogeneity in Tumors and Changes following Antiangiogenic Treatment. *Cancer*
1387 *Res* **78**, 2370-2382, doi:10.1158/0008-5472.CAN-17-2728 (2018).

1388 133 Kim, N. *et al.* Single-cell RNA sequencing demonstrates the molecular and cellular
1389 reprogramming of metastatic lung adenocarcinoma. *Nature Communications* **11**,
1390 2285, doi:10.1038/s41467-020-16164-1 (2020).

1391 134 Hida, K. *et al.* Tumor endothelial cells with high aldehyde dehydrogenase activity
1392 show drug resistance. *Cancer Science* **108**, 2195-2203,
1393 doi:<https://doi.org/10.1111/cas.13388> (2017).

1394 135 Ohmura-Kakutani, H. *et al.* Identification of Tumor Endothelial Cells with High
1395 Aldehyde Dehydrogenase Activity and a Highly Angiogenic Phenotype. *PLOS ONE* **9**,
1396 e113910, doi:10.1371/journal.pone.0113910 (2014).

1397 136 Hlushchuk, R. *et al.* Tumor Recovery by Angiogenic Switch from Sprouting to
1398 Intussusceptive Angiogenesis after Treatment with PTK787/ZK222584 or Ionizing
1399 Radiation. *The American Journal of Pathology* **173**, 1173-1185,
1400 doi:10.2353/ajpath.2008.071131 (2008).

1401 137 Angara, K. *et al.* Vascular mimicry in glioblastoma following anti-angiogenic and anti-
1402 20-HETE therapies. *Histol Histopathol* **32**, 917-928, doi:10.14670/hh-11-856 (2017).

1403 138 James, M. D. & Andrew, C. D. Vascular Mimicry: Concepts and Implications for Anti-
1404 Angiogenic Therapy. *Current Angiogenesis (Discontinued)* **1**, 133-138,
1405 doi:<http://dx.doi.org/10.2174/2211552811201020133> (2012).

1406 139 Zirlik, K. & Duyster, J. Anti-Angiogenics: Current Situation and Future Perspectives.
1407 *Oncology Research and Treatment* **41**, 166-171, doi:10.1159/000488087 (2018).

1408 140 Rini, B. I. *et al.* Randomized Phase III Trial of Temsirolimus and Bevacizumab Versus
1409 Interferon Alfa and Bevacizumab in Metastatic Renal Cell Carcinoma: INTORACT
1410 Trial. *Journal of Clinical Oncology* **32**, 752-759, doi:10.1200/JCO.2013.50.5305
1411 (2013).

1412 141 Ager, A. & May, M. J. Understanding high endothelial venules: Lessons for cancer
1413 immunology. *Oncoimmunology* **4**, e1008791-e1008791,
1414 doi:10.1080/2162402X.2015.1008791 (2015).

1415 142 De Bock, K. *et al.* Role of PFKFB3-Driven Glycolysis in Vessel Sprouting. *Cell* **154**, 651-
1416 663, doi:10.1016/j.cell.2013.06.037 (2013).

1417 143 Dumas, S. J., García-Caballero, M. & Carmeliet, P. Metabolic Signatures of Distinct
1418 Endothelial Phenotypes. *Trends in Endocrinology & Metabolism* **31**, 580-595,
1419 doi:10.1016/j.tem.2020.05.009 (2020).

1420 144 Schoors, S. *et al.* Partial and Transient Reduction of Glycolysis by PFKFB3 Blockade
1421 Reduces Pathological Angiogenesis. *Cell Metabolism* **19**, 37-48,
1422 doi:10.1016/j.cmet.2013.11.008 (2014).

1423 145 Schoors, S. *et al.* Fatty acid carbon is essential for dNTP synthesis in endothelial cells.
1424 *Nature* **520**, 192-197, doi:10.1038/nature14362 (2015).

1425 146 Kalucka, J. *et al.* Quiescent Endothelial Cells Upregulate Fatty Acid beta-Oxidation for
1426 Vasculoprotection via Redox Homeostasis. *Cell Metab* **28**, 881-894 e813,
1427 doi:10.1016/j.cmet.2018.07.016 (2018).

1428 147 Eelen, G. *et al.* Endothelial Cell Metabolism. *Physiol Rev* **98**, 3-58,
1429 doi:10.1152/physrev.00001.2017 (2018).

1430 148 Teuwen, L. A., Geldhof, V. & Carmeliet, P. How glucose, glutamine and fatty acid
1431 metabolism shape blood and lymph vessel development. *Dev Biol* **447**, 90-102,
1432 doi:10.1016/j.ydbio.2017.12.001 (2019).

1433 149 Lu, T. *et al.* Single-cell transcriptome atlas of lung adenocarcinoma featured with
1434 ground glass nodules. *Cell Discovery* **6**, 69, doi:10.1038/s41421-020-00200-x (2020).

1435 150 Rivello, F. *et al.* Probing single-cell metabolism reveals prognostic value of highly
1436 metabolically active circulating stromal cells in prostate cancer. *Science Advances* **6**,
1437 eaaz3849, doi:10.1126/sciadv.aaz3849 (2020).

1438 151 Cantelmo, A. R. *et al.* Inhibition of the Glycolytic Activator PFKFB3 in Endothelium
1439 Induces Tumor Vessel Normalization, Impairs Metastasis, and Improves
1440 Chemotherapy. *Cancer cell* **30**, 968-985, doi:10.1016/j.ccell.2016.10.006 (2016).

1441 152 Bruning, U. *et al.* Impairment of Angiogenesis by Fatty Acid Synthase Inhibition
1442 Involves mTOR Malonylation. *Cell metabolism* **28**, 866-880.e815,
1443 doi:10.1016/j.cmet.2018.07.019 (2018).

1444 153 Seguin, F. *et al.* The fatty acid synthase inhibitor orlistat reduces experimental
1445 metastases and angiogenesis in B16-F10 melanomas. *Br J Cancer* **107**, 977-987,
1446 doi:10.1038/bjc.2012.355 (2012).

1447 154 Calderon, D. *et al.* Inferring Relevant Cell Types for Complex Traits by Using Single-
1448 Cell Gene Expression. *Am J Hum Genet* **101**, 686-699, doi:10.1016/j.ajhg.2017.09.009
1449 (2017).

1450 155 Sierksma, A. *et al.* Novel Alzheimer risk genes determine the microglia response to
1451 amyloid- β but not to TAU pathology. *EMBO Molecular Medicine* **12**, e10606,
1452 doi:<https://doi.org/10.15252/emmm.201910606> (2020).

1453 156 Hong, J. *et al.* Single-Cell Study of Two Rat Models of Pulmonary Arterial
1454 Hypertension Reveals Connections to Human Pathobiology and Drug Repositioning.
1455 *American Journal of Respiratory and Critical Care Medicine* **203**, 1006-1022,
1456 doi:10.1164/rccm.202006-2169OC (2020).

1457 157 Lamb, J. *et al.* The Connectivity Map: using gene-expression signatures to connect
1458 small molecules, genes, and disease. *Science* **313**, 1929-1935,
1459 doi:10.1126/science.1132939 (2006).

1460 158 Skinnider, M. A. *et al.* Cell type prioritization in single-cell data. *Nature Biotechnology*
1461 **39**, 30-34, doi:10.1038/s41587-020-0605-1 (2021).

1462 159 Gawel, D. R. *et al.* A validated single-cell-based strategy to identify diagnostic and
1463 therapeutic targets in complex diseases. *Genome Medicine* **11**, 47,
1464 doi:10.1186/s13073-019-0657-3 (2019).

1465 160 Jakab, M. & Augustin, H. G. Understanding angiodiversity: insights from single cell
1466 biology. *Development* **147**, dev146621, doi:10.1242/dev.146621 (2020).

1467 161 Gauberti, M. *et al.* Ultra-Sensitive Molecular MRI of Vascular Cell Adhesion
1468 Molecule-1 Reveals a Dynamic Inflammatory Penumbra After Strokes. *Stroke* **44**,
1469 1988-1996, doi:10.1161/STROKEAHA.111.000544 (2013).

1470 162 Patel, N., Duffy, B. A., Badar, A., Lythgoe, M. F. & Årstad, E. Bimodal Imaging of
1471 Inflammation with SPECT/CT and MRI Using Iodine-125 Labeled VCAM-1 Targeting
1472 Microparticle Conjugates. *Bioconjugate Chemistry* **26**, 1542-1549,
1473 doi:10.1021/acs.bioconjchem.5b00380 (2015).

1474 163 Marcos-Contreras, O. A. *et al.* Selective targeting of nanomedicine to inflamed
1475 cerebral vasculature to enhance the blood–brain barrier. *Proceedings of the National*
1476 *Academy of Sciences* **117**, 3405, doi:10.1073/pnas.1912012117 (2020).

1477 164 Li, R. *et al.* Endothelium-targeted delivery of dexamethasone by anti-VCAM-1 SAINT-
1478 O-Somes in mouse endotoxemia. *PLOS ONE* **13**, e0196976,
1479 doi:10.1371/journal.pone.0196976 (2018).

1480 165 Shuvaev, V. V. *et al.* Factors modulating the delivery and effect of enzymatic cargo
1481 conjugated with antibodies targeted to the pulmonary endothelium. *J Control*
1482 *Release* **118**, 235-244, doi:10.1016/j.jconrel.2006.12.025 (2007).

1483 166 Dahlman, J. E. *et al.* In vivo endothelial siRNA delivery using polymeric nanoparticles
1484 with low molecular weight. *Nat Nanotechnol* **9**, 648-655, doi:10.1038/nnano.2014.84
1485 (2014).

1486 167 Sago, C. D. *et al.* High-throughput in vivo screen of functional mRNA delivery
1487 identifies nanoparticles for endothelial cell gene editing. *Proceedings of the National*
1488 *Academy of Sciences* **115**, E9944, doi:10.1073/pnas.1811276115 (2018).

1489 168 Halpern, K. B. *et al.* Paired-cell sequencing enables spatial gene expression mapping
1490 of liver endothelial cells. *Nature biotechnology* **36**, 962-970, doi:10.1038/nbt.4231
1491 (2018).

1492 169 Aran, D. *et al.* Reference-based analysis of lung single-cell sequencing reveals a
1493 transitional profibrotic macrophage. *Nature Immunology* **20**, 163-172,
1494 doi:10.1038/s41590-018-0276-y (2019).

1495 170 Pasquini, G., Rojo Arias, J. E., Schäfer, P. & Buskamp, V. Automated methods for cell
1496 type annotation on scRNA-seq data. *Computational and Structural Biotechnology*
1497 *Journal* **19**, 961-969, doi:<https://doi.org/10.1016/j.csbj.2021.01.015> (2021).

1498 171 Shao, X. *et al.* scCATCH: Automatic Annotation on Cell Types of Clusters from Single-
1499 Cell RNA Sequencing Data. *iScience* **23**, 100882,
1500 doi:<https://doi.org/10.1016/j.isci.2020.100882> (2020).

1501 172 Abdelaal, T. *et al.* A comparison of automatic cell identification methods for single-
1502 cell RNA sequencing data. *Genome Biology* **20**, 194, doi:10.1186/s13059-019-1795-z
1503 (2019).

1504 173 Hao, Y. *et al.* Integrated analysis of multimodal single-cell data. *Cell*,
1505 doi:<https://doi.org/10.1016/j.cell.2021.04.048> (2021).

1506 174 Schupp, J. C. *et al.* Integrated Single Cell Atlas of Endothelial Cells of the Human
1507 Lung. *Circulation* **0**, doi:10.1161/CIRCULATIONAHA.120.052318 (2021).

1508 175 Papatheodorou, I. *et al.* Expression Atlas update: from tissues to single cells. *Nucleic*
1509 *Acids Research* **48**, D77-D83, doi:10.1093/nar/gkz947 (2020).

1510 176 Svensson, V., da Veiga Beltrame, E. & Pachter, L. A curated database reveals trends
1511 in single-cell transcriptomics. *Database* **2020**, doi:10.1093/database/baaa073 (2020).

1512 177 Franzén, O., Gan, L.-M. & Björkegren, J. L. M. PanglaoDB: a web server for
1513 exploration of mouse and human single-cell RNA sequencing data. *Database* **2019**,
1514 doi:10.1093/database/baz046 (2019).

1515 178 Cao, Y., Zhu, J., Jia, P. & Zhao, Z. scRNASeqDB: A Database for RNA-Seq Based Gene
1516 Expression Profiles in Human Single Cells. *Genes (Basel)* **8**, 368,
1517 doi:10.3390/genes8120368 (2017).

1518 179 Ner-Gaon, H., Melchior, A., Golan, N., Ben-Haim, Y. & Shay, T. JingleBells: A
1519 Repository of Immune-Related Single-Cell RNA–Sequencing Datasets. *The Journal of*
1520 *Immunology* **198**, 3375, doi:10.4049/jimmunol.1700272 (2017).

1521 180 Yuan, H. *et al.* CancerSEA: a cancer single-cell state atlas. *Nucleic acids research* **47**,
1522 D900-D908, doi:10.1093/nar/gky939 (2019).

1523 181 Regev, A. *et al.* The Human Cell Atlas. *eLife* **6**, e27041, doi:10.7554/eLife.27041
1524 (2017).

1525 182 Snyder, M. P. *et al.* The human body at cellular resolution: the NIH Human
1526 Biomolecular Atlas Program. *Nature* **574**, 187-192, doi:10.1038/s41586-019-1629-x
1527 (2019).

1528 183 Lee, J. *et al.* BioBERT: a pre-trained biomedical language representation model for
1529 biomedical text mining. *Bioinformatics* **36**, 1234-1240,
1530 doi:10.1093/bioinformatics/btz682 (2020).

1531 184 Simon, C. *et al.* BioReader: a text mining tool for performing classification of
1532 biomedical literature. *BMC Bioinformatics* **19**, 57, doi:10.1186/s12859-019-2607-x
1533 (2019).

1534 185 Marx, V. Method of the Year: spatially resolved transcriptomics. *Nature Methods* **18**,
1535 9-14, doi:10.1038/s41592-020-01033-y (2021).

1536 186 Okochi, Y., Sakaguchi, S., Nakae, K., Kondo, T. & Naoki, H. Model-based prediction of
1537 spatial gene expression via generative linear mapping. *Nat Commun* **12**, 3731,
1538 doi:10.1038/s41467-021-24014-x (2021).

1539 187 Ren, X. *et al.* Reconstruction of cell spatial organization from single-cell RNA
1540 sequencing data based on ligand-receptor mediated self-assembly. *Cell Research* **30**,
1541 763-778, doi:10.1038/s41422-020-0353-2 (2020).

1542 188 Nitzan, M., Karaiskos, N., Friedman, N. & Rajewsky, N. Gene expression cartography.
1543 *Nature* **576**, 132-137, doi:10.1038/s41586-019-1773-3 (2019).

1544 189 Abdelaal, T., Mourragui, S., Mahfouz, A. & Reinders, M. J. T. SpaGE: Spatial Gene
1545 Enhancement using scRNA-seq. *Nucleic Acids Research* **48**, e107-e107,
1546 doi:10.1093/nar/gkaa740 (2020).

1547 190 Cang, Z. & Nie, Q. Inferring spatial and signaling relationships between cells from
1548 single cell transcriptomic data. *Nature Communications* **11**, 2084,
1549 doi:10.1038/s41467-020-15968-5 (2020).

1550 191 Stoeckius, M. *et al.* Simultaneous epitope and transcriptome measurement in single
1551 cells. *Nature Methods* **14**, 865-868, doi:10.1038/nmeth.4380 (2017).

1552 192 Peterson, V. M. *et al.* Multiplexed quantification of proteins and transcripts in single
1553 cells. *Nature Biotechnology* **35**, 936-939, doi:10.1038/nbt.3973 (2017).

1554 193 Spitzer, Matthew H. & Nolan, Garry P. Mass Cytometry: Single Cells, Many Features.
1555 *Cell* **165**, 780-791, doi:10.1016/j.cell.2016.04.019 (2016).

1556 194 Gault, J. *et al.* Combining native and 'omics' mass spectrometry to identify
1557 endogenous ligands bound to membrane proteins. *Nature methods* **17**, 505-508,
1558 doi:10.1038/s41592-020-0821-0 (2020).

1559 195 Katzenelenbogen, Y. *et al.* Coupled scRNA-Seq and Intracellular Protein Activity
1560 Reveal an Immunosuppressive Role of TREM2 in Cancer. *Cell* **182**, 872-885.e819,
1561 doi:10.1016/j.cell.2020.06.032 (2020).

1562 196 Specht, H. *et al.* Single-cell proteomic and transcriptomic analysis of macrophage
1563 heterogeneity using SCoPE2. *Genome Biology* **22**, 50, doi:10.1186/s13059-021-
1564 02267-5 (2021).

1565 197 Deng, J. *et al.* Single-cell gene profiling and lineage tracing analyses revealed novel
1566 mechanisms of endothelial repair by progenitors. *Cell Mol Life Sci* **77**, 5299-5320,
1567 doi:10.1007/s00018-020-03480-4 (2020).

1568 198 Zhao, G. *et al.* Single-cell RNA sequencing reveals the cellular heterogeneity of
1569 aneurysmal infrarenal abdominal aorta. *Cardiovasc Res* **117**, 1402-1416,
1570 doi:10.1093/cvr/cvaa214 (2021).

1571 199 Mathys, H. *et al.* Single-cell transcriptomic analysis of Alzheimer's disease. *Nature*
1572 **570**, 332-337, doi:10.1038/s41586-019-1195-2 (2019).

1573 200 Wirka, R. C. *et al.* Atheroprotective roles of smooth muscle cell phenotypic
1574 modulation and the TCF21 disease gene as revealed by single-cell analysis. *Nat Med*
1575 **25**, 1280-1289, doi:10.1038/s41591-019-0512-5 (2019).

1576 201 DePasquale, E. A. K. *et al.* DoubletDecon: Deconvoluting Doublets from Single-Cell
1577 RNA-Sequencing Data. *Cell Rep* **29**, 1718-1727 e1718,
1578 doi:10.1016/j.celrep.2019.09.082 (2019).

1579 202 Bernstein, N. J. *et al.* Solo: Doublet Identification in Single-Cell RNA-Seq via Semi-
1580 Supervised Deep Learning. *Cell Syst* **11**, 95-101 e105, doi:10.1016/j.cels.2020.05.010
1581 (2020).

1582 203 Bais, A. S. & Kostka, D. scds: computational annotation of doublets in single-cell RNA
1583 sequencing data. *Bioinformatics* **36**, 1150-1158, doi:10.1093/bioinformatics/btz698
1584 (2020).

1585 204 Wolock, S. L., Lopez, R. & Klein, A. M. Scrublet: Computational Identification of Cell
1586 Doublets in Single-Cell Transcriptomic Data. *Cell Syst* **8**, 281-291 e289,
1587 doi:10.1016/j.cels.2018.11.005 (2019).

1588 205 McGinnis, C. S., Murrow, L. M. & Gartner, Z. J. DoubletFinder: Doublet Detection in
1589 Single-Cell RNA Sequencing Data Using Artificial Nearest Neighbors. *Cell Syst* **8**, 329-
1590 337 e324, doi:10.1016/j.cels.2019.03.003 (2019).

1591 206 Stoeckius, M. *et al.* Cell Hashing with barcoded antibodies enables multiplexing and
1592 doublet detection for single cell genomics. *Genome Biol* **19**, 224,
1593 doi:10.1186/s13059-018-1603-1 (2018).

1594 207 Feng, C. *et al.* Dimension Reduction and Clustering Models for Single-Cell RNA
1595 Sequencing Data: A Comparative Study. *Int J Mol Sci* **21**, doi:10.3390/ijms21062181
1596 (2020).

1597 208 Huang, M. *et al.* SAVER: gene expression recovery for single-cell RNA sequencing.
1598 *Nat Methods* **15**, 539-542, doi:10.1038/s41592-018-0033-z (2018).

1599 209 van Dijk, D. *et al.* Recovering Gene Interactions from Single-Cell Data Using Data
1600 Diffusion. *Cell* **174**, 716-729 e727, doi:10.1016/j.cell.2018.05.061 (2018).

1601 210 Lytal, N., Ran, D. & An, L. Normalization Methods on Single-Cell RNA-seq Data: An
1602 Empirical Survey. *Front Genet* **11**, 41, doi:10.3389/fgene.2020.00041 (2020).

1603 211 Tran, H. T. N. *et al.* A benchmark of batch-effect correction methods for single-cell
1604 RNA sequencing data. *Genome Biology* **21**, 12, doi:10.1186/s13059-019-1850-9
1605 (2020).

1606 212 Chazarra-Gil, R., van Dongen, S., Kiselev, Vladimir Y. & Hemberg, M. Flexible
1607 comparison of batch correction methods for single-cell RNA-seq using BatchBench.
1608 *Nucleic Acids Research*, doi:10.1093/nar/gkab004 (2021).

1609 213 Haghverdi, L., Lun, A. T. L., Morgan, M. D. & Marioni, J. C. Batch effects in single-cell
1610 RNA-sequencing data are corrected by matching mutual nearest neighbors. *Nature*
1611 *Biotechnology* **36**, 421-427, doi:10.1038/nbt.4091 (2018).

1612 214 Stuart, T. *et al.* Comprehensive Integration of Single-Cell Data. *Cell* **177**, 1888-
1613 1902.e1821, doi:10.1016/j.cell.2019.05.031 (2019).

1614 215 Lähnemann, D. *et al.* Eleven grand challenges in single-cell data science. *Genome*
1615 *Biology* **21**, 31, doi:10.1186/s13059-020-1926-6 (2020).

1616 216 Slyper, M. *et al.* A single-cell and single-nucleus RNA-Seq toolbox for fresh and frozen
1617 human tumors. *Nature medicine* **26**, 792-802, doi:10.1038/s41591-020-0844-1
1618 (2020).

1619 217 Sokol, L. *et al.* Protocols for endothelial cell isolation from mouse tissues: small
1620 intestine, colon, heart, and liver. *STAR Protocols* **2**, 100489,
1621 doi:<https://doi.org/10.1016/j.xpro.2021.100489> (2021).

1622 218 Conchinha, N. V. *et al.* Protocols for endothelial cell isolation from mouse tissues:
1623 brain, choroid, lung, and muscle. *STAR Protoc* **2**, 100508,
1624 doi:10.1016/j.xpro.2021.100508 (2021).

1625 219 Dumas, S. J. *et al.* Protocols for endothelial cell isolation from mouse tissues: kidney,
1626 spleen, and testis. *STAR protocols* **2**, 100523-100523,
1627 doi:10.1016/j.xpro.2021.100523 (2021).

1628