© 2020. Published by The Company of Biologists Ltd | Development (2020) 147, dev146621. doi:10.1242/dev.146621

REVIEW

Understanding angiodiversity: insights from single cell biology

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ABSTRACT

Blood vessels have long been considered as passive conduits for delivering blood. However, in recent years, cells of the vessel wall (endothelial cells, smooth muscle cells and pericytes) have emerged as active, highly dynamic components that orchestrate crosstalk between the circulation and organs. Encompassing the whole body and being specialized to the needs of distinct organs, it is not surprising that vessel lining cells come in different flavours. There is calibre-specific specialization (arteries, arterioles, capillaries, venules, veins), but also organ-specific heterogeneity in different microvascular beds (continuous, discontinuous, sinusoidal). Recent technical advances in the field of single cell biology have enabled the profiling of thousands of single cells and, hence, have allowed for the molecular dissection of such angiodiversity, yielding a hitherto unparalleled level of spatial and functional resolution. Here, we review how these approaches have contributed to our understanding of angiodiversity.

KEY WORDS: Angiodiversity, Endothelial cell, Smooth muscle cell, Single cell biology

Introduction

The limited diffusion distance of oxygen in tissues (100-150 µm) implies that all cells of the body – with few exceptions (e.g. cartilage) – are in close proximity to the nearest capillary. The vascular system can thereby be considered as a systemically disseminated organ. In fact, the vascular endothelium forms one of the body's largest surfaces and it is estimated that an 80 kg adult harbours about 1 kg of endothelial cells (ECs), making them one of the most abundant cell populations of the body.

Structurally, blood vessels come in different flavours and show distinct morphological features that reflect their functional specialization as arteries, arterioles, capillaries, venules and veins (Fig. 1). These different types of blood vessels are exposed to varying physical and biochemical milieus, which are organ- and situation-specific and affect the molecular properties of their cellular building blocks. Conceptually, blood vessel-forming cells have long been viewed as passive bystander cell populations that facilitate barrier-function, demarcating blood and solid tissues. However, recent work has shown that vascular and perivascular cells do not just respond to exogenous cytokines, but rather perform active gatekeeper roles and secrete growth factors and signals into their microenvironment. Such vascular cell-derived paracrine-acting growth factors have been designated as 'angiocrine' factors (reviewed by Rafii et al., 2016). Moreover, as vascular cells are directly exposed to the circulating blood, they are highly responsive and sensitive to changes in the physical and biochemical properties of the blood. This not only determines the genetic, transcriptomic and proteomic profiles of distinct types of vessels in an organ-specific manner (Augustin and Koh, 2017), but also impacts the signalling of individual vascular cells within vessels. We refer to this heterogeneity as 'angiodiversity' (Fig. 2).

A prototypic example of intravascular angiodiversity is seen in the sinusoidal capillaries of the liver. Oxygen-rich blood from the hepatic artery and nutrient-rich blood from the portal vein converge in the hepatic sinusoidal capillaries and exit the liver via the central vein, leading to a strong gradient of available nutrients and oxygen along the vessel. Conversely, hepatocytes are molecularly and functionally zonated along the axis of the liver lobule. Indeed, recent elegant work has established that EC-derived angiocrine signals exert instructive functions to orchestrate liver zonation (Wang et al., 2015; Halpern et al., 2017). Central vein ECs and sinusoidal ECs near the central vein express the short-range signals Wnt2 and Wnt9b, which are essential for creating a niche in which self-renewing Lgr5-positive hepatocytes are harboured. Loss of Wnt expression consequently leads to a loss of self-renewing hepatocytes, with subsequent perturbation of liver zonation and impaired regenerative capacity of the liver (Leibing et al., 2018; Preziosi et al., 2018; Wang et al., 2015).

This example of liver zonation is merely a prototypic stage-setting example. It highlights, on the one hand, the need and, on the other hand, the enormous scientific opportunities of deconvoluting angiodiversity at the highest possible resolution. Indeed, recent technological advances in the field of single cell biology have allowed for the transcriptional, epigenetic and genetic profiling of thousands of individual cells, thereby providing insights into angiodiversity in various other contexts. These studies have enabled the molecular dissection of single cells in individual vessels and the identification of crucial subsets of vascular cells as well as rare subpopulations, which may not be captured in bulk approaches. In this Review, we discuss how single cell RNA-sequencing (scRNA-seq; see Boxes 1 and 2) approaches have contributed to our understanding of cellular angiodiversity. We first review how scRNA-seq studies of individual organs have uncovered a high degree of heterogeneity in vascular cells. We then highlight how such studies have provided insights into spatial aspects of angiodiversity. Finally, we discuss how angiodiversity contributes to vascular function and responsiveness in various contexts.

Assessing vascular heterogeneity and building cellular atlases

Comparison of vascular cells in adult mice at single cell resolution

An initial effort to assemble a single cell atlas exclusively of vascular cells was made by the group of Christer Betsholtz (Vanlandewijck et al., 2018). In this study, ECs, mural cells and fibroblast-like cells were isolated from the brain and the lungs of transgenic mouse reporter lines. As a result, 3436 single cell transcriptomes from the adult mouse brain and 1504 single cell

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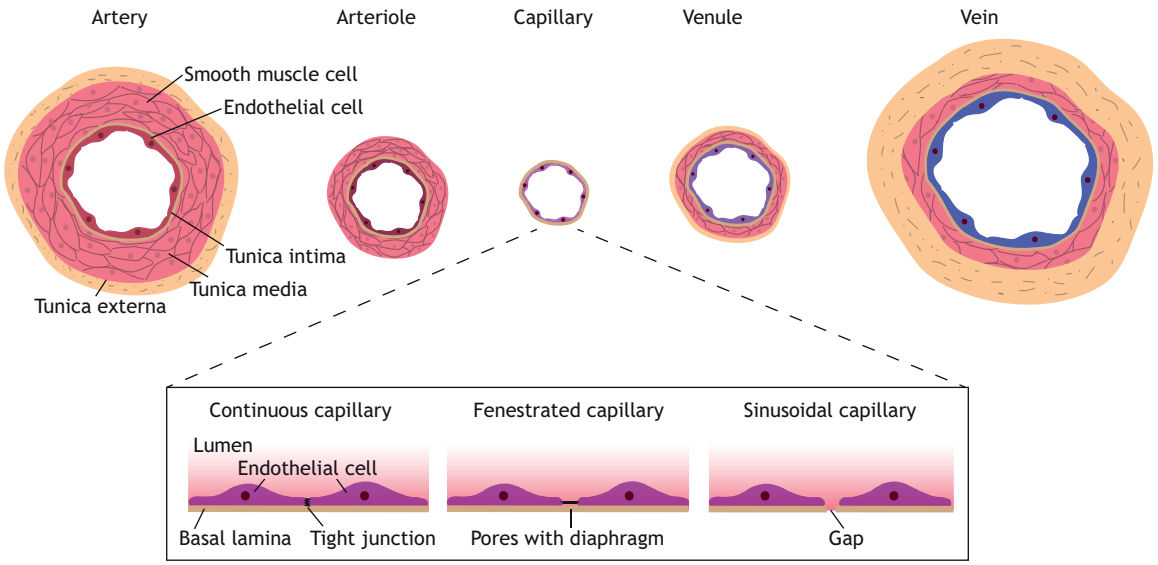

	Artery	Arteriole	Capillary			Venule	Vein
			Continuous	Fenestrated	Sinusoidal		
Pressure	↑ ↑ ↑ ↑	↑ ↑	↑			~	↑↑
Oxygen	↑↑↑	↑↑↑	↑			~	~
Velocity	↑↑↑	↑	~			↑	↑↑
Diameter/ lumen (in µm)							
Human	~4000/2000	~30/10	~8/6			~20/16	~5000/4000
Mouse	~150/50	~18/9	~5/4			~14/12	~250/150
Primary rheological function	Supply of oxygenated blood	Major site of vascular resistance	Demarcation, passive water and solute exchange	Accelerated fluid and solute exchange	Free fluid and solute exchange	Reabsorption of interstitial fluid	Collection of deoxygenated blood

Fig. 1. Schematic of the key structural and rheological features of blood vessels. Arteries (except for pulmonary and umbilical arteries) carry oxygenated blood away from the heart. Arterioles consist of only tunica media and intima, and represent the primary site of vascular resistance. Capillaries are lined by a single layer of ECs. Typically, three major types of capillaries can be distinguished: continuous, fenestrated, and sinusoidal. Continuous capillaries are present in most organs and perform distinct barrier functions permitting diffusion of water, small solutes and lipid-soluble material. Fenestrated and sinusoidal capillaries both display discontinuity in their EC lining. Whereas fenestrated capillaries have intracellular pores that are spanned by radially oriented fibrils forming a diaphragm, sinusoidal endothelia are characterized by flattened, irregular shapes and are devoid of a basement membrane. Fenestrated ECs are found in organs with intense exchange (e.g. endocrine organs for the secretion of macromolecules) and in organs with filtration and absorption functions (e.g. the kidney). Sinusoidal ECs form the endothelium in haematopoietic organs including the bone marrow, spleen and liver. Blood from capillaries is collected in venules and drained into veins. Compared with arterioles, venules have a tunica externa and a media, although their tunica media is poorly developed so that venules have thinner walls and a larger lumen compared with arterioles. Compared with arteries, veins have a thick tunica externa, but generally a much thinner media.

transcriptomes from adult mouse lungs were profiled. A substantial diversity of vascular cell transcriptomes could be identified in both organs. The brain dataset formed 15 clusters corresponding to distinct cell types and cell subtypes by means of coherent marker expression, whereas the lung dataset formed 17 cell clusters. The compiled data have been made publicly available in an interactive searchable database (see Table 1) that allows for the analysis of gene expression distribution across vessel-associated cells (He et al., 2018). Recently, the group of Peter Carmeliet created a comprehensive atlas of ECs from 11 different organs, revealing key elements of angiodiversity (Kalucka et al., 2020). On the macroscopic level, angiodiversity was found to be preserved across all tissues, as capillary, venous, arterial and lymphatic ECs grouped together in similarity analyses, regardless of their tissue of origin. On the microscopic and molecular level, however, ECs revealed unprecedented heterogeneity. Although markers for arteries, veins and lymphatics were shared by most tissues, capillary ECs were found to be highly heterogeneous, showing strong tissue specialization (Table 2). The metabolic needs and general functions of a given tissue were also reflected in the transcriptomes of capillary ECs, and ECs from organs with similar function showed stronger marker overlap. To facilitate the assessment of angiodiversity, the group has made the dataset publicly available (see Table 1), but also launched the endothelial database EndoDB (Khan et al., 2019), which aims to collect bulk EC and single cell EC transcriptomic datasets and will be continuously updated. In addition to these large data compilations, several cellular maps of single organs and purified vascular cells, including more thorough analyses, have been published recently (Table 1).

Development of the brain vasculature at single cell resolution

A more detailed analysis of CNS vascular cells revealed that postnatal brain ECs isolated from postnatal day (P) 7 mice display

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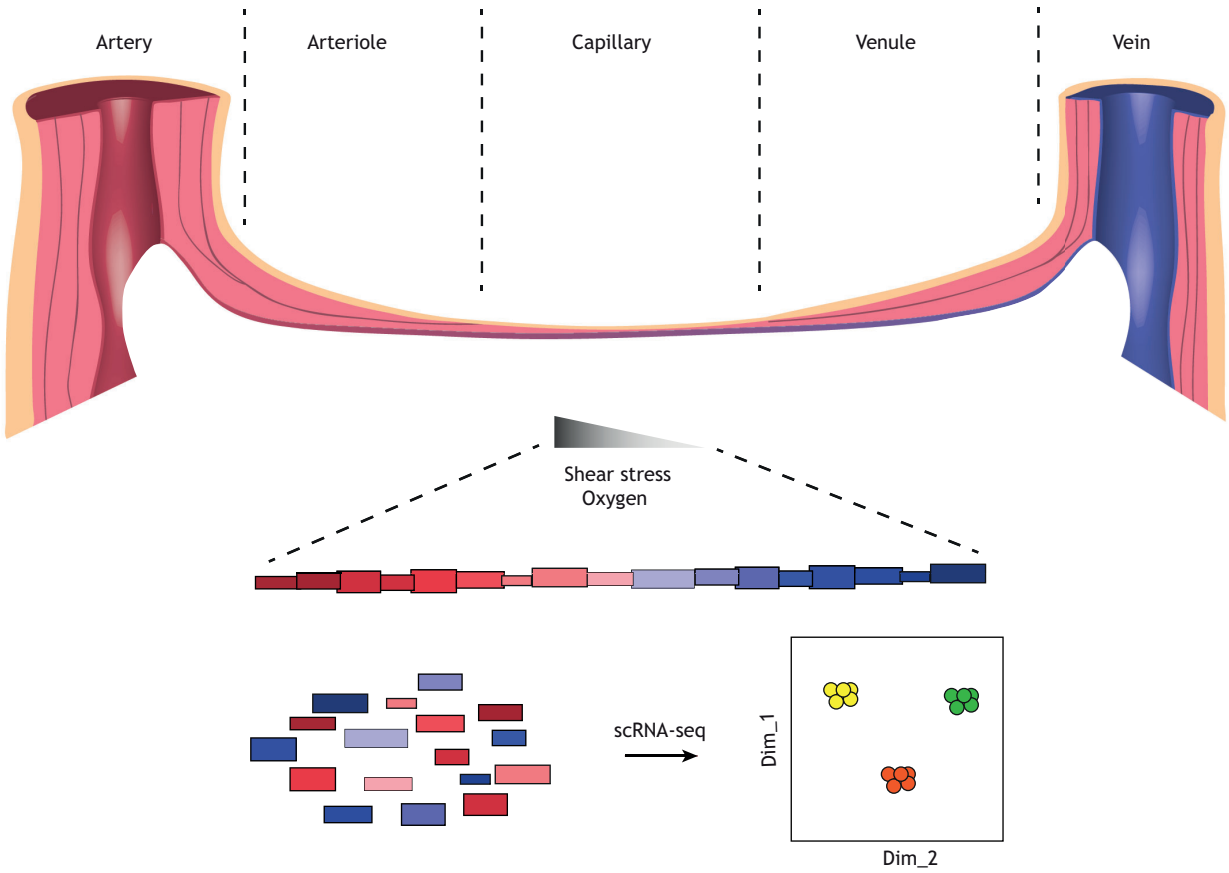

Fig. 2. Angiodiversity at the macroscopic and molecular level. High pressure blood flows from arteries via arterioles and capillaries to supply oxygen and nutrients to organs and tissues (top panel); low pressure deoxygenated and nutrient-depleted blood is then collected by venules and veins and recirculates to the heart. This assembly generates a gradient of physical parameters, such as oxygen availability and shear stress, within a vessel (middle). Gradual changes in oxygen availability and shear stress impact the transcriptomes of individual ECs lining the vessel, thereby giving rise to heterogeneity in ECs (bottom). This heterogeneity can be assessed by scRNA-seq: ECs are extracted from the tissue and subjected to scRNA-seq, and clustering approaches can be used to group cells with similar transcriptomes.

remarkable heterogeneity, closely resembling the situation observed in adult brains (Sabbagh et al., 2018). Profiling of 3946 fluorescence-activated cell sorting (FACS)-purified ECs using the 10x Genomics platform revealed six EC subpopulations that, based on known marker genes, could be classified as tip, mitotic, venous and arterial cells, as well as two distinct types of capillary cells. The two capillary clusters could further be identified as capillary ECs near veins or near arteries. Interestingly, mitotic ECs expressed an array of venous EC markers, adding to the notion that proliferating cells arise from veins during sprouting angiogenesis. Extracting specifically expressed genes from the tip cell cluster, additional potential marker genes were validated by *in situ* hybridization (ISH) using the retina of 5- to 8-day-old mice as a model of postnatal angiogenesis. As predicted by the scRNA-seq analysis, the expression of the presumed tip cell marker genes *Apln*, *Mcam*, *Lamb1* and *Trp53i11* was specifically enriched at the angiogenic front of the superficial vascular plexus (Table 2).

In a separate study, unsupervised hierarchical clustering of embryonic brain ECs isolated from the forebrain of embryonic day (E) 14.5 mice revealed only two distinct populations, which were marked by the differential expression of blood-brain barrier-associated genes (Hupe et al., 2017). This may indicate that the vasculature of the brain matures relatively early. Similarly, in the heart, valve leaflet EC heterogeneity seems to be established at an early postnatal stage: ECs isolated from valve leaflets of P7 mice could be separated into three endothelial subclusters, consisting of lymphatic ECs, vascular ECs and ECs stemming from the leaflet coaptation site (Hulin et al., 2019). Notably, the heterogeneity and location of these cell types are conserved in P30 and adult mice, as well as in humans and pigs.

Single cell analyses of the kidney vasculature

ScRNA-seq of 1.556 glomerular filter ECs identified a previously unappreciated level of EC heterogeneity in the mouse kidney (Karaiskos et al., 2018). Clustering of ECs revealed four subpopulations defined by distinct molecular signatures. Gene set enrichment analyses of the clusters unravelled stress response, cell adhesion, cell maturation and proliferation as gene sets defining the distinct EC subpopulations.

More recently, high-throughput bulk and scRNA-seq were used to create a detailed map of ECs in the developing mouse kidney (Barry et al., 2019). Notably, the kidney EC transcriptome changes dramatically after birth, indicating that post-gestation stimuli drive vascular specialization. Using pseudotime approaches, vascular heterogeneity within the kidney was found to be driven by progenitor cells that give rise to early ‘generic’ capillaries, which in pseudotime branch first into pre-glomerular large arteries and then, at a second branch point, into glomerular capillaries and efferent arteries. The remaining generic capillaries eventually mature into peritubular capillaries, which reside in the kidney

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Box 1. Visualizing single-cell RNA-seq data

The classical way to assess heterogeneity in scRNA-seq data is by means of cell clustering. One common way to do so involves using a combination of principal component analysis (PCA), graph-based nearest neighbour approaches and clustering algorithms to identify underlying substructures in the dataset and to cluster individual cells into communities of high similarity. Non-linear dimension reduction methods, such as *t*-distributed stochastic neighbour embedding (*t*-SNE) or uniform manifold approximation and projection (UMAP), are then employed to visualize the clustering results in the low dimensional space (reviewed by Wagner et al., 2016).

Although this approach is well suited to differentiate between cell types and definite cell states, transitions and gradual changes in gene expression will not be captured (see Fig. 3). As outlined in this Review, gene expression profiles in vascular cells (especially ECs) are mostly defined by gradual and continuous changes alongside the vessel. Clustering approaches therefore tend to suggest subpopulations that do not have obvious biological function. Furthermore, the number of identified clusters depends on the resolution parameters used for clustering. High resolution will yield more populations, whereas low resolution will identify fewer communities. This can be useful to distinguish between transcriptomically highly similar cells in a supervised manner, but can also lead to the overestimation of heterogeneity. Identified subpopulations therefore require careful and thorough validation, particularly because scRNA-seq is a low sensitivity technique, which at times renders it difficult to distinguish rare subpopulations from technical outliers.

Trajectory analyses preserve the continuous nature of vascular cell gene expression. Here, cells are ordered alongside a one-dimensional axis, reflecting the major axis of variability of the sample, while the endpoints of the trajectory represent the most extreme cellular states. Typically, trajectory analyses represent cell differentiation statuses ('pseudotime') but, depending on the sample, can also reflect spatial location ('pseudospace') (reviewed by Wagner et al., 2016; Tritschler et al., 2019). For the analysis of vascular scRNA-seq data, low resolution clustering to identify distinct vessel types and biologically relevant subpopulations would ideally be combined with trajectory analyses to assess changes in the continuous gene expression patterns within a vessel (Fig. 3).

cortex. Throughout development, kidney ECs establish vascular bed-specific gene sets, which are driven by the restricted and abundant expression of transcription factors (TFs). Among them, Gata5, Prdm1, Pbx1 and Tbx3 were identified as key TFs with restricted expression in glomerular capillaries and efferent arteries. Correspondingly, the EC-specific deletion of *Tbx3* resulted in disrupted EC-to-parenchymal cell crosstalk and ultimately led to glomerular hypoplasia, microaneurysms and the disappearance of fenestrations leading to fibrosis (Barry et al., 2019).

Another recent study assessed kidney EC heterogeneity and specialization of the adult mouse renal endothelium during physiological homeostasis and dehydration (Dumas et al., 2019), reporting eight previously unrecognized renal EC subpopulations. On an inter-compartment level, Gata5 was found to be a glomerulus-specific TF, as also described in Barry et al. (2019). Among other TFs, Irf1, Foxp1 and Sp1 marked cortical ECs, whereas Pparg, Lef1 and Nfe2l2 were restricted to medullary ECs (Table 2). In addition to creating a vascular map, this study focused on the adaptation of medullary ECs to dehydration, showing that they quickly respond to hyperosmolarity by upregulating gene sets associated with hypoxia response, glycolysis and oxidative phosphorylation. Surprisingly, although medullary ECs are glycolysis addicted, oxidative phosphorylation was found to be crucially involved in maintaining fundamental renal functions such as urine concentration *in vivo* and EC survival *in vitro*. These mouse studies have recently been expanded to the analysis of ECs in the human kidney (Menon et al., 2020).

Angiodiversity in the developing lung

One major difference in comparing human to mouse lung ECs was that CA4 expression was found to be a general marker for capillary ECs in humans (Table 2), but its expression was restricted to a distinct subset of ECs in mice (Goveia et al., 2020; Kalucka et al., 2020; Vila Ellis et al., 2020). Anatomically, these Car4-positive mouse ECs lined alveoli and were separated from alveolar type 1 (AT1) cells by a basement membrane without intervening pericytes. Interestingly, Car4-positive ECs were also enriched in VEGFR2 expression and were dependent on AT1 cell-derived VEGFA. Consequently, deletion of epithelial VEGFA led to a loss of Car4-positive ECs, which in turn was manifested in an aberrantly enlarged alveolar space (Vila Ellis et al., 2020).

Comparing different lung cell types in human lung cancer, MAF and MLX were identified as candidate TFs underlying lymphatic EC gene expression (Lambrechts et al., 2018). This is noteworthy as another study also identified MAF, along with PROX1 and HOXD8, as the top hits for TFs driving lymphatic EC gene expression in postnatal lungs (Guo et al., 2019). Here, whole lung tissue from P1 mice was subjected to scRNA-seq and >8000 cells were profiled. In contrast to the earlier mentioned work of He et al. (2018), postnatal lung ECs in this case did not display major diversity and clustered together in one cluster. As expected, signature genes enriched in postnatal ECs were associated with angiogenesis and vascular development, which might explain the lack of heterogeneity, as the early postnatal endothelium was probably mostly angiogenic and not yet mature. Unsupervised clustering of pericytes, by contrast, revealed two subpopulations. This observed cellular diversity appears to be lost in the adult mouse; in the He et al. study (2018), pericytes formed one cluster using either t-SNE or the BackSPIN algorithm. Moreover, *Map3k7cl* and *Acta2*, marker genes of one of the postnatal pericyte clusters, appeared to be exclusively expressed by vascular smooth muscle cells (VSMCs) in the adult mouse, whereas the marker gene for the other cluster, *Vsnl1*, was specifically expressed by pericytes. This could point towards a misassigned cell type or could represent a pericyte subpopulation that expresses VSMC markers and disappears in the adult mouse. Postnatal pulmonary smooth muscle cells (SMCs), in contrast to adult SMCs, grouped in three clusters. These subpopulations, however, seemed to reflect developmental plasticity, as the clusters likely represented myofibroblasts, cells transitioning from the myofibroblast phenotype to the SMC phenotype, as well as SMCs.

Angiodiversity in the ovary

Another highly dynamic organ is the ovary, in which cyclic follicular growth is followed by tissue regression and involution. To elucidate the molecular mechanisms underlying tissue homeostasis, the cellular landscape of growing and regressing human ovarian follicles has recently been characterized (Fan et al., 2019). Although this study mostly focused on the association between the complement system and follicular remodelling, it also revealed heterogeneity of ECs and perivascular cells in the inner cortex of the ovary. Specifically, three distinct EC subpopulations could be observed. Whereas one represented lymphatic ECs, the others were associated with the expression of angiogenesis genes and genes involved in apoptosis regulation, indicating a dynamic interplay between vessel growth and regression. In line with this observation,

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Box 2. Caveats in vascular scRNA-seq

A typical workflow to generate single cell data from a biological sample encompasses three major steps: single cell isolation, library preparation and next generation sequencing. Below, we highlight peculiarities and pitfalls when performing scRNA-seq on vascular cells.

Cell isolation and RNA extraction: Most of the technical variation in scRNA-seq datasets is introduced via cell isolation and RNA extraction protocols (van den Brink et al., 2017). For ECs, we observed that minor deviations in the isolation protocol can induce the expression of Klf2 and Klf4, two transcription factors that control a large segment of the endothelial transcriptome. We further experienced dramatically different cell recovery rates depending on the scRNA-seq platform. Plate-based approaches yielded recovery rates of roughly 80% of the original cell input; recovery rates reduced to 25% for droplet-based platforms. Given the sensitivity of ECs towards mechanical stress, this phenomenon likely results from stress-induced cell death. Building on these empirical findings, isolation protocols need to be thoroughly tested by assessing RNA integrity and induction of stress-induced gene expression. Ideally, biological replicates should be included to account for technical variability during cell isolation and processing.

Library preparation: One major lesson learnt from scRNA-seq on vascular cells is their low RNA content. We found that ECs from multiple organs contain an average of 0.5 pg of RNA per cell, which may have two major implications: (i) shotgun approaches, in which whole tissues are digested and subjected to droplet-based scRNA-seq, may lead to insufficient amplification of vascular cell cDNA and therefore to low recovery rates; (ii) high PCR cycle numbers may introduce a stronger PCR bias during library preparation.

Next generation sequencing: In most recent scRNA-seq studies, there is a tendency towards less depth and higher cell numbers per multiplex. We found a strong correlation between sequencing reads per library and genes identified, with saturation occurring at >1 million reads per library (in contrast to other cell types). In general, single cell data obtained from vascular cells during homeostatic conditions are rather shallow, which is why the sequencing depth should be critically considered.

SMCs also showed features of growth and remodelling, as two distinct subpopulations could be identified. One was enriched in genes involved in the regulation of immune responses and apoptosis, whereas the other expressed smooth muscle cell maturation signature genes.

Identification of vascular stem and progenitor cells by single cell approaches

FACS analyses based on the differential expression of the EC surface markers CD31 and VEGFR2 have led to the identification of vessel-resident endovascular progenitors (EVPs) that exhibit self-renewing capacity and can give rise to differentiated ECs, presumably through a transit amplifying cell state (Patel et al., 2017). Building on this work, scRNA-seq of aortic ECs has been used to characterise vascular stem and progenitor cells (Lukowski et al., 2019). Unsupervised clustering revealed three major EC subpopulations, suggestive of a progenitor population that displays a more mesenchymal phenotype, quiescence and high mitochondrial content, and two differentiated EC populations. Spearman correlation of the single cell data with previously conducted RNA-seq analysis of EVPs and differentiated ECs revealed a strong correlation in gene expression between the progenitor cluster and the EVP population, as well as a strong correlation between differentiated ECs. Pseudotime analysis and single cell global fate potential of subpopulations (scGPS) approaches confirmed bioinformatically that progenitor cells were likely to transition into either of the differentiated cell types, whereas differentiated ECs were restricted.

A similar EC progenitor cell (EPC) population was identified as a side population within CD200- and CD157 (Bst1)-positive liver ECs that exhibited higher efflux of Hoechst dye in flow cytometry (Wakabayashi et al., 2018). These progenitors have stem cell-like features, as evidenced by the capability of single EPCs to generate functional blood vessels *in vitro*. Furthermore, these EPCs strictly gave rise to ECs but not to cells of mesenchymal or haematopoietic lineages. In addition, EPCs were found to be present in most organs, usually covered by α-SMA-expressing adventitial cells in large vessels.

Similar side populations in the aorta were characterized more than a decade ago (Sainz et al., 2006). Here, the side population was found to be enriched in Sca-1 (Ly6a)-positive cells, which were capable of giving rise to ECs and VSMCs *in vitro* (Table 2). Yet, these cells did not form myeloid or lymphoid colonies. Employing scRNA-seq, a rare subpopulation of Sca-1-positive VSMCs in the aorta of healthy animals was recently identified that exhibited partially overlapping marker gene expression with the previously reported aortic side population (Dobnikar et al., 2018). Accordingly, *Sca-1* expression marks VSMCs that are undergoing phenotype switching under pathological conditions. Indeed, lineage-tracing experiments revealed that the Sca-1-positive VSMC lineage constituted a heterogeneous cell population, such that some cells displayed a contractile phenotype and others expressed a VSMC response signature, including markers of synthetic VSMCs. Thus, Sca-1-positive VSMC lineage cells may serve as a pool of cells with enhanced plasticity that can rapidly respond to injury or inflammation.

In an effort to characterize the overall cellular composition and underlying heterogeneity of the aorta, a recent study profiled >10,000 cells isolated from four healthy mice (Kalluri et al., 2019). Here, ECs were shown to be the most heterogeneous cell population, as clustering analysis revealed three distinct EC subclusters. One subpopulation showed enriched lymphatic gene expression, whereas the other two were associated with lipoprotein processing, and angiogenesis and ECM production, respectively. Interestingly, chronic stress, such as high caloric intake, did not markedly affect the ECs as all three subpopulations were activated in a similar manner and maintained their identity.

Limitations and bottlenecks in clustering cellular heterogeneity

The above discussion of angiodiversity in different organs highlights the strength of clustering approaches to unravel cellular heterogeneity and to identify rare subpopulations. Nevertheless, it should be noted that scRNA-seq results may also be misleading (Box 1), as clusters might contain cells of distinct phenotypes for which the differences in gene expression might not be strong enough to be partitioned into subclusters. This could especially be the case if only a few genes are regulated. An example of such a phenomenon was seen in a study employing scRNA-seq of human carotid arteries collected from patients undergoing carotid endarterectomy (Yao et al., 2018). Similar to previous studies, this report revealed that VSMCs did not show major heterogeneity and were present as one cell cluster. However, by manually partitioning the cell population according to an arbitrary threshold of ACTA2 expression, a canonical marker for VSMC identity, the histone variant H2A.Z could be identified as key regulator of VSMC identity. H2A.Z expression was shown to facilitate nucleosome turnover and to be crucially involved in the recruitment of SMAD3 and MED1, which then activated a VSMC-specific gene expression programme.

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Table 1. Interactive online databases

Vascular single cell databases

Name	Organ	Platform	Age	Mouse strain	Sex	Reference
scLAB	Lung	Drop-Seq/ Fludigm C1	P1	C57BL6/J	Female	Guo et al., 2019
Link to scLAB: https://research.cchmc.org/pbge/lunggens/SCLAB.html						
VascularSingleCells	Lung, brain	SMART-Seq2	10-19 weeks	C57BL6/J	Male & female	Vanlandewijck et al., 2018; He et al., 2018
Link to VascularSingleCells: http://betsholtzlab.org/VascularSingleCells/database.html						
VECTRDB	Brain	10× Genomics	P7	FVB	Male	Sabbagh et al., 2018
Link to VECTRDB: https://markfsabbagh.shinyapps.io/vectrdb/						
Single Cell Database	Heart	SMART-Seq2	E12.5, E14.5	Mixed	Mixed	Su et al., 2018
Link to Single Cell Database: http://redhorselab.com/single-cell-rna-seq-database						
Livermesenchyme	Liver	10× Genomics	10-16 weeks	C57BL6/J	Male	Dobie et al., 2019
Link to Livermesenchyme: http://livermesenchyme.hendersonlab.mvm.ed.ac.uk/						
LiverCellAtlas	Liver	10× Genomics	Mixed	Human	Mixed	Ramachandran et al., 2019
Link to LiverCellAtlas: www.livercellatlas.mvm.ed.ac.uk						
EC Atlas	Multi organ	10× Genomics	8 weeks	C57BL6/J	Male	Kalucka et al., 2020
Link to EC Atlas: https://endotheliomics.shinyapps.io/ec_atlas/						
Nephrocell	Kidney	10× Genomics	Mixed	Human	Mixed	Menon et al., 2020
Link to Nephrocell: http://nephrocell.miktmc.org/						
LungEC Tax	Lung	10× Genomics	7-10 weeks/mixed	C57BL6/J and human	Male/mixed	Goveia et al., 2020
Link to LungEC Tax: https://endotheliomics.shinyapps.io/lung_ectax/						
Glomerulus single-cell atlas	Glomerulus	Drop-Seq	8 weeks	CD1	Unspecified	Karaiskos et al., 2018
Link to Glomerulus single-cell atlas: https://shiny.mdc-berlin.de/mgsca/						
nichExplorer	Bone marrow	10× Genomics	6-22 weeks	C57BL/6	Male & female	Tikhonova et al., 2019
Link to nichExplorer: http://aifantislab.com/niche						
EndoDB	Compilation of existing vascular scRNA-seq datasets					Khan et al., 2019
Link to EndoDB: https://vibcancer.be/software-tools/endodb						

Online tutorials

Online introduction to scRNA-seq course: https://scrnaseq-course.cog.sanger.ac.uk/website/introduction-to-single-cell-rna-seq.html

Online introduction to scRNA-seq course: https://broadinstitute.github.io/2019_scWorkshop/index.html

Compilation of different scRNA-seq tutorials: https://github.com/SingleCellTranscriptomics

Comprehensive list of scRNA-seq tools and packages

https://github.com/seandavi/awesome-single-cell

https://www.scrna-tools.org/

Another limitation of clustering approaches is the lack of spatial information. As such, it has been difficult to ascertain how heterogeneities in gene expression might be linked to heterogeneities in cell function with regard to space. For example, in the lymph node (LN), high endothelial venules (HEVs; the entry point for lymphocytes transmigrating from the blood into the LN) display considerable cellular diversity, as well as major spatial heterogeneity, which is conserved in inflamed lymph nodes (Veerman et al., 2019). A recent scRNA-seq study of HEVs under steady state, de-differentiation and inflammation revealed lymphotoxin-β receptor (LTβR) signalling as a crucial pathway for maintaining HEV maturity. Interestingly, LTβR was shown to be activated in a dose-dependent manner and controlled genes that either required high LTβR stimulation or were low dose targets. Similar observations were made in a study of lymphatic ECs in human LNs (Takeda et al., 2019), in which a remarkable heterogeneity within lymphatic ECs was identified. In this case, heterogeneity could be mapped to particular locations within the LN using immunofluorescence microscopy. Notably, lymphatic ECs of the subcapsular and medullary sinus were shown to establish a molecular axis to attract and retain neutrophils in the LN. Although these examples highlight that heterogeneities in gene expression can be linked to spatial differences in cell function, gaining a complete picture of how gene expression varies spatially still presents a major challenge in single cell biology. However, and as we discuss below, recent technological advances and combinatorial approaches have helped to provide initial insights in how vessels are spatially organized at the molecular level.

Deconvoluting angiodiversity in space

Spatial analysis of ECs in the liver

One organ that serves as a prototypic example for understanding spatial changes in gene expression is the liver. A landmark paper published in early 2017 combined single molecule fluorescence *in situ* hybridization (smFISH) and scRNA-seq to spatially reconstruct the distribution of hepatocytes along a hepatic sinusoid (Halpern et al., 2017). In this study, smFISH of known zonated genes was used to define a set of marker genes for metabolically distinct zones ranging from the central vein to the portal vein. The individual transcriptomes obtained from scRNA-seq were then mapped to the defined zones according to their zonation marker set expression (Fig. 3). This approach resulted in a high-resolution map of hepatocyte gene zonation and revealed that around 50% of hepatocyte genes were significantly zonated.

The liver endothelium is also assumed to be strongly zonated. Yet, it has been challenging to determine zonation patterns of cells with low RNA content and without apparent landmark gene expression, as evidenced by the first attempt to generate a concise human liver cell atlas. Here, liver sinusoidal ECs (LSECs) could be partitioned into two distinct subpopulations, each of which likely represents the peri-portal or peri-central fraction (MacParland et al., 2018). Peri-portal LSECs displayed significant enrichment in pathways associated with vessel development, cell-cycle arrest, cell junction and apoptosis, whereas peri-central LSECs were enriched in genes associated with immune functions. Nevertheless, except for CD32b (FCGR2B), which was identified as a novel peri-central LSEC marker, clustering analysis did not recover zonation patterns or gradual gene expression.

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Table 2. Integrated vascular cell marker genes

Tissue	Subpopulation	Marker genes	Reference
Vessel types			
	Big vessel ECs	*Vwf*, *Vcam1*	Vanlandewijck et al., 2018; Kalucka et al., 2020
	Arterial ECs	*Bmx*, *Efnb2*, *Vegfc*, *Sema3g*, *Fbln2*, *Hey1*, *Dll4*, *Cxcr4*	Vanlandewijck et al., 2018; Sabbagh et al., 2018; Su et al., 2018; Kalucka et al., 2020
	Venous ECs	*Nr2f2*, *Slc38a5*, *Ephb4*	Vanlandewijck et al., 2018; Sabbagh et al., 2018; Su et al., 2018; Kalucka et al., 2020
Tissue-specific vessel types			
Heart	Venous ECs	*Aplnr*	Su et al., 2018
	Coaptation ECs	*Hapln1*, *Wnt9b*	Hulin et al., 2019
Brain	Capillary ECs	*Mfsd2a*, *Tfrc*, *Slc16a1*	Vanlandewijck et al., 2018
	Choroid plexus ECs	*Plvap*, *Plpp1*, *Plpp3*, *Cd24c*, *Esm1*	Kalucka et al., 2020
Kidney	Glomerular ECs	*Gata5*, *Prdm1* and *Pbx1*, *Tbx3*	Barry et al., 2019; Dumas et al., 2019
	Cortical ECs	*Irf1*, *Foxp1*, *Sp1*	Dumas et al., 2019
	Medullary ECs	*Pparg*, *Lef1*, *Nfe2l2*	Dumas et al., 2019
Lung	Lymphatic ECs	*Maf*	Lambrechts et al., 2018; Guo et al., 2019
	Tumour ECs	*IGFBP3*, *SPRY1*	Lambrechts et al., 2018; Goveia et al., 2020
	Non-tumour ECs	*MT2A*, *EDNRB*	Lambrechts et al., 2018; Goveia et al., 2020
	Alveolar lung ECs	*Car4*	Vila Ellis et al., 2020; Goveia et al., 2020
Intestine	Lacteal-like intestinal ECs	*Aqp7*	Kalucka et al., 2020
Systemic subpopulations			
	Tip cells	*Apln*, *Mcam*, *Lamb1*, *Trp53i11*, *Col4a2*, *Plaur*	Sabbagh et al., 2018; Zhao et al., 2018; Goveia et al., 2020
	Vascular endothelial stem cells	*CD157* (*Bst1*)	Wakabayashi et al., 2018
	VSCM progenitors	*Sca-1*	Sainz et al., 2006; Dobnikar et al., 2018
	Proliferative/clonally expanding ECs	*Plvap*	Li et al., 2019; Ramachandran et al., 2019; Zhao et al., 2018
	Endovascular progenitors	*Sox9*, *Sox18*, *Il33*	Lukowski et al., 2019
Tissue-specific subpopulations			
Liver	Activated myofibroblasts	*S100a6*	Krenkel et al., 2019
Brain	Inflammatory ECs	*Vcam1*	Yousef et al., 2019
Skin systemic sclerosis	Injured ECs	*HSPG2*, *APLNR*	Apostolidis et al., 2018
Lung tumour	Immature ECs	*HSPG2*	Goveia et al. 2020
	Tip cells	*TCF4*, *SOX4*, *SMAD1*	Goveia et al., 2020

This problem was solved by using the previously published hepatocyte zonation markers as a scaffold to spatially order LSECs. For this purpose, a technology referred to as paired-cell sequencing was developed, which uses cell doublets instead of single cells as input for scRNA-seq analysis (Halpern et al., 2018). As LSECs and hepatocytes are physically separated (by the space of Disse), incomplete digestion of the liver was necessary to enrich LSEC-hepatocyte cell pairs that are directly adjacent in the tissue. Given that hepatocytes have a high RNA content and are transcriptionally very active, the cell doublets could be mapped to the previously defined metabolic zones. A core LSEC transcriptome was then bioinformatically extracted from the cell doublets and could serve as an anchor to spatially align the whole LSEC transcriptomes obtained from scRNA-seq of total non-parenchymal cells. In this way, 35% of detected LSEC-specific genes were shown to have zonated gene expression in the liver. This study further revealed a plethora of new LSEC zonation markers that could be used in FACS experiments for specific enrichment and in-depth characterization of spatially distinct LSECs (Halpern et al., 2018).

In a recently published second effort to build a human liver cell atlas, the authors employed a different strategy of defining space and identifying gradual gene expression (Aizarani et al., 2019). Hepatocytes and LSECs were ordered spatially by a method referred to as diffusion pseudotime, which was initially developed to order single cell gene expression profiles of differentiating cells temporally (Trapnell et al., 2014). Based on observations made in the mouse, the authors reasoned that the major axis of variability for a cell type in the liver would not be reflective of the differentiation status of a cell, but would rather be indicative of its location and therefore of gene expression changes associated with zonation (Fig. 3). This approach was validated by analysing the expression of known zonated human marker genes and eventually revealed that about 67% of LSEC genes were significantly zonated. Pathway enrichment analysis for different zones revealed shared functions of LSECs and hepatocytes, suggesting a co-zonation pattern across the liver. Interestingly, only limited conservation of zonated gene expression could be identified between mouse and human, pointing towards divergent evolutionary changes.

Spatial analysis of the brain vasculature

Using a similar bioinformatic approach, zonation of EC gene expression could be described in the brain of adult mice (Vanlandewijck et al., 2018). The authors used the SPIN algorithm to re-order ECs with a biased distribution of known arteriovenous markers into a one-dimensional range. A set of 1798 transcripts was found to be significantly differentially expressed across that range. Nested patterns of gradually changing gene expression were indicative of a seamless continuum of ECs. Interestingly, the described arteriovenous continuum was also observed in heart capillaries (Su et al., 2018), reinforcing the idea that gene expression within a vessel changes gradually and continuously alongside its axis. In the brain vasculature, TFs were found to be overrepresented at arterial locations, whereas transporters were enriched in capillary and venous ECs.

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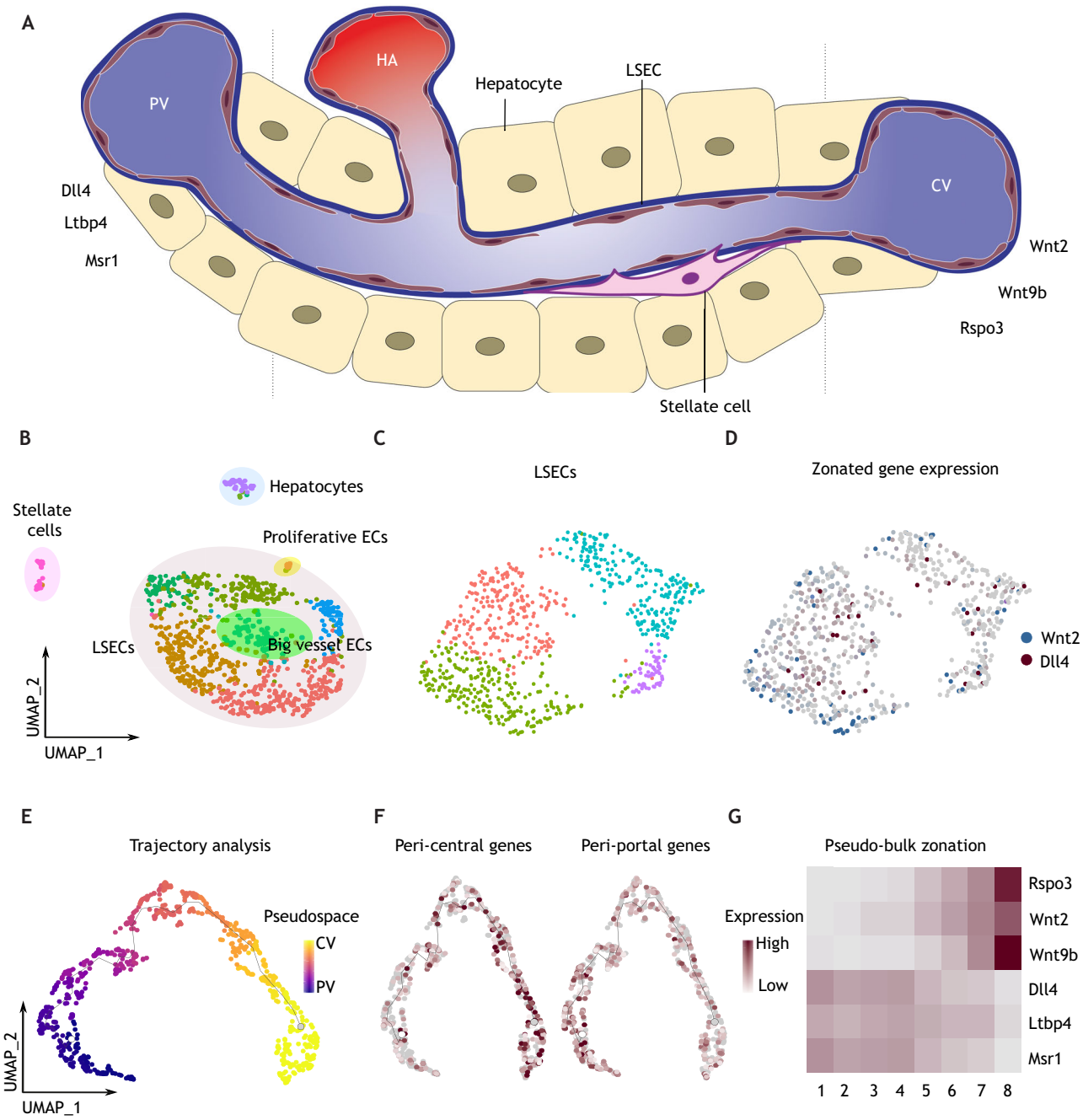

Fig. 3. Visualizing angiodiversity. (A) The liver endothelium serves as a prototypic example for angiodiversity. Nutrient-rich blood from the portal vein (PV) mixes with oxygen-rich blood from the hepatic artery (HA) and flows through the sinusoidal vasculature into the central vein (CV), generating a biochemical gradient, which is reflected at the transcriptomic level. PV-associated liver sinusoidal endothelial cells (LSECs) are enriched in, among other genes, *Dll4*, *Ltbp4* and *Msr1*, whereas LSECs close to the CV are defined by a Wnt signature. (B) Visualizing scRNA-seq data of murine liver cells using clustering in Seurat leads to a clear separation of distinct liver cell types. Cell clusters are annotated by enriched marker gene expression. (C) Reclustering of the LSEC population using default parameters reveals four distinct subpopulations. (D) Cells that express known zonated genes Wnt2 and Dll4 cluster together and are present in all subpopulations. (E) Trajectory analysis using Monocle reveals a linear trajectory from PV- to CV-associated LSECs, reproducing the gradual change in gene expression alongside the vessel axis and, thus, reflecting a 'pseudospace'. (F) Peri-central and peri-portal marker gene expression is enriched at opposing ends of the trajectory, confirming the spatial arrangement of cells. (G) Spatial resolution can also be achieved in pseudo-bulk approaches. Here, known marker gene sets are used to define the spatial distribution of gene expression patterns and to define zones ranging from one end of the vessel to the other. For panels B-F, analyses were performed and cells were mapped to the respective zones according to their landmark gene expression pattern, as first described by Halpern et al. (2017).

Contrarily, the gene expression of mural cells followed a punctuated continuum pattern, whereby two phenotypically distinct continua could be identified (Vanlandewijck et al., 2018). This is interesting insofar as, on a more macroscopic level, individual brain capillary pericytes occupy discrete territories by forming thin processes that extend hundreds of micrometres along the capillary bed and that do not overlap with processes of adjacent pericytes (Berthiaume et al., 2018). This patchy pattern seemed to be recovered using BackSPIN analysis, as hierarchical clustering of brain mural cells revealed two predominant gene expression patterns: whereas pericytes formed a

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continuum with VSMCs by gradually losing pericyte markers and acquiring SMC identity, arteriole SMCs progressively obtained arterial SMC identity (Vanlandewijck et al., 2018).

Deconvoluting angiodiversity in time

Most bioinformatic tools to study spatial relationships were initially developed to assess cell trajectories through distinct differentiation states in complex tissues. Simplified pseudotime algorithms identify the most extreme cells in a cell population and use these as starting and end points, respectively (Fig. 3). The rest of the cells are then ordered alongside the established pseudotime axis and, thus, allow for the reconstruction of sequential gene expression patterns and branching lineages.

Temporal analysis of EC development

Studying developmental relationships between ECs using pseudotemporal approaches is particularly interesting as embryonic ECs show a high degree of plasticity and can also give rise to haematopoietic stem cells (HSCs). Using nine sequential time points ranging from E6.5 to E8.5 to generate serial scRNA-seq snapshots of the developing mouse embryo (Pijuan-Sala et al., 2019), cells arising during the first wave of haematopoiesis were found to transit through a molecular state that does not exhibit classical characteristics of mature ECs, such as high expression of the endothelial cell markers *Cdh5* and *Pecam1*. Compared with the second wave of haematopoiesis, the first wave produces primarily primitive red blood cells and macrophages, which could indicate that EC maturity is necessary to give rise to HSCs. Furthermore, Tal1 was validated as a crucial TF driving the transition; disruption of Tal1 blocked cells at a transcriptional state similar to that of one of the earliest-appearing EC subtypes and activated other mesodermal programmes as a consequence of not being able to proceed towards a haemogenic phenotype. Interestingly, clustering analysis of embryonic ECs revealed their spatial origin, as the three identified subclusters could be mapped to three distinct EC formation sites, namely the yolk sac, allantois and the embryo proper. During the second wave of haematopoiesis, HSCs are generated from haemogenic ECs through the formation of intra-aortic haematopoietic clusters (IAHCs). Using scRNA-seq, IAHCs were found to already contain pre-HSCs and committed progenitors that showed transcriptional similarities to cells in the yolk sac (Baron et al., 2018). As expected, most transcriptional changes in IAHCs occurred in parallel to endothelial-to-haematopoietic transition (EHT) and pre-HSC maturation, between E10 and E11 (Baron et al., 2018). The underlying mechanisms of the transition were further dissected and a model was proposed in which the initial EC fate programme is driven by the TFs Erg and Fli1. The subsequent expression of Runx1 and Gata2 initiates a haematopoietic transcriptional programme, and EC genes are progressively downregulated. By completion of the EC-to-HSC transition, Erg expression is completely suppressed and the EC programme shut down, and Fli1 transitions from promoting EC identity to driving an HSC fate programme together with Runx1 (Bergiers et al., 2018).

Further insights into EC plasticity

The plasticity of embryonic ECs has also been reported in the context of coronary artery formation. Sinus venosus-derived ECs gradually decrease their expression of venous-specific genes to simultaneously acquire arterial identity. The vein-specifying TF COUPTF2 (also known as NR2F2) acts as a crucial regulator in this process, as high levels of COUPTF2 drive a gene programme associated with cell cycle and proliferation, which retains ECs in their venous identity. Sharp downregulation of COUPTF2 is necessary for ECs to exit the venous-associated proliferative programme and acquire arterial identity (Su et al., 2018).

Mature vascular cells can be driven into more plastic states during injury and pathology. These (mal-)adaptive processes are of vital importance for human health, as dysfunction of the inner lining of blood vessels reflects the single most common cause of human mortality. Indeed, ECs play crucial roles in major fatal human diseases including stroke, cardiac ischemia, hypertensive diseases and cancer. Single cell analyses of ECs in adult healthy and diseased tissues therefore promise to shed unparalleled pathophysiological insights into the mechanisms of disease in the years to come.

Angiodiversity during vascular responsiveness

As discussed above, vascular cells come in different flavours and show tremendous heterogeneity within the circulatory system. Combining scRNA-seq with models of vascular injury or activation has unravelled previously unappreciated functional specialization of distinct vascular cell subsets in different pathological contexts. Below, we focus on the role of angiodiversity during adaptive processes in the context of tissue repair, inflammation and aging. However, it is worth mentioning that substantial progress has also been made in deconvoluting the properties of tumour-associated ECs at single cell resolution; these developments cannot be covered here in detail due to space constraints so the reader is referred to recent ground-breaking literature on this topic (Sun et al., 2017; Lambrechts et al., 2018; Zhao et al., 2018; Goveia et al., 2020).

Vascular regeneration during cardiac repair

Neovascularization is an important step during tissue repair and regeneration. Recent reports identified a progenitor-like EC subpopulation that clonally expands and drives neovascularization upon heart injury (He et al., 2017). Likewise, tracing single cells upon myocardial infarction in a multispectral lineage-tracing mouse model confirmed the structural integrity of the cardiac endothelium by clonal expansion of resident ECs without significant contribution from progenitor cells from the bone marrow (Li et al., 2019). ScRNA-seq of cardiac ECs in this context revealed major heterogeneity, suggesting that clonal expansion was likely not driven by endothelial-to-mesenchymal transition (EndMT) but rather through the expression of *Plvap*, an EC gene linked to the formation of stomatal and fenestral diaphragms of caveola. Silencing of *Plvap* directly inhibited proliferation *in vitro*, hence making it an interesting candidate for therapeutic strategies to promote cardiac regeneration.

Similar to the heart, the aorta harbours a subset of quiescent cells with high proliferative capacity that can undergo rapid transcriptional changes to initiate a robust mitotic response that can fully regenerate the inner lining of the aorta in mice upon injury (McDonald et al., 2018). Sequential bulk RNA-seq experiments of total aortic ECs revealed *Myc* as a crucial gene driving this healing process. Interestingly, *Myc* targets are significantly enriched in tumour ECs (Lambrechts et al., 2018), pointing towards a more general role for *Myc* in EC activation. Although the broad initial mitotic response in the aorta was niche-independent, a hierarchy within the regenerative capacity could be observed, as subsequent rounds of proliferation included fewer cells with enhanced proliferative capacity. In addition, comparative scRNA-seq of aortic ECs from young versus old mice revealed *Atf3* as a marker of ECs with regenerative potential. The number of *Atf3*-positive cells in aged mice was significantly reduced, which translated into

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impaired wound healing, pointing towards a pool of pre-existing resident ECs with high proliferative properties.

Adaptive plasticity of VSMCs

A similar level of plasticity was also described for VSMCs during atherosclerotic plaque-formation (Dobnikar et al., 2018; Kalluri et al., 2019). In a process referred to as 'phenotype moulding', VSMCs de-differentiate to acquire proliferative and migratory capabilities. TCF21, a gene causally involved in coronary artery disease, was found to be expressed in VSMCs and promote phenotype moulding. During embryogenesis, TCF21 is involved in crucial cell fate decisions and serves as a determining factor for the divergence of coronary VSMC and cardiac fibroblast lineages. In response to atherosclerotic plaque-formation, TCF21-positive VSMCs displayed a continuous trajectory from a contractile SMC state towards a fibroblast-like identity, which seemed to exert protective functions (Wirka et al., 2019). A spontaneously de-differentiating VSMC subtype had previously been identified in healthy aortae (Kaur et al., 2017). Interestingly, in this study VSCMs and ECs could be defined according to a subtype-specific set of G-protein-coupled receptors (GPCRs), and inflammatory activation of vascular cells during atherosclerosis or sepsis was shown to alter the GPCR repertoire of VSMCs and ECs. This is of interest insofar as VSMCs in atheroprone niches have been characterized by *Gprc5b* expression. Correspondingly, selective targeting of *Gprc5b* was sufficient to significantly increase inflammatory gene expression in de-differentiating SMCs (Kaur et al., 2017).

Adaptive vascular processes during inflammation and aging

It will be interesting to study whether age-related impairments are also reflected by *TCF21* expression and/or GPCR repertoire. However, a major obstacle in age-related studies is the question of whether changes in gene expression are due to cell intrinsic properties (e.g. changes in their epigenetic status) or dictated by extrinsic factors (e.g. chronic inflammatory signals) or a combination of both. As ECs are in direct contact with blood, age-related phenotypes likely also result from changes in circulating levels of signalling molecules. Blood transfusion experiments, in which blood from young mice was given to old mice, seemed to mitigate age-related phenotypes, whereas aged blood negatively affected the cognitive functions of young mice (Villeda et al., 2011, 2014). A recent study characterized the role of aged brain ECs in this process and concluded that factors present in aged plasma induced brain EC expression of VCAM1, which led to leukocyte tethering and sustained inflammation (Yousef et al., 2019). Inflammation then triggered brain EC signalling that activated microglia and inhibited neuronal progenitor cell activity, which eventually impaired cognition. Notably, ECs seem to generate a positive feedback loop, as upregulation of VCAM1 concomitantly led to its shedding into the circulation. Plasma VCAM1 in turn fuelled leukocyte tethering and completed the vicious circle. Clearing VCAM1 from the plasma appeared to mitigate the phenotype, as aged mice showed enhanced cognition.

Chronic inflammatory responses probably more likely reflect aging, as the phenotypes manifest over time and are not due to acute stimulation that eventually is resolved. In the liver, chronic inflammation is accompanied by fibrosis, which may lead to cirrhosis (scarring of the liver). During cirrhosis, a specific subset of ACKR1- and PLVAP-positive ECs expands in the fibrotic niche (Ramachandran et al., 2019). As mentioned earlier, Plvap expression also drives the proliferation of clonally expanding progenitor ECs upon myocardial infarction (Li et al., 2019), pointing towards a more conserved mechanism underlying EC activation. In the liver, niche LSECs express pro-fibrogenic genes and exhibit immunomodulatory phenotypes, facilitating leukocyte transmigration (Ramachandran et al., 2019). Furthermore, fibrotic niche LSECs resemble highly connected, instructive signalling hubs. For example, cultured primary LSECs from human cirrhotic livers express high levels of the Notch ligand JAG1, which in turn activates Notch-mediated fibrillar collagen production in hepatic stellate cells. These hepatic stellate cells act as pericytes in the liver and, similar to aortic VSMCs, acquire myofibroblast (MFB) identity during chronic inflammation. ScRNA-seq of these cells in healthy and fibrotic mouse livers revealed a striking heterogeneity of MFBs *in vivo* compared with resting hepatic stellate cells (Krenkel et al., 2019). In this analysis, hepatic stellate cells appeared to transdifferentiate through an intermediate MFB state that is associated with immune regulation. Mature activated MFBs on the other hand were enriched in genes involved in matrix formation and collagen fibril organization and could be marked by S100A6 expression. Moreover, hepatic stellate cell activation appeared to be a site-specific process. In an effort to dissect hepatic stellate cell zonation across the liver lobule, scRNA-seq was used to profile cells from healthy and fibrotic mouse livers (Dobie et al., 2019). A combination of independent component analysis to identify highly variable genes, which were used to extract two opposing gene signatures, and a supervised clustering approach helped to define four spatial zones ranging from central to portal vein. Carbon tetrachloride (CCl_4)-induced liver damage revealed a bias of collagen production towards central vein-associated hepatic stellate cells, which could have two ramifications: either the hepatic stellate cell response towards hepatocyte damage is zonated towards the central vein, or hepatic stellate cell activation is locally restricted to the damage site, as CCl_4 mainly damages central venous hepatocytes.

Surprisingly, chronic liver damage led to an expansion of lymphatic ECs in human liver, likely in an attempt to maintain tissue homeostasis (Tamburini et al., 2019). In this case, liver lymphatic ECs exhibited an active cell cycle and CCL21 expression. Moreover, chronic liver damage was associated with oxidized low-density lipoprotein-induced lymphatic EC IL13 production and led to enhanced lymphatic instability by diminishing PROX1 expression.

EC adaptation to hypoxia

Hypoxia is an environmental determinant that has a major impact on EC gene programmes. The mammalian central nervous system (CNS) appears to adapt particularly well to hypoxic conditions. For example, the integrity of the cortical blood-brain barrier is maintained during periods of hypoxia, likely due to adaptive response programmes of blood-brain barrier-forming ECs. A recent study employing scRNA-seq of cortical and retinal ECs isolated from mice with chronic CNS hypoxia and healthy controls identified a substantial fraction of cortical ECs acquiring tip cell identity (Heng et al., 2019). Interestingly, retinal ECs displayed a similar shift towards tip cells, pointing to a general mechanism as part of an adaptive neoangiogenic programme to maintain vascular density and blood flow. The authors concluded that glia-derived VEGFA may be the activator of the tip cell programme in both the cortex and retina. Of note, the study employed the previously published dataset of P7 brain ECs (Sabbagh et al., 2018; see above) to classify EC subtypes. As P7 brain ECs appeared to recapitulate adult brain EC heterogeneity and marker expression, this seemed like a valid approach. Other organs, however, show strong

differences in cellular diversity with age, which needs to be considered to avoid misassignment of subpopulations.

Conclusions

Single cell biology per se is not a novel discipline: experimental pathologists have, in principle, always worked at single cell resolution, e.g. when studying the expression of a gene in a given tissue by means of ISH or immunohistochemistry. Such approaches have already yielded important insights into the heterogeneity of vascular cells in different organs and in different vessel calibres. Yet, although the resolution of microscopic techniques is high, their throughput and parallel processing capabilities are low. These limitations have been overcome in recent years by major advances in omics technology. In particular, scRNA-seq now enables the simultaneous analysis of the transcriptomes of thousands of cells, making it possible to deconvolute organ architecture and individual and collective cellular behaviours and effector functions at single cell resolution. Single cell epigenomics and proteomics are lagging behind a little, but it can be expected that such approaches will also be advanced to routine use in the years to come.

Although these approaches offer tremendous opportunity, there are also major challenges ahead. Single cell technology will no doubt become more affordable and generally available as technological developments materialize. Yet, it continues to be a costly technology and it may be a major challenge for the vascular community to generate sufficient funds to fully exploit the power of single cell approaches. In addition, data are being generated at an unprecedented pace, but it requires the concerted action of the community to push for data integration in order to maximally exploit the available data on a systems level and to identify novel potential therapeutic targets (Muus et al., 2020 preprint). One of the major hurdles for data integration is the lack of standardization, making it difficult to integrate data coming from different laboratories and platforms. A concerted effort for increased standardization is therefore urgently needed. Finally, vascular biology has historically been a wet lab science, although there are nowadays considerable data science experts in vascular biology labs. Still, we require a fertile crosstalk culture between wet lab scientists and data scientists in order to translate hypotheses into answerable questions for the maximum exploitation of available data.

With little more than two years in the making, vascular single cell biology is still in its early stages. Although much of the early work was aimed at generating snapshot maps of vascular cells in different organs, mostly focusing on ECs, the focus has – as spelled out in this Review – rapidly moved towards the analysis of biologically relevant differentials (e.g. temporal changes, spatial comparisons, adaptation to challenge including genetic manipulation, response to pathologic insult, species comparative approaches). Such approaches enable unparalleled insight into molecular mechanisms and guide the identification of relevant subpopulations and niche cells. Ultimately, vascular single cell biology may contribute to the long dream of selectively targeting subsets of vascular cells for therapeutic applications. After all, no cell is as readily accessible through the systemic circulation as the EC. Targeting selective subsets of ECs (‘precision angioscience’) may therefore offer fundamentally novel approaches towards the treatment of organ diseases including cancer.

Acknowledgements

The authors regret that, owing to space limitations, they could not discuss and cite all original research articles and related references on this topic.

Competing interests

The authors declare no competing or financial interests.

Funding

Work in the authors’ laboratory is supported by funds from the Baden-Württemberg Stiftung special programme ‘Angioformatics Single Cell Platform’, the Deutsche Forschungsgemeinschaft (CRC1366, CRC1324, CRC-TR209, RTG2099), the European Research Council (ERC AdG ‘Angiomature’), the Helmholtz Association and Universität Heidelberg.

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