Single Cell Analysis in Vascular Biology

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The ability to quantify DNA, RNA, and protein variations at the single cell level has revolutionized our understanding of cellular heterogeneity within tissues. Via such analyses, individual cells within populations previously thought to be homogeneous can now be delineated into specific subpopulations expressing unique sets of genes, enabling specialized functions. In vascular biology, studies using single cell RNA sequencing have revealed extensive heterogeneity among endothelial and mural cells even within the same vessel, key intermediate cell types that arise during blood and lymphatic vessel development, and cell-type specific responses to disease. Thus, emerging new single cell analysis techniques are enabling vascular biologists to elucidate mechanisms of vascular development, homeostasis, and disease that were previously not possible. In this review, we will provide an overview of single cell analysis methods and highlight recent advances in vascular biology made possible through single cell RNA sequencing.

Keywords: vascular development, genomic analysis, vascular disease, single cell analysis, bioinformatics

INTRODUCTION

Recent advancements in single cell analyses have enabled researchers to investigate tissue development, cell heterogeneity, and cellular response to injury and disease in unprecedented ways. Such analyses require continued development of techniques that separate and label single cells isolated from tissues, quantify components in each cell (RNA, DNA, protein, etc.), and analyze resulting data on a single cell level. These bioinformatic pipelines can cluster similar cells, compare gene expression patterns among clusters, and predict their signaling and lineage progression pathways. Comparing individual cells from a tissue-wide perspective has revealed previously unappreciated levels of cellular heterogeneity, identified unknown cell types and elucidated cellular differentiation and interaction pathways.

Obtaining such novel insights will be particularly useful for the vascular biology field, as vascular cells are abundantly distributed throughout all tissues and must establish and maintain different functions to ensure tissue homeostasis. Thus, via single cell analyses, we can define tissue-specific phenotypes and functions of vascular cells, and provide needed insight into how they cross-talk with tissue-specific cells (**Figure 1**).

To date, the most abundantly used single cell analysis technique has been single cell RNA sequencing (scRNA-seq) that involves generating barcoded converted DNA from RNA of single cells, transcriptome sequencing, and raw data processing. However, emerging techniques in single cell analysis that are focused on chromatin availability and protein abundance have recently allowed for exciting experiments that will deepen our understanding of vascular cell heterogeneity beyond

FIGURE 1 | Applications of single cell analysis to understanding aspects of vascular biology.
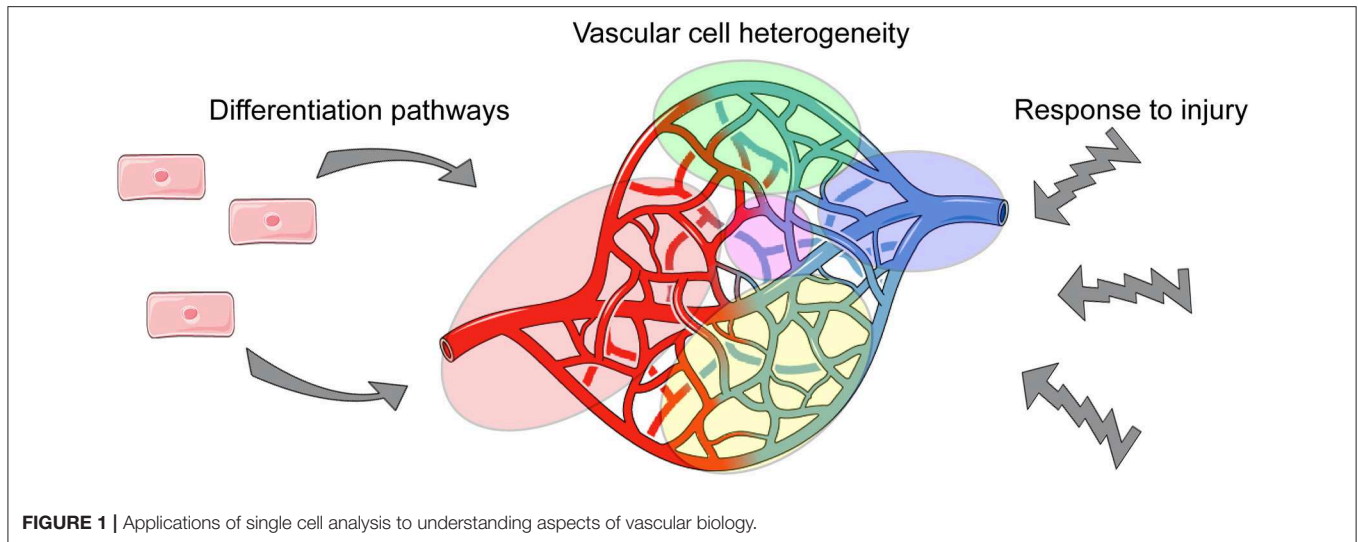
RNA expression. In this review, we provide an overview of the scRNA-seq process, discuss the unique advantages of single cell analysis that have been used in vascular biology, and highlight emerging new single cell techniques.

OVERVIEW OF SINGLE CELL RNA SEQUENCING

In the vascular biology field, single cell RNA transcriptome sequencing has become standard for understanding cellular heterogeneity and lineage progression. The first description of whole transcriptome sequencing on the single cell level was published in 2010 by Tang et al., who dissociated cells from tissues, manually selected single cells to isolate RNA and amplify cDNA to sequence the entire transcriptome of each single cell, analyzed the data using basic bioinformatics approaches, and experimentally validated the single cell data (1). Since then, technical advances have updated some aspects of this process, but the general scRNA-seq pipeline remains the same (**Figure 2**): tissue dissociation, single cell barcoding and RNA sequencing, bioinformatics analysis and experimental validation.

Single Cell Isolation
The very first step toward single cell analysis is enzymatic digestion of tissues into single cell suspensions. Isolation of healthy single cells from tissues is required to obtain high quality, reproducible data, and optimizing digestion of specific tissues is critical (2, 3). Over-digestion can induce cell death, and under-digestion can exclude subpopulations of cells from the analysis (4). Cell damage associated with enzymatic digestion can also induce changes in gene expression in specific populations of cells within tissues (5). Therefore, a digestion protocol that optimizes enzyme concentration and time of digestion is needed to isolate cell types with minimal induction of apoptosis and aberrant gene expression. Recent publications have described digestion methods for specific tissues, including heart (6), brain (7), lung (8), kidney (9, 10), and cultured cells (11, 12), which have distinct

extracellular matrix densities that require different enzymes and digestion times. Digestion cocktails can include enzymes such as trypsin, collagenase and elastase, or build on predesigned cocktails like Liberase (Sigma). Recent advances in digestion techniques also include cold-activated protease methods that better preserve RNA expression patterns compared to traditional digestion methods (2). Optimizing this process to obtain a healthy suspension of single cells from the desired tissue leads to more reliable data.

Single Cell RNA Sequencing
Delineating the transcriptome of single cells from a population requires a method for identifying sequencing reads generated from each cell. Two methods have emerged as the most-used by researchers: plate-based sequencing (Smart-seq) and droplet-based sequencing (Drop-seq). Smart-seq, as well as more recently developed Smart-seq2 (13), is a plate-based single cell isolation technique that employs flow cytometry to sort single cells into individual wells of a tissue culture plate. In each well, RNA is lysed, converted to cDNA by reverse transcriptase with barcoded primers to distinctly label reads from each cell, and then the DNA is amplified to allow for next-generation sequencing (14). Plate-based sequencing methods are commercially available through products like the Fluidigm C1 platform (Fluidigm, San Francisco, CA, USA). Additionally, variations on plate-based sequencing have been developed, such as Cell Expression by Linear Amplification and Sequencing [CEL-seq (15) and CEL-seq2 (16)], which combines the barcoded cDNA libraries and amplifies them together. The other method for scRNA-seq is droplet-based sequencing, Drop-seq. This method employs a microfluidics approach to suspend single cells within droplets containing reverse-transcriptase enzymes and barcoded primers (17). Drop-seq can be used to quickly emulsify large numbers of single cells and generate cDNA libraries, and has a commercially available pipeline through 10× Genomics machines and reagents (Chromium, 10X Genomics, Pleasanton, CA, USA). Comparing the scRNA-seq methods, plate-based single cell sequencing has
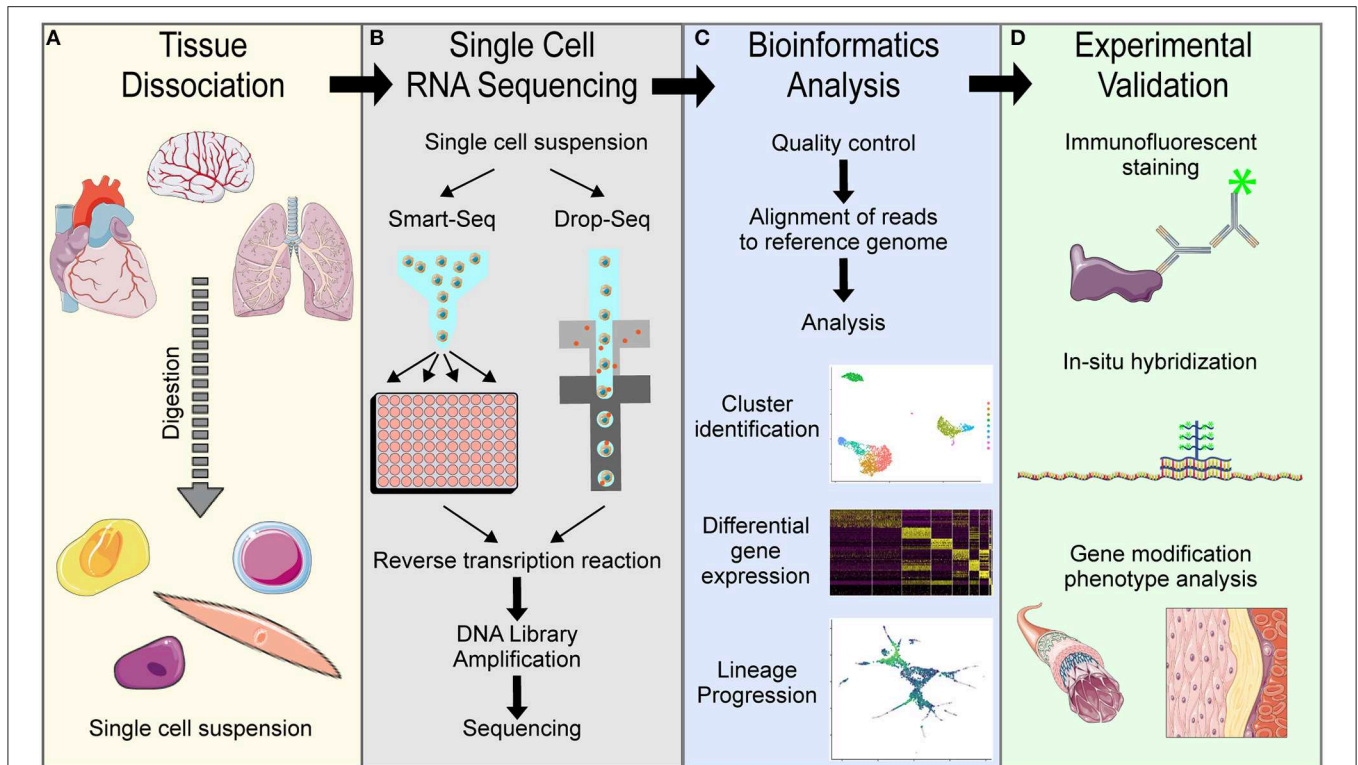
FIGURE 2 | Overview of general single cell RNA sequencing pipelines. **(A)** Tissue dissociation is achieved using digestion enzymes to generate a single cell suspension. **(B)** Single cell RNA sequencing of the single cell suspension in either Smart-Seq or Drop-Seq methods to obtain single cell transcriptomes. **(C)** Bioinformatics analysis of the raw sequencing data is needed to interpret results. **(D)** Experimental validation is necessary to confirm computational findings.

more coverage of the transcriptome and can identify more genes per cell; whereas, Drop-seq has less amplification noise and can analyze more cells per sample with a more cost-effective approach (18). Since each method has strengths and limitations, the choice of scRNA-seq method is determined by the experimental design and research goals. The sequencing efficiency and depth of genomic analysis will vary based on the reagents used and the quality/experience of the sequencing facility.

Computation Analysis of Single Cell RNA Sequencing Data

Understanding the data generated from scRNA-seq requires bioinformatics approaches. Many different computational-based methods have been generated for different types of analyses of raw scRNA-seq datasets. Before transcriptome analysis, it would be useful to determine whether the raw sequencing data are of sufficient quality with regard to the raw read output (per base content, Phred scores, GC and N content, sequence duplication, adapter content, etc.). Thus, using programs such as FastQC (Babraham Bioinformatics) could prevent loss of time and resources on extensive analyses of low-quality sequencing data. Additionally, sequence reads per cell and genomic coverage should be sufficient for each experiment, which can be checked in scRNA-seq analysis programs CellRanger (10× Genomics) and Seurat v3 (19) (explained below). Once high-quality

sequence data has been verified, the first step in analysis is to align the reads to a reference transcriptome to convert the raw sequencing data to transcript counts. This can be done with more classic bulk-RNA sequencing approaches, including Bowtie2 (20), BWA (21), and Kallisto (22). Subsequently, the data must be de-multiplexed to assign transcript reads to individual cells.

Similarities and differences among cells can be visualized and quantified using different methods depending on the desired outcomes. The Seurat v3 program can be used to group cell types into clusters and identify key genes that are significantly different among the clusters (19), and programs such as the Monocle2 can be used to identify potential lineage connections among cell types (23, 24). Other well-defined analysis programs include BackSPIN (25) and TSCAN (26). Additionally, 10× Genomics reagents allow for straight-forward conversion from sequencing data to aligned reads and generated transcript counts using their CellRanger analysis tool. Finally, it is also important to be aware of batch effects. That is, when comparing datasets from different experiments, large variations in the data may be explained by technical differences rather than biological differences, but batch effects can be computationally corrected using software programs such as Seurat v3. Other reviews have described single cell RNA sequence analysis pipelines in more detail (27).

Validation of Computational Results

The final step in single cell RNA sequence analysis is to validate results. Each step in the pipeline can add unintended bias in the data, so false positive or negative results must be ruled out through experimental validation. Validation experiments could include tissue immunostaining or RNA spatial patterning to verify expression results, or genetic modification to test functional relevance. Fluorescent *In Situ* Hybridization (FISH) employs probes to identify RNA strands in tissue slices, and this technique is the most direct method for validating RNA expression levels in tissues. FISH protocols have now been optimized to identify small populations or low-abundance RNA constructs (28), which is necessary for many scRNA-seq validation experiments. Validated FISH-based methods of RNA spatial analysis are also commercially available from Advanced Cell Diagnostics (Bio-Techne, Advanced Cell Diagnostics, Hayward, CA, USA) through their RNAscope products and reagents (29). RNAscope methods have also been applied to whole-mount tissues (30). Overall, experimental validation is critical for confirming single cell bioinformatics analysis.

Online Databases of Single Cell RNA Sequencing Results

An exciting consequence of the collective genomic efforts within many fields has been the push for publicly available sequencing datasets. The Mouse Cell Atlas is a project that aims to sequence mouse tissues from as many different sources as possible, and is publishing the findings online (31). PanglaoDB is a searchable online database of single cell datasets with over 1,000 mouse and 300 human samples integrated into an easy-to-use search tool that incorporates unbiased cluster annotations (32). More specific to vascular biology, EndoDB is an online database that includes 360 datasets from bulk and scRNA-seq covering six species (33). In addition, EC Atlas is a searchable database of endothelial cell scRNA-seq data from 12 different mouse tissues (34). As more studies are performed and datasets are made public, a more complete collection of species- and tissue-specific single cell data will be searchable and usable by all researchers.

SINGLE CELL ADVANCEMENTS IN MECHANISMS OF VASCULAR DEVELOPMENT

Vascular development requires the differentiation of endothelial cells from mesodermal progenitors, and their specification toward diverse phenotypes including arterial, capillary, venous, lymphatic, and hemogenic endothelial cells (35). These developmental pathways have multiple transition states at different times during gestation. The cell signaling mechanisms driving these specification events have been somewhat defined, but *in vivo* studies have been limited by our lack of understanding of the phenotypic transitions of endothelial cells during these specification events and the low numbers of cells that can studied in developing embryonic tissues. Development of supporting mural cells in the vasculature, including pericytes and vascular smooth muscle cells (VSMC), has also been difficult to characterize. Pericytes and VSMC derive from multiple embryonic sources and have different phenotypes in adult tissues and disease (36, 37). However, scRNA-seq allows for analysis of low-abundant populations of vascular cells that exist during the transition toward mature fates, which will lead to a better understanding of vascular development.

Primordial Endothelial Cell Characterization

Primordial endothelial cells are derived from mesodermal progenitors in the early mouse embryo at around embryonic day (E)7.5–8 (35) via a process referred to as vasculogenesis. They are then specialized to become arterial, venous and capillary endothelial cells, and primordial endothelial cells are difficult to study, *per se*, because they represent an intermediate endothelial cell state that is short-lived in the embryo and varies based on location. ScRNA-seq of mouse embryos at E8.25 revealed characteristics of primordial endothelial cells, described as branching away from mesodermal cells and containing allantois- and non-allantois-derived populations (38). Thus, delineating the transcriptome of primordial endothelial cells within developing tissues, and predicting and validating their lineage trajectory, will provide needed insights into mechanisms of vasculogenesis (39, 40). Toward this goal, stem cell culture models have also been used to recapitulate the process of endothelial cell differentiation from mesodermal progenitors and study underlying mechanisms. One such study used scRNA-seq of mouse embryonic stem cells undergoing differentiation to better characterize hemangiogenic progenitors as derived from FLK1-positive mesodermal progenitors, and identified SRC kinase as a key regulator of this transition (41). Furthermore, a recent study using scRNA-seq to analyze human pluripotent stem cell-derived hematopoietic organoids suggests that RAG1 could be a novel marker of hemogenic progenitors derived from primordial endothelial cells, as RAG1 is co-expressed with known endothelial/hematopoietic progenitor markers (CD34, VE-Cadherin and CD90) at the single cell level (42).

Arteriovenous Endothelial Cell Specification

To form a functional blood circulatory system, primordial endothelial cells must be specified into arterial and venous fates (43). Arterial and venous blood vessels have different functions within the circulatory network, and the endothelial cells that line arteries and veins reflect those differences in gene expression, structure, and response to cytokines and growth factors (44, 45). Some of the molecular mechanisms underlying the development of mature arteries and veins have been revealed [reviewed in (43)]; however, the process is still poorly understood and further complicated by the fact that arteriovenous plexi are further phenotypically specialized to perform tissue-specific functions (46, 47), as discussed below. Nonetheless, recent studies applying scRNA-seq to heterogeneous populations of endothelial cells isolated from adult tissues, such as the cortex and lung (25, 48, 49), have revealed a surprising heterogeneity, even among endothelial cells with known arterial and venous characteristics.

Thus, more work is needed to define endothelial cell subtypes throughout the body and gain insights into their differentiation pathways and molecular regulation.

Studies using human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC) can lend further insight into the process of endothelial cell specification in a controlled environment. Endothelial cell differentiation from hESC and iPSC has been described [reviewed in (50)], and iPSC-EC appear to be functionally heterogeneous (51). ScRNA-seq confirmed phenotypic differences among iPSC-EC; e.g.„ one differentiation protocol yielded four distinct endothelial subtypes (52). Differentiation protocols to promote arterial and venous specification of human stem cell-derived endothelial cells have also been established (53, 54). ScRNA-seq of such human endothelial cells suggests differentiation pathways consistent with previous *in vivo* studies including TGF/BMP signaling, Notch activation, and shear stress (43, 55, 56). Further evaluation and validation of scRNA-seq data from human stem cell-derived endothelial cells is likely to reveal new mechanisms of arteriovenous differentiation, as well.

Endothelial-to-Hematopoietic Transition

During development, another distinct type of endothelial cells, termed hemogenic endothelial cells, are also specified and give rise to the hematopoietic stem and progenitor cells that serve as the foundation of the hematopoietic system. Hemogenic endothelial cells are known to form in the extraembryonic yolk sac and placenta, as well as within the aorta-gonad-mesonephros (AGM) region of the embryo (57). The number of hemogenic endothelial cells in each of these tissues is very small [∼1–3% of endothelial cells; (35, 58, 59)], and the process of endothelial-to-hematopoietic (EHT) involves a progression through multiple intermediate cell types; thus, hemogenic endothelial cells and the EHT process are difficult to study without scRNA-seq. The same experiment that investigated primordial endothelial cells in the E8.25 embryo also found a small population of endothelial cells that had potential to generate hemogenic endothelium, which were characterized by expression of leukotriene production controlling Alox5 gene expression (38). A separate study investigated endothelial cells at E11.5 via scRNA-seq and found that the small population of hemogenic endothelial cells in the AGM exhibited enriched expression of transcription factors Elk3, Jun, and Mecom (60). Thus, the differential expression of genes in the small population of hemogenic endothelial cells compared to non-blood forming vascular endothelium will likely be better characterized using single cell analysis approaches.

Pericyte and Vascular Smooth Muscle Cell Differentiation

Mural cells, including pericytes and VSMC, are required for blood vessel formation and maturation (61). Understanding the differentiation of these cells that make up the vessel wall is therefore critical for understanding vascular development. For example, in the brain, pericytes are critically important for the development and maintenance of the blood-brain barrier (62, 63); however, pericytes, in general, have been poorly defined and lack universal markers for identification. Thus, they have

been thought to represent a heterogeneous population of cells with different functions and their differentiation pathways have not been clearly defined (64, 65). However, recent scRNA-seq of mural cells isolated from adult cortex suggest that pericytes within this tissue reside within one clearly defined population (48); thus, similar studies of pericytes derived from other tissues should provide needed insights into tissue-specific phenotypes and functions of these vascular cells.

VSMC are known to be derived from multiple embryonic lineages *in vivo* (66), which has led to difficulty in understanding their developmental pathways. To address this issue, a recent study performed scRNA-seq of the cardiac outflow tract at early-, mid-, and late-stage development. These studies revealed that although the VSMC within this tissue are derived from different sources (mesoderm and neural crest), they converge to the same phenotype [i.e., myocardial-to-VSMC and mesenchymal-to-VSMC (67)] through different developmental pathways. Further study of VSMC from many tissues is also needed to understand the diversity of their phenotypes, functions and molecular regulation. Additionally, studies using human pluripotent stem cells could provide more needed insight; e.g., one such study suggests that pericytes, stromal cells, and vascular smooth muscle cells could originate from a clonal mesenchymal progenitor cell type, referred to as mesenchymoangioblasts (PDGFRβ$^+$CD271$^+$CD73$^-$) (68). Clearly, more work is needed in multiple complementary model systems.

TISSUE-SPECIFIC DIFFERENTIATION OF VASCULAR CELLS

Vascular cell phenotypes and functions vary among tissues due to tissue-specific microenvironments and requirement for diverse vascular functions (69, 70). This need for tissue-specific function leads to tissue-specific differentiation and specialization of vascular cells, which has been highlighted by recent analyses of tissue development through scRNA-seq.

Cardiac Vascular Differentiation

One very important endothelial specification event occurs in the developing coronary vasculature where it has been shown that endothelial cells from the sinus venosus give rise to the coronary arteries. It was previously reported that sinus venosus endothelial cells proliferate in response to VEGF-C stimulation and migrate in response to shear stress-induced activation of the CXCL12-CXCR4 pathway (71–73). However, their differentiation into arterial endothelial cells was not well-described until coronary artery development was analyzed using scRNA-seq (74). This study showed a gradual specification from a venous to pre-arterial fate, and subsequent generation of coronary arteries in response to the onset of blood flow. Additionally, this study confirmed that the venous-enriched transcription factor, NR2F2 (COUP-TFII), actively inhibits Notch-induction of arterial identity (75–77). Pre-arterial cells that give rise to coronary arteries are too few in number with no previously known markers to be found in conventional analyses. This novel mechanism of venous-to-arterial phenotype transition

also provides insights needed to optimize vascular interventions that induce the same transition, including arteriovenous fistulas in hemodialysis patients (78, 79), deep vein arterialization in claudicated limb ischemia patients (80, 81) and autologous vessel grafts in coronary bypass surgery patients (82).

Neurovascular Differentiation

Endothelial cell specialization is also critically important in the brain for the generation and maintenance of the blood-brain barrier. Vascular endothelial cells in the brain contain specialized tight junctions that are more restrictive to cell and soluble factor trafficking (83, 84). The maintenance of tight junctions in brain endothelial cells is known to be dependent on Wnt signaling (85–87); however, the signaling pathways responsible for the development of endothelial barrier function has been difficult to study *in vivo*. Performing scRNA-seq of E14.5 brain endothelial cells, and comparing these data to bulk RNA sequencing of wild-type and Beta-catenin deficient brain endothelial cells, revealed that Wnt signaling is also required for the establishment of barrier function in brain endothelial cells (88). This study also identified brain endothelial-specific transcription factors Foxf2, Foxl2, Foxq1, Lef1, Ppard, Zfp551, and Zic3, and bioinformatic analysis was then used to identify subsets of brain endothelial cells with low- and high-expression of these newly discovered transcription factors. Further analyses of such cells will continue to advance our understanding of the regulation of blood-brain barrier formation and maintenance.

Lung Vascular Differentiation

Vascular cells in the lung are unique because the flow of oxygenated blood in the lung is opposite in arteries and veins relative to other tissues in the body. In addition, there is increased need for gas exchange in the lung vasculature. ScRNA-seq analysis of cells in the lung showed that the vascular cells are comprised mainly of blood vascular endothelial cells, a small population of lymphatic endothelial cells, and two sub-populations of pericytes (49). The subpopulations of pericytes could be divided into Acta2-high and Pdgfrb-high, and the distinct endothelial cell populations exhibited differences in transcriptional regulators. Lymphatic endothelial cells were enriched for Prox1, Hoxd8, and Maf; whereas, blood vascular endothelial cells were enriched for Epas1, Klf4, Gata2, Klf2, and Sox17 (49). Further study of these potential key drivers of lung endothelial cell differentiation will advance our understanding lung vascular development. Other single cell analyses of whole lung cells will also lend to our understanding of vascular diversity in this tissue. For example, a recent scRNA-seq study of whole lung cells revealed multiple endothelial cell types in this tissue, as well as endothelial cell crosstalk with, and regulation by, alveolar epithelial type I cells and mesenchymal cells that is conserved in multiple mammalian animals (89).

VASCULAR HETEROGENEITY

Large-scale tissue-specific vascular gene expression profiles have been revealed through comparative next-generation RNA sequencing studies (90). A *post-hoc* analysis of published single cell studies revealed that organ-specific endothelial cells were more variable between organs than within organs (91). However, recent scRNA-seq studies have also shown that vascular cells, even within the same tissue, are broadly heterogeneous. Vascular cells can be generally defined as arterial, capillary or venous depending on their location within a vascular plexus (44). In a recent scRNA-seq study, endothelial cells from 12 different mouse tissues were analyzed and compared to identify tissue-specific gene expression. The analysis revealed a broad diversity of endothelial-expressed genes among arterial, capillary and venous populations in distinct tissues (34). There were uniquely upregulated genes and functional pathways in endothelial cells from each tissue analyzed; e.g., membrane transporters in the brain; genes that control sugar metabolism in the liver; and interferon signaling in the kidney. Consistent with previous scRNA-seq reports, this new study also suggests that endothelial cells exist on a continuum from arterial to venous phenotypes, making it difficult to identify capillary-specific endothelial cell markers. Nonetheless, by comparing endothelial cells from different tissues, genes consistently specific to arterial, capillary, and venous endothelial cells among all tissues were identified. These scRNA-seq data are summarized, along with data from other studies highlighted in this review, in **Table 1**. ScRNA-seq can be used to further define these cell types and highlight their similarities and differences. Additionally, scRNA-seq and analysis can be used to compare sub-populations of vascular cells between tissues to identify tissue-specific phenotypes and functions. Collectively, such studies have led to a greater appreciation and understanding of different types of endothelial and mural cells and the roles that they play in tissue-specific vascular function.

Neurovascular Heterogeneity

Vascular cell heterogeneity in the brain contributes to the maintenance of critical neurovascular functions. Endothelial cells, vascular smooth muscle cells, and pericytes all provide oxygen and nutrients to neurons, and also support neurovascular stem cell niches (94, 95). Maintenance of proper blood perfusion requires control of vascular cell fate specification (96, 97). Dysregulated arteriovenous specification of brain endothelial cells results in cerebral cavernous malformations that disrupt normal blood distribution and can rupture causing hemorrhage (98–101). Decreased blood perfusion in the brain can impair neural function and cause neurovascular disorders (102), and complete blockage of blood and vascular failure results in stroke (103). In addition, vascular cells respond to ischemic injury, which promotes angiogenesis, vascular remodeling, and activation of the neurovascular stem cell niche (104–107). Therefore, maintenance of proper blood circulation via mature arteries and veins is critical for healthy brain function.

Since blood vessels within the brain are widely distributed and interconnected, previous understanding of vascular subpopulations in the brain was limited by an inability to physically dissect the vasculature. Previous studies enabled the identification of a limited number of pericyte and vascular smooth muscle cell markers (92, 93) (**Table 1**); however, recent scRNA-seq and computational analysis of brain endothelial and

TABLE 1 | Novel and previously identified markers of vascular cell identity.

	Vascular cell identity	Previously identified markers	References	Vanlandewijck et al. (48)	Kalucka et al. (34)
Endothelial cells	Arterial ECs	Ephrin B2	(45)	Bmx	Clusterin
		Connexin40		Gastrokine-3	Crip1
		Hey1			Fibulin-2
		Hey2			Mecom
		Neuropillin-1			Sat1
		Sox17			Sema3G
		Alk1			
	Capillary ECs	VegfR2		Mfsd2a	Rgcc
				Car4	Sgk1
					Sparc
	Venous ECs	Ephb4	(45)	Slc38a5	ApoE
		COUP-TFII			Biglycan
		Neuropillin-2			Ctla2a
		Endomucin			IL-6st
					Ptgs1
					Thymosin Beta 10
Mural cells	Arterial VSMCs	Calponin 1	(92)	Myf9	
		Acta2			
		Tagln			
		Myh11			
	Venous VSMCs			Kcnj8	
	Pericytes	NG2	(93)	Vitronectin	
		PdgfRβ		Higd1b	
		Des		S1pr3	
				Mcam	
				Ifitm1	
				Baiap3	
				EH3	

mural cells enabled the identification and evaluation of brain-specific arterial-, capillary-, and venous-specific gene regulation (48). This study revealed that brain endothelial cells exist on a continuum from arterial to capillary to venous phenotypes, but mural cells exhibit more distinct arterial- and venous-specific phenotypes (48). This work also revealed a difference in the phenotypic regulation of arterial vs. venous endothelial cells and vascular smooth muscle cells. Interestingly, other studies have suggested that vascular cells differ within various sections of the brain, and recent studies of mouse brain vascular cells showed endothelial-specific gene expression patterns in the cortex and hippocampus (25). Even more specialized in function is the subventricular zone of the brain, which contains a neurovascular niche that maintains neural stem cells (108, 109). The population of vascular cells within the neural stem cell niche has been difficult to study, limiting our understanding of their role in neural stem cell maintenance; however, a recent scRNA-seq study showed that endothelial cell regulation of neural stem cells in the subventricular zone is mediated via Wnt and BMP signaling pathways (110). Thus, scRNA-seq studies of the brain

vasculature have helped to identify specialized subtypes and their regulatory pathways.

Cardiac Vascular Heterogeneity

In myocardial tissue, vascular cell heterogeneity is important for maintaining cardiomyocyte function. Endothelial cells are the most abundant cell type in the heart (111), and a high blood vessel density is important to keep nutrient-rich blood feeding contracting myocytes (112, 113). ScRNA-seq of healthy murine cardiac cells identified two distinct endothelial cell clusters (114); whereas, a broader analysis of multiple cardiac scRNA-seq datasets revealed three distinct endothelial cell populations: endocardial, coronary and aortic endothelial cells (91). Interestingly, vascular smooth muscle cell and pericyte heterogeneity was found to be less complex than endothelial cell heterogeneity in the heart (114, 115), and many subsets of cardiac cells were also found to express fibroblast genes (115). Single cell analysis also revealed extensive crosstalk between cell types through paracrine signaling, with abundant crosstalk pathways evident among endothelial cells, smooth muscle cells, pericytes, fibroblasts, and macrophages (115). These studies suggest that there are novel sub-populations of vascular cells in the heart that exhibit cross-functionality that may not be observed in other tissues.

Aorta Heterogeneity

Another vascular tissue that maintains a broad range of cell types with various functions is the aorta. Aortic vascular cells must accommodate high pulsatile flow forces, and also respond to changes in blood pressure (116), flow rate (117), and lipid levels (118, 119). Thus, endothelial cells in the aorta have very specialized functions. A recent scRNA-seq study of aortic cells identified three endothelial cell clusters with distinct gene expression patterns suggesting specialization for lipoprotein handling, angiogenesis and extracellular matrix production, and lymphatic function (120). Interestingly, aortic smooth muscle cells showed surprisingly little transcriptional variation compared to endothelial cells (120). Thus, consistent with the results in the brain and heart, healthy large blood vessels appear to have more endothelial cell heterogeneity while mural cells exhibit less transcriptional variability.

Renal Vascular Heterogeneity

Vasculature in the kidneys is not only important for maintaining renal tissue function, but also for controlling blood pressure throughout the body. Renal vascular integrity is critical for maintaining blood filtration function, where blood passes through the glomeruli and nephrons to filter out metabolites and other compounds that are then excreted through urine [reviewed in (121)]. The vasculature in the kidneys also transports renal hormones that control blood pressure through the renin-angiotensin system [reviewed in (122)]. Recent scRNA-seq analysis of adult mouse renal endothelial cells revealed extensive heterogeneity; 24 different endothelial subtypes were characterized (123). This degree of endothelial cell heterogeneity is necessary for establishing and maintaining diverse types of blood vessels in the kidney that enable distinct functions,

including the renal artery and vein, as well as the fenestrated vasculature of the glomerulus, and the vasa recta in the medulla. Learning more about the unique characteristics of each of these endothelial cell subpopulations will enable a better understanding of the regulation of their distinct structures and functions. For example, this work discovered that specific medullary endothelial cell subtypes respond to water deprivation via upregulation of hypoxia response, glycolysis and oxidative phosphorylation to preserve cell viability. As proper kidney function is critical for the function of many other organs, further investigation into these renal endothelial cell types may reveal novel regulatory mechanisms that are critical for vascular functions throughout the body.

UNDERSTANDING POPULATION CHANGES IN DISEASE

Chronic and acute injury to the vascular system can induce a broad range of responses. The observed heterogeneity of vascular cells suggests that these responses may be specific to sub-types of vascular cells. Additionally, vascular cells may differentiate into disease-specific phenotypes that have new functions. Advances in scRNA-seq have allowed for the study of the differential effects of injury and disease on subpopulations of vascular cells.

Neurovascular Disease
Maintenance of vascular integrity in the brain is critical for cognitive function. Hypoxic conditions in the brain induce rapid vascular responses to increase blood flow, including angiogenesis to form new blood vessels (106). Specialized endothelial tip cells are the leading cells in an angiogenic sprout and control the rate of angiogenesis (124). These endothelial tip cells are rare, yet one single cell analysis study of the mouse brain identified a subset of neurovascular endothelial cells that activate tip cell genes during chronic tissue hypoxia (125). This finding suggests that, in a specific population of endothelial cells in the brain, hypoxia induces a tip cell phenotype to promote angiogenesis and reperfusion. It would be interesting to apply similar approaches to other ischemic-injured tissues to determine whether such endothelial cells are ubiquitously distributed throughout the body to facilitate tissue repair.

Responses to Myocardial Infarction
Another tissue in which ischemia induces angiogenesis is the heart. The cardiac vascular cells are very important for supplying nutrients to cardiomyocytes, and lack of proper blood supply leads to heart failure (126, 127). Myocardial infarction is the most dramatic form of myocardium tissue ischemia and results in a large amount of cell death and subsequent angiogenesis (128–130). Single cell analysis of hearts after myocardial infarction shows separation of arterial, capillary and venous endothelial cell clusters, and identifies strong interactions between vascular endothelial cells and fibroblasts (131). Additionally, following myocardial infarction, endothelial cells within the heart were analyzed using scRNA-seq, which identified Plasmalemma vesicle-associated protein (Plvap) as a novel gene upregulated specifically in a subset of cardiac

endothelial cells that clonally expand near the fibrotic scar (132). In subsequent validation studies, Plvap was confirmed to regulate endothelial cell proliferation, suggesting a pro-angiogenic role in this endothelial subset during neovascularization following myocardial infarction. This study also showed the upregulation of some genes involved in endothelial-to-mesenchymal transition following myocardial infarction; however, this upregulation was not accompanied by downregulation of endothelial genes leading the authors to conclude that endothelial-to-mesenchymal transition did not play an obvious role in post-infarction neovascularization. Together, these studies show that, just as in the brain, different types of endothelial cells respond differently to cardiac injury, and suggest that cardiac endothelial cell heterogeneity is important for managing the response to injury.

Arteriovenous Specification in Disease
Specification and maintenance of arterial and venous endothelial cells is critical for tissue survival and function; however, dysregulation of arteriovenous specification can occur spontaneously or can be induced. For example, an arteriovenous fistula (AVF) occurs when an artery and vein are shunted so that blood flow avoids a capillary network. AVF are usually created surgically to improve access for hemodialysis patients with end-stage renal disease (133). This causes large increases in shear flow force in the venous blood vessel, which requires remodeling of both endothelial and mural cells to strengthen the venous structure and enable arterial function (134, 135). But the surgical AVF can become stenotic and fail. Interestingly, single cell isolation and sequencing of endothelial cells from patients with healthy or stenotic arteriovenous fistulas showed four separate endothelial cell clusters, each containing cells from both healthy and stenotic vessels; however, stenotic vessels had differential regulation of SMAD4 (136). Arteriovenous specification is strongly linked to SMAD4 and TGF/BMP signaling, as disruption of this signaling pathway results in arteriovenous malformations during development (137, 138). This single cell analysis suggests that dysregulated arteriovenous specification via SMAD4 may contribute to failing AVF.

Arterial Diseases
Large arteries are critical for maintaining blood pressure and flow regulation to tissues. Injury to large arteries can include damage to the endothelial cell layer and VSMC-mediated atherosclerotic plaque formation. VSMC contribute to atherosclerotic plaque formation via proliferation and trans-differentiation (139), and interestingly, VSMC contribution to plaque formation is a clonal process, typically arising from a single VSMC in the medial layer of large vessels (140). ScRNA-seq has also revealed an arterial Sca1-positive cell population that derives from the arterial adventitia and contributes to VSMC expansion in response to arterial injury (141). Additionally, endothelial cells in the lumen of large arteries respond to injury by clonal expansion (142). These cells may be tissue-resident endothelial progenitor cells with greater regenerative properties (143), suggesting that endothelial cell heterogeneity can contribute to vessel repair after injury (144).

Vascular endothelial and smooth muscle cell heterogeneity is also apparent within arterioles in different tissues, which may also play a role in tissue response to injury. Maintenance of proper blood vessel diameter in arterioles requires signal transduction through G-protein coupled receptors (GPCR), but there are many different GPRC with varying functions and downstream signaling targets (145). Single cell analysis showed that expression of these GPCR is highly variable within endothelial and smooth muscle cells of arterioles within different tissues, but also among vascular cells of the same tissue (146). Patterns of GPCR expression unique to endothelial cells in the lungs (Glp1r, Ccbp2, Lphn3, Celsr2), skeletal muscle (Ednrb, Ptger4, Adora2a, F2r), and brain (Gpr4, Gpr124, Gpr30, Lpar4) were identified. Similarly, in VSMC, the expression of some GPCR was specific to aorta (e.g., Lphn2, Cmklr1, Lpar1) and skeletal muscle (e.g., Ednrb, Adora2, Gpr30). This observed cell type and tissue-specific variability in GPCR expression suggests that arterioles may respond to injury through different signaling pathways. Additionally, the anti-inflammatory and anti-proliferative GPCRs Ptgir and Vipr2 were found to be upregulated specifically in dedifferentiated VSMC in atherosclerotic plaques suggesting they may play a role in clonal vascular cell response to injury.

Tumor Vasculature
Vasculature in tumors is structurally and functionally different from vasculature in healthy tissues. Endothelial cells within tumor blood vessels have increased VEGF and Dll4-Notch signaling, which leads to increased proliferation and permeability, enabling leakage and metastasis (147–149). The consequences of treating tumors in mice with anti-VEGF or anti-Dll4 treatments was analyzed by scRNA-seq to find that the number of tip cells, a marker of angiogenesis, was reduced in anti-VEGF, but not anti-Dll4, treatment (150). These results suggest that initiation of angiogenesis via tip cell activation in tumor vasculature is specifically mediated through VEGF signaling. This novel understanding of tumor vascular regulation can help optimize anti-cancer treatments that target the dysregulated vasculature for drug delivery or attempt to mature the tumor vasculature to inhibit metastasis (151).

Age-Related Macular Degeneration
Occurring in 8.7% of people aged 45–85, Age-Related Macular Degeneration (AMD) is one of the most prevalent disorders that leads to blindness (152). In AMD, damage to the retina progresses gradually and results in loss of vision and eventual blindness (153). The most common treatment for AMD is anti-VEGF therapy, suggesting neovascularization may play a role in disease progression (154–156); however, the underlying cause of AMD is not well-defined. A recent scRNA-seq study evaluated human AMD tissues and found that, among all vascular cells, one specific cell type exhibited functional changes associated with AMD progression, along with glia and cone photoreceptor cells (157). Further single cell studies of the functional changes in specific vascular cell types responsible for AMD progression could lead to improved therapies that target putative driver cell types.

FUTURE APPLICATIONS OF SINGLE CELL ANALYSIS IN VASCULAR BIOLOGY

Novel single cell analysis techniques are being continually generated. Advances in sequencing and computational analyses are helping to improve scRNA-seq, but new approaches are not limited to evaluating RNA expression. Chromatin accessibility and high-dimensionality protein assays are also being optimized for single cell analysis. All of these techniques can be combined to understand cell heterogeneity on multiple molecular levels.

New Single Cell RNA Sequencing Methods
New advances in scRNA-seq techniques include the development of Quartz-Seq2, a new method that allows for 30–50% more gene reads per cell compared to established methods (158). Novel advanced computational analysis techniques are also being developed to address limitations of scRNA-seq. One limitation, the abundance of technical noise that can lead to reduced gene reads, is being addressed with a new computational technique termed Markov affinity-based graph imputation of cells (MAGIC), which uses statistical approaches to recapitulate the transcript values lost from technical errors and dropout (159). An additional limitation to scRNA-seq is the lack of spatial recognition of scRNA-seq. A novel imaging-based approach attempts to solve this limitation. The fluorescent *in situ* RNA sequencing (FISSEQ) method starts with fixed cells and tissues, converts RNA to cDNA, and uses sequencing reagents and sensitive confocal microscopy to manually read the sequence (160, 161). In this manner, the spatial aspect of transcript heterogeneity is preserved.

Chromatin Accessibility With Single Cell ATAC Sequencing
Beyond scRNA-seq, advancements in other types of single cell analyses are emerging. For example Assay for Transposase-Accessible Chromatin (ATAC) sequencing can determine regions of open chromatin by using transposase to insert specific primers to sequence open regions of DNA (162). This technique has been optimized for low cell numbers (163), and has recently been applied to single cells. Single cell ATAC sequencing has been described with a microfluidics platform (163), and a sorting-based method to isolate single cells (164). A bioinformatics approach called Destin has been designed specifically to analyze single cell ATAC sequence reads (165). More advanced techniques and standardized protocols will help the vascular biology field define cell heterogeneity on the DNA epigenetic level.

Protein Heterogeneity With CyTOF and Cell Signaling
Single cell protein analysis has also had some exciting recent advances. Traditional single cell approaches to protein analysis have used flow cytometry to define populations of cells, but the degree of variation described in flow cytometry experiments is limited by availability of excitation and emission ranges. However, a new method of flow cytometry, Mass cytometry time-of-flight (CyTOF), uses metallic-bound antibodies to read the

metal ions attached to cells through the antibody interactions, allowing for many more antibodies to be used in a single experiment (166). This method adds more variables to better describe and understand cell heterogeneity on a protein level. However, CyTOF still requires dissociation of cells. A new method called multiplexed ion beam imaging (MIBI) applies the metal ion-based approaches of CyTOF to fixed tissue slices, allowing for spatial resolution of protein heterogeneity (167). Together, these approaches will help define protein heterogeneity among vascular cell types.

DISCUSSION

Single cell analysis has revolutionized our understanding of vascular biology. Vascular cell differentiation, functional heterogeneity, and population-specific response to disease have all been characterized in greater detail than previously possible. Such studies have also suggested trends among vascular cells; e.g., endothelial cells appear to have a high degree of heterogeneity, while mural cells exhibit less phenotypic diversity. Additionally, we have gained insights into tissue-specific differentiation of vascular cells that plays a key role in establishing diverse functions including blood-brain barrier, cardiac vascular density and aortic vascular cell responses.

The process of vascular cell differentiation (or dedifferentiation) and specialization in development and disease represents a continuum of fate changes, and single cell analysis is particularly well-suited for identifying transitional phenotypes along a trajectory. Additionally, the novel hypothesis that vascular cells in an established plexus may exist in a continuum of arteriovenous identity can be more rigorously tested using advanced single cell analysis (25). Finally, single cell analysis will provide needed insight into clonal regenerative

potential of specific vascular cell subtypes in response to injury or disease (144). Thus, future studies using different types of single cell approaches will enable a more in-depth investigation of the establishment of vascular cell types and regulation of their functions. Although most current studies are focused on transcriptional analysis, gaining further understanding of the role of epigenetic and protein regulation in vascular cell differentiation, tissue-specific function, and disease response is also needed. Thus, continued single cell analysis studies of all types will lead to a better understanding of the diverse phenotypes and functions of vascular cells.

Advances in single cell analysis will ultimately improve our ability to maintain health and treat disease. Understanding cell heterogeneity and differential cell population-specific response to disease has large implications for vascular therapies. Targeted medicine approaches can take advantage of vascular cell heterogeneity to manipulate precise cell types for drug delivery or gene therapy (168–170). For example, drug delivery via homing peptides could potentially target tissue-specific vasculature (171) or tumor vasculature (172). Overall, future therapeutic strategies will need to understand vascular cell heterogeneity on multiple levels to generate targeted, specific therapies for prevalent diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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