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REVIEW ARTICLE

Single-cell RNA Sequencing: In-depth Decoding of Heart Biology and Cardiovascular Diseases

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Abstract: ***Background*:** The cardiac system is a combination of a complex structure, various cells, and versatile specified functions and sophisticated regulatory mechanisms. Moreover, cardiac diseases that encompass a wide range of endogenous conditions, remain a serious health burden worldwide. Recent genome-wide profiling techniques have taken the lead in uncovering a new realm of cell types and molecular programs driving physiological and pathological processes in various organs and diseases. In particular, the emerging technique single-cell RNA sequencing dominates a breakthrough in decoding the cell heterogeneity, phenotype transition, and developmental dynamics in cardiovascular science.

***Conclusion*:** Herein, we review recent advances in single cellular studies of cardiovascular system and summarize new insights provided by single-cell RNA sequencing in heart developmental sciences, stem-cell researches as well as normal or disease-related working mechanisms.

Keywords: Singe-cell RNA sequencing, cardiovascular disease, cardiogenesis, stem cells, vascular system, heart transcriptome.

1. INTRODUCTION

The mammalian heart is a complex organ, in which the myocardium, conduction system, conduits, and valves are collaborating to maintain systemic circulation [1]. Because of the essential role of the cardiovascular system, any dysfunctions of heart will lead to damaging outcomes. In the past few decades, we have been fighting against various common cardiovascular diseases such as coronary heart disease, arrhythmia, cardiomyopathies, heart failure, aortic dissection, and valve diseases. Even though diagnosis and therapy of these diseases have been significantly improved along with the cumulative anatomical, cellular and genomic knowledge and techniques [2-7], limitations of understanding endogenous cellular heterogeneity and gene interplay within the development of normal hearts and relevant diseases hinder us in deciphering pathogenesis and introducing better therapeutic strategies.

Since single-cell RNA sequencing (scRNA-seq) technique has been applied to several organs/systems [8-10], we can conduct the research through individual cell-based resolution, decipher integrated cell-map for organs to gain insights into understanding the cellular heterogeneity of diseases and organism biology. Moreover, scRNA-seq enables us to profile the whole transcriptomic landscape of various cell groups, identify previously unknown cell subtypes, elucidate the phenotypic transitions, unveil different cell fates, as well as confirm or expand previous knowledge of various organs and different systems [11-25].

Recently, the flourishing scRNA-seq has also shed light on the area of cardiovascular research. Apart from promoting the discovery of novel cell types, this technique enables the interpretation of endogenous principles in cellular gene patterns, the detections of potential cell interactions and the trajectories of cell fate [26]. In both animal-based researches and human histological studies, scRNA-seq has been revolutionizing the definition of cardiogenesis, normal heart composition, stem-cell differentiation, vasculature development, cardiovascular diseases, and associated immune landscape. Herein, we summarized the advances of scRNA-seq platforms and underlined the latest scRNA-seq studies in the area of cardiovascular research. We also highlighted the potential significance for a better understanding of heart biology and pathology, as well as looked into the prospect of scRNA-seq in further cardiovascular researches.

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2. PLATFORMS AND ADVANCED PROTOCOLS OF scRNA-SEQ IN HEART

Recent studies have shown that the single-cell sequencing techniques and platforms have been optimized significantly, which allows it to be used on multiple organs and tissues. Main techniques that have been developed worldwide include Fludigm C1, iCell8 Single-Cell system, Illumina Bio-Rad Single-Cell Sequencing Solution, Drop-seq, InDrop, 10X Genomics Chromium system, Microwell-seq, and SMART-seq2, *etc*. and each technique has its own advantages [27-32]. Specifically, in terms of the major used platforms, Smart-seq2 could detect more genes per cell. Due to its high efficiency, it is appropriate to be used on discovering isoform or allelic expression [33]. For example, through a downstream single-cell cDNA library constructed by manual SMART-seq2 in 396 cells, Nomura, *et al*., has identified different transcriptomic features and related regulators or signaling pathways that encode morphological and functional phenotype between normal and failing hearts [34]. However, as SMART-seq2 restricts massively parallel sequencing, the reaction chamber-based technologies such as Fludigm C1, iCell8 Single-Cell system show their priority. It has been proven that these platforms exhibited higher sensitivity and lower dropout frequency [35]. In addition, it is reported that the combination of an optimized digestion protocol and iCell8 Single-Cell system had a relatively high throughput (>20,000 cells), according to a subsequent study [36]. Alternatively, Drop-seq, InDrop, 10X Genomics Chromium are economical since they have high throughput while the lowest experimental cost per cell, despite the lower sensitivity. Moreover, since they are suitable to detect cell subpopulation, droplet-based scRNA-seq platforms are becoming more popular to profile the organ atlas and characterize adult heart non-myocyte populations in recent studies. However, due to the difficulties in the dissociation of live cardiomyocytes (CMs) and their large and irregular shape, the applications of scRNA-seq in heart tissue have been delayed compared with that in other organs. In earlier studies of heart-related scRNA-seq, experts focused on the embryonic hearts, in which the CMs were accessible for isolation, and their size was small for single-cell selection [37, 38]. More efforts have been taken to optimize the dissociation protocols and single-cell platforms to sequence adult CMs at a single cellular level recently. Due to the high frequency of failure based on simple enzymatic dissociation to ventricular tissue, some researchers resort to fluorescence-activated cell sorting (FACS). In the study of Gladka *et al.*, they used a simple enzymatic dissociation protocol followed by a tailored FACS to select various single CMs from adult ventricular tissue [39]. Nevertheless, CMs in this study were fragmented and mitochondrial fractions were high (80%). In addition, as previously reported, almost no viable CMs could be identified in other studies by using the same dissociation protocol of myocardium, which indicates the intolerance of adult CMs for FACS [40]. Therefore, traditional enzymatic dissociation and FACS may not be considered as the optimized choice. To obtain vigorous CMs from the adult human heart, other protocols were also developed. Recently, a slice dissociation protocol based on Ca^{2+} free perfusion and enzymatic digestion have isolated CMs with intact morphology and dynamic metabolism [41]. A combination of Langendorff perfusion and chunk-based dissociation methods was also proven to isolate viable and intact adult mammalian myocytes [34]. More importantly, the single nuclear RNA-seq (snRNA-seq) technique, which used to be widely applied in the brain and kidney [42-45], now becomes another popular solution in decoding mammalian heart. It is more convenient to use cardiac nuclei to do sample preparation because they are less fragile than their cellular counterparts, and they can be isolated even from frozen banked tissue. Studies have also confirmed a high consistency of snRNA-seq and scRNA-seq in the characterization of cell identity. Moreover, snRNA-seq has exhibited substantial advantages, including reduced dissociation bias and elimination of dissociation-induced transcriptional stress responses [44, 45]. Several studies have reported the whole population of the postnatal and adult mammalian heart using high throughput snRNA-seq by Drop-seq or 10X Genomics platforms, convincing a promising application of this strategy [46-50]. Optimized protocols for snRNA-seq and head-to-head comparison for cardiac snRNA-seq and scRNA-seq require subsequent studies in the future. In summary, considering the previous experience and reported studies, the high throughput snRNA-seq *via* the 10X Genomics platform might be more efficient and stable in studying transcriptome signatures of whole cells population of the myocardium, while the 10X Genomics or Drop-seq based scRNA-seq are more valuable for non-myocytes or immune cells analysis. The SMART-seq2 is still widely applied for deep sequencing of a small number of cells.

3. scRNA-SEQ IN DECIPHERING NORMAL HEART COMPOSITION

scRNA-seq studies in the mammalian adult hearts have been updating our knowledge in the intricate cellular milieu of the heart [51] (Table **1**). Different from the traditional histology and bulk transcriptome, CMs are not restricted to a single group. Instead, they show gradient gene expression and heterogeneous function. According to Gladka *et al.*, though sharing well-known CMs markers, the variation of enriched gene expression pattern in normal murine CMs have differentiated them into 4 clusters named CM-cluster 1, 3, 4, 9. CM-cluster 1 showed enriched makers of *Casq2* and *Atp2a2*, CM-cluster 3 displayed higher expression of *Ttn* and *Ryr2*, CM-cluster 4 was characterized by *Myl2* and *Myoz2*, while CM-cluster 9 had distinctly enriched expression of *Myom1* and A*ctc1*. Notably, the anti-hypertrophy gene, *Myoz2*, was limited to only the CM-cluster 4, indicating that the cluster 4 might be more resistant to hypertrophy. Although they belong to a specific cell type, the study broke the previous stereotype and provides the possibility that various subpopulations of CMs might have different responses to disease [39]. In addition to defining new clusters of CMs, scRNA-seq helps figure out the heterogeneity of non-cardiomyocytes (NCMs), which also play an important role in the functional regulation of the adult heart. Skelly *et al.* employed scRNA-seq to analyze 10,519 cells within mouse heart and characterized 12 distinct subpopulations of NCMs, comprising fibroblasts, smooth muscle cells, endothelial cells (ECs), pericytes, natural killer (NK) cells, T cells, group 2 innate lymphoid cells (ILC2), Schwann cells, macrophages, dendritic cells (DC)-like cells, B cells, and

Table 1. Single-cell RNA sequencing analyses in decoding normal heart architecture.

References	Species	Tissue	Topic	Technological Platform	Number of Cells/Nuclei	Major Findings
DeLaughter, D.M., *et al.* (2016). [37]	mouse	primordial heart tube and mature heart	mouse cardiogenesis	Fluidigm C1	1,200 cells	Dynamic spatiotemporal gene expression directs CMs maturation during development.
Li, G., *et al.* (2016). [38]	mouse	embryonic heart	embryonic cardiac cells	Fluidigm C1	2,233 cells	Providing anatomical patterns of gene expression among single embryonic cardiac cells.
Linscheid, N., *et al.* (2019). [49]	mouse	adult sinus node	sinus node and pacemaking	10x Genomics	5,357 nuclei	Membrane clock proteins but not calcium clock proteins are responsible for the pacemaking abilities of the sinus node.
Tucker, N.R., *et al.* (2020). [50]	human	adult heart	normal human heart	10x Genomics	287,269 nuclei	Mapping a transcriptional and cellular landscape in the normal human heart.
Cui, Y., *et al.* (2019). [59]	human	embryonic heart	human cardiogenesis	STRT-seq	4,948 cells	Revealing transcriptomic landscapes of human fetal heart development.
Li, G., *et al.* (2019). [58]	mouse	embryonic heart	mouse cardiogenesis	10x Genomics Fluidigm C1	>10,000 cells	Explaining cell cycle and cell crosstalk in spatial-specific proliferative differences during heart development.
Asp, M., *et al.* (2019). [60]	human	embryonic heart	human cardiogenesis	10x Genomics	3,717 cells	ScRNA-seq and spatial transcriptomic approaches construct gene landscape and 3D organ cell atlas.
Xiao, Y., *et al.* (2018). [61]	mouse	embryonic heart	fibroblast development	Drop-seq	18,166 cells	*Hippo* kinases *Lats1/2* promoted epicardial-fibroblast transition during cardiac fibroblast development.
Hulin, A., *et al.* (2019). [62]	mouse	postnatal valve	heart valve development	DropSeq	2,840 cells	Maturation of heart valve cell populations during postnatal remodeling.
Sereti, K.I., *et al.* (2018). [63]	mouse	embryonic heart	CMs clonal expansion during cardiogenesis	Fluidigm C1	122 cells	A "progenitor-like" subpopulation of CMs may have the potential for limited proliferation during late embryonic development and shortly after birth.
Xiong, H., *et al.* (2019). [64]	mouse	embryonic heart	intraorgan crosstalk during cardiogenesis	Smart-seq2	1,230 cells	Chemotaxis mediates intraorgan crosstalk during cardiogenesis.
Lescroart, F., *et al.* (2018). [65]	mouse	embryonic heart	cardiovascular lineage segregation	Smart-seq2	892 cells	Identifying distinct populations of *Mesp1*$^{+}$ CPCs and molecular features associated with early lineage restriction and regional segregation.
Sahara, M., *et al.* (2019). [66]	human	hiPSCs and fetal heart	human cardiogenesis from hiPSCs	Smart-seq2	842 cells	*LGR5*$^{+}$ ventricular progenitors promote human-specific cono-ventriculogenesis
Jia, G., *et al.* (2018). [67]	mouse	embryonic heart	cardiac progenitor cells transition	Fluidigm C1	498 cells	Providing a model of transcriptional and epigenetic regulations during cardiac progenitor fate decisions.
de Soysa, T.Y., *et al.* (2019). [68]	mouse	embryonic heart	cardiac progenitor cells transition	10x Genomics	73,926 cells	Transcriptional factor, *Hand2* specifies fate and differentiation in individual cardiac progenitor cells
Goodyer, W.R., *et al.* (2019). [69]	mouse	embryonic heart	conduction system development	10x Genomics	22,000 cells	Providing a comprehensive gene landscape of the cardiac conduction system in developing hearts.
Skelly, D.A., *et al.* (2018). [52]	mouse	adult heart	normal mammalian cardiac cellulose	10x Genomics	10,519 cells	Comprehensively profiling the gene landscape of NCMs and showed the complex and diverse intercellular communication.

(Table 1) contd….

References	Species	Tissue	Topic	Technological Platform	Number of Cells/Nuclei	Major Findings
Vidal, R., *et al.* (2019). [54]	mouse	adult heart	aging heart	10x Genomics	27,808 nuclei	Molecular changes of cardiac fibroblasts may contribute to declined heart function in the aging heart.
Su, T., *et al.* (2018). [79]	mouse	embryos vessel	formation of coronary arteries	Smart-seq2	843 cells	Delineating progenitor cells in sinus venosus -to-coronary transition.
Liu, X., *et al.* (2019). [80]	mouse	embryonic outflow tract	development of cardiac outflow tract	10x Genomics	55,611 cells	Identifying convergent development of VSMCs during cardiac outflow tract development.
Kalluri, A.S., *et al.* (2019). [81]	mouse	adult aorta	normal mouse aorta	10x Genomics	>10, 000 cells	Delineating an atlas of all cells in the murine aorta and the heterogeneity of ECs.
Lukowski, S.W., *et al.* (2019). [82]	mouse	adult aorta	transcriptional profiling of aortic endothelium	10x Genomics	7,671 cells	Suggesting the heterogeneity of the aortic endothelium and hierarchy between progenitor and differentiated cells.
Dobnikar, L., *et al.* (2018). [83]	mouse	adult aorta	VSMCs in healthy mouse vessel	Fludigm C1 Smart-seq2 10xGenomics	>3,000 cells	Exploring disease-relevant transcriptional signatures in VSMCs lineage cells in healthy blood vessels.
Friedman, C.E., *et al.* (2018). [92]	human	hiPSCs	hiPSC-CMs	10x Genomics	43,168 cells	Heterogeneity of hiPSC-CMs, and the key role of HOPX in CMs maturation and hypertrophy.
Churko, J.M., *et al.* (2018). [93]	human	hiPSCs	hiPSC-CMs	10x Genomics	10,367 cells	Differentiation-associated heterogeneity and hierarchy exist in hiPSC-CMs.
McCracken, I.R., *et al.* (2019). [94]	human	hiPSCs	hiPSC-ECs	10x Genomics	56,998 cells	Identifying the major directions of early and later hiPSC-ECs differentiation and indicating their difference from isolated ECs.
Paik, D.T., *et al.* (2018). [95]	human	hiPSCs	hiPSC-ECs	10x Genomics	5,673 cells	Uncovering identities of hiPSC-ECs subpopulations and their biological function.

granulocytes. Besides, some major cell populations can also be further separated due to distinct gene features. For example, the ILC2s expressed T cells-like genes like *Gata3* and *Areg*, and a unique population exhibiting signatures of both macrophages and fibroblasts were also observed. It should be noted that, as such existence of multi-faceted subsets in different NCMs, using several traditional markers to generalize the characteristics and function of particular cell types appears to be incomplete and might lead to a loss of information. And the biological functions of these novel subsets deserve further exploration. More importantly, as a critical factor, gender-related cell transcriptomic characteristics have also been proposed by scRNA-seq. It was found that despite similar cluster patterns in male and female NCMs, genes showed sexual dimorphism within cell types. Particularly, male-upregulated genes were inclined to respond to foreign antigens in macrophages, while female-upregulated genes were associated with response to stress in the same cell type, revealing that sexual transcriptomic dimorphism might govern the organ function [52]. The study gives us a dynamic landscape of NCMs and evidence that normal heart function can also be influenced by gender. Apart from animal-based studies, the latest publication offers us a blueprint of healthy human adult hearts in single-cell resolution [50]. By performing snRNA-seq with a total of 287,269 single cardiac nuclei, 9 major cell types were separated. In addition to the CMs and aforementioned NCMs, neuronal cells that might participate in the intrinsic cardiac autonomic network and epicardial adipocytes with enriched immune marker CD96 were also identified. The robust study marks a new achievement in understanding the human heart. Firstly, neuronal cells were detected throughout all four chambers besides CMs, which confirmed the universality of an intrinsic cardiac autonomic network within the heart. Furthermore, apart from the chamber-specific CMs, the chamber-specificity was more striking among NCMs, which equipped exquisite NCMs transcriptome with specialized spatial features. Strikingly, the gene expression in most cell types was different across the chambers, with the right atrial cells displaying the most distinct gene transcriptomic features. For example, the right atrial macrophages foster another subcluster, which was different from those of other chambers. Analogously, the side distinction in ECs was also driven by the right atrium, raising interest in deciphering the role of these specificities in atrial disease. Last but not least, the study has reshaped our view about cardiovascular diseases. By integrating the

database of the healthy human heart and genome-wide association studies (GWAS), the particular disease was likely to be attributed to specific cell types. For example, more than a quarter of the cardiomyopathies and arrhythmia associated genes were enriched in the CMs population. Remarkable myocardial infarction (MI) linked gene enrichment was found in pericytes, while low-density lipoprotein (LDL) cholesterol-related gene enriched in the adipocyte. These results indicate that cardiovascular diseases might partly be cell type-dominated and the targeting cells might be an alternative strategy for disease management. Besides, scRNA-seq also filled the blanks of the complex cardiac conduction system. Previously, like a pacemaker for the heart, the sinus node remained mysterious due to the obscure molecular underpinnings of its specialized function. More importantly, since both of the membrane clock and Ca^{2+} clock could regulate heart automatic electrical activity, which one of them dominates the pacemaking is a trending topic nowadays [53]. Again, the scRNA-seq analysis has recently provided detailed insights into the unique molecular composition of sinus nodes from adult mice. By combining quantitative proteomics and single-nucleus transcriptomics analysis, Linscheid *et al.* added new evidence to the long-lasting dispute about the mechanism of pacemaking. Through proteomic analysis, despite the abundance of Ca^{2+} clock proteins in the sinus node, their expression was similar across the whole atrium. By contrast, the membrane clock held a significant difference between the sinus node and atrial muscle. Moreover, the scRNA-seq further re-affirmed the expression of the particular genes in specific cell type and it confirmed that it is membrane clock proteins rather than the Ca^{2+} clock proteins predominantly expressed in the sinus node myocytes, which indicated that the membrane clock contributed to pacemaking. In addition, scRNA-seq unveiled the complexity of the cell types in the mouse sinus node. The result demonstrated that macrophages, ECs, endocardial cells, epithelial and epicardial cells were also located in the sinus node besides sinus node myocytes and fibroblasts, which laid a foundation of exploring physiological or pathological functions of more cell types and their molecular changes in sinus node [47].

In addition to younger hearts, scRNA-seq helps to explain the complex and profound changes in healthy aging hearts. Aging hearts hold a cell-specific background to facilitate the presence of cardiovascular diseases. For instance, in terms of fibroblasts, compared with 12-week-old murine hearts, 18-month-old murine heats exhibited a significant change in the expression pattern of fibroblasts. The snRNA-seq analysis demonstrated that aged fibroblasts from hearts exerted antiangiogenic effects on ECs *via* secreting increased paracrine cytokines like Serpine1 and Serpine2. In addition, an enlarged subpopulation of aged fibroblasts expressed proinflammatory genes and osteogenic genes, paving the way for calcification and inflammation in the aging heart [54].

In a nutshell, beyond the classical major cell types composition in normal hearts, decoding cell subtypes and specified gene expression patterns within the healthy adult heart by scRNA-seq has provided a profound interpretation of heart biology. Unfamiliar cell types like immune cells and neuron cells were also confirmed in the heart, and some major cell types can be further divided into function-specific subpopulations, which yields the intrinsic potential for maintaining normal function or participating in heart pathologies. Moreover, spatial and sex-specific distinctions in cells arouse new topics in future studies. Further studies in biological functions of novel cell types and cellular distinct transcriptomic mechanisms will help us understand the cell-targeted therapies better and it is reasonable to believe that the precise cell-target therapies can soon be approached.

4. scRNA-SEQ IN TRACING CARDIOGENESIS

Studies before the application of scRNA-seq identified the dynamic changes in RNA expression during the development of a mature heart [55, 56]. Even more complicated, cell development neither strictly obeyed the constructed rules nor irreversibly followed certain branches [57]. However, due to the limited resolution and inability of decoding multiple cell populations within the heart, intrinsic mechanisms related to cellular differentiation and heart maturation remain unclear. With the utilization of scRNA-seq, spatial and temporal cellular characteristics, signal determined phenotype transition and cardiac progenitor cell differentiation underlying normal cardiac development have become increasingly clear. Modern studies have mainly focused on spatial and temporal single-cell genomic features and proved that cardiac cells within different anatomical distribution and developing stages were diverse. DeLaughter *et al.* performed scRNA-seq of >1,200 cells within primordial and mature murine hearts, identified markers of cell-lineage and depicted temporal and chamber-specific developmental programs of CMs. It was found that ventricles transcriptomic difference mainly existed in CMs instead of ECs or fibroblasts. The right ventricle (RV) CMs featuring genes related to dilated CMs and arrhythmogenic RV CMs, while left ventricle-derived CMs were enriched with genes implicated in congenital heart disease. Data from the study also provided a repertoire of genes during cell maturing and evidenced the immaturity of stem cell-derived CMs and ECs, thus detailing the limitation of stem cell-based therapeutics [37]. In the same year, Li *et al.* described some zone-specific transcriptome markers of CMs based on scRNA-seq of 2,233 single cardiac cells in the murine embryonic heart and developed a new bioinformatics platform to identify region-specific features of single CMs, which might also assist the assessing the anatomical identities of *in vitro* differentiated CMs [38]. Furthermore, Li's research group mapped the specific locations of more than 10,000 mouse embryonic cells in given ventricular chambers and illuminated the proliferative difference of CMs between distinct regions [58]. The research provided that CMs in the G2/M phase displayed downregulated sarcomeric and cytoskeletal markers, revealing the cell cycle phase might induce chamber specific transcriptomic difference. Moreover, by ligand-receptor analysis, it is suggested that endocardium-secreted Tgfβ1 and epicardium-derived Rspo1 may have the potential to regulate the proliferation of different types of myocardium. The developmental track of human fetal hearts (from 5 weeks to 25 weeks) have also been deciphered at single-cell resolution. In accordance with murine fetal CMs, the gene expression in CMs also displayed distinctions in various chambers by as early as 5 weeks in the fetal period. Additionally, CMs experienced dynamic gene variations regulated by NOTCH and BMP

pathways during functional maturing stages. However, the development of specific cell types in human fetal hearts is not synchronized with that in mouse hearts. Some genes, such as *SALL4* and *CITED1* in CMs, which were responsible for disease in mouse fetal hearts did not act as influencers any more in human fetal hearts. This reminded us to consider the molecular characteristics of specific cell types when using mouse models to imitate human heart disease [59]. The latest study tends to focus on constructing 3D organ-wide atlas of human heart development at single-cell resolution *via* spatial transcriptomic approaches. Such integration of both cellular information and spatial context help detail the functions, networks, and interactions of various cell types throughout organ and disease development [60]. Moreover, in terms of cell differentiation during development, single scRNA-seq has made a remarkable contribution. According to Xiao *et al.*, scRNA-seq in *Lats1/2* conditional knockout murine embryonic day 13.5 (E13.5) and embryonic day 14.5 (E14.5) embryotic cardiac tissues revealed that *Lats1/2* mutant epicardial cells were stagnated in an intermediate stage between epicardial cells and fibroblast. Remarkably, the intermediate stage cells feature a higher expression of *Yap* targets *Dhrs3* and *Dpp4* instead of activated fibroblast differentiation, confirming the essential role of *Hippo* signaling in cell state transition during heart development [61]. These profound studies offer new insights into decoding heart development and enrich our perception of the diverse cardiac cells.

Apart from the myocardium, scRNA-seq was also used to explore postnatal valve maturing. Hulin *et al.* evaluated heterogeneous cells during postnatal extracellular matrix (ECM) remodeling and leaflet morphogenesis. The findings confirmed that from early stage to maturity, valve interstitial cell (VIC) populations displayed alteration in functions. In fact, the postnatal day7-VICs showed higher synthetic activity with prevalent ECM gene expression while postnatal day30-VICs became more quiescent as valve maturation and stratification proceeds [62]. The study concluded that VICs underwent constant gene expression and cellular functional changes throughout postnatal development, which encourages us to further investigate the mechanism of valve dysfunction brought by VICs differentiation.

The characteristics, differentiation regulators, molecular features and fate determinants of cardiac progenitor cells (CPCs) make up another essential part of cardiogenesis. Hence, scRNA-seq is also adapted to explore CPCs during heart development. By applying scRNA-seq in mouse embryonic hearts, It is interesting to note that the "progenitor-like" population was found abundantly in early embryonic stages and minimized postnatally. Moreover, cardiac injury activated CMs proliferation was observed in neonatal mice but not in adult mice, which implied the existence of remnant proliferation potential in late embryonic or neonatal hearts [63]. ScRNA-seq analysis in *Nkx2-5* and *Isl1* lineages at E7.75, E8.25, E8.75, and 9.25 also discovered that chemotaxis-mediated intra-organ crosstalk oriented CPCs positioning and promoted cardiogenesis [64]. ScRNA-seq also strengthens the previous definitions, for its application in verifying the conventional recognition that the development of the heart of mouse originated from *Mesp1*-expressing CPCs by profiling early murine embryotic CPCs. On top of that, not only did the study show molecular heterogeneity of *Mesp1*-expressing CPCs but also identify specific *Mesp1*$^{+}$ subpopulations associated with early lineage restriction during mouse gastrulation temporally and spatially [65]. Furthermore, scRNA-seq on human embryonic stem cell-derived cardiac cells from specific fetal compartments identified a unique human ventricular progenitor with positive expression of *LGR5*. It proved that the *LGR5*$^{+}$ population exclusively lied in the proximal outflow tract of human embryonic hearts and promoted human-specific cono-ventriculogenesis [66]. Based on the newly discovered subpopulations, we have access to drive chamber-specific CMs for dealing with particular developmental dysfunction. Additionally, single-cell transcriptomic is also a reliable strategy to determine the cellular mechanisms of CPC's fate choices during development. For example, Jia *et al.* have demonstrated that CPC's fate transitions were linked to distinct open chromatin states critically based on *Isl1* and *Nkx2-5* through scRNA-seq in mouse CPCs from E7.5 to E9.5 [67]. Similarly, by using scRNA-seq to investigate murine CPCs, De Soysa *et al.* identified *Hand2* as a transcriptional determinant that decided the fate and differentiated directions of individual cardiac progenitor cells [68]. By regulating these fate determinants, we might have access to interfere in the initiation of congenital heart defects. Besides, scRNA-seq provides a comprehensive landscape for molecular and cellular identification of unique cell types within the developmental conduction system. By combining microdissection and scRNA-seq in E16.5 mouse hearts, Goodyer *et al.* uncovered a series of novel genes and confirmed the existence of transitional sub-groups in the conduction system. For example, *Smoc2* was a unique novel marker with specific expression in the sinoatrial node (SAN), while *Cpne5* was found significantly expressed in the atrioventricular node (AVN) region. Besides, beyond confirming the previously observed transitional cells between the SAN and surrounding atrial myocardium, they also provided the molecular and cellular identity of the rare cell type and indicated that *Lgfbp5*, *Cpne5, Rgs6*, and *Ntm* were novel markers enriched in both the compact SAN and transitional subpopulation. This study laid a foundation for further exploration of the molecular mechanism in the conduction system and related diseases [69]. Furthermore, through a detailed atlas of cardiogenesis, we have opportunities to compare and explore disorders throughout human heart development. Suryawanshi *et al.* studied healthy and autoimmune-associated congenital heart block (CHB) fetal hearts by applying scRNA-seq. Apart from deciphering the cell type landscape, it was noted that all CHB cell types, especially macrophages and monocytes, showed overexpressed interferon (IFN)-stimulated genes (ISGs) as well as upregulated "interferon signaling" process. Also, the enrichment of matrixsome transcripts in CHB stromal cells indicates that stromal cells may be the potential contributor to extracellular matrix deposition and fibrosis in CHB [70]. In summary, the application of scRNA-seq not only delineates a new landscape of heart development in an upgraded view but also provides novel genes and mechanisms for the subsequent endeavor in elucidating the congenital heart diseases.

5. scRNA-SEQ IN ELABORATING VASCULAR SYSTEM DEVELOPMENT

It is well known that the heart contains vastly heterogeneous microvessels and arteries [71]. Since vascular dysfunction initiates coronary artery disease [72], hypertension [73], cardiomyopathy [74] and congenital heart disease [75], it is essential to acquire a comprehensive knowledge of the cardiac vascular system. However, based on previous bulk sequencing, we knew a little about the cell fate change during angiogenesis and outflow track formation [76, 77]. Modern scRNA-seq helps overcome these limitations [78], which promotes an updated understanding of vascular generation.

Given that murine coronary arteries may originate from sinus venosus (SV), Su *et al.* performed scRNA-seq on murine embryotic heart vessels, which confirmed that SV cells would differentiate into pre-artery cells. The conclusion showed that expression of artery-specific genes and significantly downregulated venous genes before blood flow. After blocking the pre-artery specific genes, artery formation was inhibited. Furthermore, the transition from SV endothelium to pre-artery cells was found to be a gradually changing process, in which venous genes experienced declining expression, while arterial genes were gradually upregulated [79]. These findings reveal that a pre-artery cell stage is determinate for artery formation and the process will be governed by a series of genes. Thus, these findings provide the possibility of regulating the artery development *in vitro*.

Additionally, another study deciphered the analogous gradual transition of cells during the formation of the outflow tract. Leveraging on scRNA-seq, a high incidence of outflow tract malformations was re-explained based on complex interaction and transitions among various cell populations. According to scRNA-seq data that contained 55,611 mouse outflow tract cells from three outflow tract formation stages, it has been demonstrated that vascular smooth muscle cells (VSMCs) lineage can originate from either CMs or mesenchymal cells. Additionally, pseudo-time analysis and network analysis showed a gradual loss of myocardial markers and gain of VSMCs markers during the progression of myocardial-to-VSMCs trans-differentiation, while the loss of mesenchymal marker expression and gain of VSMCs marker during the process of mesenchymal-to-VSMCs transition was observed. The latter process was accompanied by the upregulation of the NOTCH signal pathway characterized by *Heyl* and *Tbx20* as the top upregulated and downregulated regulators, respectively [80]. Above all, scRNA-seq helps decode cell fates during vascular formation and uncover key regulators that govern the cellular transition, which paves the way for future researches in conventional heart vascular malformations. Extraordinary scRNA-seq studies also mark profound advances in understanding physiological vascular complexity. Different from conventional stereotypes within vascular biology, scRNA-seq allows a comprehensive interpretation of cellular heterogeneity and special subtypes associated with vascular diseases. For example, Kalluri *et al.* employed scRNA-seq in over 10,000 mouse aortic cells and identified 3 major cell types, including ECs, VSMCs, and fibroblasts. Through in-depth analysis, ECs were re-clustered into three subgroups with distinct functions including lymphocyte-like function, lipoprotein handing, angiogenesis, and extracellular matrix production. Interestingly, after exposure to high-fat diets, these subpopulations shared similar genetic change, with upregulated contractile markers such as *Myl9, Tagln*, and *Acta2,* which confirmed the endothelial-mesenchymal transition during atherosclerosis (AS) might be initiated by increased contractile transcripts. Furthermore, in terms of the pathogenesis of specific arthropathies, the three major cell types showed different contributions. It was found that VSMCs and fibroblasts might mediate contraction or extracellular matrix metabolism-related defect in arthropathies, whereas aorta dysfunction arising from alterations in transforming growth factor-β signaling could be attributed to all of the three types [81]. Also, a combination of scRNA-seq and bulk sequencing data helps to reaffirm the hierarchy of ECs in normal adult mouse aortas, confirmed the existence of two major transitioning subpopulations of ECs. In particular, one cluster showed the specific vascular signature of differentiated cells with significantly higher expression of *Fabp4* and *Jchain*, while the other displayed mesenchymal phenotype in endovascular progenitors with markers such as *Ccl11* and *Pdgfrα*. The study proved dynamic alterations in rest of the adult aortas and provides new angles to understand the molecular mechanism underlying the progenitor- to differentiated-cell transition in the adult aorta [82]. Besides, it is necessary to note that VSMCs in healthy murine vessels show heterogeneity as well. According to Dobnikar *et al.*, scRNA-seq profiled single VSMCs transcriptomic characteristics and detected their region-specific developmental history. Similar to the aforementioned study, vascular inflammation, adhesion, and migration-related genes were identified in subgroups of VSMCs. Moreover, a rare VSMCs population with a phenotype switching hallmark marker *Sca1* was identified in both healthy vessels and atherosclerotic lesions and upregulated *Sca1* represented the response of VSMCs when confronted with stimuli such as culture conditions and vascular injury. Such advanced analysis extends our knowledge of VSMCs' function, paving the way for cell population-targeting clinical therapeutics in vascular disease [83]. Altogether, scRNA-seq redefines the mammalian vascular system and gives us an intriguing landscape of previous familiar vascular cells. However, it is only the beginning. By more comprehensive investigations in the particular stage or novel population of the vascular cells, we might address the pathological or therapeutic mechanisms of vessel development.

6. scRNA-SEQ IN APPROACHING STEM CELL RESEARCHES

CMs and ECs derived from human induced pluripotent stem cells (hiPSCs) have been extensively studied in the area of cardiovascular research as they have the potential of imitating the genetic features of cardiovascular disease, testing drug safety and replacing the damaged cardiac tissue [84-88]. Though stem cells appear to be direct and efficient therapeutic tools, as evidenced by previous studies, the pluripotent stem cell-derived cardiac cells are different from mature *in vivo* counterparts because they often fail to integrate within the host cardiac network [89-91]. Therefore, Friedman *et al.* applied scRNA-seq analysis to investigate the gene regu-

lation and fate choices that result in the incomplete transcriptomic activation of hiPSC-CMs [92]. Impressively, they found the widely distributed HOPX, a key development regulator of CMs, only expressed in a small fraction of *in vitro*-derived CMs. Afterward, by applying gain and loss of function models, it was proved that HOPX could be activated by hypertrophic stimulation through activation of its distal transcriptomic start site. Also, they suggested stimulating the activation of HOPX might be a tool for promoting the maturity of *in vitro* hiPSC-CMs. Furthermore, to accurately direct the hiPSCs differentiation, beyond regulators of cell maturity, heterogeneity among hiPSC-derived cells also draws concentration. Another study clarified the gene signatures of distinct CMs derived from hiPSCs through combining analysis of scRNA-seq and bulk RNA-seq. Interestingly, hiPSC-CMs showed atrial like gene expressions in the early stage and ventricular gene expressions afterward. Moreover, transcription factor *NR2F2* promoted an earlier atrial-like gene signature while *HEY2* promoted a more mature ventricular-like gene expression feature in hiPSC-CMs [93]. Based on these findings, regulating these cell may improve the efficiency of generating specific CMs subtypes, which can be increasingly promising in the future.

Apart from CMs, stem cell-derived ECs also make great contributions to vascular disease, hence researchers also unraveled the transcriptional signatures of endothelial-directed differentiation, which indicated that hiPSC-ECs would be first differentiated into non-specified endothelial and mesenchymal populations, then undergo further maturing. The study also proved that different inducing protocols will result in diverse heterogeneity. For example, by using the "five-factor protocol", more heterogeneity during differentiation was observed and unwanted non-ECs were also induced. In addition, compared with freshly isolated human ECs, these induced ECs lacking organ-specific phenotypes were suggesting that future studies should also focus on driving the organ-specific ECs specification in a lineage or organ-specific nature of these cells in order to apply them in clinical therapy [94]. Furthermore, due to the inefficiency of current ECs differentiation protocol, Paik *et al.* also performed scRNA-seq to further study the characteristic of hiPSC-ECs as well as limitations of the protocol. Notably, in terms of pure hiPSC-ECs, 4 heterogeneous subgroups were defined by the enriched expression of *CLDN5*, *APLNR*, *GJA5*, and *ESM1* respectively, and each of the subsets displayed distinct biological functions. *CLDN5*-expressing ECs showed activated metabolic activity while *APLNR*-specific ECs equipped with an enriched inflammation response. *GJA5*-positive ECs were atrial like while *ESM1*-expressing ECs were functional in angiogenesis. The study also revealed that apart from hiPSC-ECs, a large number of non-ECs including CMs and other cell types of mesodermal lineage can also be generated, which prevents the use of these cells in decoding mechanisms of specific vascular diseases [95].

The scRNA-seq based studies explain the difference between hiPSC-ECs/CMs and *in vivo* mature cells, which defines enriched genes and molecular signaling pathways that will be used as cues in future studies. Thus, it boosts further downstream studies in promoting the functional specificity of these cells.

7. scRNA-SEQ IN UNRAVELING CARDIOVASCULAR DISEASES

With the increasing concentration on pathogenesis and novel therapeutic targets of heart disease, comes the prosperity of scRNA-seq in disease exploration. The new technique scRNA-seq has been revolutionizing our knowledge of common heart diseases such as AS, MI, heart failure, cardiomyopathy and valve diseases [96-99] (Table **2**).

AS was recognized as a chronic inflammatory process, in which several cells underwent profound alteration. According to the *in vitro* study and gene knockout mouse models, VSMCs appear to experience the "phenotype modulation" in response to stimuli during the progress of AS, which forms the fibrous cap and the underlying necrotic core [100]. Meanwhile, macrophages are also recruited to the intima and take up oxidized LDL, which leads to the formation of foam cells and accelerates atherosclerotic progression [34]. However, with the complicated environment of *in vivo* situation, investigating the real contribution of these cell phenotypic plasticity was difficult. Gratifyingly, scRNA-seq has advanced the previously established multi-factor-involving and diverse cell-interacting atherosclerotic process. In particular, a study conducted by Wirka *et al.* provided new insights into VSMCs phenotype modulation [101]. It was found that, instead of traditionally expected macrophage phenotype, VSMCs in atherosclerotic lesions would transform into unique "fibromyocytes" under the regulation of *Tcf21*. After confirming the "fibromyocyte" population in both mouse and human, they have also evidenced the association between higher expression of *TCF21* and lower risk of coronary artery disease in human tissues, which demonstrated a protective role of the "fibromyocyte" in AS. Furthermore, loss of *Tcf21* results in fewer fibromyocytes at the protective fibrous cap, which suggested the protective role of the gene might be achieved by promoting the infiltration of fibromyocytes into the lesion and fibrous cap. As *in vivo* VSMCs phenotype modulation was such a different story, our traditional prospective was updated by the striking results. According to the protective role of the novel "fibromyocytes" and the regulator *TCF21*, further research in regulating such VSMCs phenotype might provide an effective approach for preventing AS. Besides, the scRNA-seq analysis also renewed the conventional understanding of the core status of macrophages by showing a comprehensive transcriptional landscape, which elucidated heterogeneity and depicted their interplay with other cell types. According to the research conducted by Kim *et al.*, it is impressive to find newly recruited macrophages rather than foamy macrophages that were pro-inflammatory and it was these recruited macrophages that contributed to the progress of the AS. The authors performed scRNA-seq and bulk sequencing in both the $CD45^{+}$ leukocytes from the murine atherosclerotic aorta and the isolated live foam cells [102]. In fact, foamy macrophages displayed significantly reduced expression of inflammatory genes and upregulated genes related to cholesterol uptake, processing, and efflux. By contrast, intimal nonfoamy macrophages showed elevated levels of genes encoding chemotactic cytokines and exacerbation of inflammation. Again, the scRNA-based study challenges the typical ideas, which promote an in-depth understanding of innate immune response in the lipid-loaded atherosclerotic lesion. Moreover,

Table 2. Single-cell RNA sequencing analyses in decoding cardiovascular diseases.

References	Species	Tissue	Disease Paradigm	Technological Platform	Number of Cells/Nuclei	Major Findings
Gladka, M.M., *et al.* (2018). [39]	mouse	adult heart	MI	SORT-seq	932 cells	Cytoskeleton associated protein 4 was a new modulator of fibroblasts activation after ischemic injury.
Nomura, S., *et al.* (2018). [34]	Mouse/ human	adult heart	heart failure	Smart-seq2	396 cells	CMs identity is encoded in transcriptional programs that orchestrate morphological and functional phenotype.
Wang, L., *et al.* (2020). [36]	human	adult heart	heart failure	iCell8 system	21,422 cells	Cellular composition and interaction networks in normal failed and recovering human heart.
Hu, P., *et al.* (2018). [46]	mouse	postnatal hearts	pediatric mitochondrial cardiomyopathy	sNucDrop-seq	20,000 nuclei	Uncovering profound cell type-specific modifications of pediatric mitochondrial cardiomyopathy.
See, K., *et al.* (2017). [47]	Human/ mouse	adult heart	heart failure	unclear	359 nuclei	LincRNA regulates de-differentiation and cell cycle stress-response in the CMs
Zhang, Y., *et al.* (2019). [48]	mouse	adult heart	MI	10x Genomics	31,542 nuclei	Dedifferentiation may be an important prerequisite for CMs proliferation after MI.
Suryawanshi, H., *et al.* (2019). [70]	human	fetal hearts	CHB	10x Genomics	>17,000 cells	Increased interferon responses contribute to autoimmune-associated CHB fetal hearts.
Winkels, H., *et al.* (2018). [105]	mouse	adult aorta	AS	10x Genomics	3,541 cells	Diverse immune cells participate in regulating AS.
Gu, W., *et al.* (2019). [104]	mouse	adult aorta adventitia	AS	10x Genomics	5,424 cells	Profiling dynamic interplay between macrophage and mesenchyme in the vascular adventitia.
Cochain, C., *et al.* (2018). [103]	mouse	adult aorta	AS	Drop-seq	1,226 cells	Decoding the heterogeneity of macrophages and discovering a novel type of macrophage.
Wirka, R.C., *et al.* (2019). [101]	mouse human	adult aorta coronary artery	AS	10x Genomics	unclear	Revealing the protective roles of VSMCs phenotypic modulation and the role of *TCF21* in AS.
Kim, K., *et al.* (2018). [102]	mouse	adult aorta	AS	10x Genomics	3,781 cells	Nonfoamy rather than foamy macrophages are proinflammatory in atherosclerotic lesions.
Ruiz-Villalba, A., *et al.* (2019). [106]	mouse	adult heart	MI	10x Genomics	29,176 cells	*Cthrc1*$^{+}$ cardiac fibroblasts regulate ventricular remodeling after MI.
Li, Z., *et al.* (2019). [107]	mouse	adult heart	MI	10x Genomics	>7,000 cells	*Plvap*, the endothelial-specific marker, regulated human ECs proliferation, and angiogenesis.
Bajpai, G., *et al.* (2019). [108]	mouse	adult heart	MI	10x Genomics	17,931 cells	Tissue Ccr2^{+} cardiac macrophage orchestrates monocyte recruitment after MI.
Calcagno, D.M., *et al.* (2020). [109]	mouse	adult heart	MI	In Drop 10X Genomics	120,000 cells	Type I interferon responses to ischemic injury begin in the bone marrow of mice and could be regulated by Ccr2^{-} cardiac resident macrophages.
Kretzschmar, *et al.* (2018). [111]	mouse	infant and adult heart	MI	CEL-Seq2	1,939 cells	Cycling CMs were only robustly observed in the early postnatal growth phase and no cardiac stem cells were observed in infant or adult heart.
Martini, E., *et al.* (2019). [114]	mouse	adult heart	heart failure	10x Genomics	17,853 cells	Various immune cell types and specific immune molecules participate in pressure overload-driven heart failure.

(Table 2) contd….

References	Species	Tissue	Disease Paradigm	Technological Platform	Number of Cells/Nuclei	Major Findings
Li, J., *et al.* (2019). [113]	mouse	neonatal heart	MI	10x Genomics	unclear	Tregs regulate neonatal heart regeneration by potentiating CMs proliferation in a paracrine manner.
Vafadarnejad, E., *et al.* (2019). [110]	mouse	adult heart	MI	10x Genomics	1,405 cells	Uncovering dynamics of cardiac neutrophil diversity in murine MI.
Wünnemann, F., *et al.* (2020). [115]	mouse	embryo hearts	valvular heart disease	Drop-seq	55,152 cells	The loss of *Adamts19* leads to progressive non-syndromic heart valve disease.

novel types and interactions of macrophages were also discovered by scRNA-seq. Cochain *et al.* applied scRNA-seq to aortic $CD45^+$ cells from the non-diseased and atherosclerotic aorta of $Ldlr^{-/-}$ mice, they then detected three macrophage populations at different time points of lesion formation. Among which, the resident macrophages existed in both the healthy and diseased aortas, while the inflammatory population and the newly defined "*Trem2*" macrophages were exclusively detected in atherosclerotic aortas [103]. They further indicated that the *Trem2* macrophage held a highly specialized function in lipid handling and catabolic processes. Their similar gene signature to osteoclasts was also shown to indicate their potential role in atherosclerotic lesion calcification. Such observations enriched our knowledge about the heterogeneity of macrophage, which can inspire further exploration and target particular functional macrophage populations in AS.

Furthermore, Gu *et al.* [104] depicted the cell atlas of the heterogeneous cell populations within vascular adventitia among wild type and ApoE deficient mice, which identified enhanced activation of resident macrophages, stem /progenitor cells like resident mesenchyme cells and pro-inflammatory mesenchyme subpopulation (Mesen II). They then elaborated closely, the interaction between a resident mesenchyme cluster and inflammatory macrophages. It has been proven that the resident macrophage cluster showed an enriched pathway about cell-cell adhesion and leukocyte migration besides the inflammatory macrophage cluster, which suggested its potential role in priming adventitia inflammation. Furthermore, intercellular communications suggested enrichment of chemotaxis, cytokine, and inflammatory response between the macrophage (particularly inflammatory macrophages) and Mesen II cells (particularly inflammatory macrophages), implying the cell communication in vascular adventitia played a crucial role in the early formation of AS [104]. Another comprehensive study based on $Apoe^{-/-}$ and $Ldlr^{-/-}$ mouse models was conducted by Winkels H. *et al.*, which highlights the role of B cells and T cells in AS [105]. Specifically, loss of tissue-resident memory T cells would add risk to ischemic events while targeting a subset of B cells with enhanced TNF-α pathway could help to reduce the plaque lesions. Therefore, apart from concentrating on *in vitro* experiment and mono cell type, scRNA-seq studies represent a useful resource for future functional work, which reveals that targeting novel cell types and cell interplay should also be considered in the future studies.

Because scRNA analysis identified genomic characteristics and special functions of disease-specific cell subpopulations, as well as tracked the cell fate transition during the initiation, progression and recovery stage of MI, advancements in understanding the mechanisms driving MI have also been provided. scRNA-seq in MI studies mainly focus on decoding the biological properties of cardiac non-myocyte subpopulations including fibroblasts, ECs, and immune cells. After the myocardial injury, fibroblasts will be activated to orchestrate a fibrotic response that leads to the generation of a collagen scar to prevent cardiac rupture. Hence, the identification of activated fibroblasts would improve our understanding of their roles in cardiac homeostasis. By employing scRNA-seq, Villalba *et al.* described a fibroblast subpopulation with high expression of *Cthcr1* localized in the infarcted zone of the murine heart [106], which proved that *Cthcr1* regulated fibrotic response and suggested that cardiac *Cthcr1* might be a biomarker of cardiac dysfunction after MI. Akin to this study, Gladka *et al.* also unveiled *Ckap4* as a novel marker inactivated fibroblasts emerging after MI. However, according to a loss of function experiment, unlike *Cthcr1*, *Ckap4* itself was shown to dampen the expression of genes that related to activated fibroblasts. These specifically activated fibroblasts deserve further investigation in human *in vivo* experiments so that we will get hang of the pathogenesis and mechanisms in MI better [39].

Neovascularization after MI is also a critical process underlying structural repair in the injured heart. To figure out the underpinning mechanisms, Li *et al.* coupled scRNA-seq with an EC-specific multispectral lineage-tracing mouse. They observed that resident cardiac ECs rather than bone marrow cells contribute to angiogenesis after MI, and they further proved that *Plvap*, the endothelial-specific marker, regulated human ECs proliferation and angiogenesis after MI [107]. The study provides a promising therapeutic target for MI induced heart injury through regulating vascular perfusion and generation.

In terms of heart remodeling after MI, innate immune response served as an important regulator. Of note, monocyte recruitment in injured tissue is regarded as predominately a maladaptive response associated with adverse outcomes. Also, the regulation of monocyte recruitment is a pivotal mechanism for post-MI immune response. Using scRNA-seq, Bajpai *et al.* revealed that $Ccr2^+$ resident cardiac macrophages acted as critical upstream mediators of monocyte recruitment, fate specification and the inflammatory response to myocardial injury, which resulted in adverse left

ventricle remodeling after MI. As shown in the heart transplantation model, loss of tissue-resident $Ccr2^{+}$ macrophages substantially reduced the recruitment of recipient monocytes, monocyte-macrophages, as well as lower expression of inflammatory chemokines and cytokines. But the depletion of tissue-resident $Ccr2^{-}$ macrophages induced a remarkable increase in monocyte and macrophage recruitment. The study established the mechanistic basis and new avenues for modulating inflammation in MI [108]. Another interesting theory indicates that prior to infiltrating the infarcted heart, monocytes could be activated by the immune pathway remotely. Based on the strength of scRNA-seq in discriminating ISG within myeloid, the work of Calcagno *et al.* demonstrated that part of monocytes in peripheral blood induced by the type I IFN responses, which was initiated in the bone marrow after MI, can be negatively regulated by $Ccr2^{-}$ resident macrophages in a *Nrf2*-dependent manner. Similar to Bajpai's results, they also revealed the protective roles of $Ccr2^{-}$ resident macrophages by providing a relevant mechanism [109]. Besides, the study also started a new chapter in decoding the heterogeneity of other important immune cells, neutrophils, which are the first responders to tissue injuries during MI. Surprisingly, apart from monocyte-derived macrophages, neutrophils also expressed ISGs before entering the infarcted heart, and such ISGs expressing did not interfere with their intracardiac differentiation. In addition, they discovered a unique subset of neutrophils that expressed the eosinophil marker *Siglecf* during the late phase after MI, which was confirmed in another study [110]. The study of Vafadarnejad *et al.* further decoded the characteristics and function of *Siglecf* expressing neutrophils. The study revealed that $Siglecf^{high}$ neutrophils accounted for half (51.01%) of the total neutrophils at day 5 after MI, and the *Siglecf* gene was acquired in ischemic tissues rather than bone marrow, blood or spleen, which proved that this subtype was paralleled by features of aging and activation. However, comprehensive descriptions about the function of these immune responses and biology of the newly detected cells are still limited, further investigations are required for clinical purposes. Through scRNA-seq, recent studies also tried to test the proliferative cardiac cells after heart damage through a single cell view. According to Kretzschmar *et al.*, though no cardiac stem cell population was observed in a mature heart, the early postnatal CMs do preserve cycling features, which means that some CMs in the heart hold the potential for proliferation [111] and Zhang *et Al.* provided a deeper explanation about it by emphasizing the role of dedifferentiation as a prerequisite [48]. Based on snRNA-seq, they found a significant increase in dedifferentiated and cycling CMs in post-infarct hearts. These adult CMs showed downregulated gene networks for cardiac hypertrophy, contractile, and electrical function, while upregulated signaling pathways and gene sets for active cell cycle, proliferation, and cell survival. The study provided activated new signaling networks underlying CMs dedifferentiation, including focal adhesion, integrin/ECM receptor interaction, Rap1 signaling, and actin cytoskeleton regulation. Moreover, despite the classical *S100a6* and *Tmsb10*, *Knl1*, *Kif11*, and *Cdk14* were also upregulated in dedifferentiation-like CMs. Thus, this detailed knowledge of the specific regulators of both dedifferentiation and cell cycle reactivation promoted the understanding and advanced exploration of the CMs proliferation. Finally, it is necessary to note that regulatory T cells (Tregs) play an essential role in repairing the injury after ischemic [112], and a recent study identified its contribution to CMs regeneration after MI in a paracrine manner [113]. By performing single-cell RNA-sequencing of Tregs in neonatal hearts, Li *et al.* identified the role of Tregs as a source of regenerative factors after MI, as they secreted factors such as Ccl24, Gas6 or Areg, which potentiate neonatal CMs proliferation. Interestingly, they also found the difference of Tregs between regenerating and non-regenerating neonatal heart lies in the absolute number of the Tregs instead of the transcriptomic features, which indicates that raising the amount of Tregs could also be a promising research project for dealing with the injury after MI.

Remarkably, in models of heart failure attributed to pressure overload, coronary heart disease or diastolic cardiomyopathy scRNA-seq offers insights into the cellular background of cardiac homeostasis, laying a foundation for therapeutic strategies in disease. Nomura *et al.* performed scRNA-seq in murine and human CMs to elucidate specific CMs gene programs deciding morphological and functional signatures from the early stage of cardiac hypertrophy to failure. They clarified that cell size and Erk1/2 and Nrf1/2 linked transcriptional networks are activated in CMs during the early stage of cardiac hypertrophy. After long-time pressure overloading, the CMs will develop into adaptive and failing CMs with a P53-dependent mitochondrial inhibition, morphological elongation, and heart failure gene program activation, which offers molecular mechanism and therapeutic targets for cardiac hypertrophy and heart failure [34]. SnRNA-seq analysis in both mouse and human failing adult hearts provided information of cell cycle activators and inhibitors about sub-populations of stress-response CMs and predicted long intergenic non-coding RNAs (lincRNA) for regulating the change, which deeply explained why targeting key lincRNA may be a promising choice for assisting cardiac repair [47]. From an immunological perspective, induction of pressure overload leads to immune activation involving far more cell types than previously recognized, including macrophages, B cells, T cells, and Treg, dendritic cells, NK cells, neutrophils, and mast cells. The upregulation of key subset-specific molecules, such as OSM in pro-inflammatory macrophages and PD-1 in Tregs, helped to explain the possibility of using anti-TNF therapy and cardiac toxicity during anti-PD-1 cancer immunotherapy to relieve heart failure [114]. Beyond the murine models, scRNA-seq in profiling transcriptomes of failed human hearts caused by diastolic cardiomyopathy and coronary heart disease highlight the essential role of NCMs in heart homeostasis and disorders, which implies that targeting heart muscle contraction and metabolism is a proper therapeutic choice. In particular, a type of $ACKR1^{+}$ ECs acted as the hub in cell crosstalk and secreted ligands implicated in maintaining heart contraction, which suggested that the ECs subtype has a protective function in the adult human heart. Indeed, *in vitro* experiments also proved that novel ECs enhance cardiac function through increasing vessel density, which reduced cardiac fibrosis. Moreover, despite the severity of heart failure, patients received left ventricular assist device treatment exhibited restored heart function-inclined transcriptomic features of CMs, ECs, and fibroblasts, indicating cardiac cells in the

adult human heart still have plasticity and substantial recovery potential [36]. The study offers insights into the cellular foundation of cardiac homeostasis and disease and provides regulating NCMs that could help to treat heart failure.

Additionally, heart valve disease also has shown a high frequency among the average population. Due to the non-syndromic clinical features, the early diagnosis was difficult, which resulted in delayed interference until the occurrence of severe hemodynamic alteration. To date, only a handful of genes have been identified as causes of disease. Nevertheless, through scRNA-seq, we have access to investigate the potential gene contributor and elaborate the confusing molecular pathogenesis of valvular diseases. By combining patient genome information and scRNA-seq data in the *Adamts19* knockout murine models, a new regulation, *Adamts19*, in non-syndromic heart valve disease was clarified. Compelling evidence revealed that *Adamts19* not only serve as a novel marker of VICs but also loss of *Adamts19* induced valve disease in mice by prompting hemodynamic perturbation in a *Klf* dependent fashion, which suggested that a balanced *Wnt–Adamts19–Klf2* axis is necessary for maintaining proper valve maturation and maintenance. The study shed light on further incentive to examine the therapeutic role of *Adamts 19* in valvular disease [115]. The pathogenesis of pediatric mitochondrial cardiomyopathy can also be explained from a single-cell resolution. SnRNA-seq analysis of both healthy and pediatric mitochondrial cardiomyopathy mouse models found distinct changes in cell-type-specific transcriptional remodeling. Overall, CMs, fibroblasts, and ECs play a dominant role in metabolic and functional changes during the disease model, which displayed the down-regulation of the mitochondrial Oxphos pathway and increased expression of ribosome related genes. Moreover, increased expression of fibrosis-associated extracellular matrix genes was observed in both the fibroblast and nonfibroblast-cells, which suggested that tissue fibrosis might involve a multi- participation of diverse cell types. Such observations provided a detailed cell alteration in the fibrosis of mitochondrial cardiomyopathy and indicated that further studies should also focus on multi-faceted pathways and cell types beyond fibroblast itself [46].

Collectively, scRNA-seq is a promising tool for disease investigation, by which we can identify specialized cell types, define the disease inducing cell response in detail, and develop potential therapeutic targets for heart diseases.

8. PROSPECTS IN SINGLE-CELL SEQUENCING WITHIN THE CARDIOVASCULAR SYSTEM

The previously reported single-cell research was mostly based on animal models such as mice, while the studies on the healthy or diseased heart from human or non-human primates are still inadequate, especially for the adult heart due to inaccessibility of human specimens. Moreover, the comparison of cross-species is still necessary to illuminate heart constitutions. The objective of the forthcoming research is to achieve greater mRNA capture rates and sensitivity. The rationale for the improvement includes identifying rare cell populations such as progenitor cells, immune like cells, and discriminate cell subtypes such as immune cells. Another intrinsic issue is to characterize the heterogeneity and capacity for regeneration of CMs in adult hearts, which depends on the improvement in sensitivity and throughput of scRNA-seq/snRNA-seq techniques. Therefore, to optimize the dissociation of viable cardiac myocytes and non-myocytes from the myocardium, particularly from healthy and diseased human hearts, is still necessary for scRNA-seq studies. Alternatively, the extraction of cardiac nuclei, protection of nuclear RNA and the optimized selection and sequencing platform are promising approaches to improve heart cell atlas studies.

Emerging multiplexed sequencing methods are extending the cell biology study of the cardiovascular systems such as spatial transcriptome sequencing [60] and single-cell transposase-accessible chromatin profiling (ATAC-seq) [67, 116]. The spatial transcriptome analysis provides a possibility to map cell distribution and spatial organization in the heart at a near single-cell resolution [60]. This is a powerful approach to study cardiac development and various disease models, such as MI. The scATAC-seq is an assay for transposase-accessible chromatin, which offers a power of resolution and generates epigenetic information about gene regulatory processes of diverse cell types in a composited organ.

CONCLUSION

With the development of sequencing platforms and protocols, scRNA-seq marks a new era in cardiac research. It helps us to understand the interactive gene regulation and specialized cell type functionalities, providing new insights into heart architecture and cardiac diseases. In subsequent decades, the optimization of scRNA-seq and combination of other emerging techniques is expected to offer a detailed landscape and promising therapeutic targets for various cardiac diseases.

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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