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STATE OF THE ART REVIEW
Genetics

Single-cell technologies to decipher cardiovascular diseases

Wesley Tyler Abplanalp [1,2,3], Nathan Tucker [4,5], and Stefanie Dimmeler [1,2,3]*

[1]Institute for Cardiovascular Regeneration, Centre of Molecular Medicine, Goethe University Frankfurt, Theodor Stern Kai 7, 60590 Frankfurt, Germany; [2]German Center for Cardiovascular Research DZHK, Partner site Frankfurt Rhine-Main, Berlin, Germany; [3]Cardiopulmonary Institute, Goethe University Frankfurt, Frankfurt, Germany; [4]Masonic Medical Research Institute, Utica, NY, USA; and [5]Cardiovascular Disease Initiative, The Broad Institute of MIT and Harvard, Boston, MA, USA

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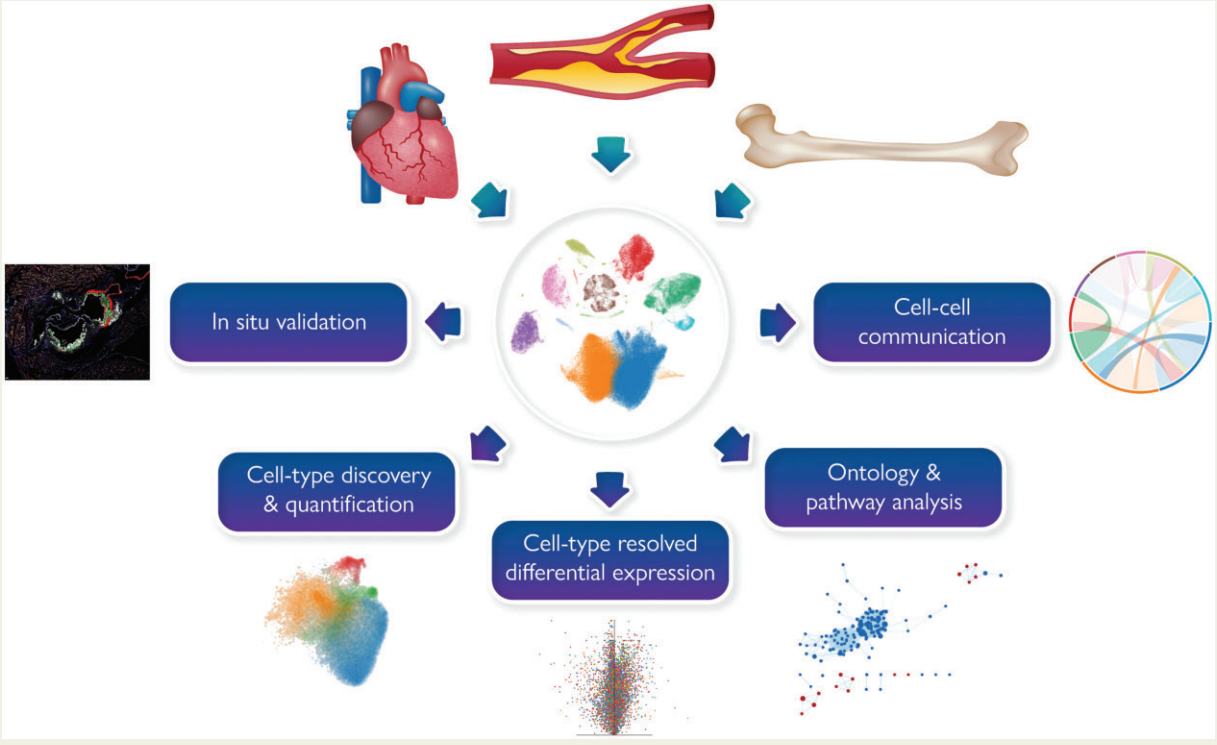
Graphical Abstract



Single-cell analysis of tissues studied in cardiovascular disease. Opportunities for in-depth analysis of tissue heterogeneity, transcriptional responses, and cell communication are enabled with scRNA or snRNA sequencing.

* Corresponding author. Tel: +49 69 6301 5158, Fax: +49 69 6301 83462, Email: dimmeler@em.uni-frankfurt.de

Abstract

Cardiovascular disease remains the leading cause of death worldwide. A deeper understanding of the multicellular composition and molecular processes may help to identify novel therapeutic strategies. Single-cell technologies such as single-cell or single-nuclei RNA sequencing provide expression profiles of individual cells and allow for dissection of heterogeneity in tissue during health and disease. This review will summarize (i) how these novel technologies have become critical for delineating mechanistic drivers of cardiovascular disease, particularly, in humans and (ii) how they might serve as diagnostic tools for risk stratification or individualized therapy. The review will further discuss technical pitfalls and provide an overview of publicly available human and mouse data sets that can be used as a resource for research.

Keywords Single-cell sequencing • Single-nuclei sequencing • Diagnostic • Hypertrophy • Heart failure • Atherosclerosis

Introduction

Despite progress in pharmacological and interventional treatments, cardiovascular disease is still the primary cause of death globally. To gain insights into disease mechanisms, researchers focused on the cell types most associated with the physiological function of the organ system, such as cardiomyocytes in the heart and endothelial and smooth muscle cells (SMCs) in the vessels. In the last decades, it became evident that the interplay with mural cells, and the various subtypes of inflammatory cells play crucial roles in the pathogenesis of cardiovascular disease and the view of cell types as homogeneous entities were challenged. In fact, heterogeneity within cell populations drives a remarkable network of cellular cross-talk that maintains tissue integrity and function. During disease, some cells acquire an overt dysfunctional state, impairing cellular function and contributing to altered cellular cross-talk.

Scientists experienced a moon landing sense of awe when the first single-cell RNA-seq studies emerged as high-throughput, standardized, accessible techniques. Today, the technology has become more widespread as it serves as the lens through which we now expect to regularly receive high-dimensional data. The interconnectedness of such technology with content is described by Marshall McLuhan, who famously stated, 'The medium is the message'. Solutions for how to utilize such high-dimensional data are rapidly evolving, and patterns in basic cardiovascular biology and pathology are surfacing. In this review, we will focus on the insights this technological leap has provided, while envisioning a path forward.

Single-cell technologies

Altering the transcriptional output of the genome is a fundamental mechanism through which cells adapt to differentiation clues and stressors. Our ability to assess changes in the transcriptome advanced rapidly over the past decades, with critical impacts on basic discovery and translational science. The improvement of the reference human genome and gene annotations, combined with short-read sequencing, enabled genome-wide transcriptome assessment in the late 2000s. However, a major challenge remained: the resultant RNA output was a combination of cell composition and cell state. For example, when a decrease in the abundance of a myosin family member is observed in cardiac hypertrophy, is this a sign of reduced cardiomyocyte number? Or instead a change in the transcriptome of cardiomyocytes in response to the pro-hypertrophic environment? A combination of both? Single-cell RNA sequencing (scRNA-seq) and other single-cell technologies offer a solution to such types of questions (*Graphical Abstract*). Also, addressing transcriptomes of single cells allows dissection of the multicellular composition of the myocardial tissue, where cardiomyocytes only comprise from 25 to 45% of the cells by number.[1,2] While cardiology has long been aware of supporting cells in the heart and their roles in cardiac function, identifying and quantifying their full diversity have remained a challenge until recently.

Overview of single-cell sequencing technology platform utility

Conceptually, all single-cell sequencing approaches are similar in that cells are isolated and molecularly barcoded prior to sequencing (*Figure 1*). After sequencing, the barcode allows for each transcript to be assigned to its cell of origin, whereby transcriptome similarity between individual cells allows them to be grouped by type.

The first approaches followed the format of bulk RNA-seq, where each cell is processed independently prior to sequencing. Library generation protocols improved,[3] integrating template switching oligos for greater library construction efficiency and complexity. Molecular advances were integrated with throughput of automated single-cell capture platforms, whereby thousands of cells can be compared with each other. Then, in the late 2010s, microfluidics enabled massive increases in scale and decreases in per-cell cost, making possible the achievements discussed hereafter.

For the reader's benefit, direct comparisons of single-cell sequencing platforms are shown below. However, for further details and full bioinformatic pipeline discussions, we refer the reader to well-written reviews covering the technical aspects of this topic.[4,5]

Droplet sequencing (as in 10x Genomics workflows) utilizes isolated single cells or nuclei (for large cells or complex tissue) which are moved by a fluidic pump through ~40 μm microfluidic devices wherein they are individually partitioned with beads loaded with capture oligonucleotides containing poly dT (for the capture of mRNA), cell barcodes, and unique molecular identifiers (UMIs, identifying unique transcripts) (*Figure 1*). After lysis of cells within an emulsion, reverse transcription is initiated, which links transcripts to their cell of origin after sequencing. Then, the emulsions are broken, and typically the product is amplified, fragmented, indexed for multiplexed Illumina sequencing runs. One advantage of droplet sequencing is the high number of cells per experiment (5000–10 000 cells are typical). This might be critical if looking for rare/new cells or cell subpopulations within a given cell class, as these cells might not be highly abundant in a given tissue. Also, there is a wide availability of standardized kits for droplet sequencing, which

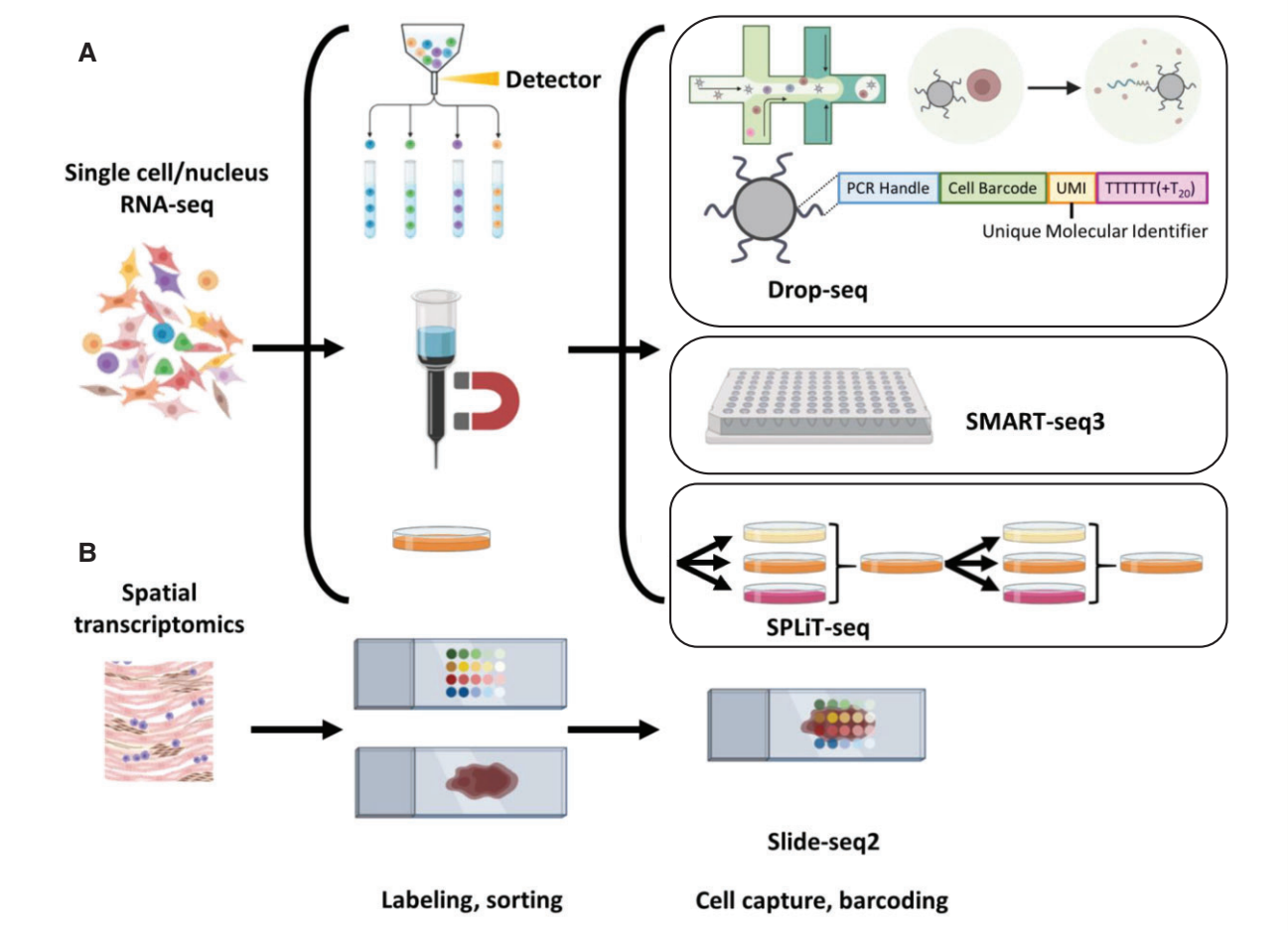


Figure 1 Summary of sequencing approaches. (*A*) Following isolation of cells or nuclei, labelling and/sorting strategies are often employed via flow cytometry, magnetic sorting, or antibodies to enrich a cell-type or deplete unwanted material. Next, droplet sequencing approaches generate gel emulsions to capture single cells in high-throughput manner, whereas cells sorted into plates (e.g. SMART-seq3) are lysed in their component wells. SPLiT-seq uses multiple rounds of barcoding where an iterative process, wherein samples are split and labelled/barcoded, then pooled, and the process is repeated. (*B*) Spatial transcriptomics applies fixed tissue to a slide with spatially barcoded wells, allowing for relative anatomical location of RNA which is captured following RNA liberation.

can be acquired from several companies (e.g. 10x Genomics, BD). However, this does not preclude the need to establish protocols of isolation of the cells from a given tissue, which is particularly important for cardiac tissue, which needs to be digested to gain a single-cell suspension (*Figure 2*). Since the matrix differs in hearts from development to adulthood, and particularly after injury (scar tissue), incubation time, and concentration of proteases matter. Moreover, during tissue digestion, RNA-degradation may occur, requiring the addition of RNase inhibitors.

When analysing cardiomyocytes, investigators may avoid droplet approaches (see size constraints above) and select to sort cells into plates (e.g. Smart-Seq3),[6] or repeatedly splitting and pooling samples for sequential molecular barcoding (i.e. SPLiT-seq).[7] Following sorting, Smart-seq3 lyses cells with reverse transcription reagents in wells and typically results in fewer cells per experiment.[6] This approach allows sequencing full-length transcripts and detects more genes per cell than droplet approaches.

Alternatively, cardiomyocyte analysis (and that of whole tissues) can be performed by isolating nuclei from tissue (e.g. DroNc-seq).[8] Gene expression is well correlated between single-nuclei and single-cell sequencing, though nuclear-localized pre-mRNA, non-coding, and architectural RNAs are enriched in snRNA-seq data sets. Here, pre-mRNAs can provide additional snRNA-seq transcriptomic insights.[9–11] Furthermore, snRNA-seq enables analysis of biobanks with available frozen tissues: cell membranes are lysed during flash freezing, while nuclear membranes remain intact.

Spatial transcriptomics (e.g. Slide-seq2, Visium) is rapidly improving and library preparation protocols are similar to that for droplet sequencing (3′ mRNA capture). With spatial transcriptomics, fixed tissue is dissociated on slides having wells with spatially coded beads. As RNA is released from tissue, it is deposited within the wells. The advantage is knowing the locations of regulated genes. However, the size of wells and thickness of tissue section typically result in multiple cells captured per well. While commercially available platforms like the 10x Genomics Visium have improved resolution when compared with predecessors, the diameters of the capture wells, the diffusion of mRNA before capture preclude these data being truly 'single cell.' Therefore, the

A

Tissue	Complication	Impact on sc/snRNA seq
Heart / Vasculature	Cell isolation from tissues / High cell debris (panel **B**) Fibrotic, calcified tissue disturb cell liberation	May clog apparatus, risk for ambient RNA contamination and high mitochondrial content (panel **C**/**D**) Cell recovery may not match tissue proportions; increased likelihood of doublets (see panel **D**)
Vasculature/Biopsies	Few cells	Insufficient statistical power
Immune cells	Low RNA content Bone marrow cell isolation Ficoll gradients	Fewer cells to isolate, risk for reduced gene and high mitochondrial content (see panel **C**) Activation of cells, results may vary with isolation protocols Hands on isolation may yield variable inclusion/exclusion of cell populations
All Tissue	Enrichment of cell populations by FACS/MACS	Risk for cell activation; variability of purity of populations

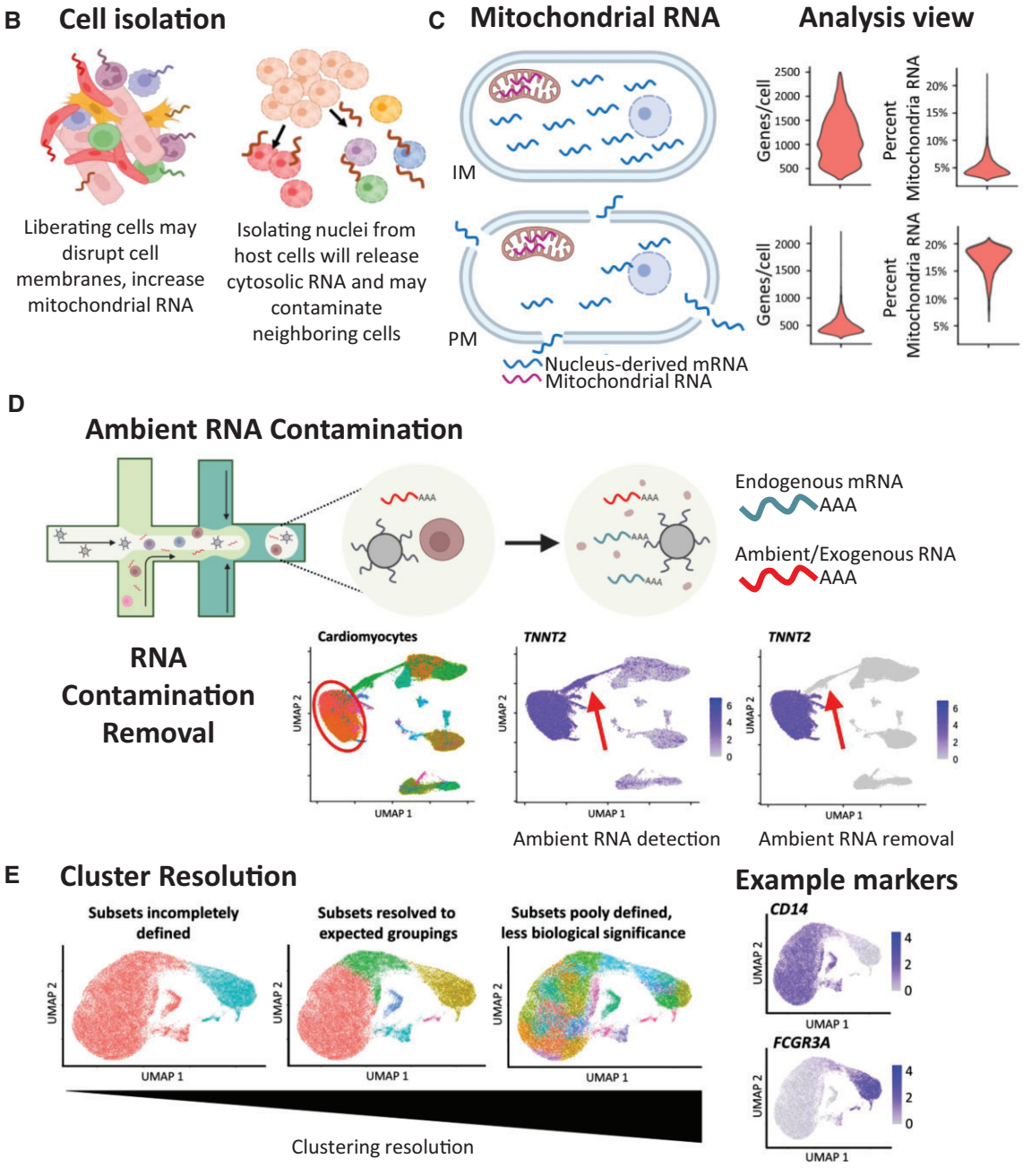


Figure 2 Quality control in scRNA-seq experiments. (*A*) Overview of typical complications arising by cell and tissue type in cell preparation and impacts on quality of data. (*B*) Representation of how cell isolation may disrupt cell membranes, leach RNA, and how ambient RNA contamination arises from disrupted cells. (*C*) Depiction of how cytoplasmic RNA loss can modulate the ratio of cytoplasmic/mitochondrial RNA and

Continued

utility of the approach may lie in conditions with clear transitions in cell state, such as that moving from unaffected myocardium through the border zone post-myocardial infarction (MI). Excitingly, advances in this field are rapid with approaches Slide-seqV2 and sci-Space nearing true cellular spatial resolution.[12,13]

Quality control assessment for single-cell sequencing experiments

With all scRNA-seq analyses, it is critical to ascertain the quality of the data set. An early step is to ensure these data, which are defined by the 'cell barcodes', correspond to single live cells. This is determined by the total number of transcripts, genes, and proportion of mitochondrial genes per-cell barcode. An informed approach must be taken during the quality control assessment, as each of these variables can lead to misunderstanding. For example, increasing mitochondrial genes might indicate a contamination of the samples with fragmented mitochondria (particularly occurring upon isolation of nuclei) (*Figure 2*). On the other hand, high levels of mitochondrial expressed transcripts may be indicative of a higher mitochondrial content or activation. Cells with low gene abundance could be senescent cell populations, and cells with high RNA abundance could be larger cells. Cells with the expression of diverse marker genes might be transition cell states or represent doublets or contaminated cells. So called 'ambient' gene expression represents transcripts that do not come from the barcoded cell, but from other lysed cells whose RNA is contaminating the target cell in the analysis (*Figure 2*). This is especially relevant in nuclei liberated from cardiac tissue, where cardiomyocyte markers can be found in various cell types. Cell doublets can be identified by using bioinformatics analysis tools.[14]

Setting classical *a priori* inclusion criteria can be exceedingly challenging for scRNA-seq datasets and revisiting quality control inclusion criteria may need to be considered after initial clustering and annotation steps are performed. When working with new cell profiles, it is beneficial to first set more inclusive filters to avoid excluding viable cell populations by accident. Over the course of the next sections, we will discuss insights these technologies enabled.

Single cell/nuclei RNA-Seq to uncover cardiac cellular composition

Classical methods for cell identification and quantification, including *in situ* stereology and tissue dissociation with cell sorting, depend on knowledge of cell types and specific markers. Until the mid-2010s, the availability of cell surface marker genes and the necessary tools for identifying them limited advanced sorting-based approaches based on transgenic mouse lines and multiplexed immunolabelling.[15] Surveys of cell-type diversity in cardiac research were at the level of cardiomyocytes, mesenchymal, and endothelial lineages, and rarely included immune cells.[16]

Sc/snRNA-seq is not similarly constrained. The method assesses a sample of each cell's transcriptome, examining the similarity between all sampled cells in high-dimensional space. Then, graph-based clustering segregates cells into 'clusters' with similar transcriptomes, which can be assigned an identity (for example, using prior knowledge or *in situ* analysis of anatomical location). In 2018, Skelly *et al.* provided an early picture of cellular diversity in the murine heart,[17] profiling over 10 000 non-myocytes and identifying 12 distinct cell types. In addition to the expected endothelial, mural, and fibroblast components, there were resident macrophages, T cells, NK cells, and B cells. In 2020, a pair of studies employed single-cell sequencing approaches to identify cellular diversity within hundreds of thousands of cells from over 20 non-failing human hearts.[1,2]

Utilizing snRNA-seq technology to assess cardiac tissue,[18] both studies identified nine major cell types in comparable proportions.[1,2] Cardiomyocytes comprised ~30% of all cells but were more numerous in the ventricles than atria. By proportion, these were followed by fibroblasts, mural cells, endothelial cells, myeloid cells, lymphoid cells, adipocytes, and neuronal-like cells. The composition of the adult heart described above is similar to that observed during embryonic development by scRNA-Seq,[19] although the latter retains neural crest cells at the first 9 weeks post-conception. Also, transcriptomic definition of cell types in the heart through the studies above provides the platform for the analysis of transcripts of interest in emerging diseases. Soon after the identification of important receptors and proteases for the internalization of SARS-CoV-2 in early 2020, several studies identified cell types that might be at the highest risk of direct infection in the entire body.[20–23] While direct infection of cardiomyocytes or endothelial cells appears to be a minor source of cardiac involvement in COVID-19, single-cell analysis provided detailed insights into the expression of the SARS-CoV-2 receptors in the different cardiovascular cells, showing the relatively low expression of the receptor in cardiomyocytes and negligible expression in endothelial cells.

An important feature of single-cell analysis is the ability to set a clustering resolution, which allows increased granularity on cell-type definitions, uncovering diversity in seemingly monolithic cell

Figure 2 Continued
impact genes per-cell ratios. IM, intact membrane; PM, perforated membrane. (*D*) Example of droplet sequencing with ambient RNA in fluidic channels and gel emulsions, following cell lysis and barcoding, ambient RNA will be associated to a particular cell. Examples of situations with high ambient RNA, e.g. after nuclei isolation of hearts, wherein cardiomyocytes will cluster in one region, but with cardiomyocyte-specific markers appearing in other non-cardiomyocyte clusters. Ambient RNA removal minimizes such artefacts (right panel). (*E*) Example of degrees of clustering resolution is shown for incompletely resolved, improved cluster resolution, and over resolved clusters. Example of well-defined clusters of myeloid cells, which can be assigned to classical (red: $CD14^{high}/FCGR3A^{low}$), intermediate (green: $CD14^{mid}/FCGR3A^{mid}$), non-classical monocytes (gold: $CD14^{low}/FCGR3A^{high}$), and dendritic cells (three clusters in dark blue, light blue, pink). Cell-type-specific markers are shown in right panels.

populations (*Figure 2*). An example is cells of mural lineages, which combine microvasculature-lining pericytes with larger vessel-lining SMCs. The increased resolution is required to resolve these populations from each other. Further cellular diversity has been uncovered in the non-failing heart by increasing resolution. Litviňuková *et al.*[2] reported 10 cardiomyocyte subtypes, 17 populations of endothelial/stromal/mesothelial cells, 7 populations of fibroblasts, and 21 immune cell types. Also, subclustering of human myocardial fibroblasts in a companion study revealed a previously undescribed population of fibroblasts, which are devoid of canonical fibroblast activation markers *POSTN* or *FAP*, and were distributed widely in a heart with no overt signs of clinical dysfunction.[1] Given the user-defined nature of clustering resolution thresholds, subtypes should be interpreted with care (a theoretical example is given in *Figure 2*). Importantly, several cell states defined by these two studies were appropriately validated using *in situ* hybridization, a critical step before undertaking any follow-up experiment.

Deconvolving transcriptomic responses to myocardial injury, remodelling, and failure

The feature of foremost interest to single-cell transcriptome research is the ability to deconvolve signals within samples at the cell-type level. Applications of scRNA-Seq to cardiac pathology and available data sets are described hereafter (*Table 1*, Supplementary material online, *File S1*) with the intent of displaying the potential for discovery of biological and translational insights that will grow as the technique becomes more widely adopted.

Cardiomyopathies and cellular response to pressure overload models of heart failure

Application of scRNA-seq provided unique insights into the cellular response of the heart to pressure overload in experimental models and humans[24–26] (*Figure 3*).

Table 1 Examples for single cell/nuclei sequencing data sets of human and mice studies

Disease/model	Reported cells	Tissue	Platform	Measurement	Cell source
Atherosclerosis	9490	Carotid endarterectomy	10x Genomics	scRNA-seq, CITE-seq, CyTOF	All cells, PBMC
Atherosclerosis	3700	Right coronary artery	10x Genomics	scRNA-seq	All cells
Heart failure	21 422	Heart	10x Genomics	scRNA-seq	CM and non-CM
Heart failure (paediatric DCM)	18 211	Heart	10x Genomics	snRNA-seq	All cells
Heart failure, hypertrophy	1190	Heart	Smart-seq2	RNA-seq	Cardiomyocytes
Heart failure	181 712	CD31+ circulating cells	10x Genomics	scRNA-seq	CD31+
Heart failure (ICM)	77 278	PBMCs	10x Genomics	scRNA-seq	PBMC selection
Aging	27 808	Heart	10x Genomics	snRNA-seq	All cells
Atherosclerosis	3541	Aorta	10x Genomics	scRNA-seq, CyTOF	CD45+
Atherosclerosis	3700	Aortic root and ascending	10x Genomics	scRNA-seq	SMC (Myh11-tracing)
Atherosclerosis	1266	Aorta	10x Genomics	scRNA-seq	CD45+
Development	1000	Heart	10x Genomics	scRNA-seq	EC
Development	21 366	Heart	10x Genomics	scRNA-seq	All cells (E7.75, 8.25, 9.25)
Development	1378	Heart	Fluidigm/Smart-seq	RNA-seq, ATAC	Nkx2.5 and Isl1 sort
Heart failure, Ang II model	29 558	Heart	10x Genomics	scRNA-seq	CM, non-CM
Heart failure, pressure overload	17 853	Heart	10x Genomics	scRNA-seq	CD45+
Myocardial infarction	1334	Heart	10x Genomics	scRNA-seq	CD11b
Myocardial infarction	30 000	Heart	10x Gen. and SMARTer	scRNA-seq	Non-CM
Myocardial infarction	7150	Heart	10x Genomics	scRNA-seq	EC (Pdgfb-lineage traced)
Myocardial infarction	50 677	Heart	10x Genomics	scRNA-seq	Non-CM and EC (Cdh5-traced)
Myocardial infarction	8283	Heart	10x Genomics	scRNA-seq	Macrophage and DC sort
Myocardial infarction	38 600	Heart	10x Genomics	scRNA-seq	Non-CM
Postnatal injury model	21 737	Heart	10x Genomics	scRNA-seq	All cells

An extended, searchable list of studies including technical details and link to downloading the data is available as Supplementary material online, *File S1*. CM, cardiomyocytes; EC, endothelial cells; PBMC, peripheral blood mononuclear cells.

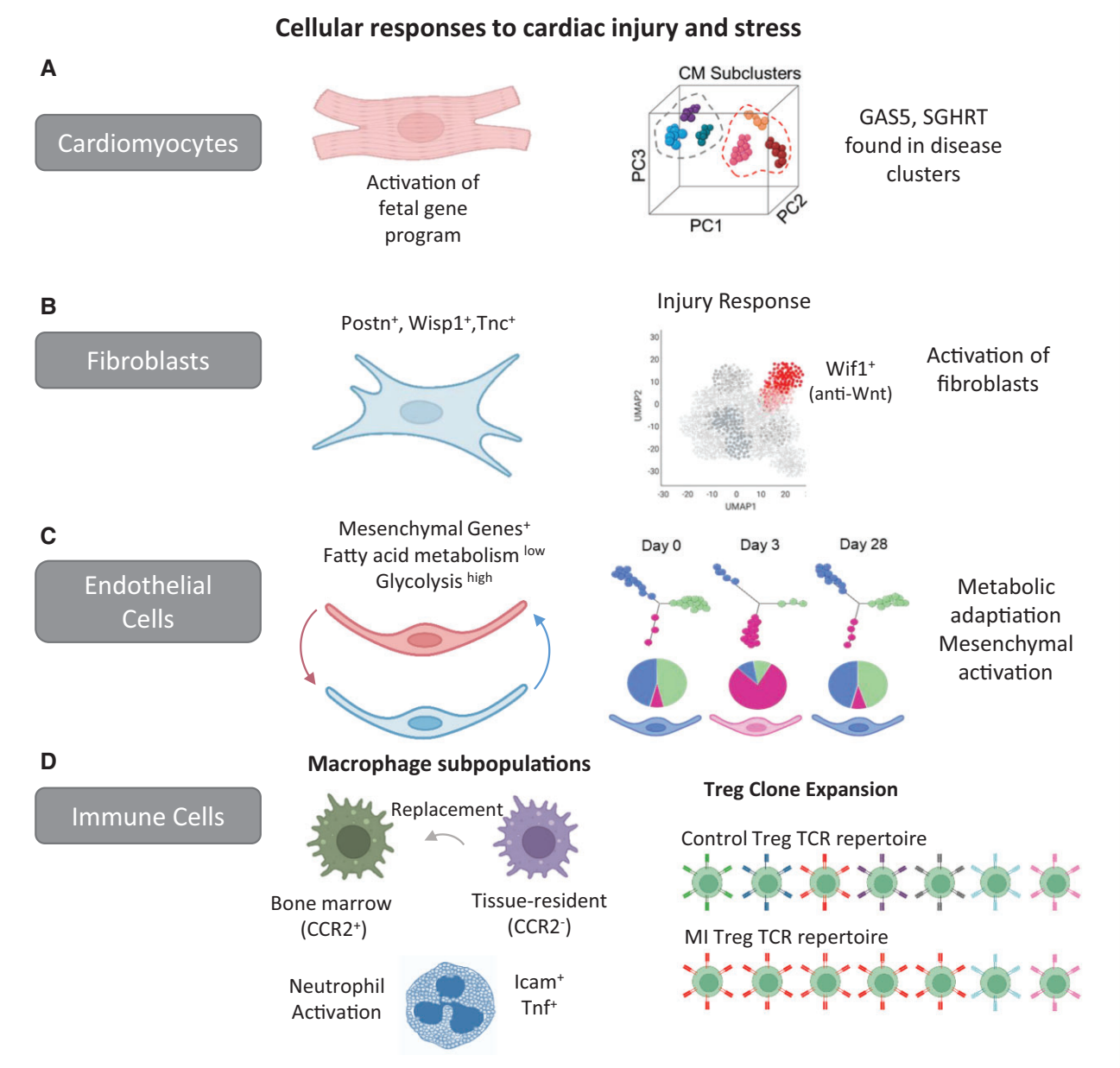


Figure 3 Cellular responses to cardiac injury. Representation of changes in cardiac cells after injury and stress identified by sc/snRNA-seq. (*A*) Cardiomyocytes express lncRNAs involved in re-expression of foetal genes, whereas fibroblast (*B*) showed a heterogenic response to injury or aging. (*C*) Endothelial cells show metabolic adaptations that coincide with transient enhanced mesenchymal gene signatures following myocardial infarction. (*D*) Immune cell alterations after myocardial infarction. Bone marrow vs. tissue-resident macrophages following cardiac insult, along with neutrophil and T-cell responses.

Cardiomyocyte response

An early, foundational snRNA-seq study of mouse and human failing cardiomyocytes leveraged single-cell analysis in two interesting ways.[24] For the first time, distinct subpopulations of human cardiomyocytes could be distinctly resolved based on health status [healthy v. dilated cardiomyopathy (DCM)], giving promise that altered gene networks might arise (*Figure 3*). Building on this, the assessment of nodal hubs of gene regulatory networks identified lincRNAs (i.e. *Gas5*, *Sghrt*) that target fetal gene programme activation programmes and were validated to play a role in cardiomyocyte cycling during the endogenous myocardial stress response, providing key, cell-specific insights into non-ischaemic DCM. Recently, the response of cardiomyocytes to pressure overload in human clinical samples disclosed major alterations in cellular cross-talk, by using tools to assess ligand–receptor pairing in sc/snRNA-seq experiments.[26] Cardiomyocytes showed a reduction of incoming connections via Ephrin receptors (i.e. EPHB1) preventing cardiac endothelial cell-derived activation by ligand Ephrin-B2. Interactions between EPHB1 and Ephrin-B2 inhibited cardiomyocyte hypertrophy *in vitro*.

In a paediatric DCM study, it was found that infants up to 1 year of age revealed a primarily regenerative profile and thus may benefit from treatment strategies supporting cardiac regeneration, while older children showed patterns associated with cardiac fibrosis and may benefit from early antifibrotic therapy.[27] Identified alterations in β-adrenergic signalling gene expression also additionally provide hints for a personalized treatment of paediatric DCM.[27]

Endothelial cell/endocardial response

Single-cell RNA sequencing revealed interesting insights into how the endothelium controls cardiomyopathies, such as non-compaction cardiomyopathy, a tragic genetic disease resulting in poor consolidation of the ventricular wall and decreased cardiac function.[28] Rhee *et al.* showed that in left ventricular non-compaction pathologies, the endocardial cell populations displayed dysregulated angiocrine factors such as Col15a1. Diminished Col15a1 decreased cardiomyocyte proliferation and was associated with elevated secreted factors like Tgfbi, Igfbp3, Isg15, and Adm—further decreasing cardiomyocyte proliferation.[28] The data support the hypothesis that coronary endothelial cells control myocardial compaction and non-compaction by altered secreted factors.

Immune cell response

Martini *et al.* demonstrated in the early stages of heart failure upon pressure overload infiltration of oncostatin M-positive, M1 pro-inflammatory monocytes/macrophages along with robust activation/expansion of PD-1 in $CD4^+$ regulatory T cells (Tregs).[29] These data may provide insights related to cardiac toxicity during anti-PD-1 cancer immunotherapy and lack of response in persons with heart failure to anti-tumour necrosis factor therapy.

Future perspectives

Single-cell analysis will likely help to decipher the heterogeneity of cardiomyopathies, particularly by disclosing the consequences originating from specific mutations. The combination of single-cell technologies with machine learning may yield novel, targeted therapeutic options.

Cellular responses to myocardial infarction

The majority of single-cell analyses have been studying cardiac responses to MI or ischaemia/reperfusion injury (*Table 1* and *Figure 3*). In the earliest application of the technique, scRNA-seq profiled the response of 935 murine myocardial cells following ischaemia–reperfusion injury,[30] while meanwhile, large data sets are available (*Table 1*).

Fibroblast cell response

Comparison of fibroblast transcriptomes post-injury revealed fibroblasts with a specific up-regulation of *Ckap4*, which could have a modulating effect on fibroblasts during ischaemic injury by attenuating myofibroblast activation.[30] This study stood as one of the first to clearly display the translational potential for such scRNA-seq-mediated discoveries modulating fibrosis.

A uniquely activated fibroblast cell state defined by a strong anti-WNT transcriptome signature was identified in response to acute injury at 3–7 days post-MI. Among other specifically enriched genes, this subset of fibroblasts expresses *Wif1*, which is essential for the heart's response to injury.[31] Another study showed that modulation of fibroblast subpopulations predicts cardiac rupture and pathological remodelling in 129S1/SvImJ mice, which are prone to rupture, and provides mechanistic insights into the previously documented therapeutic benefits of fibrinolytic and anti-renin–angiotensin system treatments.[32] Future assessment of the networks that control the fibroblast injury response state may reveal unique traits that confer sensitivity and resilience to cardiac rupture, allowing for patient stratification and the creation of precision therapeutics.

Activation of cardiac fibroblasts was also detected in a comprehensive analysis of the aging heart, which provided the first insights into a disturbed interaction of fibroblasts with endothelial cells during aging.[33] Age-induced fibroblast activation resulted in the release of Serpins, which induced endothelial cell inflammation and dysfunction in a paracrine manner.

Cardiomyocyte response

Novel insights in cardiomyocyte biology were obtained via cell–cell communication analysis of post-MI snRNA-seq data, wherein it was shown that cardiomyocytes expressed and released increased quantities of beta-2 microglobulin (B2M), which can activate fibroblasts in a paracrine manner.[34] Such findings could lead to the development of new therapeutic approaches by modulation of B2M in ischaemic heart disease patients.

Endothelial cell response

Two complementing investigations studied the vascular response to MI in mice. Both utilized endothelial-specific lineage tracing mice to determine the transcriptomes of endothelial cells and their progeny using Pdgfb-iCreERT2[35] or Cdh5-CreERT2 (VE-cadherin) lines.[36] Both studies found clusters primarily populated by endothelial cells in post-infarct mice, which maintained genes associated with proliferation, remodelling, and endothelial cell–extracellular matrix interactions (*Figure 3*). Interestingly, both groups found plasmalemma vesicle-associated protein (*Plvap*) enriched after MI, which may promote endogenous cardiac tissue repair after MI.[35]

The studies revealed that endothelial cells show metabolic adaptation and plasticity. The endothelial cluster at Day 3 post-MI was shown to be enriched in glycolytic genes and some mesenchymal markers along with a diminution of endothelial markers, suggesting that endothelial cells are undergoing an endothelial-to-mesenchymal transition (EndMT).[36] Endothelial-to-mesenchymal transition was first described in 2007[37] and was considered as a detrimental response to injury, which leads to cardiac fibrosis.[38] Surprisingly, however, single-cell resolution of lineage traced endothelial cells showed that the induction of EndMT is transient and most cells later revert back to an endothelial transcriptomic state.[36] Similar findings were shown in another study, in which researchers documented the partial acquisition of mesenchymal signatures (e.g. *Vim*, *Fn1*) in ECs at Day 7 post-MI but

not full conversion into fibroblasts suggesting that the mesenchymal activation is transient and contributes to clonal expansion and vessel growth.[35,36,39] The transient nature of this process, resembling 'mesenchymal activation' rather than long-term transition, may explain the controversial findings in the past, which failed to demonstrate a contribution of endothelial cells to fibroblasts.[40]

Recent studies showed that other vascular beds outside the heart are activated post-MI in humans and mice.[41] Researchers elucidated the response of the bone marrow vascular niche by scRNA-seq of selected $CD31^+$ endothelial cells and, by subclustering, found endothelial cells with a high expression of endomucin (previously described as 'type H' cells) are lost via IL-1β-dependent pyroptosis post-MI. Loss of this cell type correlated with $CD41^+$ myeloid progenitor cell expansion and was prevented by anti-inflammatory inflammasome inhibitors. The data may provide support for therapeutic benefits of the anti-inflammatory strategies in patients with cardiovascular diseases.

Immune cell response

A series of studies focused on the immune contribution to cardiac homeostasis and injury repair within the heart[31,32,42] (*Figure 3*). One such group identified the complexity of monocytes and macrophages in the myocardium, where a self-renewing *Ccr2*(−) resident macrophage population is supplemented by those derived from, and replaced by, infiltrating monocytes after MI.[42] The resident macrophage held a critical role in response to MI, where inducible ablation led to severe adverse remodelling and reduced cardiac function. Time-dependent examination of the heart and blood of infarcted animals allowed the in-depth characterization of neutrophils in response to MI. The authors uncovered diversification of transcriptomic signatures in the heart (*Icam1*+/*Tnf*+) which speaks to a specific neutrophil phenotype at the tissue injury site which may lead to increased phagocytosis and oxidative damage.[43] In addition, immunosuppressive Tregs were shown to generate unique T-cell receptor clones found in the heart after MI.[44] The investigators further hypothesized a role for Treg-derived Sparc to be critical for damage control in the post-infarcted heart.

Further chronic, systemic effects following MI have been documented in circulating monocytes.[45] This study found that ischaemic heart failure had transcriptomic impacts on gene expression patterns in monocyte subclass subpopulations (i.e. classical, intermediate, and non-classical monocytes), separating the cell subpopulations by health status. Markers driving this bifurcation were metabolic genes such as *FABP5* (which can potentiate IL-1B signalling). Fluorescence-activated cell sorting was used to validate these findings and might be used as a potential diagnostic or stratification tool for heart failure patients. Modulation of the metabolism of the inflammatory cells may help to ameliorate chronic inflammation in these patients.

Future perspectives

While the transcriptional response to MI has been rigorously investigated in mouse models, further insights in human hearts will be essential. Longitudinal studies analysis of acute and chronic remodelling after MI in humans may provide insights into what drives chronic ischaemic heart failure. Increasing the depth of cellular resolution may further define particularly affected smaller cell populations and local mosaic responses. Possibly, the technology may help to unravel or elucidate the mystery of cardiac regeneration.[46]

Single-cell analysis of atherosclerosis and other vascular disease

Some of the most striking insights into novel cell identities derived from scRNA-seq come from atherosclerosis research (*Table 1* and *Figure 4*). Whereas first studies focused on dissecting the heterogeneity of immune cells in atherosclerotic plaques, additional reports soon followed elucidated the regulation of endothelial and SMCs.

Immune cell response

The use of scRNA-seq in mouse and human plaque identified an unexpectedly diverse range of specialized leucocyte subpopulations consistently found between species, and therefore are useful for translational studies.[47] Plaques were populated largely from macrophage/myeloid (≈60–75%) and T-cell (≈25%) subsets. The frequency of genetically defined T-cell populations in carotid plaques inversely correlated with cardiovascular events in patients, suggesting new avenues for T-cell involvement in plaque stabilization. Adding insight on T-cell subsets in plaques, a later study found a greater proportion of CD8+ T cells in carotid artery plaques relative to the blood of symptomatic patients. Interestingly, a particular CD4+ T-cell profile associated with T-cell migration (RhoGTPase), activation (PDGFR-β), and differentiation (Wnt, IL-2) predicted cerebrovascular events. Conversely, selective accumulation of *Nrp1*-Tregs is a signature for plaque regression, a highly debated phenomenon in the atherosclerosis field.[48] A parallel study focusing on myeloid cells showed specific macrophage subtypes almost exclusively detectable in atherosclerotic aortas.[49] These myeloid cells showed inflammatory profiles and had elevated interleukin-1β (*Il1b*) and the poorly described *Trem2*.

From B cells of atherogenic and control mice, one study found 56 antibodies from *in vivo* expanded clones, with one-third of these being reactive against atherosclerotic plaques.[50] A promising candidate targeted ALDH4A1 and slowed plaque formation, reduced free cholesterol, and LDL. This approach could be utilized in many other contexts such as myocarditis, acute MI, and others to expedite immune-based therapies for cardiovascular disease.

Endothelial cell response

As disturbed flow is critical to atherogenesis, researchers have utilized scRNA-seq and single-cell sequencing assay for transposase-accessible chromatin (scATAC-seq) in a murine carotid artery ligation model of disturbed vs. stable flow.[51] Single-cell sequencing assay for transposase-accessible chromatin is a method for assessing genome-wide regulatory landscapes in single cells.[52] Transcriptomic switching from atheroprotective phenotypes to pro-inflammatory, mesenchymal-like, haematopoietic-like, and immune cell-like phenotypes were found in the study. Single-cell sequencing assay for transposase-accessible chromatin data

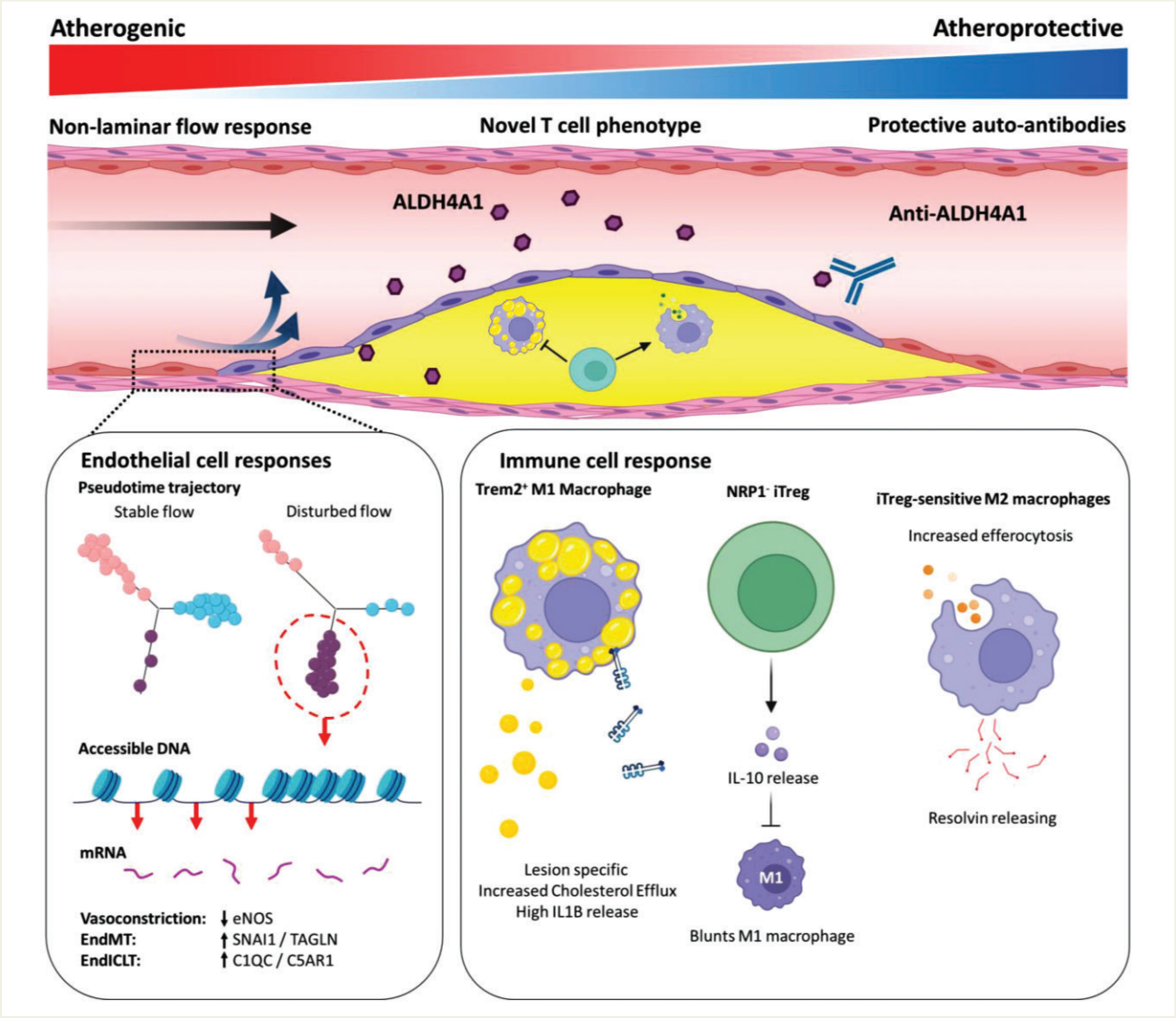


Figure 4 Atherogenic and atheroprotective responses. Representation of endothelial cells exposed to laminar (pink) vs. disturbed flow (purple) in a physiological setting (left portion of figure). Such responses were assessed in endothelial cells using scATAC-seq and scRNA-seq wherein accessible chromatin and RNA expression found signatures associated with vasoconstriction, EndMT, and EndICLT in disturbed flow endothelial cells. *Trem2*$^{+}$ macrophages were detected in lesions, showing high expression of *Il1b* and specialized lipid metabolism. Naive T-cell-induced Tregs were critical for the maintenance of an atheroprotective environment. These iTregs diminished pro-inflammatory M1 monocyte signatures and sensitized M2 macrophages to anti-inflammatory responses. B cells and plasma cells demonstrated elevated clonality of antibodies targeting atherosclerotic lesions. Autoantibodies against ALDH4A1 were shown to be atheroprotective. EndMT, endothelial to mesenchymal transition; EndICLT, endothelial to immune cell-like transition; iTreg, induced (from Naive T cell) T regulatory cell; NRP1, Neuropilin; ALDH4A1, aldehyde dehydrogenase 4 Family Member A1.

confirmed that transcription factor binding sites like KLF4/KLF2 remained open during stable flow, while conversely showing that RELA, AP1, STAT1, and TEAD1 binding sites increased in accessibility following disturbed flow. Furthermore, the luminal face of diabetic atherogenesis was studied to assess endothelial cell plasticity during diabetic atherogenesis.[53] The group assessed ECs from mouse heart and aorta with standard chow or diabetogenic high-fat diet with cholesterol. Interestingly, the authors found subsets of endothelial cells expressing mesenchymal markers, suggesting an endothelial-to-mesenchymal transcriptomic activation.

Aortic endothelial regeneration following a clamp-based injury model was elucidated by McDonald *et al.*,[54] wherein a biphasic response was reported. The authors showed that regeneration is determined by specific populations arising from differentiated endothelial cells. Interestingly, cells immediately proximate to the site of injury enter into cell cycle, and a second step is driven by a highly proliferative subset of cells. This is in agreement with

another study,[55] wherein they showed CD157^{+} endothelial cells may act as resident vascular endothelial stem cells that clonally expand in response to injury. This process is dependent upon genes like Atf3 (e.g. stress response genes). Importantly, aortas from aged mice had diminished regenerative capacity and expressed less *Atf3*. Atf3 involvement in endothelial repair was confirmed with an Atf3 deletion model. A recent study further assessed the contribution of reactive oxygen species in endothelial injury responses.[56]

These studies indicate that endothelial regeneration involves collective cell behaviour and speaks to the importance of cell–cell communication and the need for further insights. The findings in McDonald *et al.* indicate that endothelial cells retain their junctional complexes and remain connected throughout the entire repair process. Interestingly, these studies provide no support for circulating endothelial progenitor cells or bone marrow-derived cells playing a central role in endothelial repair, though this was not completely ruled out.

Smooth muscle cell response

One of the early scRNA-Seq studies in 2017 dissected the heterogeneity of G-protein-coupled receptors in the vascular wall, and specifically in isolated SMCs.[57] An additional study further assessed SMC phenotypic switching in atherosclerosis in mice and humans.[58] Smooth muscle cells phenotypic switching leads to dedifferentiation, migration, and transdifferentiation of SMCs into other cell types. The authors found that SMCs of human atherosclerotic plaques transited to an intermediate, multipotent state capable of differentiating into macrophage-like and fibrochondrocyte-like cells. This transition was associated with activation of retinoic acid signalling, which blocked SMC phenotypic switching and promoted fibrous cap stability.

A study in humans and mice (Fbn1C1041G/+ model) showed temporal and spatial subtleties during aortic aneurysm development in Marfan syndrome, and established Marfan syndrome-specific signatures of plasminogen activator inhibitor-1 (Serpin E1), Kruppel-like factor 4, and enhanced expression of TGF-β-responsive genes.[59]

Future perspectives

Given the heterogeneity of immune cells and the known major difference between mice and humans, one would expect single-cell approaches to gain important additional insights into vascular inflammation and cellular responses in humans.

Insights in clonal haematopoiesis of indeterminate potential by single-cell approaches

Somatic mutations leading to clonal haematopoiesis of indeterminate potential (CHIP) promote atherosclerosis and the progression of heart failure.[60,61] While more insights are needed to assess whether CHIP is a novel risk factor or may be a therapeutic target for precision treatment, a few studies provide insights as to how haematopoietic cells with somatic mutations may drive disease.

Immune cells harbouring mutations in one of the CHIP-driver genes, *DNMT3A*, were shown to express high levels of genes encoding the inflammasome complex, pro-inflammatory cytokines and Resistin, which augments monocyte-endothelial adhesion.[62] This was confirmed in another scRNA-seq study of patients with chronic ischaemic heart failure or aortic valve stenosis having either DNMT3A or TET2 CHIP-driver mutations.[63] A study of *JAK2* somatic mutations suggests that increased proliferation and glycolytic metabolism in mutated macrophages lead to DNA replication stress and activation of the AIM2 inflammasome, which exacerbates atherosclerosis.[64] Moreover, insights into a role for T-cell blockade therapies are implicated in CHIP patients, suggested by elevated T-cell costimulatory molecules in antigen-presenting cells.[62] These studies suggest that specific interventions targeting interleukin-1β, interleukin-6, inflammasome activation, or T-cell costimulation with respect to CHIP mutation status could potentially reduce cardiovascular risk.

Future perspectives

Current studies so far did not allow to identify the mutation site in the individual cell, thus, precluding the analysis of direct vs. indirect effects of somatic mutations in humans. Increasing coverage of the mutated gene in combination with long-read sequencing (to allow for detection of mutations across the entire gene) will provide an option to gain further insights in the consequences of CHIP in humans.

Identification of therapeutic targets and cell populations

While many scRNA-seq studies to date have been descriptive, more detailed human studies and the inclusion of transgenic models, especially those combined with surgical or pharmacological interventions, offer an exciting opportunity to analyse cell-specific and time-dependent effects on pathology. Alongside this, Xiao *et al.*[65] found silencing of Hippo signalling stimulated cell-autonomous effectors of cardiac fibroblast fate transitions, with proliferation leading to specification and interstitial fibrosis suggesting clear implications for therapeutic intervention. The identification of previously unknown cardiac fibroblast populations as fibrosis drivers[66] can similarly lead to novel therapeutic approaches, e.g. by using recently developed CAR-T cells to deplete such detrimental population.[67] Another example is the identification of ACKR1+-endothelial cells, which were found as a novel subpopulation with a high pro-angiogenic gene expression profile in human hearts.[68] Injection of this subpopulation indeed promoted regeneration in mice after MI.[69]

Perhaps atherosclerosis has been most impacted by the enhanced understanding of leucocyte diversity in plaques found by scRNA-seq data. Meta-analysis of nine scRNA-seq studies found 17 leukocyte clusters that included all main immune cells.[70] Macrophages, which included Trem2+ foamy macrophages that were not aggressive and tissue-resident macrophages that expressed Pf4, were the most common cell type in the atherosclerotic aorta of mice. Vascular SMCs were suggested to make up a

significant fraction of all foam cells. Thus, scRNA-seq has also allowed for a broadening of biological definitions within cell types and activities.

Finally, identification of cell population in combination with bioinformatics analysis of putative ligand–receptor interaction highlight the importance of cellular interactions in cardiac disease,[26,31] particularly heart failure, where traditionally research was focused on cardiomyocyte-intrinsic defects. Here, changes in cardiomyocyte–endothelial interactions and altered interaction between other cells were shown to occur in pathological settings[26,28,31] and might be therapeutically approached as well.

Another way to consider using scRNA-seq was realized in Phase I clinical trial assessing target derepression induced by an antimiR therapy that has been shown to effectively induce angiogenesis.[71] Here, scRNA-seq helped to identify cell-type-specific responses to treatment and to determine the modulation of effector pathways.[71] Less than 20 clinical trials registered in ClinicalTrials.gov use scRNA-seq in their analysis, leaving room for expansion to use this technology to analyse the efficiency of a given therapy to target the respective pathways.

Conclusion and the path forward

Single-cell RNA sequencing has provided important and novel insights into pathophysiological processes underlying cardiovascular disease. Most studies have evaluated murine cellular transcriptomes to gain understanding of the disease progression. While often helpful, the ability to extrapolate the data to humans is debated, notably with respect to immune responses.[72] Going forward, sc/snRNA-seq may help assess, which human diseases and subclinical states a particular animal model best represents.

Likely, single-cell analysis of tissue specimens and circulating blood will allow to more precisely define unique signatures of human disease pathologies. First examples include the deconvolution of healthy and diseased hearts[1,23,27] and the single-cell analysis of human atherosclerotic lesions.[47] Also, with increasing numbers of samples available, the data may be used to profile diseases. While only recently established, bioinformatic approaches to integrate large numbers of single-cell data sets (for example Harmony[73]) will in time generate references and enable reanalysis of large cohorts of bulk RNA-seq. In these methods, single-cell reference datasets deconvolve bulk sequencing, revealing biomarkers or disease strata, which may be below the detection limit in the smaller cohorts typically analysed in single-cell work.[74–76] Then, such data sets may be used to define specific disease-associated genes or cell types. Disease scores (integrating pattern of informative genes) may link gene expression patterns with disease phenotypes or outcomes. Bioinformatic tools to define such disease scores are already available in current Seurat packages (AddModuleScore[77]). Such approaches might be used to profile heart failure, which is a complex chronic disease of often unclear aetiology, and may guide an accurate and tailored therapy of heart failure subtypes. For example, single-cell analysis may better define the pathophysiology of heart failure with preserved ejection fraction or better characterize dilative cardiomyopathies, where current routine diagnostic is limited to cardiac biomarkers, imaging, conventional histology, and viral examination. Ultimately, the integration of scRNA-seq with clinical metadata and molecular data may yield the greatest translational therapeutic value. Published examples are in kidney function and Alzheimer's disease, where hundreds of target genes and their relevant cell types were identified for putative translational investigation.[78,79] Similar efforts for cardiovascular disease should prove equally fruitful.

Even with success, one should not ignore the current limitations of the single-cell analysis tools, which go beyond the low per-cell transcript complexity and technical pitfalls (see Chapter 2). Further studies are required to document the biological relevance of cellular subpopulations and transcriptional signatures. Also, cardiovascular research is hindered by the limited availability of tissues (and anatomical specificity) and kinetic responses to disease. Additionally, despite often small biopsy sizes, single-cell technologies provides valuable information from these sources, enhancing the understanding of tissue complexity and heterogeneity.

Yet, methodological and analytical advancement in the field continues at a rapid pace. Assessment of chromatin accessibility and chromatin conformation and histone modifications are all on the horizon.[80] Certain modalities allow simultaneous measurement of RNA abundance and regulatory DNA, as in sci-CAR and SHARE-seq, eliminating data alignment complications and enabling inference of regulatory events underlying differential gene expression.[81,82] Accurate spatial transcriptomics techniques could elucidate responses following injury and identify local alterations. Such advances will fuel the next years of scientific discoveries.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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