Title
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Journal
Cardiovascular Research, 119(1)

ISSN
1015-5007

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Publication Date
2023-03-17

DOI
10.1093/cvr/cvac040

Peer reviewed

ESC European Society of Cardiology

Cardiovascular Research (2023) **119**, 64–78
https://doi.org/10.1093/cvr/cvac040

INVITED REVIEW

Single-cell transcriptomes in the heart: when every epigenome counts

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Received 6 October 2021; revised 3 February 2022; accepted 14 February 2022; online publish-ahead-of-print 24 March 2022

Abstract

The response of an organ to stimuli emerges from the actions of individual cells. Recent cardiac single-cell RNA-sequencing studies of development, injury, and reprogramming have uncovered heterogeneous populations even among previously well-defined cell types, raising questions about what level of experimental resolution corresponds to disease-relevant, tissue-level phenotypes. In this review, we explore the biological meaning behind this cellular heterogeneity by undertaking an exhaustive analysis of single-cell transcriptomics in the heart (including a comprehensive, annotated compendium of studies published to date) and evaluating new models for the cardiac function that have emerged from these studies (including discussion and schematics that depict new hypotheses in the field). We evaluate the evidence to support the biological actions of newly identified cell populations and debate questions related to the role of cell-to-cell variability in development and disease. Finally, we present emerging epigenomic approaches that, when combined with single-cell RNA-sequencing, can resolve basic mechanisms of gene regulation and variability in cell phenotype.

Keywords Chromatin • Epigenomics • Transcriptomics • Cardiovascular

1. Introduction

Nearly a decade after single-cell RNA-sequencing (scRNA-seq) became Nature's 'Method of Year', this technology has become a staple technique in most molecular biology fields. Over 500 single-cell studies were published by the end of 2019[1] and the number continues to climb as the technology expands into epigenetic and proteomic approaches[2] to examine biological systems at single-cell resolution. scRNA-seq is particularly relevant to many studies in the heart because it is conducive to research in systems with few cells, rare cell types, vast cell heterogeneity, and scarce samples. In studies of the developing human ventricular myocardium, for example, the small number of cells that can be obtained from each time point in embryonic development and the scarcity of the sample not only means that sample collection can be arduous, but also that the RNA-seq analysis of the collected samples can be noisy and prone to artefacts, making only the evaluation of the topmost differentially expressed genes appropriate.[3] Additionally, the heterogeneity of cell types within each sample obscures the detection of transcripts from rarer cell types, as collapsing heterogeneous gene programmes into a single expression profile will cause the most common cell type's transcriptome to dominate the analysis. For example, the cardiac conducting system—specialized cells that establish the rhythmic beating of the heart—has been a challenge to study because of the small number of cells, large cell-type heterogeneity, and difficult mechanical dissection. scRNA-seq has allowed a clearer view of these vital cell populations and helped identify novel conduction markers and unique molecular signatures that were often lost during bulk sequencing.[4,5] The power of scRNA-seq to address the limitations of bulk RNA-seq while increasing the resolution and exploratory power of transcriptomic analysis makes the use of this technique in the heart invaluable.

One of the most striking conclusions from scRNA-seq analysis is a concept observed many times but often ignored in bulk 'omics analyses: not all the cells in a given organ system behave the same, even if they all have the same genetic manipulation or arise from the same developmental lineage. This is also true in the heart. Consider this observation: despite a mountain of literature characterizing changes in the regulation of beta-myosin heavy chain in diseased hearts, *Myh7* expression marks only a subpopulation of cells in the hypertrophying heart—indeed a population that itself does not hypertrophy.[6] Or the observation that ploidy plays a key role in the regenerative capacity of myocytes, with mononuclear diploid myocytes (a minority in most mammalian hearts) having an enhanced regenerative capacity in comparison with multinucleated myocytes.[7] We can trace the same theme in epigenomic analyses of the heart. If the entire genome is examined in the setting of myocyte

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hypertrophy and failure, the correlation between gene expression and either accessibility or DNA methylation is poor;[8] this correlation is predictive only for a subset of genes, suggesting cell type- or locus-specific context determines the relationship between chromatin marks and transcription. These cell-type and cell subtype differences are evident in single-cell epigenomic studies.

A single-cell epigenomic investigation of the neonatal heart, which unlike the adult heart is capable of scarless regeneration after injury, has provided a resource of the changes in accessible chromatin regions across different cell populations in response to injury following myocardial infarction (MI).[9] Specifically, multiple populations of myocytes, fibroblasts, and other cardiac cells can be deconvolved on the basis of distinct accessibility profiles as measured by single-cell Assay for Transposase Accessible Chromatin and sequencing (ATAC-seq).[10] To justify performance of a single-cell study and for its findings to be biologically meaningful, there should be evidence of different functionality among cells from a nominally identical population. Epigenomics and proteomics techniques can allow us to connect newly identified cell populations to reproducible phenotypes that can be shown to be distinct from other members of the larger, parent population.

In this review, we examine the use of single-cell technologies in the cardiovascular field and provide an overview of the new cell populations identified through scRNA-seq. We will attempt to make sense of the heterogeneity that is observed through single-cell transcriptomics during cardiac development, injury, and *in vitro* differentiation and reprogramming (*Figure 1*). Finally, we will highlight the rising field of single-cell epigenomics and how it encourages reassessment of accepted wisdom on gene regulation through chromatin from the past decade, addressing the extent to which cell-to-cell variation reflects biological function and determining under what circumstances individual chromatin features dictate phenotype. An overview of scRNA-seq technology and associated analytical pipelines has been recently described.[19] Please see Supplementary material online, *Table S1* for a comprehensive list of sRNA-seq studies published to date in the mammalian heart, including targeted cells, protocols, disease models, and chromatin endpoints.

2. Discovering new cellular populations

In the heart, early scRNA-seq investigators were surprised by, and even periodically attributed to error, instances where a cell simultaneously expressed markers from two distinct cell types and cases where single populations expressed known markers at variable levels.[11,20] As new scRNA-seq studies in the heart are conducted, researchers continue to define and dissect these subpopulations to provide precise characterization of cell types and the transition between types, sometimes referred to as cell states.[21]

By achieving such a high-resolution view of specific cell types, single-cell methods have facilitated the discovery of more specific cell-type markers. This has been especially useful in the case of cardiac fibroblasts. Because cardiac fibroblasts are the primary cell type responsible for fibrosis, studying their function is key to identifying potential therapeutic targets for heart failure or post-infarction remodelling. However, simply identifying these cells can pose a significant challenge, as cardiac fibroblasts have long been identified vaguely as flat, adherent, spindle-shaped cells that produce connective tissue.[11] The proteins that make up this connective tissue include collagens and fibronectin, but marking cardiac fibroblasts by the expression of these genes is problematic: other cells in the heart, mainly including pericytes and vascular smooth muscle cells can also produce these extracellular matrix components.[11,22,23] Additionally, cardiac fibroblasts maintain only a low level of expression of these extracellular matrix genes in homeostasis, further impeding the use of these genes to identify cardiac fibroblasts in healthy adult hearts.[24] Various other markers for cardiac fibroblasts have been considered, including Fsp1, Tcf21, the cell membrane proteins Sca1, Ddr2, Thy1, and Pdgfra, or the matricellular protein Postn; however, all of these markers lack specificity and some such as Thy1 and Sca1 underrepresent the fibroblast population.[24–26] Even for activated cardiac fibroblasts, the commonly accepted marker α smooth muscle actin (αSMA, *Acta2*) is not only co-expressed by vascular smooth muscle cells, but also marks only a small subset of activated fibroblasts in a mouse model of pressure overload-induced cardiac hypertrophy.[24,27] Therefore, conclusively characterizing cardiac fibroblasts remains a significant challenge in the field.

Single-cell expression analysis has allowed for the identification of more precise fibroblast markers. Using fluorescence-activated cell sorting, researchers were able to identify a novel marker for fibroblast populations, *Mefsk4*, which although not perfectly exclusive to cardiac fibroblasts (it is also expressed in pericytes and leucocytes), includes all cells identified by *Col1a1*, *Pdgfra*, and *Tcf21* expression.[25] As a further improvement, scRNA-seq protocols have allowed researchers to re-evaluate markers of activated fibroblasts that arise after injury (*Figure 2*). Using a microfluidic platform to sort single cells from adherent cells of digested mouse ventricles, investigators were able to use quantitative reverse transcriptase–polymerase chain reaction to quantify transcripts of several candidate markers of activated cardiac fibroblasts and found that *Postn* may be a better marker than *Acta2* for activated fibroblasts in mice subjected to either angiotensin II infusion or MI.[24] By performing SORT-seq on digested adult mouse hearts subjected to ischaemia-reperfusion surgery, researchers identified a novel marker of activated fibroblasts, *Ckap4*, which is co-expressed with other activated cardiac fibroblast markers *Postn*, *Ctrc1*, and *Fn1*.[20] Single-cell transcriptomic investigations have thus advanced our understanding of what defines a cardiac fibroblast and can be equally useful for identifying markers of other, potentially novel, cell type subpopulations.

Single-cell transcriptomics has allowed some consistency in the populations and markers identified by different studies. In general, a *Wnt*-expressing population is found at basal levels. After infarction, an inflammatory cluster and a 'reparative'[29] or matrifibrocyte cluster[15] are found, which are involved in scar maturation and collagen crosslinking later after injury. Other frequently observed populations of cells have been described but do not have common markers. For example, many single-cell sequencing data sets describe a proliferating population of cells after injury. Although the genes driving the proliferation phenotype vary between studies, *Mki67* is one gene often found in common.[30]

scRNA-seq can also be used to validate previously identified cell markers. It was long thought that *c-kit*+ cells may have marked cardiac progenitors in the adult heart. After the isolation of *c-kit*+ *Nkx2.5*+ progenitor cells from mouse embryos,[31] several groups attempted to observe this population in the adult heart. Initial insights into the true origin and role of *c-kit*+ cells in the adult heart were revealed from lineage tracing studies,[32,33] which showed that the majority of *c-kit*+ cardiomyocytes are in fact the result of fusion events.[33] The first single-cell papers on the subject identified this population of cells but did not test their replicating capabilities.[34] A few years later, several studies used scRNA-seq to examine cardiomyocyte cell populations in the adult heart post injury and gathered evidence that the *c-kit*+ cells in the

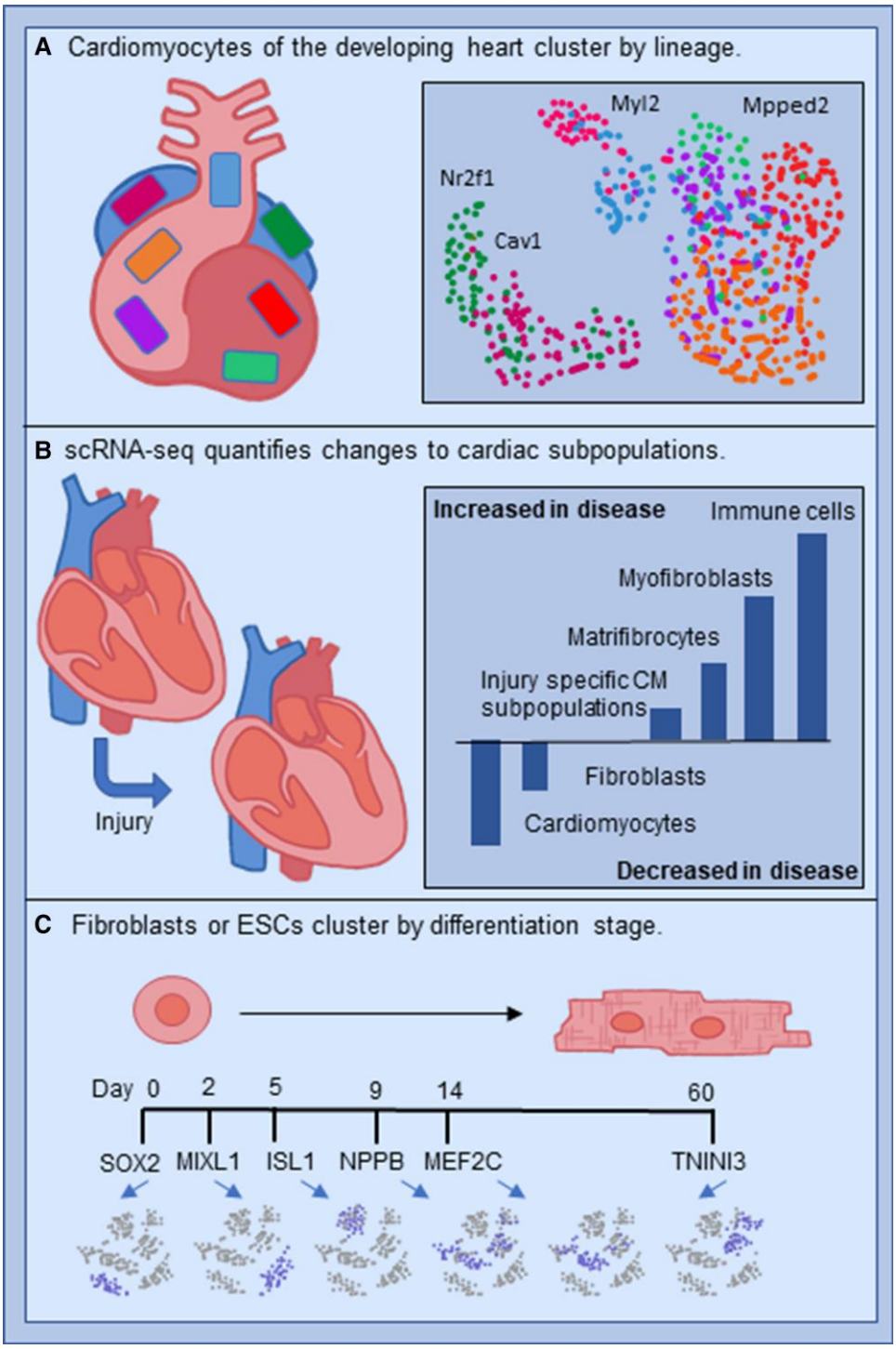

Figure 1 scRNA-seq defines distinct types of cellular heterogeneity. (*A*) Zone classification algorithms reconstruct spatial origins of individual cardiomyocytes during development. scRNA-seq data (right t-SNE plot) combined with dissections of mouse hearts at e8.5, e9.5, and e10.5 (left graphic) identified chamber-specific genes such as *Nr2f1* and *Cav1* for the right atrium and left atrium and *Myl2* and *Mpped2* for right ventricle, left ventricle, the proximal outflow tract, and the right and left ventricular septum.[11] (*B*) scRNA-seq of cardiac tissue from diseased patients or mouse injury models tracks the decline of healthy cardiomyocytes and fibroblasts while identifying cardiomyocyte subpopulations associated with specific injuries,[12] as well as novel immune cell populations,[13,14] myofibroblasts,[15] and matrifibrocytes.[16] (*C*) hiPSC-CMs from time points during *in vitro* differentiation cluster by stages of differentiation (depicted by cluster plots), which are heterogenous even at a single time point.[17,18] scRNA-seq defines differentiation subpopulations and allows comparison to *in vivo* development models, identifying key differences that could improve *in vitro* cardiomyocyte differentiation.

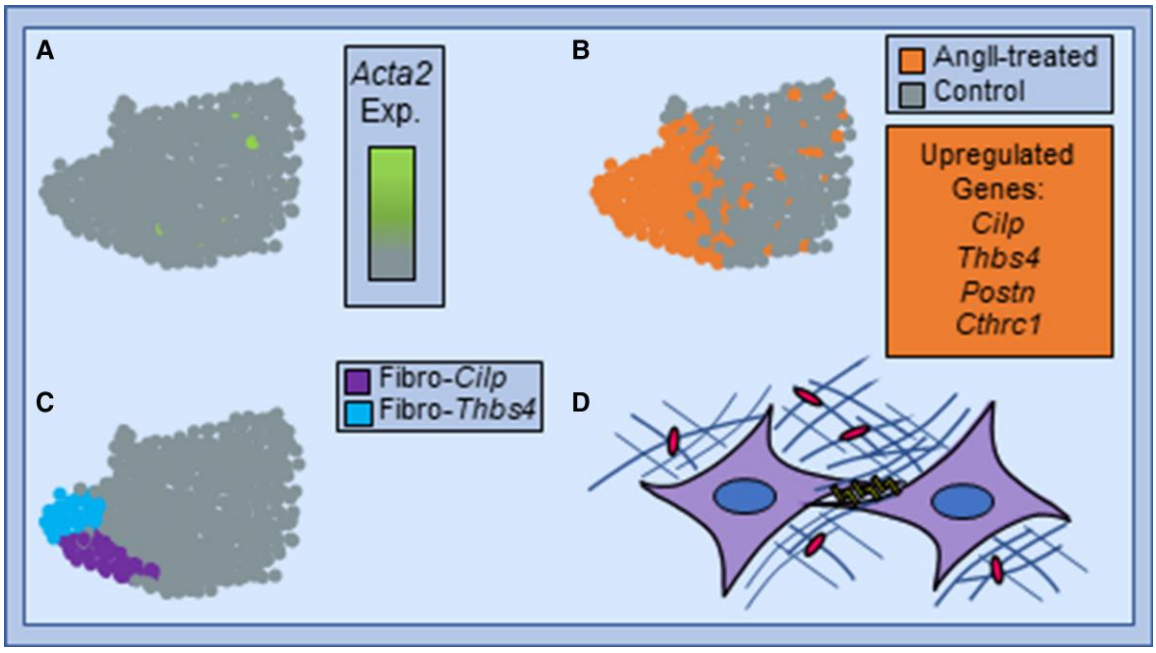

Figure 2 scRNA-seq identifies new markers of activated cardiac fibroblasts. (*A*) Expression of a classical marker of fibroblast activation, *Acta2*, in a dataset of fibroblasts isolated from hearts of healthy and angiotensin II (AngII)-treated mice identifies no clear subset of activated fibroblasts. (*B*) Single-cell transcriptomic analysis of AngII-treated and control hearts reveals that AngII treatment causes significant up-regulation of genes Cilp and Thbs4, indicating that these genes may be alternative markers of activated fibroblasts. (*C*) Querying the fibroblast population for expression of *Cilp* and *Thbs4* shows that the cells expressing high levels of *Thbs4* and *Cilp* are found in two subpopulations of fibroblasts, Fibro-*Thbs4* and Fibro-*Cilp*, respectively. (*D*) Gene ontology analysis of the Fibro-*Thbs4* and Fibro-*Cilp* subclusters reveals enhanced extracellular matrix organization (lines: collagen fibrils, ovals: matricellular proteins) and cellular adhesion (hooks: cadherins) functions compared with other fibroblasts.[28]

murine heart are endothelial cells and that cardiomyocyte progenitor cells do not exist in the adult heart.[35–37] Notably, *in vitro* cell cultures contained a 1000-fold higher amount of *c-kit*+ *Nkx2.5*+ cells than those isolated directly from the heart.[38] Thus, scRNA-seq was uniquely able to help resolve a longstanding debate in the cardiovascular field.

3. Unprecedented resolution of the developing heart

scRNA-seq has forced a re-examination of the transcriptional programmes in the developing cardiovascular system, revealing unappreciated nuance to the various lineages. scRNA-seq studies identified substantial heterogeneity in cardiac progenitor populations which had been viewed as labels of different cell lineages. Most notably, *Mesp1*+ mesoderm, which gives rise to all heart cells, was found to contain different lineage-primed subpopulations.[39] *Mesp1*+ cells exhibit marked expression at different times in development, thus specifying the lineage of left ventricle progenitors at E6.5 and right ventricle, atrial, and other progenitors at E7.5.[40] *Tbx6*, a known early development transcription factor, was also shown to play a role in determining cardiovascular and somite lineage specification via its temporal expression.[41] Thus, the heterogeneity of early heart cells likely corresponds to cardiac progenitors committed to different cardiovascular lineages as well as regions of the heart (*Figure 1*A). Cell cycle genes and cell location in the heart were often found to be the largest contributor to pre-natal heart heterogeneity, driving transcriptional shifts in each cardiac cell type.[42]

Single-cell studies allow investigators to retain the maximum transcriptomic information from every cell that can be isolated from difficult-to-access systems. For example, scRNA-seq of human foetal tissue has successfully identified new cellular populations and their dynamics throughout human heart development.[43–45] scRNA-seq allows a greater preservation of a greater extent of transcriptional diversity because the signal from individual cells is not averaged out like in bulk studies. Thus, these approaches are able to identify novel cell populations, like NOTCH-signalling-sensitive proepicardial cells,[44] *LINC00520*+ migratory endothelial cells,[45] *LGR5*+ outflow tract progenitors,[46] and *TNNT3*+ endothelial cells that recruit perivascular cells.[45] Correlation-based analyses of single-cell transcriptomes at several developmental time points can also reveal cellular dynamics and potential interactions between these populations. For example, researchers found that transcription of extracellular matrix components sharply increases in fibroblast and cardiomyocyte clusters from 5 to 6 gestational weeks, which corresponds to an increase in cardiomyocyte maturation and suggests that extracellular matrix proteins can affect cellular maturation.[44] Ligand–receptor analysis of second-trimester human foetal tissue scRNA-seq data has likewise revealed extensive cellular interactions in the developing heart.[45]

Dissecting scarce samples like foetal cardiac tissue into anatomical zones before performing scRNA-seq allows for higher resolution datasets. Using this technique, cardiomyocytes were shown to have region-specific gene expression profiles: atrial cardiomyocytes specifically express *MYH6*, *MYL7*, and *ULK4*, whereas ventricular cardiomyocytes express *MYH7*, *S100A4*, *LBH*, and higher levels of extracellular matrix genes (*Figure 1*A).[44] Comparing these atrial and ventricular cardiomyocyte

populations across developmental time revealed that these spatial transcriptional differences were driven by the expression of different sets of transcription factors at 5 gestational weeks.[44] Furthermore, some of these transcription factors were expressed specifically on the left or right side of the heart. *PITX2*, for example, a gene known to be associated with atrial arrhythmias, is expressed exclusively in left atrial cardiomyocytes.[44] Indeed, the increased dimensionality provided by combining single-cell and spatial transcriptomics is undoubtedly valuable in our understanding of the development of a complex organ like the human heart.

4. Expanding the utility of *in vitro* cardiomyocyte differentiation

As well as harvesting human foetal tissue samples, researchers have also paired scRNA-seq with cardiac differentiation protocols of human induced pluripotent stem cells (hiPSCs) to model cardiac development and disease *in vitro*. More than 10 years after hiPSCs were first developed, these cells have become a widely utilized platform to study the genetic mutations and developmental pathways associated with cardiovascular pathologies. hiPSCs allow researchers to culture and expand patient-derived cells to allow molecular and phenotypic analysis, as well as therapeutic screening.[38] The first scRNA-seq experiments on hiPSCs that were differentiated into cardiomyocytes (hiPSC-CMs) revealed cell heterogeneity, with five subpopulations of cardiomyocytes, and also provided explanations for these clusters as researchers determined that these subpopulations were related to different stages of cardiomyocyte differentiation as well as atrial or ventricular gene signatures (*Figure 1C*).[17] The technique has also identified crucial markers of cardiac lineage commitment. scRNA-seq identified crosstalk between cardiac progenitor cells and endoderm cells, showing that cellular microenvironments may support lineage commitment through factors like ETS1:[18] this crosstalk was identified by correlating differentially expressed markers with gene ontology and pathway analysis.

These combined methods have been used to track the trajectory of cardiomyocyte diseases using an approach called pseudotime analysis. This strategy takes high-dimensional scRNA-seq data and maps them on to a series of one-dimensional features. Trajectory analysis is calculated through a dimensionality reduction step followed by a trajectory modelling step that aims to identify cell states.[47] The resulting analysis measures the relative progression of each individual cell alongside the biological process of interest, such as disease progression. Pseudotime analysis dissolves the familiar cell cluster plots and replaces them with inferred trajectory paths, elucidating key branching pathways and distinct populations post injury.[48] Infant Death Syndrome was modelled with mitochondrial HADHA-deficient hiPSC-CMs and showed with pseudotime analysis that a distinct cardiomyocyte subpopulation developed into mature cardiomyocytes in healthy cells but diverged into a pathological state defined by decreased beta-oxophosphorylation in the hydratase subunit A (HADHA)-deficient cells.[49] In hiPSC-CMs derived from pulmonary atresia with intact ventricular septum patients, scRNA-seq showed that patient cells lagged in the cardiomyocyte maturation cue trajectory but not in calcium handling.[50] Both studies provide novel insights into pathologies that currently have no cure.

5. Understanding dynamics of cardiac cells in pathology

Some of the most therapeutically promising questions addressed by single-cell transcriptomics involve investigating the behaviour of various cell types in the heart in response to injury and disease (*Figure 1B*). The resolution offered by scRNA-seq is useful for examining cell populations in the adult human heart in the setting of disease or failure. For example, scRNA-seq of PCM1+ cardiomyocyte nuclei in humans with dilated cardiomyopathy found differential expression of a dedifferentiation marker, *DSTN*, in diseased cardiomyocytes compared with age-matched controls, which could not be seen by the use of bulk RNA-sequencing.[37] This study also identified several lncRNAs associated with foetal gene expression, regulation that was further investigated with knockdown experiments.

More recent single-cell studies of heart failure in dilated cardiomyopathy and coronary heart disease patients have revealed that both diseases affect the transcriptomes of cardiomyocytes in the left ventricle more than the left atrium. Cardiomyocytes from all heart failure samples also showed shared down-regulation of genes like *SPP1, HSPA1B,* and *HADHB*, but other genes varied by aetiology. Cardiomyocytes from coronary heart disease patients showed enrichment of genes involved in protein targeting in energy metabolism, whereas cells from dilated cardiomyopathy patients displayed up-regulation of genes related to muscle system processes, potentially identifying several disease-specific targets for therapy.[12] In addition to these studies in humans, single-cell work from animal models has contributed to a new appreciation for the role of distinct cell populations in the specific causes, and temporal development, of cardiovascular disease.

5.1 Pressure overload

Cardiac pressure overload causes hypertrophy and eventually leads to heart failure. In mice, this condition is commonly modelled using transverse aortic constriction (TAC) surgeries and disease progression is usually measured by echocardiography in the days and weeks following surgery. scRNA-seq can map the changes in the transcriptional programmes that orchestrate morphological and functional phenotypes of the cardiomyocytes as they progress from acute insult, to hypertrophy and then to heart failure.

One study investigating transcriptional changes in cardiomyocytes at several time points after TAC demonstrated that cell-to-cell heterogeneity increases during pressure overload, *Myh7* expression being one of the most variable markers. Trajectory analysis of cardiomyocyte remodelling after pressure overload revealed a distinct population bifurcation 2 weeks after TAC surgery. The population of failing cardiomyocytes were characterized by transcriptional profiles relating to heart contraction, actin binding, and contractile fibre while repressing mitochondrial ribosome and oxidative phosphorylation.[51] A separate single-cell study examined *Myh7* expression after TAC showed that its expression is inversely correlated with cell size,[52] in agreement with previous conventional approaches using cell sorting, immunohistochemistry, and western blotting.[6]

A third study showed dynamic changes in cardiomyocyte subpopulations exhibited throughout hypertrophy and heart failure progression. This paper also found ample evidence of fibroblast subtype switching at different time points after TAC surgery, evolving from predominantly negative regulation of inflammatory response in healthy hearts, to promotion of muscle cell development in heart failure. These cell changes in pressure overload begin to paint a picture of the complex cell-to-cell communication between cell types. Non-cardiomyocyte-secreted factors were found to correlate with biological behaviour of cardiomyocytes through ligand analysis.[23] Cell-to-cell communication may highlight a biological explanation for the existence of subpopulations of cardiac cell types: distinct subsets of cells have different functions in

response to injury and these functions must be co-ordinated at the organ level, necessitating varied subpopulations of cells that can all communicate among each other. Indeed, drugs that prevent activation of a pathology-associated population can prevent disease progression, as shown with anti-inflammation drugs targeting macrophage activation after TAC surgery.[23] Targeting injury-responsive cell populations, rather than all populations of a given cell type, may be a promising therapy.

5.2 Myocardial infarction

Single-cell methods in mouse models of MI have helped researchers understand the scope of the heart's response to MI, identifying the cellular populations that arise at various time points post-MI to preserve the heart's structure and function, and revealing possible targets for therapy. Cardiac remodelling post-MI can be divided into three phases: first, the inflammatory phase, including cardiomyocyte cell death and immune cell recruitment; second, the proliferative phase, consisting of myofibroblast activation and fibrosis; and finally, the maturation phase, in which the fibrotic scar matures, remote regions of the heart experience inflammation, and heart function is impaired.[53] Below we will discuss the insights that single-cell techniques have provided about the heart's cellular dynamics throughout these phases.

5.3 MI: inflammatory phase

The inflammatory phase of MI includes massive cardiomyocyte cell death due to ischaemic injury. Therefore, re-activating cardiomyocyte proliferation is seen as a potential avenue for treatment. To this end, several studies have leveraged a combination of lineage tracing and scRNA-seq to examine the surviving cardiomyocyte populations after MI to determine their regenerative potential. Single-cell sequencing of embryonic and post-natal cardiomyocytes in mice has revealed a proliferative, 'progenitor-like' αMHC+ cardiomyocyte population that decreases in number after birth. Clonal analysis shows that MI can reactivate these cardiomyocytes' proliferative potential in neonatal hearts[36] in contrast to the majority of adult cardiomyocytes which exhibit limited regenerative capacity. Using a triple-labelling method to mark the nuclei of mature and dedifferentiated adult cardiomyocytes, investigators concluded that the genes up-regulated in these dedifferentiated cells were related to the cell cycle, indicating that it is the dedifferentiation of adult cardiomyocytes, not the differentiation of cardiac progenitor cells, that allows for limited regeneration post MI.[54]

Another possible therapeutic route involves regulating the healing potential of immune cells that appear in the heart in the inflammatory phase of MI. Immediately upon MI, the heart responds with a strong pro-inflammatory response: single-cell studies of the infarcted heart note the appearance of new immune populations in the heart between 1 and 7 days post-MI.[13–15] Characterizing these subpopulations of immune cells and generating a diffusion map (a type of pseudotemporal analysis)[55] of these cell states reveals the temporal shift from early infiltrating M1 monocytes to classical inflammatory monocyte-derived M1 macrophages at Day 3 post-MI, and another shift to non-classical reparative, anti-inflammatory M2 macrophages at Day 7 post-MI.[15] These studies were also able to identify subpopulations of cells and associated signalling pathways that may be targeted to improve health outcomes: for example, the identification of a population of infiltrating leucocytes called interferon inducible cells, has allowed investigators to disrupt the cGAS–STING–IRF3 pathway and improve cardiac contraction and decrease dilation and incidence of rupture post-MI.[13]

5.4 MI: proliferative phase

The proliferative phase of MI includes the activation and differentiation of cardiac fibroblasts into a myofibroblast phenotype to provide structure and support contractile function in the infarcted area. Characterizing the differentiation trajectory and the regulators of the cardiac fibroblast to myofibroblast transition is the focus of many studies, because targeting the overactivation of cardiac fibroblasts is a therapeutic strategy to prevent cardiac fibrosis. Single-cell studies in lineage-traced cardiac fibroblast cells in normal and infarcted hearts at various time points post-MI has identified disease-specific cardiac fibroblast populations, as well as the differentiation trajectory of cardiac fibroblasts into the myofibroblast phenotype and the heterogeneity of myofibroblast themselves. One study used a Pdgfra-GFP reporter mouse line to isolate the cardiac fibroblast lineage from hearts 3 days and 7 days post-MI for scRNA-seq.[15] The researchers found 11 fibroblast populations, 5 of which were seen exclusively after MI, including proliferative, activated, and myofibroblast clusters. Trajectory analysis of the GFP+ population at 3 days post-MI showed a continuum from homeostatic clusters to activated and proliferative ones. Trajectory analysis of the myofibroblast population in these hearts, which only appeared at 7 days post-MI, showed 3 separate subclusters of myofibroblasts, termed MYO-1, MYO-2, and MYO-3. MYO-2 and MYO-3 expressed pro-fibrotic genes, while MYO-1 expressed an anti-fibrotic gene signature, expressing inhibitors of WNT and TGF-β signalling. The heterogeneity of the myofibroblast population revealed by scRNA-seq suggests that myofibroblast differentiation may not be linear and may contain cell states with varying fates in the maturing scar.[15]

Beyond characterizing the cell states in infarcted hearts, single-cell studies of MI in genetic knockout animals are beginning to add greater mechanistic insights into the transition from cardiac fibroblast to myofibroblast. For example, investigators were able to implicate the Hippo pathway in regulating the differentiation of fibroblasts to myofibroblasts, as the conditional deletion of *Lats1/2* in Tcf21+ cells led to a spontaneous transition into the myofibroblast state with a highly pro-fibrotic and pro-inflammatory phenotype, even in non-infarcted, healthy mice.[56] This phenotype can be seen in the scRNA-seq data as separate clusters of cardiac fibroblasts on a UMAP plot and increased interactions with macrophages in ligand–receptor analyses. MI enhanced this fibrotic phenotype, and trajectory analysis of the scRNA-seq data showed that wild-type resting cardiac fibroblasts were on the opposite end of a trajectory with the MI-induced knockout myofibroblasts, with wild-type MI-induced activated fibroblasts residing between them. Reducing downstream effectors of the Hippo pathway in the conditional knockout mice attenuated the fibrosis and heart failure after MI, definitively linking the Hippo pathway to the maintenance of the resting cardiac fibroblast state.[56]

5.5 MI: maturation phase

Several studies have reported interesting, and sometimes conflicting, findings about the subpopulations of cardiac fibroblasts involved in scar maturation. For example, a scRNA-seq study on cardiac fibroblasts at 14 days post-MI identified 11 cardiac fibroblast subpopulations, including an activated *Fstl1*+ population that persisted in the scar and was necessary for scar formation to prevent cardiac rupture.[35] Additionally, single-cell studies of the mouse heart at the maturation phase of MI have allowed for the continued characterization of the cell type known as the matrifibrocyte, which expresses extracellular matrix and tendon genes, like *Chad*, *Comp*, and *Cilp2*, that specifically support scar maturation.[16]

More recently, investigators isolated cardiac fibroblasts from hearts at later time points post-MI (7, 14, and 30 days), revealing 11 subpopulations of cardiac fibroblasts, 1 of which was pericyte-like, 4 of which were distinct fibroblast subtypes, and 7 of which represented intermediate fibroblast cell types. One of these subclusters, marked by expression of *Cthrc1*, was dubbed a 'Reparative Cardiac Fibroblast' (RCF) cluster. The RCF cluster, enriched in gene expression related to extracellular matrix organization and assembly and collagen fibril organization, was shown by RNA velocity analysis and latent time analysis to be the final activation stage of a subset of *Postn*+ cells in response to MI.[29] It is possible that RCF cells are similar to, or the same as, those previously characterized as matrifibrocytes. The use of epigenetic assays can begin to untangle some of these cell type differences and transitions and answer more mechanistic questions with scRNA-seq datasets. The use of chromatin immunoprecipitation with sequencing (ChIP-seq) and ATAC-seq paired with motif analysis has allowed investigators to begin to identify the possible regulators of RCF cells, including candidates like SOX9, SMAD, JUN, TEAD, and RUNX1, among others.[29]

Cardiac cell-to-cell heterogeneity is most relevant to models of disease because different stresses on the heart often lead to the activation of specific subpopulations of cardiac cells which promote the pathology in an otherwise healthy organ. This phenomenon can be seen in hypertension, where fibrosis incited by AngII treatment leads to the emergence of two distinct cardiac fibroblast populations marked by *Cilp* and *Thbs4* (*Figure 2*). Flow cytometry was used to show that the heart did not experience a net increase in fibroblasts and pseudotime analysis demonstrated that these subpopulations emerged from existing fibroblast cells.[28]

6. Relevance of epigenetics to heterogeneity of cell populations

Chromatin structure and accessibility are the readouts of the actions of various chromatin remodelling enzymes, histone-modifying enzymes, and histone binding proteins (including the writers, erasers, and readers of histone modification language).[57,58] Some of these proteins exhibit cell type specificity in terms of their own expression. Many of the chromatin modifiers, however, are cell type independent and rely on the activities of cardiac transcription factors or other as yet unidentified processes (perhaps cell type-specific lncRNAs) to produce specific transcriptomes.[59] Much of our knowledge about the role of histone-modifying enzymes in cardiac biology has come from myocyte-specific gain and loss of function studies, primarily the alpha-myosin heavy chain promoter.[60] The histone demethylase JMJD2A was examined in mouse hearts using alpha-MHC-mediated gain and loss of function: depletion of JMJD2A blocked, and overexpression accentuated, pressure overload-induced hypertrophy, with minimal effects on basal phenotype.[61] Similarly, loss of the histone methyltransferase Smyd1 in cardiomyocytes-induced hypertrophy[62] and impaired mitochondrial energetics,[63] precipitating heart failure. Myocyte-specific overexpression of KDM4D, a histone demethylase that targets the silencing mark H3K9Me3, reduced this mark and activated cell cycling, leading to myocyte hyperplasia and increased muscle mass.[64] Depletion of the histone methyltransferase G9A/EHMT2 induced hypertrophy and activated the expression of foetal genes in adult cardiomyocytes.[65,66] The histone methyltransferase Setd2 was necessary for proliferation of myoblasts in culture[67] and for proper cardiac development *in vivo*.[68] Alternatively, chromatin readers such as the BET-bromodomain containing Brd4, have been shown to exert powerful effects in the setting of hypertrophic stimuli, such that loss of Brd4 or its chemical inhibition serve and methods to prevent heart failure.[69] The role of demethylases in the heart has recently been reviewed in detail.[70]

The first insights into cardiac myocyte chromatin accessibility and structure came from techniques that measured these features indirectly or not at all. Genetic and pharmacologic manipulation of histone deacetylases (HDACs) has been shown through extensive studies from many laboratories to regulate cardiac development and disease-associated remodelling.[71,72] Using ChIP-seq against known histone marks, two early papers in embryonic stem cell-derived mouse[73] or human [74] cardiac cells identified temporal changes in lineage specific transcription and histone modification. Adaptation of the heart to pressure overload is associated with alterations in histone stoichiometry[75] and aberrant expression of chromatin structural proteins associated with chromatin compaction.[76,77] Imaging-based approaches to cardiac chromatin organization have shown that adrenergic stimuli induce changes in global chromatin compaction over time scales associated with hypertrophic growth of neonatal myocytes in culture.[78] These approaches also show that myocyte growth is associated with focal increases in RNA polymerase II activity and locus-specific reorganization of genes within the nucleus:[79] genes are silenced or activated by increasing or decreasing (respectively) their association with the nuclear periphery, central heterochromatin centres, or both.

The most comprehensive analysis of cardiac transcription factor occupancy to date was carried out using biotinylated knock-in alleles to enable endogenous evaluation of protein localization while maintaining normal stoichiometry throughout development.[80] This study examined 7 essential transcription factors involved in cardiac lineage commitment and proper organ development (GATA4, NKX2-5, MEF2A, MEF2C, SRF, TBX5, and TEAD1) finding that, akin to what has been shown in other organs, occupancy at individual loci is often characterized by more than one transcription factor, often operating in a co-operative manner, and chromatin accessibility around genes expressed in the heart is facilitated by the localization of multiple tissue specific transcription factors.[80]

What remains unclear from these experiments is to what extent the appearance of co-operative binding or co-occupancy is due to the actions of different proteins in different subpopulations of cells—i.e. whether a single copy of a locus is occupied by multiple proteins in the same cell, or the result of multiple distinct subpopulations experimentally averaged in a bulk sequencing experiment. Distinguishing between these two possibilities requires single-cell experiments. Likewise, histone variants and histone modifications are mechanisms to control accessibility, operating in combination rather than through the actions of single proteins or post-translational modifications. This observation cautions pause in the interpretation of findings from genetic gain or loss of function targeting the actions of a single reader, enzyme, or other chromatin-binding protein: whether the resulting phenotypes are the result of genome-wide changes in chromatin features—and in which populations of cells these changes occur—must be measured.

7. Combining single-cell transcriptomics with epigenomics

Epigenomics is the next frontier in understanding maintenance and interconversion among developmental and injury-induced cell populations in the heart. Development and cellular reprogramming studies

ask distinct mechanistic questions about heterogeneity, focusing on lineage control. In contrast, studies in disease models describe and interrogate the mechanisms of adaptive cellular heterogeneity within an ostensibly terminally differentiated organ in response to stimuli (*Figure 3*).

7.1 Cellular reprogramming

DNA methylation, the addition of a methyl group to the fifth carbon of a cytosine, is an epigenetic mark found throughout the genome and which is associated with repression when enriched in regulatory regions.[84] One of the first examples of epigenetic approaches coupled with scRNA-seq examined the dedifferentiation of adult cardiomyocytes into cardiac progenitor-like cells using bisulfite sequencing to examine methylation. Compared with freshly isolated cardiomyocytes, 3-day cultured and dedifferentiated cardiomyocytes exhibit vastly different global methylation profiles, with cardiac progenitor-like cells having more hypermethylated regions.[81] These data correlate well with scRNA-seq which show that cardiomyocyte genes are down-regulated while embryonic genes and growth factors like *Sox4* and *Ereg* are hypomethylated (*Figure 3B*). These experiments suggest that methylation regulates some aspects of cardiac cell plasticity.

These findings were expanded upon in studies examining the direct reprogramming of human fibroblasts into induced cardiomyocytes (hiCMs). scRNA-seq of these cells during the reprogramming process revealed an up-regulation in immune response genes. Knocking down candidate immune regulatory genes like *TLR3* greatly decreases cardiomyocyte induction.[85] An shRNA loss-of-function screen identified several epigenetic modifiers that mediate the effect of the immune response on hiCM reprogramming, including *TET1*. A knockdown of *TET1* resulted in a four-fold increase in percentage of hiCM compared

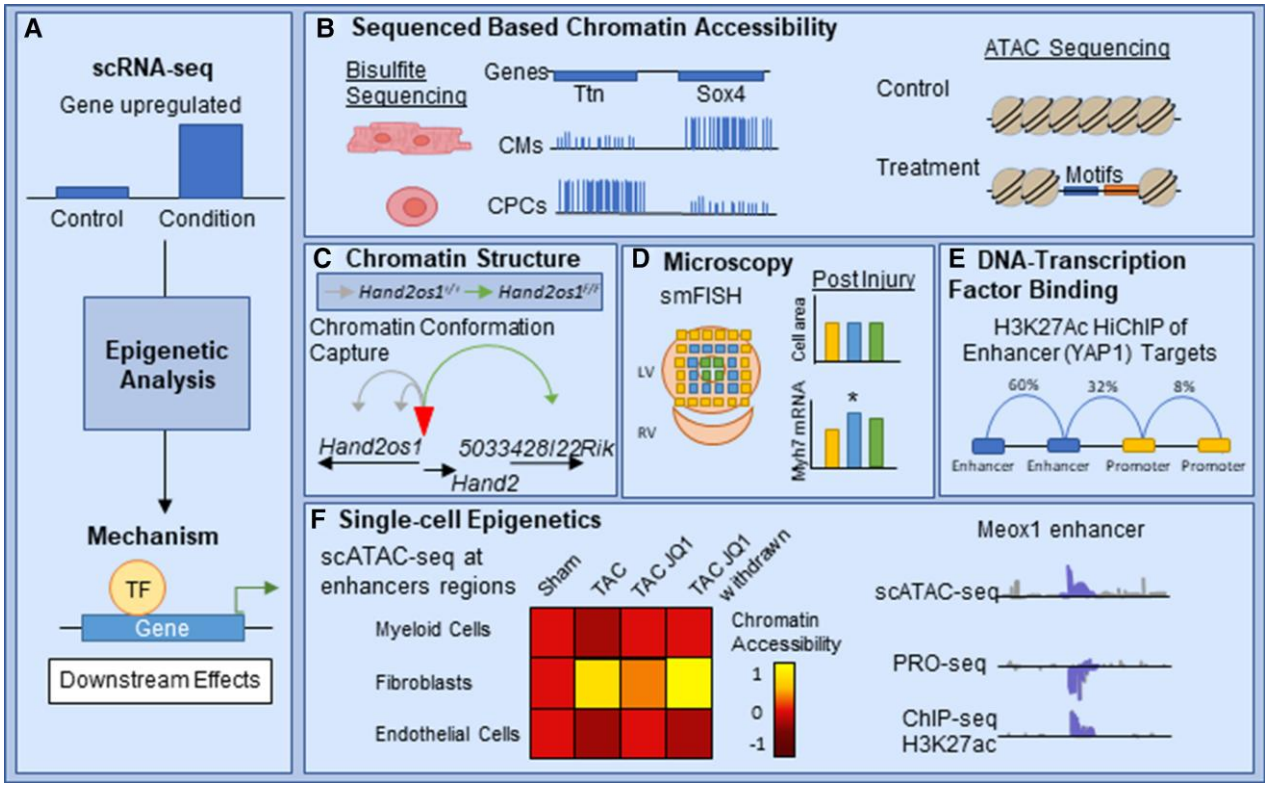

Figure 3 Integrating epigenomic with transcriptomic analysis. (*A*) Many types of epigenetic analysis are used in conjunction with scRNA-seq data to elucidate the mechanism of transcriptional interactions. (*B*) Sequencing-based methods of chromatin accessibility. Left: bisulfite sequencing data (vertical lines) performed on freshly isolated cardiomyocytes (top track) and reprogrammed CPCs (bottom track) reveal hypomethylation at promoters of genes associated with contraction (*Ttn*) and hypermethylation at promoters of embryonic transcription factors (*Sox4*) in mature CMs (top track), a signature that is reversed as cells are reprogrammed into cardiac progenitor CPCs.[81] Right: Motif analysis of regions of differential chromatin accessibility between control and treatment conditions may reveal transcription factors at play.[29] (*C*) Chromatin conformation capture of murine cardiomyocytes with deletion of the *Hand2os1* locus shows that the *Hand2* promoter, which normally uses upstream enhancers (left arrows), uses an alternative downstream enhancer to drive expression (right arrow).[82] (*D*) smFISH of mouse hearts exposed to TAC shows that only the middle layer of myocardium (blue squares on tissue diagram) re-expresses foetal genes (*Myh7*) in response to injury.[52] (*E*) HiChIP of H3K27Ac identifies which relevant enhancers and promoters are interacting. Yap1 was shown to primarily occupy enhancer loops (60%) and enhancer–promoter loops (32%), elucidating Yap1's mechanism as a master regulator in response to myocardial infarction.[56] (*F*) Single-cell epigenomics can reveal the cell types responsible for a specific response, potential factors of interest within these cell types, and their mechanism of action. Left: single-cell ATAC-seq (scATAC-seq) in murine hearts in pressure overload revealed a strong reaction to JQ1 treatment and withdrawal only in the cardiac fibroblast subpopulation (chromatin accessibility heat map). Right: Further investigation *in vitro* with PRO-seq (middle track) and histone ChIP-seq (bottom track) allowed the identification of Meox1, a regulator of fibroblast activation.[83] CPCs, cardiac progenitor cells; CM, cardiomyocytes.

with control treatment. Bisulfite sequencing in these *TET1* and *TLD3* knockdown cells showed reduced methylation rates at the promoter regions of cardiac genes. These data suggest that the immune response is critical for myocyte fate acquisition during hiCM reprogramming, possibly through impacting DNA methylation status of cardiac loci. By factoring in methylation into their analysis, the researchers were able to identify previously unexplored mechanisms that potentially restrict cells from reprogramming. Recent single-cell studies have also revealed the genome-wide nature of chromatin accessibility changes and epigenetic mechanisms that underpin cardiac cell reprogramming via *Mef2c*, *Gata4*, and *Tbx5*,[86] identifying Smad3 as a key regulatory node. This discovery is a promising new direction to improve the reprogramming process as well as to tailor it to the production of a specific type of cardiomyocytes.

Beyond DNA methylation, recent work in direct reprogramming has focused on the contribution of chromatin structure and combinatorial transcription factor binding to cellular reprogramming (*Figure 4A*). Although it has long been known that ectopic expression of transcription factors like *Gata4*, *Mef2c*, and *Tbx5* (GMT) induce cardiomyocyte formation from cardiac fibroblasts,[89] the mechanisms by which these factors open heterochromatin and induce reprogramming has only recently been illuminated.[87] ATAC-seq was employed to infer regions of increased accessibility, as well as to map regions of transcription factor binding and nucleosome density. Performing scRNA-seq and ATAC-seq on cardiac cells at various stages of reprogramming showed that the cells begin expressing transcriptional signatures of cardiomyocytes, as well as undergo a rapid gain in chromatin accessibility in distal gene regions, within 48 hours. Using a machine learning algorithm that predicts the transcription factor motifs correlated with the transcriptional changes occurring within these first 48 hours, the study found that Tbx5 is a key driver of the reprogramming process, followed closely by Mef2c. ChIP-seq of GMT (alone or in combination with each other) demonstrated that each transcription factor acts in a locus-specific manner to induce expression or repression of their target genes (*Figure 4A*). Integration of GMT ChIP-seq and ATAC-seq data revealed that for some loci, either Mef2c or Tbx5 binding is sufficient to increase accessibility, while other loci require binding of all three factors. Integration of GMT ChIP-seq and scRNA-seq data also showed a varied interdependency that suggests that GMT can direct lineage conversion via mechanisms not limited to strict synergistic activation among these transcription factors.

7.2 Development

Epigenetic techniques have been used extensively to study mechanisms driving cell fate transitions under cardiac embryonic development. Tet-mediated DNA demethylation is also involved in heart development, playing a role in modulating long-range chromatin interactions to co-ordinate high-order chromatin organization. One study demonstrates that both human and mouse embryo tissue show stable global levels of methylation throughout development, examining both 5-mC methylation using bisulfite-seq and 5-hydroxymethylcytosine using CMS-IP-seq. Interestingly, the authors detected dynamic changes at focal regions that are stage specific, with the majority of these changes enriched at cis-regulatory elements for Bmp10 and Tnnt2.[90]

One of the first scATAC-seq experiments in mouse cardiac tissue isolated cardiac progenitor cells from E7.5 to E9.5 development time points.[88] Correlating chromatin accessibility with scRNA-seq found that the RNA expression of transcription factors directly matches accessibility of corresponding transcription factor binding sites in cardiomyocytes. The analysis also determined that expression of

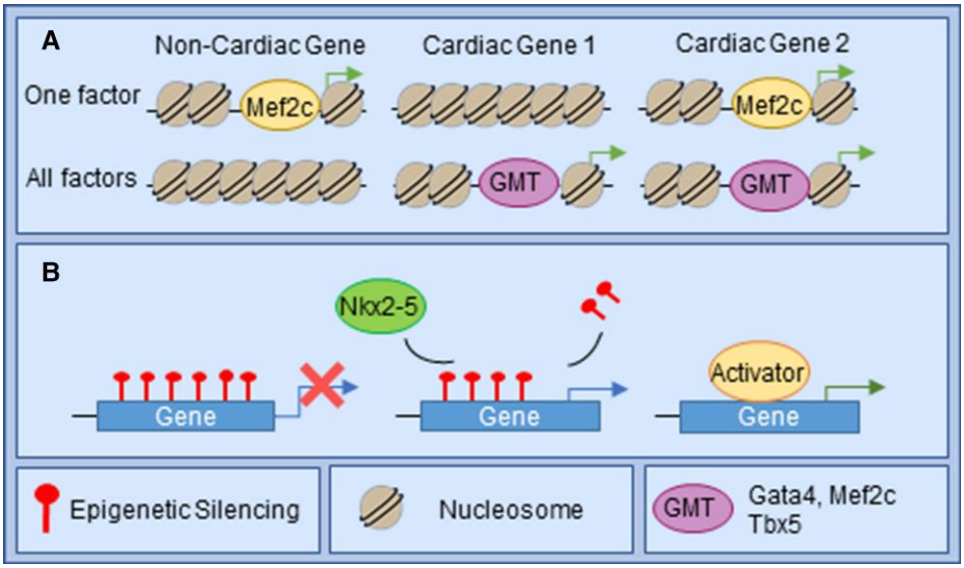

Figure 4 Mechanisms of transcriptional activation revealed by transcription factor ChIP-seq. (*A*) ChIP-seq of Gata4, Mef2c, and Tbx5 (GMT) during cardiac fibroblast reprogramming into a cardiomyocyte-like state showed that cardiac transcription factors exhibit extensive collaborative binding[80] that drives chromatin accessibility (visualized through ATAC-seq). Individually, the GMT transcription factors increased accessibility in a subsection of genes in and outside the heart (top row). In combination, the GMT transcription factors demonstrated a specific increase in chromatin accessibility of cardiac reprogramming factors, generating a robust response (bottom row).[87] (*B*) scATAC-seq analysis examining cardiac embryonic development showed that expression of transcription factors precedes accessibility of the corresponding motifs, suggesting that transcription factors can trigger epigenetic reorganization.[88]

transcription factors precedes accessibility of the corresponding motif, implying that transcription factor activity triggers epigenetic reorganization that opens the corresponding motif (*Figure 4B*). Follow-up analysis with bulk ATAC-seq on more developmental time points examined overexpression of *Nkx2-5*, a canonical cardiac development transcription factor that is a core component of transcriptional complexes that positively regulate the cardiac programme.[88] *Nkx2-5* overexpression increases global accessibility in a striking example of a single transcription factor altering significant fractions of the cell's chromatin structure. However, these *de novo* openings are not sustainable and disappear at later time points, indicating strong parallel regulatory control of cardiac development.

Upstream of *Nkx2-5*, a constellation of histone marks was shown to correlate with cardiac development. The methyltransferase SETD7 has been shown to bind the activating histone mark H3K36me3 at targets of distinct sets of genes to drive their stage-specific expression during cardiomyocyte differentiation.[91] Using ChIP-seq, this study shows that SETD7 promotes transcription of SWI/SNF targets during the mesodermal stages and then *Nkx2-5* targets in cardiac progenitor cells, correlated with the pattern of histone methylation.

The role of long non-coding RNAs (lncRNAs) has also been examined in cardiac development using epigenetic and single-cell methods. lncRNAs have been proposed to exert locus-specific control of gene expression. Various cardiac-specific lncRNAs have been described, including several involved in disease progression in the adult heart.[92–98] lncRNAs have been accorded a range of possible behaviours to directly regulate chromatin structure or transcription, including the formation of triple helices with DNA (thereby potentially affording locus-specific targeting)[99] and recruitment of silencing machinery like Polycomb repressive complexes or DNA methylation machinery (primarily on the basis of work from one of the most well characterized lncRNAs, *Xist*, which silences the X chromosome during Barr body formation).[100] lncRNAs may also contribute to nuclear organization and chromatin compartmentalization based on the immiscibility of protein and nucleotides or so-called phase separation, whereby RNA and/or protein accretes on DNA at specific loci to alter gene expression or to form membraneless suborganelles.[101,102]

None of these mechanisms, to our knowledge, has been definitively tested in cardiac cells, although there is some limited evidence that several lncRNAs can bind cardiac chromatin and alter transcription. Using HiCap (genome-wide promoter capture method[103]) and 3C (Chromosome Conformation Capture[104]), researchers examined the mechanism of lncRNAs in cardiac development, focusing on *Hand2os1/Uph*, located on the opposite strand of *Hand2*, a gene that encodes a key cardiac development transcription factor.[82] HiCap on mouse embryonic stem cells revealed that the lncRNA and the *Hand2* gene sit on the boundary between two topologically associated domains: one open and gene-rich, the other closed and gene-poor. Despite the limited scope of 3C—which examines interactions between loci selected by the experimenter—chromatin contacts in ESCs with partial lncRNA deletion suggested engagement of alternative downstream enhancers with the *Hand2* promoter, in addition to regulatory elements embedded in the *Hand2os1* locus. These data demonstrate that lncRNAs may regulate genes through control of chromatin spatial organization. However, based on current available evidence, it is doubtful that there is a single mechanism of action for all chromatin regulatory lncRNAs: they likely perform a wide array of molecular functions as determined by tertiary structure, localization and interaction with other molecules.

7.3 Injury and disease

A key unanswered question from transcriptome analyses is how changes in gene expression become entrained in different populations of cells (and indeed whether there is such a thing as epigenetic memory in the context of a multifactorial, evolving disease like heart failure). The Hippo signalling pathway has been implicated in restraining the transition of cardiac fibroblasts to myofibroblasts in response to injury.[56] Conditional deletion of *Lats1/2* in adult murine hearts showed a spontaneous transition of cardiac fibroblasts to a myofibroblast phenotype, a transition exacerbated after MI. scRNA-seq and gene regulatory network analysis of sham *Lats1/2* conditional knockout hearts showed an increase in fibrotic gene expression among the cardiac fibroblasts and an increase in myeloid cellular heterogeneity that recapitulates those seen in injured hearts. ATAC-seq showed that open chromatin regions in the *Lats1/2* knockout cells were enriched in motifs for Tead and Yap. A combination of CUT&RUN,[105] a method to identify protein interactions with DNA, and HiChIP,[106] a method that can identify promoter-enhancer chromatin interactions, revealed that Yap is directly activating the myofibroblast cell identity genes, such as *Myc*, and pro-inflammatory genes. Knocking down Yap in *Lats1/2* mutant cardiac fibroblasts attenuated the fibrotic phenotype post infarction. Thus, Hippo signalling limits cardiac fibroblast fate transitions and proliferation.

The molecular regulators of cellular heterogeneity in response to cardiac stress have also been investigated in the context of a transition of a subpopulation of cardiac fibroblasts from an activated, pro-fibrotic phenotype to a RCF phenotype.[29] In this study, the authors used published ChIP-seq datasets to identify transcription factors that were bound in the vicinity of RCF-up-regulated genes, identifying SOX9 and SMAD3 as potential candidates. Overexpression of SOX9 partially replicated the RCF transcriptional signature. ATAC-seq experiments also allowed for motif analysis of differentially accessible regions in these cells to identify transcription factors that might drive RCF identity. The authors identified several other transcription factor candidates, like RUNX1 and JUN, but the overexpression of *Runx1* was not sufficient to induce the transcription signature of RCFs. Although the authors were unable to conclusively determine the factors that induce cells into this population, the use of ChIP-seq and ATAC-seq datasets allows for identification of candidate factors and provides a starting point to answer this question.

In some cases, epigenetic analyses have determined how a subpopulation of cells can be targeted to reverse disease progression. A study used scATAC-seq to examine the chromatin accessibility changes of reversible activation of cardiac fibroblasts by the BET inhibitor JQ1. The analysis revealed transcription factor binding motifs for CEBPB, JUN, and MEOX1 were enriched in open chromatin states after TAC surgery that were transiently reversed with JQ1 treatment. To establish that chromatin dynamics occur in regions relevant to stress-activated transcription factors, the study ran a correlation analysis which ranked enhancers on the measure of correlation between chromatin accessibility and cardiac ejection fraction. One of the topmost negatively correlated enhancers was found to regulate *Meox1,* a gene expressed in low levels in a healthy heart but up-regulated during pressure overload. The scATAC-seq and scRNA-seq datasets were used to determine an upstream TGFB1 binding motif that drove the expression of this novel regulation of fibroblast plasticity and pro-fibrotic function.[83]

Researchers now have access to an entire atlas of adult human heart snATAC-seq data that highlights candidate cis-regulatory elements that allow investigation of links between transcriptional regulatory

programmes and non-coding risk variants for cardiovascular disease traits.[107] This and other such studies demonstrate the therapeutic or diagnostic potential of epigenetic investigations, that is, by allowing for testing of the hypothesis that engineered reorganization of chromatin accessibility in non-coding regions may promote beneficial gene expression and/or by enabling the use of changes in accessibility of these regions during the course of disease for more precise and informative diagnosis of risk.

An imminent advancement in single-cell technologies is multilayered sequencing from the same cell. This approach integrates different layers of 'omics information collected from individual cells using analytic tools such as Seurat's IntegrateData and TransferData functions[108] or LIGER.[109] Technologies like 10X Genomics' single-cell Multiome ATAC + Gene Expression Sequencing protocol, SNARE-seq[110] and Paired-seq,[111] now enable the measurement of chromatin accessibility and whole-transcriptome profiles from each individual cell,[112] although they have yet to be used on cardiac samples.

8. Proteomics and cellular heterogeneity

Although it was predicted by some that single-cell technologies would 'redefine cell identity,' making it less dependent on strict hierarchies and pre-defined markers,[113] most papers still highlight discovery of novel cell populations within the context of cells identified on the basis of previously known markers. An interesting question to consider: what is the evolutionary advantage proffered by cellular heterogeneity in the heart? Some heterogeneity can be explained through spatial analysis. Sampling tissue from six different regions of the healthy adult heart combined with single molecule fluorescence *in situ* hybridization revealed that cardiomyocyte subpopulations often grouped by location, indicative of different developmental origins, distinctive hemodynamic forces, and specialized functions in cardiac chambers.[114] Chamber specificity, however, did not account for all cardiomyocyte diversity as well as the heterogeneity of other cell types.

To explain this persistent heterogeneity, early hypotheses suggested either that the transcriptional heterogeneity was buffered at the protein level, or that this diversity reflected the nature of cell tissue that is poised to respond to a diverse range of stimuli[115]—two non-competing explanations that await formal proof.

Few single-cell and proteomics data sets have been jointly analysed in the heart because of the gulf in number of identified populations, such as 12 distinct cell clusters found with scRNA-seq compared to only two clusters with proteomics.[4] Outside of the cardiology field, single-cell data are often compressed to bulk before they are compared with the proteome.[116] Recently, new techniques have allowed for a direct comparison in macrophages, finding that the majority of genes exhibit qualitatively similar RNA and protein profiles, although there is significantly more variability in the scRNA-seq data.[117] Thus, while more studies are needed to definitively show that the cell transcriptomic heterogeneity is not lost at the protein level, proteomic analyses can still measure cellular heterogeneity and the continuum of proteome states within a single-cell population.[117] Technical challenges still limit the depth of such studies and larger sample sizes have to be collected before concrete conclusions can be drawn.[112]

Fewer proteomic cell clusters raise the question of the biological meaning behind the transcriptional diversity detected by scRNA-seq. Several papers have embraced a theory of intercellular competition as an explanation for heterogeneity, as hypothesized in the case of Hippo-induced cell competition between cardiac fibroblasts and other cells,[56] reflecting a 'survival of the fittest' model where cells in a tissue exhibit a degree of healthy competition that is exploited by cancer cells.[118] A different hypothesis is the 'bet-hedging' model where heterogeneity exists to prepare the organism for a variety of environmental stressors (Myh7 variability in cardiac disease being a potential exemplar of this model[6,52]), even within clonal populations.[119] Currently, it falls on individual studies to explain the source and potential biological implications of heterogeneity in cell populations. Many studies have identified cell location as a main driving factor of differential gene expression.[44,120] In the developing heart, cell cycle phase is hypothesized to drive the majority of transcriptional shift between cardiac subpopulations.[42]

9. Outlook

9.1 Clinical potential

Throughout this review, we have highlighted the therapeutic potential of single-cell technologies relating to cardiovascular disease, mostly in the realm of discovery of possible drug targets. On a broader level, the newly appreciated scale of cell-to-cell heterogeneity may explain the varied response to drugs among patients. By understanding cardiac cellular heterogeneity, basic research scientists can provide clinicians with tools to employ single-cell-based precision medicine where, for example, patient single-cell transcriptomic data would be screened to inform clinical intervention.[19] Epigenetic analysis adds another layer of information that can be harnessed for therapeutic intervention. Several drugs that erase or write epigenetic signatures are already in clinical use, such as HDAC inhibitors, methyl donors, and BET inhibitors, among others.[121] As studies continue to deploy these rapidly developing single-cell technologies, patient-specific therapies become more possible.

9.2 Drawbacks of single-cell sequencing

Single-cell sequencing techniques have vastly improved in the past 10 years. While some of the first studies analysed hundreds or thousands of cells,[34] studies today boast hundreds of thousands of individual cell transcriptomes. However, cell isolation and library preparation challenges persist. Isolation of cells from cardiac tissue, especially enzymatic digestion of highly fibrotic tissue, remains difficult and may introduce bias in the cells isolated and changes in their transcriptional profiles. To address this, several papers have compared various digestion protocols for murine and human hearts,[20,122] and research has shown that nuclear RNA isolation and profiling, rather than cytoplasmic or whole-cell, may help alleviate some of these biases.[122] Isolation of rare or hard-to-isolate cell types, like cardiac conduction cells, can also benefit from Cre-driven reporter expression to guide the dissection of these tissues.[123] Even so, the batch-to-batch and lab-to-lab variability of cell isolation and library preparation remain problematic.

Data produced by scRNA-seq are naturally sparse, but are exacerbated by a higher level of technical noise than bulk RNA-seq data.[124] Inefficiencies in the capture, reverse transcription, or amplification of a transcript can cause a transcript to go undetected by scRNA-seq, known as a 'dropout event.' Dropout events pose a significant analytical issue that must be accounted for by various imputation techniques in downstream analysis.[124–126] Indeed, the high levels of technical noise in scRNA-seq data requires investigators to take strict quality control measures to remove low-quality cells and perform appropriate batch correction at the start of every analysis.

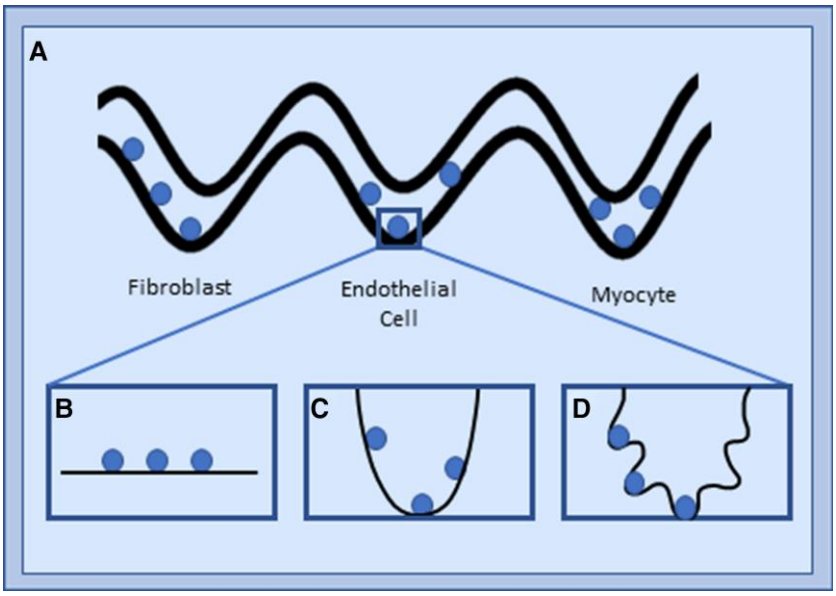

Figure 5 Limits of meaningful cellular variability. (*A*) Waddington landscape depicting three mature cardiac cell types in the adult heart: fibroblasts, endothelial cells, and cardiomyocytes. (*B–D*) Zooming in on each branch with the resolution afforded by single-cell techniques provides three possible options for the topography of each cell type. (*B*) Each cell type may be completely homogenous, with no transcriptional or epigenetic differences distinguishing each cell. (*C*) Each cell type may have heterogeneity caused by transient transcriptional differences, not indicative of distinct substates. (*D*) Each cell type may contain distinct, semi-stable subpopulations of cells that perform different functions.

To interpret single-cell sequencing data, researchers rely on non-linear dimensionality reduction and clustering algorithms that can be biased by the choice of the algorithm and specification of parameters. Non-linear dimensionality reduction methods that were once the go-to analytical method have sometimes proved ineffectual in the long-run. For example, t-SNE plots,[127] once a staple of any scRNA-seq paper, have mostly fallen out of favour due to drawbacks in the algorithm that make the plots difficult to interpret. Both t-SNE and a popular alternative, the UMAP plotting algorithm,[128] have been criticized for introducing distortion into local or global structure, which can affect downstream analyses like pseudotime.[129,130] To address such uncertainties, studies can now employ scATAC-seq in combination with scRNA-seq to validate cell types revealed by clustering in both datasets.[131]

A separate review has recently covered in-depth the challenges of single-cell technologies.[124] Like any novel technology, best practices for sample collection and data analysis of single-cell sequencing experiments are constantly evolving. Any data from these studies must still be verified with follow-up experiments to determine the relevance to *in vivo* phenotypes.

9.3 The question of heterogeneity

We reason there is an irreducible limit after which distinction among cell types becomes irrelevant, governed only by chance. This limit is the point at which we can distinguish between different cell types and different members of a cell type (*Figure 5*). Above this limit, increased resolution can result in greater biological understanding. Below this limit, greater resolution does not provide additional biological information because it is not linked to other measurable phenotypes of these cells. When we understand this limit, we will be able to answer fundamental questions about cell fate and canalization. We know that each cell type is not transcriptionally homogenous (*Figure 5B*), but are these transcriptional differences meaningless noise (*Figure 5C*) or are they distinct cell states with independent functions (*Figure 5D*)? As mentioned above, a fundamental drawback of single-cell sequencing is the user-defined delineation of cell clusters. Single-cell technologies rest on a premise of dimension reduction to a finite number of cell populations based on the expression levels of several RNAs made across thousands of possible molecules in thousands of different cells.

The assumption of this technique and the hypothesis of every single-cell study is that variation in transcript abundance is reflective of difference in cell activity or potential, even when all the cells are outwardly similar by (some) other measurements of phenotype. The question remains, are these cell subpopulations freely interconvertible (*Figure 5C*) or do they occupy unique niches and perform distinct functions (*Figure 5D*)? The goal of single-cell experiments, and greater resolution with any molecular measurement, should not be to redefine what a cell type is: rather, we should aim to understand how cells support a range of functions by adopting a range of behaviours. Development must be deterministic—hence it cannot be governed by interconvertible populations of cells at the whim of the environment. Disease has no such restriction and thus epigenomics of disease should seek to resolve the scale at which the chemistry of the cell impacts the function of the organ. This scale may be coarser than can be measured with modern single-cell techniques.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

Research in the Vondriska Laboratory is supported by the National Institutes of Health of the United States (HL105699, HL150225, and HL 150667), the David Geffen School of Medicine, and the Department of Anesthesiology & Perioperative Medicine at UCLA. T.G. was supported by T32 HL069766 and N.D.G. by T32 GM007185.

Conflicts of interest: The authors declare no conflicts of interest related to the subject matter in this article. All the artwork in the figures of this paper is the original creation of the authors.

Data availability

We do not present new data.

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