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INVITED EDITORIAL

Cardiac fibroblast heterogeneity and dynamics through the lens of single-cell dual 'omics

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This editorial refers to 'Single-cell dual-omics reveals the transcriptomic and epigenomic diversity of cardiac non-myocytes' by L. Wang *et al.*, pp. 1548–1563.

In adult hearts, fibroblasts are distributed throughout the myocardial wall, the adventitial spaces of outflow and coronary arteries, and nodal elements, where they act as master regulators of extracellular matrix (ECM) synthesis and remodelling and as lineage progenitors, electrical and mechanical transducers or insulators, and intercellular signalling hubs. As such, cardiac fibroblasts (CFs) are integral to the complex molecular dialogues and feedback circuits controlling tissue architecture, integrity, and adaptability to injury or stress. CFs are also the central actors in cardiac fibrosis. This initially protective but eventually insidiously maladaptive process accompanies most forms of heart disease, leading to chamber stiffening, heart failure, arrhythmias, and sudden death. Given the high health and economic burden of heart failure in society, understanding CF biology and finding ways to arrest or reverse cardiac fibrosis remain a high priority.

In a paper recently published in ***Cardiovascular Research***[1] (see *Figure 1*), Wang *et al.* used single-cell dual 'omics analysis to survey the heterogeneity and dynamics of the interstitial (non-cardiomyocyte) cells of the ventricles of healthy mouse hearts. Single-cell genomics has heralded a revolution in our ability to understand tissue complexity, overcoming the limitations of bulk cell analyses where insights into complex cell systems dynamics are lost. We can now address new questions about tissue structure and function, and disease mechanisms, divorced from a priori notions of cell identities, hierarchies, and dynamics.

Wang *et al.* first used single-cell RNA sequencing (scRNA-seq) to reveal the major subtypes of the fibroblast, vascular and immune lineages of the uninjured mouse heart, and define signature marker sets and candidate functional pathways.[1] Results align with other cardiac studies[2–4] which have revealed unanticipated lineage subtype heterogeneity underpinning cardiac tissue homeostasis and disease responses.

A significant strength of the Wang *et al.* study is the integration of single-cell transcriptomics data with single nuclear chromatin accessibility data generated using the assay for transposase accessible chromatin using sequencing (ATAC-seq). ATAC-seq displays regions of 'open' chromatin, generally found around coding regions, promoters, and enhancers of actively expressed or primed genes, making these regions accessible to regulatory factors such as transcription factors (TFs). In the study by Wang *et al.*, high general chromatin accessibility scores for lineage-defining genes correlated well with their expression scores in scRNA-seq data, and, reassuringly, single-cell accessibility scores allowed the identification of the main cardiac interstitial cell types following integration with the scRNA-seq data.

The most interesting application of ATAC-seq data in this study was the assessment of TF-binding motifs enriched in open chromatin regions. Focusing on CFs, Wang *et al.* identified a collective of 42 TFs that were proposed to participate in regulating CF character based on enrichment of their motifs in the open chromatin of lineage-defining genes. This list contained known CF-enriched TFs such as transcription factor 21 and heart and neural crest derivatives expressed 2, as well as many ubiquitously expressed factors including twist family BHLH transcription factor 1, twist family BHLH transcription factor 2, and transcription factor AP-4, which may help in maintaining the mesenchymal character of CFs. Subsequent cross-referencing with scRNA-seq data showed that 7 of the 42 TF genes were also highly expressed in CFs. Interestingly, motifs for several TFs typically associated with neurogenesis were also over-represented. ATAC-seq data also facilitated the identification of a small number of distal *cis*-regulatory elements showing differential chromatin accessibility scores in genes that help define CF sublineages, and TF motifs enriched at these putative enhancers provide the first clues to how substates might be generated or maintained.

Three main CF subtypes prominent in uninjured hearts were defined in the Wang *et al.* study. The most abundant subtypes fibroblast (FB-1 and FB-2) showed up-regulation of genes associated with extracellular stimulus responses and cytoskeletal organization and signalling, respectively, suggesting distinct functional roles in cardiac homeostasis, injury, or stress responses. As the authors point out, FB-1 and FB-2 likely correspond to populations defined previously based on low and high expression, respectively, of the stem cell marker *Sca1*.[3] The FB-2 subtype

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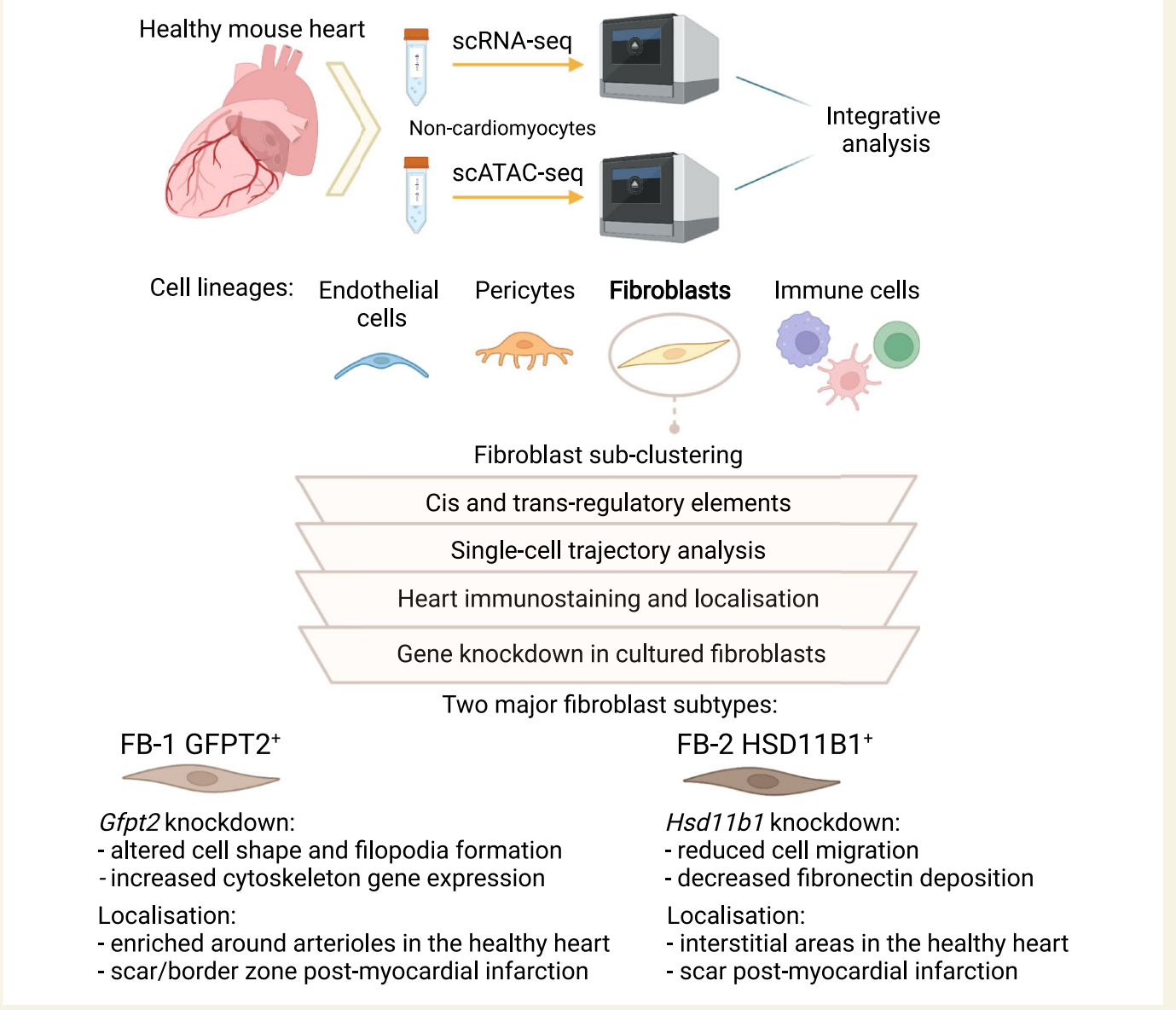

Figure 1 Summary of experimental procedures and results of knockdown experiments for signature genes for the two main fibroblast subpopulations identified. Healthy mouse hearts were profiled using scRNA-seq and scATAC-seq. Main cell lineages identified from integrative analyses including endothelial cells, pericytes, fibroblasts, and immune cells. Among fibroblasts, two major subtypes, FB-1 and FB-2, were identified, marked by *Gfpt2* and *Hsd11b1* expression, respectively. Knockdown experiments for each gene were performed *in vitro* on mouse cardiac fibroblasts. Localization experiments were performed using immunohistochemistry. Figure created with BioRender.com.

(corresponding to the *Sca1*-high population; fibroblast-sca1-high (F-SH)[3]) is of particular interest because analogous cells appear to be present in many healthy organs.[5] Furthermore, in the adult mouse heart, the FB-1/F-SH fractions are enriched in cells with mesenchymal stem cell characteristics,[6] which may represent self-renewing precursors of other homeostatic or injury-related CF subtypes,[7] including activated and proliferating CFs found after ischaemic injury.[6,8]

A limitation of commonly used single-cell genomics platforms is that the anatomical context of discovered cell types is lost during organ or tissue preparation. In the final part of Wang *et al.*, the authors identify *Hsd11b1* and *Gfpt2* as among the most highly discriminative genes for FB-1 and FB-2, respectively. *Hsd11b1*, expressed more highly in FB-1, encodes cortisone reductase, which converts cortisone to cortisol, a glucocorticoid stress hormone implicated in limiting cardiac fibrosis in inflammatory settings. Consistently, small hairpin RNA knockdown of *Hsd11b1* in CFs *in vitro* reduced their migration and fibronectin deposition in ECM. *Gfpt2*, expressed more highly in FB-2, encodes a rate-limiting enzyme that controls the flux of glucose into the hexosamine biosynthetic pathway, an off-shoot of glycolysis that governs protein glycosylation influencing many cellular processes. Knockdown of *Gfpt2* in CFs induced changes in cell shape and filopodia formation and increased the expression of genes involved in actin cytoskeleton reorganization. These findings reinforce the idea that FB-1 and FB-2 show different metabolic and/or functional states.[2,6]

Notwithstanding the fact that *Hsd11b1* expression was only partially discriminatory for FB-1 cells, immunofluorescence staining revealed

preferential localization of glutamine-fructose-6-phosphate transaminase 2 (GFPT2) compared to 11βHSD1 (encoded by *Hsd11b1*) around arterioles. Furthermore, whereas both proteins were expressed in the infarct zone at Day 7 post-myocardial infarction in mice, GFPT2 appeared more strongly expressed in the border zone. The tentative conclusion is that FB-1 and FB-2 show distinct functional states, anatomical localizations, and/or differential kinetics of activation after ischaemic injury. New technologies including 'spatial transcriptomics' are now facilitating the reconstruction of spatial information lost during cell capture and will soon enable the high-resolution spatial discrimination of CF subtypes within the heart.

Wang *et al.* and allied papers[9] have marked the beginning of the multi-'omics era of cardiac single-cell biology. Technologies for integrating single-cell transcriptomics, epigenomics, and/or proteomics data into a single workflow, and spatial transcriptomics, are now imminent. Many antifibrotic drugs have failed in cardiac disease clinical trials, speaking to the inherent complexity of the above processes. Non-linear CF lineage trajectories,[2–4] the coexistence of profibrotic and antifibrotic CF subsets,[2] bidirectional toggling between quiescent and activated states,[9] and the identification of pathways that repress spontaneous CF differentiation[8] during responses to myocardial injury have recently been revealed by single-cell 'omics studies, challenging the field to explore more nuanced approaches in identifying antifibrotic therapy targets. Experimental therapies now include epigenetic pathway inhibitors[9] and genetically engineered T cells.[10] The rapidly expanding single-cell 'omics data resources are helping to drive a rejuvenated interest in CF biology and disease pathophysiology and bring great potential for new discovery and biomedical innovation.

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