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Unravelling fibrosis using single-cell transcriptomics

Ross Dobie and Neil C Henderson

Fibrosis, the excessive accumulation of extracellular matrix, is a major global healthcare burden. Despite major advances in our understanding of the mechanisms regulating fibrosis, treatment options for patients with fibrosis remain very limited. However, recent developments in the rapidly evolving field of single-cell transcriptomics are enabling the interrogation of individual pathogenic cell populations in the context of fibrosis at unprecedented resolution. In this review, we will discuss how single-cell transcriptomics is driving this step change in our understanding of fibrotic disease pathogenesis, and how these cutting-edge approaches should accelerate the precise identification of novel, relevant and potentially druggable therapeutic targets to treat patients with fibrosis.

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Introduction

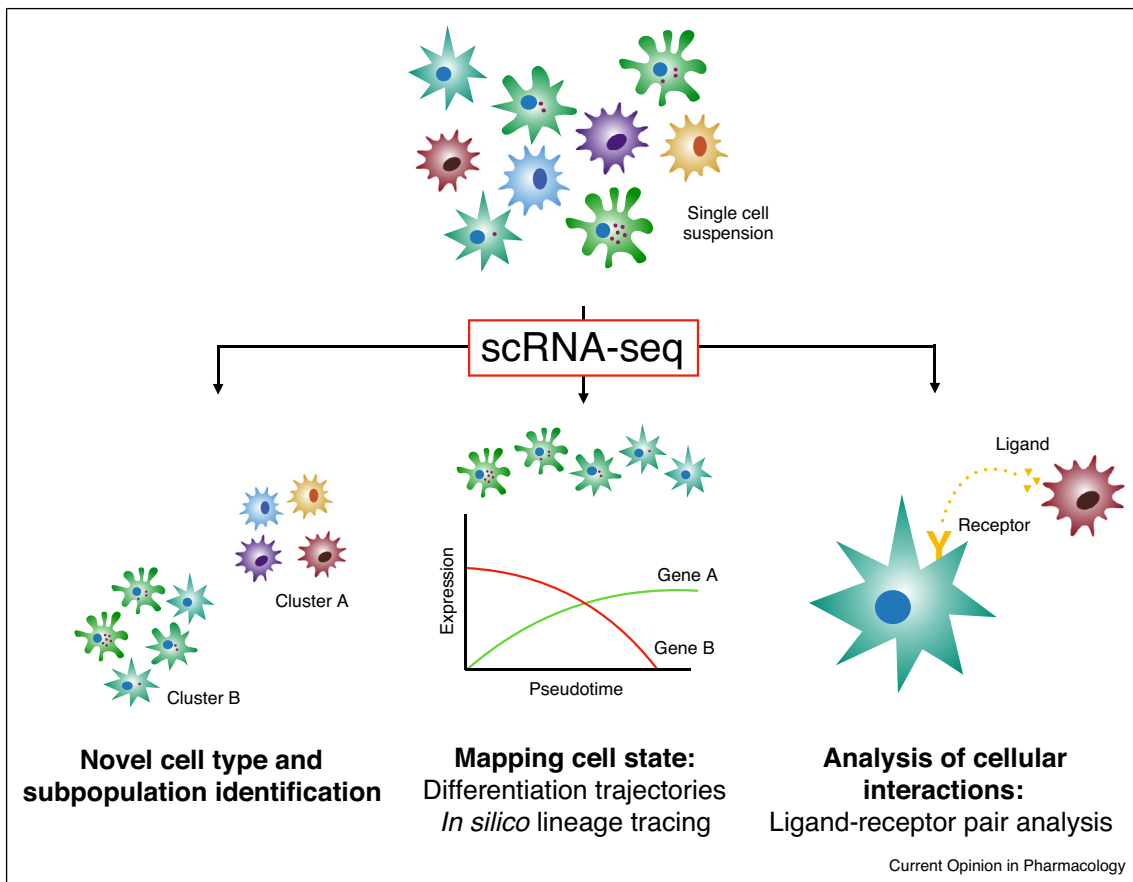
Fibrosis, characterised by the excessive accumulation of extracellular matrix, contributes to a high level of morbidity and mortality worldwide [1–3]. Despite advances in our understanding of the cellular and molecular mechanisms regulating this process, the therapeutic options available to treat fibrosis remain very limited [4–7]. However, recent advances in single-cell omic technologies, such as single-cell RNA sequencing (scRNA-seq), are allowing the interrogation of individual pathogenic cell populations at unprecedented resolution (Figure 1). The identification of novel cell types and states afforded by scRNA-seq analysis not only greatly advances our understanding of the mechanisms underlying disease, but also accelerates the precise identification of new, highly relevant and potentially druggable therapeutic targets [8]. In this review, we will discuss how single-cell transcriptomics is driving a step change in our understanding of fibrotic disease pathogenesis.

Lung

The initiation and progression of lung fibrosis is driven by a complex interplay between multiple cell lineages. Given the importance of macrophages in regulating numerous pathological processes including organ fibrosis [9], and the fact that they represent a diverse and plastic population, scRNA-seq is particularly well-suited to the further investigation of this cell type in the context of lung fibrosis. Chakarov *et al.* performed an elegant study demonstrating the value of scRNA-seq in the investigation of macrophage heterogeneity. Interrogating interstitial macrophages (IM) in healthy mouse lung they identified two distinct populations, classified as Lyve1^{lo}MHCII^{hi} and Lyve1^{hi}MHCII^{lo} IM with differing transcriptional profiles [10]. Complementary fate-mapping models identified these IM populations as two separate lineages of monocyte-derived cells which reside within different anatomical niches in the lung and have distinct functions. Interestingly, this dichotomy in IM was conserved across multiple mouse tissues including heart, fat and dermis as well as human lung. Selective ablation of Lyve1^{hi}MHCII^{lo} IM exacerbated lung fibrosis, highlighting their critical role during lung injury, and identifying these cells as a potential antifibrotic target [10**]. Similarly, using scRNA-seq in a murine model of pulmonary fibrosis, Aran *et al.* identified a profibrogenic transitional macrophage population that is intermediary between monocyte-derived and alveolar macrophages, that localise to the fibrotic niche [11**]. Orthologs of genes expressed by this transitional population were shown to be upregulated in samples from patients with idiopathic pulmonary fibrosis highlighting conserved fibrogenic pathways across species. Furthermore, these pro-fibrotic transitional macrophages were shown to regulate fibroblast biology via secretion of PDGF-AA, potentially offering insight into how nintedanib, a PDGF receptor inhibitor and one of the few therapies currently approved to treat lung fibrosis, mediates its effects [12].

During lung fibrosis mesenchymal cells are responsible for extracellular matrix production that ultimately leads to architectural distortion and decline in lung function. ScRNA-seq has been used to characterise lung mesenchymal cell heterogeneity in both healthy and fibrotic mouse lung [13–15]. In conjunction with transgenic reporter lines this has led to the generation of a spatial and transcriptional map of healthy lung mesenchyme uncovering distinct populations including mesenchymal alveolar niche cells (MANC) – responsible for supporting alveolar growth and regeneration, and myofibroblast progenitor (AMP) cells – the major contributors to myofibroblasts following injury [14*]. Studies assessing

Figure 1



Resolving cellular state, subpopulations, differentiation dynamics and inter-cellular interactions using single-cell transcriptomic approaches.

mesenchymal cells in bleomycin-induced fibrotic mouse lung have uncovered extensive cellular heterogeneity, with slightly differing conclusions. These may be secondary to differences in duration of lung injury, cell isolation techniques and the analysis approaches used [13,15]. Xie *et al.*, provide a purely computational analysis of mesenchymal cell heterogeneity in healthy and fibrotic lung disease, identifying seven distinct populations of mesenchymal cell in fibrotic mouse lung, including an injury-specific PDGFR β^{hi} population [15]. In contrast, Peyser *et al.* conclude that activated fibroblasts are not uniquely defined, but instead display a similar, albeit amplified gene expression pattern to control cells [13]. Interestingly, both studies cast doubt on the use of *Acta2* as a marker to define disease-associated fibroblasts.

Extensive scRNA-seq studies have also been performed in human lung. Reyfman *et al.* provided the first single cell atlas of human pulmonary fibrosis, analysing over 70 000 cells from eight patients with pulmonary fibrosis of varying aetiologies and eight uninjured donor lung samples [16 \bullet]. By analysing each subject separately, as

well as performing an integrated analysis of all patients combined, the authors were able to comment on patient-specific populations which were lost in the integrated analysis, highlighting the important point that subtle inter-patient differences can be lost when only assessing integrated datasets. Furthermore, they characterised an injury-specific population of profibrotic monocyte-derived alveolar macrophages which had previously been identified in mice [9]. This study also highlighted that scRNA-seq from lung tissue obtained at time of bronchoscopy is technically possible, suggesting that scRNA-seq approaches may in time have applications in the diagnosis and monitoring of treatment responses in pulmonary fibrosis [16 \bullet].

In recent years, increasing evidence suggests that epithelial cells play an important regulatory role in lung fibrogenesis [17]. To characterise the changes in epithelial heterogeneity in human fibrotic lung Xu *et al.* performed scRNA-seq on three control and six idiopathic pulmonary fibrosis (IPF) patient samples. They uncovered significant changes in the lung epithelia including the

identification of an injury-specific population of atypical ‘indeterminate’ cells that did not express signature genes associated with any known lung epithelial subtype. This population generally expressed AT2 cell-associated markers, however gene set enrichment analysis uncovered unique predicted functions including ‘activation of myofibroblasts’ [18].

Liver

To date scRNA-seq in the liver has focussed predominantly on characterising the cellular landscape during liver homeostasis [19^{••},20[•],21^{••},22[•],23]. Generation of these reference maps in healthy, homeostatic liver are a critical platform from which to develop our understanding of liver disease pathogenesis.

ScRNA-seq permits interrogation of subtle differences in gene expression within a given cell lineage allowing high resolution mapping of populations to their topographical location. In an elegant study, Halpern *et al.* used scRNA-seq along with spatially mapped landmark genes to annotate zonation of distinct hepatocyte subpopulations across the mouse liver lobule. Challenging the traditional binary classification of the liver into periportal and pericentral zones they found that the intermediate lobule is likely to have specialised functions [21^{••}]. These key findings have since been confirmed by other groups in mouse and human liver [19^{••},23]. In a further study, Halpern *et al.* harnessed the spatial information gathered from their previous hepatocyte zonation work [21^{••}], and coupled this with a novel technique called paired-cell sequencing [22[•]]. This technique sequences mRNA from pairs of attached cells, leveraging the gene expression profile of one cell lineage within the pair to allow inference of the other cell’s spatial coordinates. The authors used this approach to characterise endothelial cell zonation within the liver [22[•]]. Many pathological conditions in the liver demonstrate zonation and are spatially heterogeneous, however the role of zonation in disease initiation and progression remains largely unknown [24]. It is likely that in the coming years single-cell transcriptomics will begin to shed light on the role of cellular zonation in liver disease.

Despite the rapid progress in our understanding of the mechanisms underlying liver fibrogenesis accrued using rodent models, a significant ‘translational gap’ remains between potential therapeutic targets and effective therapies [4,25], and as such there is a real drive to gain a better understanding of the human liver cellular landscape in health and disease. To date there have been two comprehensive scRNA-seq studies of human liver [19^{••},20[•]]. MacParland *et al.* interrogated the transcriptome of over 8000 parenchymal and non-parenchymal cells obtained from five healthy human livers generating a cell atlas comprising 20 discrete cellular subpopulations derived from the main hepatic cell lineages [19^{••}]. They

describe macrophage heterogeneity in the human liver, identifying two distinct populations—recently recruited pro-inflammatory macrophages and resident Kupffer cells which have a more immunoregulatory phenotype. Similar macrophage subpopulations have since been described in other human studies [20[•]]. As a cautionary note, McParland *et al.* highlight that tissue preparation methodologies likely have a significant impact on the ability to transcriptionally profile all hepatic cell populations, with hepatocytes being particularly susceptible to dissociation effects [19^{••}]. In a second human liver scRNA-seq study, Aizarani *et al.* have used an alternative approach (diffusion pseudo-time (dpt)) to the previous studies in mouse to classify functional zonation of hepatocytes and endothelial cells in human liver. Interestingly, in a comparison of mouse [21^{••},22[•]] and human data they discovered only limited evolutionary conservation of gene expression, again highlighting the important differences between mouse and human biology. Focussed analysis of the EpCAM⁺ cellular compartment lead to classification of a potential human liver progenitor cell type with the capacity to form organoids [20[•]].

ScRNA-seq experiments on fibrotic liver have focussed on interrogating hepatic stellate cells (HSC)/myofibroblasts and immune cells. Currently HSC are thought to be the major source of myofibroblasts during liver fibrosis, independent of aetiology [26,27], however it must be noted that these data are derived from mouse models of liver fibrosis and not human studies. ScRNA-seq studies in a rodent model of liver fibrosis have shown that myofibroblasts are a heterogeneous population in the fibrotic liver [28]. While activated myofibroblasts could be identified by the universal activation marker *S100a6*, as well as upregulation of fibrillar collagens, expression of certain chemokines was restricted to specific subpopulations [28]. Non-alcoholic fatty liver disease (NAFLD) is the most prevalent liver disease worldwide [29] and as a result there is a drive to gain a deeper understanding of the mechanisms regulating this disease. Characterisation of myeloid cell subsets in the liver and bone marrow from high-fat, high-sugar, high-cholesterol ‘western diet’—fed mice revealed an adaptation to a specific inflammatory phenotype during NAFLD progression, characterised by downregulation of inflammatory calprotectin (*S100a8/a9*) in macrophage and dendritic cell subsets with effects on inflammatory polarisation and modulation of responses to acute sterile liver injury [30].

Kidney

Since the emergence of scRNA-seq there have been a number of publications investigating the cellular composition of the kidney in development, homeostasis, cancer and fibrosis [31–36]. One of the first applications of scRNA-seq in the kidney elegantly highlights the power of this technique in the investigation of disease pathogenesis. Park *et al.* generated a single-cell atlas of healthy

mouse kidney and through correlation with bulk RNA-seq kidney data from rat, microarray data from human immune cells and transgenic mouse reporter lines, they were able to provide molecular definitions for 18 previously defined kidney populations, as well as three novel cell types including a transitional population of cells with an expression profile likened to both principal cells (PC) and intercalated cells (IC) of the collecting duct [32**]. Lineage tracing experiments demonstrated plasticity between PC and IC through this newly identified transitional population. Interestingly, the authors demonstrated that Notch-mediated IC to PC transition is a possible cause of metabolic acidosis in mouse models and patients with chronic kidney disease [32**].

Fibrosis is the final common pathway of virtually all types of chronic kidney injury, and while it is accepted that myofibroblasts are the scar-producing cells in the kidney, their cellular origin is still a matter of debate [37]. Using a parabiosis model, Kramann *et al.* demonstrated that only a small percentage of myofibroblasts are derived from circulating cells. ScRNA-seq characterisation of this population (PDGFR β ⁺CD45⁺) as well as resident myofibroblasts (PDGFR β ⁺CD45⁻) confirmed these findings, identifying two distinct populations of cells. While the resident myofibroblast population was largely associated with matrix production, myofibroblasts derived from the circulation were shown to have a monocyte-like phenotype associated with immune response mechanisms [36].

Despite the immense progress that has been made with regard to understanding disease pathogenesis using scRNA-seq, there are number of factors to consider when using this technique. These include the underrepresentation of certain cell types due to cell fragility and/or difficulty in liberating specific cell types during tissue dissociation, the introduction of cell stress artefacts secondary to prolonged isolation protocols, and the inability to generate high quality whole cell scRNA-seq data from frozen, archived tissue. Single nuclei sequencing (snRNA-seq) is an emerging complementary approach that may help address a number of these issues, as it allows an unbiased assessment of nuclei from all cell lineages present within a tissue, and importantly unlocks analysis of biobanked frozen tissues. A recent study of mouse kidney has compared a number of snRNA-seq platforms with standard scRNA-seq workflows and found that snRNA-seq approaches result in detection of fewer stress related genes and increased detection of specific cell types including podocytes, endothelial cells, and intercalated cells [38*]. Furthermore, this study found that snRNA-seq of fibrotic kidney uncovered novel and rare populations, resulting in increased resolution of the cellular composition present during kidney fibrogenesis. Taking into account the advantages of snRNA-seq outlined in this study, along with the availability of frozen archived fibrotic human tissue, it is likely that the use of

snRNA-seq will increase in the coming years, further expanding our understanding of the mechanisms underlying fibrotic disease.

Conclusions

The rapidly evolving field of single-cell transcriptomics has already led to important, new discoveries in the context of tissue fibrosis. Furthermore, developing technologies such as single-cell epigenomics, single-cell proteomics, and spatial transcriptomics will allow the research community to further define the cellular and molecular mechanisms regulating fibrosis at even higher resolution. These cutting-edge approaches will advance our understanding of how the fibrotic niche operates across the different forms of tissue fibrosis, and should generate rational and potentially druggable therapeutic targets to treat patients with fibrosis.

Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

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