

Application of single-cell sequencing in autoimmune diseases

Liang-Yu Mi¹, Jin-Fang Gao², Dan Ma², Li-Yun Zhang², Gai-Lian Zhang², Ke Xu²

¹Department of Rheumatology, Bethune Hospital Affiliated to Shanxi Medical University, Taiyuan, Shanxi 030032, China;

²Department of Rheumatology, Shanxi Bethune Hospital, Taiyuan, Shanxi 030032, China.

To the Editor: The Human Genome Project decodes the mysteries of life, explores the pathogenesis of disease, and provides scientific evidence for the diagnosis and treatment of disease. Traditional sequencing methods predominantly provide genome-wide sequence information and an average of the mix of cell types, which does not take into account the heterogeneity between cells and between subtypes. Advances in genome sequencing technology have allowed the development of single cell sequencing (SCS).

SCS technology, including genome, transcriptome, and epigenetic sequencing, reveals the single cell genetic structure and gene expression status of single cells, uncovers the differences in genetic material and protein information in cells, and provides information on the different stages, functions, and characteristics of cells. First, single cells should be isolated in a manner that does not destroy their biological integrity. Furthermore, the integrity of the genome (DNA or RNA) should be preserved during cell lysis. The current methods of cell lysis include physical methods, chemical methods, and bioenzymatic degradation. Following lysis, DNA polymerase and primers are used to amplify the genome. Finally, individual cells are sequenced and the results are analyzed. SCS technology is currently widely applied in immunological research. SCS can be used for the efficient diagnosis of autoimmune diseases, the determination of the molecular susceptibility of specific cells, and the identification of candidate therapeutic targets [Figure 1].

Common rheumatic diseases that primarily involve the joints include osteoarthritis (OA) and rheumatoid arthritis (RA). OA is characterized by marked cartilage damage rather than inflammation. Cartilage consists of chondrocytes, including proliferative chondrocytes, prehypertrophic chondrocytes, hypertrophic chondrocytes, and fibrochondrocytes. Ji *et al*^[1] obtained 1464 chondrocytes from ten patients with OA undergoing knee arthroplasty surgery and used single-cell RNA sequencing to report the

molecular programs and lineage progression patterns controlling the pathogenesis of OA. They identified seven populations of chondrocytes in human OA cartilage, three of which are effector chondrocytes, regulatory chondrocytes, and homeostatic chondrocytes. Effector chondrocytes are involved in energy metabolism. Regulatory chondrocytes serve important roles in antigen presentation and B cell and T cell receptor signaling, and may play the role of master regulator during OA progression. Homeostatic chondrocytes express high levels of human circadian clock rhythm markers and favorable genes (such as period 1 [*Per1*] and sirtuin 1 [*Sirt1*]) that control the circadian clock in OA progression. RA is characterized by the abnormal synovial hyperplasia of joints and destruction of cartilage and bone. Fibroblast-like synoviocytes (FLSs) and macrophages play a central role in joint inflammation and cartilage degradation during RA progression. According to cell surface staining patterns, transcriptome and anatomic locations, cells can be divided into unique subsets.

The identification of anatomically discrete and functionally distinct cell subsets with non-overlapping functions has been instrumental for the development of cell-based therapies in RA. Single-cell RNA-seq analysis was used to study non-hematopoietic tissue-resident fibroblasts and define the subpopulations of cells found in pathological tissues, thereby providing novel insights into disease etiology and treatment options. Different FLSs subsets have distinct spatial localization and functions in synovial tissue. THY-1 (CD90) is a surface marker that can be used to distinguish fibroblast subsets. In RA and OA, THY1⁺ FLSs are present in the sublining areas, while THY1⁻ FLSs are mostly found in the synovial lining. In RA, a greater proportion of FLSs express high levels of THY1 compared with OA, and the majority of the FLSs are THY1⁻ in OA. THY-1⁺ FLSs produce cytokines that drive inflammation, and are enriched in modules associated with the production and interactions of the extracellular matrix (ECM), while THY-1⁻ FLSs induce bone and cartilage destruction.^[2] Fibroblast activation protein- α (Fap- α) is a biomarker of the

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Liang-Yu Mi and Jin-Fang Gao contributed equally to this work.

Correspondence to: Dr. Ke Xu, Department of Rheumatology, Shanxi Bethune Hospital, Taiyuan, Shanxi 030032, China
E-Mail: zhaoxuke@hotmail.com

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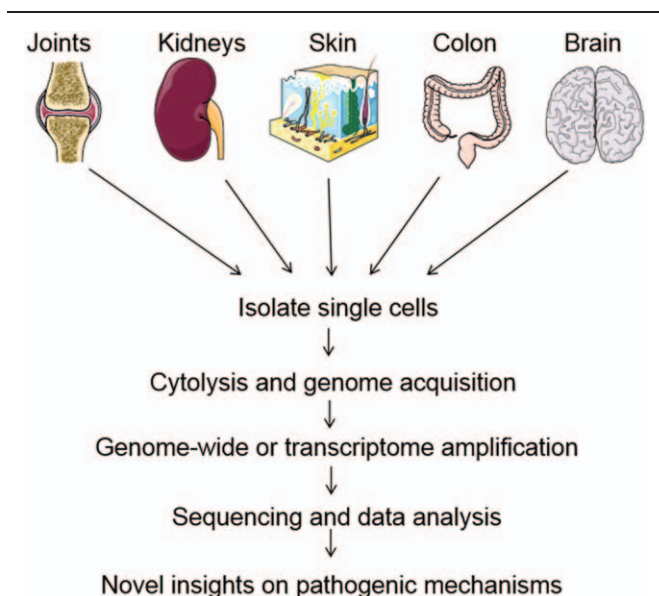


Figure 1: Harnessing SCS technology to study end-organ inflammation. SCS allows a fairly comprehensive detailing of the cellular and molecular landscapes that dominate the joints in OA or RA, the kidneys in LN, the skin in LN or SSc, the colon in inflammatory bowel diseases, and astrocytes in MS. LN: Lupus nephritis; MS: Multiple sclerosis; OA: Osteoarthritis; RA: Rheumatoid arthritis; SCS: Single cell sequencing; SSc: Systemic sclerosis.

tissue inflammatory response, and the migration of Fap- α^+ FLSs into bone and cartilage leads to joint damage. Conversely, deletion of FAP α^+ FLSs suppresses both inflammation and bone erosion. The presence of FAP α^+ THY1 $^+$ immune effector fibroblasts in the synovial sublining results in a more severe and persistent inflammatory arthritis, with minimal effect on bone and cartilage. However, FAP α^+ THY1 $^-$ destructive fibroblasts restricted to the synovial lining layer selectively mediate bone and cartilage damage with little effect on inflammation.^[3] Podoplanin (PDPN), a type of sialic acid glycoprotein, is a transmembrane protein found on the surface of FLSs. The expression level of PDPN in FLSs *in vitro* increases in a time-dependent manner following stimulation with tumor necrosis factor (TNF) α and interleukin (IL)-1 β . PDPN $^+$ FAP α^+ THY1 $^+$ FLSs serve an immune effector role and sustain inflammation through the production of a distinct repertoire of chemokines and cytokines. PDPN $^+$ FAP α^+ THY1 $^-$ FLSs are bone effector cells that mediate joint damage. PDPN $^+$ CD34 $^-$ THY1 $^+$ FLSs are present in the perivascular zone in the inflamed synovium and secrete proinflammatory cytokines. The levels of these cells are three-fold higher in patients with RA compared with those with OA, and reflect RA disease activity.^[4] PDPN $^+$ CD34 $^-$ THY1 $^+$ FLSs have an *in vitro* phenotype characteristic of invasive cells and are proliferative, which suggests that they serve pathological roles in matrix invasion, immune cell recruitment, and osteoclastogenesis.

SCS may be used to determine the dynamic evolution of FLSs. After describing the similar development trajectory of human FLS in OA and RA, Cai *et al*^[5] also clarified their functional status by using single-cell RNA sequencing technology. In both RA and OA, FLSs underwent a similar transition process, from an unstimulated state (state 4) to activated states with different functional patterns (states 1, 2, 3, and 5). The process may have two branches: (i) from

state 4 to state 5 and (ii) from state 4 to state 3 to state 1 or state 2. FLSs in states 2 and 5 are more pathologic than FLSs in states 1 and 3. FLSs in state 5 exhibit stronger proinflammatory ability, while those in state 2 are more aggressive.

Macrophages, a source of TNF- α , drive RA. Individual subsets of RA macrophages have highly specialized and tissue-specific functions, with distinct transcription and epigenetic inheritance. CX3C chemokine receptor 1 (CX3CR1) $^+$ tissue-resident macrophages and heparin-binding epidermal growth factor-like growth factor (HBEGF) $^+$ inflammatory macrophages are enriched in RA tissue. CX3CR1 $^+$ tissue-resident macrophages form an internal, locally renewing, and protective tight-junction-mediated immunological barrier to restrict the inflammatory reaction.^[6] HBEGF $^+$ inflammatory macrophages, known as “proinvasive macrophages,” produce inflammatory cytokines, such as IL-1, and the EGF growth factors epiregulin and HBEGF, which subsequently induce invasive FLSs.^[7]

According to the aforementioned studies, anatomically distinct, functionally discrete fibroblast or macrophage subsets with non-overlapping functions have important implications for cell-based therapies aimed at modulating inflammation and tissue damage in pathological conditions.

Der *et al*^[8] and Arazi *et al*^[9] subjected human renal and skin biopsy tissues to SCS, and evaluated potential diagnostic and prognostic markers for lupus nephritis (LN), as well as identified novel targets for personalized treatment. RNA expression patterns can prompt a potential effector function and development trajectory. There are various leukocyte subtypes in patients with LN, each with a characteristic gene expression pattern. CD8 $^+$ T cells are divided into two distinct effector populations, including a population of cytotoxic T cells with a high similarity to natural killer cells, and a separate population with a high expression of granular enzyme K. The CD4 $^+$ T cell population includes effector populations, Treg cells, and T follicular helper-like cell subsets that may contribute to the B-cell response. The SCS trajectory analysis indicated that naive B cells may differentiate into activated cells and age-related and/or autoimmune-related B cells, which may occur *in situ*. The innate and adaptive immune cells in the LN kidney interact with each other through receptor-ligand binding. The expression of the chemokine receptor C-X-C chemokine receptor type 4 (CXCR4) and the cytokine receptor TNF RSF10A was increased in renal immune cells. In addition, CXCR4 and CX3CR1 may play a central role in cell transport and may serve as potential therapeutic targets. The ECM-interacting proteins tissue inhibitor of metalloproteinases-1 and serine protease inhibitor family G are associated with renal fibrosis and are upregulated in the renal tubular cells of patients with LN receiving ineffective treatment. Type I interferon regulates immune system activity, and type I interferon response pathway genes are upregulated in renal tubular epithelial cells and keratinocytes in patients with LN. Type I interferon may be used to evaluate the therapeutic response to LN, as the renal tubular epithelial cells interferon response scores in patients with ineffective LN treatment are significantly higher than those receiving

effective treatment. Interferon is present in keratinocytes of non-skin lesions and non-sunburned skin biopsies obtained from LN patients, suggesting that skin may serve as a potential substitute of kidney for kidney disease. Urine is easily collected, and cells present in urine may recapitulate molecular changes occurring in the kidney, and may; therefore, be used in future biomarker studies.

Systemic sclerosis (SSc) is an autoimmune fibrotic disease. Sun *et al*^[10] identified skin cell subsets and related marker genes in patients with SSc using single-cell RNA sequencing. They revealed pericyte-specific expression of RGS5, T-cell-specific expression of IL32, endothelial-cell-specific expression of von Willebrand factor (vWf), fibroblast-specific expression of COL1A1, basal keratinocyte-specific expression of Krt14 and Krt5, and basal keratinocyte-specific expression of Krt1 and Krt10. Mesenchymal cells in the intestinal lamina propria are a heterogeneous group of cells that are crucial for maintaining homeostasis of intestinal epithelial cells. Furthermore, they are associated with the development of inflammatory bowel diseases, including ulcerative colitis and Crohn disease, by mediating the inflammatory environment. Colon mesenchymal cells have four subsets.^[11] The S1 subset is rich in non-fibrillar collagen and elastic fibers. The S2 subset is found adjacent to the intestinal epithelial crypts, and is characterized by the expression of SOX6, F3 (CD142), and WNT, which play an important role in stem cell proliferation and differentiation. The S2 subset is reduced in ulcerative colitis, and the expression of flaky collagen is decreased, leading to epithelial barrier dysfunction. The S3 subset is associated with supramolecular fibrous tissue. The S4 subset expresses inflammatory factors that regulate T cell activation and leukocyte migration, exacerbate oxidative stress, and promote disease progression.

Multiple sclerosis (MS) is an inflammatory demyelinating disease, which is strongly associated with genetics. eomesodermin (EOMES) is associated with increasing risk in Chinese Relapsing-remitting MS (RRMS) patients and might be a potential therapeutic target in RRMS.^[12] Astrocytes are involved in the pathogenesis of MS. There are multiple subsets of astrocytes with different transcriptional states in experimental allergic encephalomyelitis and MS.^[13] Cluster 4 is the most amplified subpopulation during experimental autoimmune encephalomyelitis (EAE) induction. The transcriptional regulatory factor nuclear factor erythroid-2-related factor-2 (Nfe2L2), encoding the transcription factor NRF2, has the lowest expression in Cluster 4, and is accompanied by increased MAF bZIP transcription factor G (MAFG) and MAFG interacts directly with methionine adenosyltransferase 2 (GMAT2 MAF) signaling. NRF2 acts as a negative regulatory factor related to the inflammatory response and neurotoxic pathways. If MS is not treated in a timely and effective manner, the nervous system is irreversibly damaged. Therefore, the identification of novel biomarkers is important for the early detection of MS, and may provide potential therapeutic targets.

SCS technology may improve our understanding of the function of single cells in the inflammatory microenviron-

ment in autoimmune diseases, and to define cell subsets in pathological tissues. Furthermore, SCS may allow the identification of diagnostic or prognostic biomarkers and therapeutic targets for autoimmune diseases.

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Conflicts of interest

None.

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