Functional genomics of stromal cells in chronic inflammatory diseases

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Purpose of review
Stroma is a broad term referring to the connective tissue matrix in which other cells reside. It is composed of diverse cell types with functions such as extracellular matrix maintenance, blood and lymph vessel development, and effector cell recruitment. The tissue microenvironment is determined by the molecular characteristics and relative abundances of different stromal cells such as fibroblasts, endothelial cells, pericytes, and mesenchymal precursor cells. Stromal cell heterogeneity is explained by embryonic developmental lineage, stages of differentiation to other cell types, and activation states. Interaction between immune and stromal cell types is critical to wound healing, cancer, and a wide range of inflammatory diseases. Here, we review recent studies of inflammatory diseases that use functional genomics and single-cell technologies to identify and characterize stromal cell types associated with pathogenesis.

Recent findings
High dimensional strategies using mRNA sequencing, mass cytometry, and fluorescence activated cell-sorting with fresh primary tissue samples are producing detailed views of what is happening in diseased tissue in rheumatoid arthritis, inflammatory bowel disease, and cancer. Fibroblasts positive for CD90 (Thy-1) are enriched in the synovium of rheumatoid arthritis patients. Single-cell RNA-seq studies will lead to more discoveries about the stroma in the near future.

Summary
Stromal cells form the microenvironment of inflamed and diseased tissues. Functional genomics is producing an increasingly detailed view of subsets of stromal cells with pathogenic functions in rheumatic diseases and cancer. Future genomics studies will discover disease mechanisms by perturbing molecular pathways with chemokines and therapies known to affect patient outcomes. Functional genomics studies with large sample sizes of patient tissues will identify patient subsets with different disease phenotypes or treatment responses.

Keywords
fibroblasts, functional genomics, inflammation, microenvironment, rheumatic disease, stromal cells

INTRODUCTION

The stroma is the background of every tissue in the body, and plays a critical role in supporting the normal epithelium, forming the general architecture of the tissue, and modulating the local microenvironment. Although many types of cells constitute the stroma in varying proportions across tissues, fibroblasts are the major constituent of stroma in all tissues. One of the first demonstrations of functional fibroblast heterogeneity was done 40 years ago, showing three distinct phenotypes of fibroblast response to prostaglandin [1]. Many studies since then have shown how fibroblasts in different systems support tissue homeostasis and shape immune responses [2].

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Curr Opin Rheumatol 2018, 30:65–71
DOI:10.1097/BOR.0000000000000455

KEY POINTS

- The potential to define disease-related cell types in stroma is rapidly expanding with emerging single-cell technologies such as single-cell RNA-seq and mass cytometry.
- CD90 (Thy-1)-positive synovial fibroblasts are significantly overabundant in rheumatoid arthritis, indicative of major phenotypic changes in the rheumatoid synovium.
- Functional genomics can shed light on the interaction of the stroma with the immune system.

Fibroblasts are central to tissue biology because they organize the extracellular matrix in which other cells are embedded, and they communicate with these cells [2]. Since their functions are dependent on the context in which they are embedded, we face the challenge that in vitro studies may fail to capture physiologically relevant context essential to tissue biology. In contrast to hematopoietic cells, fibroblasts lack well defined cell surface markers that distinguish different functional types of cells subsets. Only one marker, the GPI-anchored protein Thy-1 (CD90), has been studied in many tissues. Thy-1 is differentially expressed on different types of fibroblasts in the spleen [3], lung [4,5], female reproductive system [6,7], ocular orbit [8], liver [9], and prostate cancer tumors [10].

Functional genomics, or discovery of biological insights through genome-wide assays, has revolutionized the way in which we understand cell biology and tissue physiology. By assessing global measures of all genes, it has the potential to find new factors important for production and degradation of extracellular matrix, development of blood and lymph vessels, wound healing, and communication with leukocytes during inflammation.

One limitation of current genomic studies is that they effectively average signals in bulk, high-dimensional assays across millions of cells with diverse phenotypes. This complicates interpretations about the roles of specific cell types. In other words, differences in cell composition cannot be distinguished from differences in gene regulation at the whole tissue level. More recently, studies using low-input genomics on subpopulations sorted by cell surface markers and studies using single-cell genomics demonstrated that the average signals can be explained by independent contributions from different proportions of functionally distinct single cells. Further, single-cell technologies are directly addressing the challenge of discovering cell surface markers that distinguish functional subsets of cells relevant to tissue biology and disease pathology.

FIBROBLASTS MEDIATE INFLAMMATION IN CHRONIC INFLAMMATORY DISEASES

The role of stromal cells in orchestrating local inflammatory response is becoming increasingly appreciated. Recent studies suggest that the diversity of stromal cells across anatomical sites may contribute to location-specific disease development [11▪▪,12▪,13]. There are consistent patterns of anatomical distribution for diseases like RA, inflammatory bowel disease (IBD), psoriasis, ankylosing spondylitis, and various types of cancers. For example, ankylosing spondylitis often affects lower extremities or the spine, whereas RA affects small distal joints of the hands and feet [11▪▪]. These anatomical patterns might be caused by site-specific local cell types, local responses to systemic signals, or environmental factors like mechanical stress that affect cells locally [11▪▪]. For example, one recent study shows that expression of developmental *HOX* genes can influence the tumor necrosis factor (TNF)-induced activation of inflammatory molecular pathways in knee synovial fibroblasts [12▪].

The synovium is a thin membrane composed mostly of fibroblasts and macrophages that surrounds the joint capsule and contains the synovial fluid. In RA, it is the critical site of chronic inflammation [14]. A healthy synovium has a one to two cell-thick lining layer and a sublining layer with blood vessels, lymph vessels, fibroblasts, collagen fibers, nerve fibers, and few leukocytes [15]. In RA, the inflamed synovium has a hyperplastic lining layer, dramatic expansion of blood vessels in the sublining, and dense infiltration by inflammatory leukocytes [15]. Although all of the cells play some role in chronic inflammation, synovial fibroblasts orchestrate joint destruction through production of proteinases that degrade the cartilage and cytokines that activate bone resorbing osteoclasts [16].

Autoimmune diseases, such as RA, progress from an initiation phase of generating autoantibodies to an effector phase of continuous feedback between stromal cells and leukocytes [17,18]. In RA, a vicious cycle of inflammation is fueled by fibroblasts in the synovium. The fibroblasts produce chemokines that recruit leukocytes to the damaged tissue, and these two cell types then continuously stimulate each other to maintain chronic inflammation [19,20]. A recent study shows that there is a similar feedback loop between leukocytes and stromal cells in IBD. In IBD, fibroblasts are activated by TNF and other chemokines, causing them to produce IL6 and other chemokines to activate leukocytes [21▪▪]. In contrast

to the cartilage destruction in RA, the fibroblasts of the gastrointestinal tract are responsible for excessive extracellular matrix deposition, causing fibrosis [22].

As a result of the signaling feedback loops between stromal cells and leukocytes, fibroblasts undergo long-lasting epigenetic changes, become resistant to apoptosis, and maintain a proliferative, destructive, invasive, and migratory phenotype [23–25]. Cultured synovial fibroblasts from inflamed joints of RA patients, but not from healthy joints, express major histocompatibility complex (MHC) class II molecules in response to IFN-gamma [26]. This is consistent with a similar demonstration that fibroblasts within the human lung express human leukocyte antigen – antigen D-related (HLA-DR) and costimulatory molecules, modulating the CD4 T-cell response to pathogens during infection [27]. Hence, many synovial and lung fibroblasts are actually antigen-presenting cells; they are able to extract and present antigens to CD4+ T cells [26–28].

EXPLORING STROMAL CELL BIOLOGY WITH FUNCTIONAL GENOMICS

Functional genomics offers a promising path forward to understand the diversity of fibroblast functions in health and disease. Here we summarize some strategies and glimpse the future of stromal cell genomics (Fig. 1).

Three main strategies are used to apply functional genomics to understand stromal cells in rheumatic disease. First, whole tissue transcriptomics by microarray, RNA-seq, or DNA methylation is used to contrast patients and controls. This type of analysis highlights molecular markers of disease such as cytokines elevated in the disease state, or cell surface markers of differentially abundant cell types. Second, purified cell populations by fluorescence-activated cell sorting (FACS) after tissue dissociation or laser dissection is followed by transcriptomics of selected populations. This strategy reveals the distinct genomic signals underlying the average signals seen in bulk tissues, helping to focus on a smaller set of cell types that are most relevant for disease pathogenesis. Third, the latest single-cell RNA-seq techniques can be performed on mechanically or enzymatically dissociated tissue samples to provide a somewhat unbiased view of thousands of cells in patient samples. This overcomes the need for cell surface markers to sort different cell types, and can give a holistic perspective of how abundances and functions of different cell types relate to each other in patient tissues.

Another important consideration for functional genomics is whether to use cultured cell lines or fresh cells from patient specimens. A major advantage of cultured cell lines derived from patient tissues is that they can be expanded to provide enough RNA, DNA, and other cellular material necessary for most genomic assays. Cell lines, however, lack the context of the whole tissue and are altered by growing *in vitro*. In many instances, studies contrast a small number (<10) of cell lines derived from patients and controls, sometimes including perturbations by therapies or gene knockdowns. A recent study did differential gene expression analysis with microarrays comparing macrophages and fibroblasts from synovial samples from RA and osteoarthritis. The authors found that both cell types are producing inflammatory cytokines in RA [29]. They also identified POSTN and TWIST1 as key regulators of fibroblast invasiveness in RA, and siRNA

Tissue Source	Molecular Assay	Resolution	Study Design
Cell lines **Whole tissue** • **Biopsy** • **Surgical specimen** **Phenotypically sorted cells**	**Genomics** • **DNA sequencing** • **Transcriptomics (microarray, RNA-seq, etc.)** • **Epigenetics (ATAC-seq, ChIP-seq, etc.)** **Proteomics** • **Fluorescence-activated cell sorting** • **Mass cytometry (CyTOF)**	**Bulk (thousands to millions of cells)** **Low Input (hundreds of cells)** **Single-cell**	**Exploratory (small n)** **Case control** • **Disease vs healthy** • **Disease vs disease** • **Responder vs nonresponder** **Case only progression** • **Early or late disease** • **Drug response**

FIGURE 1. Functional genomics in stromal cells. All studies involve careful considerations of the costs and benefits for selection of a tissue source, molecular assay, desired resolution, and study design. Critically, effective application of high dimensional molecular assays requires effective strategies to disaggregate whole tissue sources without disturbing biological signal.

experiments confirmed this finding [29]. In other study, microarray analysis with osteoarthritis and RA fibroblasts revealed constitutive upregulation of the transforming growth factor beta (TGFB) pathway in RA fibroblasts that resulted in greater expression of MMP11 [30]. Regarding anatomical patterning, genome-wide methylation microarrays and RNA-seq data show differential methylation and expression of developmental *HOX* genes between fibroblasts taken from hips and knees from RA and osteoarthritis individuals [13]. In knee synovial fibroblasts, silencing the *HOTAIR* lncRNA in the *HOX* cluster resulted in greater expression of constitutive and TNF-induced collagenase MMP1 [12▪]. This suggests that the *HOX* genes control stromal cell phenotypes during embryonic development and the immunoregulatory phenotypes during disease pathogenesis.

Functional genomics with fresh cells from patient tissues promises a path toward understanding disease mechanisms *in vivo*. However, fresh human-derived tissue samples are a limited resource, and must be used judiciously. For RA, these samples are often taken from patients in the late stages of disease who elect for joint arthroplasty. One exciting opportunity for querying early stages of RA is the use of research synovial biopsies [31]. Although tissue quantities are limited with biopsies, they enable querying tissues under prescribed clinical conditions, and are possible in a trial setting. In a study using transcriptomics by microarrays, the authors defined a rule set for discriminating RA from osteoarthritis and healthy controls [32]. Another microarray study of RA, osteoarthritis, and normal donors found that RA patients can be divided into two subsets with high or low expression of PRG4 in the synovium. They found that low PRG4 expression is associated with more aggressive stage of disease [33]. Pathogenic fibroblast destruction of cartilage, as well as recruitment and retainment of leukocytes is amplified by hematopoietically derived cytokines such as TNF and interleukin-6 (IL-6). Transcriptomics studies with synovial biopsy tissues revealed that treatment with tocilizumab, methotrexate, adalimumab, and rituximab reduces the mRNA levels of IL-6 and many other chemokines and T-cell activation genes after 12 weeks of therapy [34].

A recent study highlighted the interaction of stromal cells with leukocytes by using functional genomics with tissue biopsies from patients with Crohn's disease and ulcerative colitis. In that study, West *et al.* [21▪▪] found a TNFi-resistant pathway specific to gut-resident stromal cells. Analysis of public transcriptomics data put the focus on oncostatin M (OSM) because it is more highly expressed in biopsy tissues from IBD patients. Next, the authors found that OSM is mostly derived from hematopoietic cells, and stromal fibroblasts have highest expression of the OSM receptor (OSMR) in the gut. These PDPN+ fibroblasts are highly abundant in the inflamed tissue from Crohn's disease and ulcerative colitis patients, and they produce inflammatory chemokines in response to TNF and OSM. This study highlights the novel system of leukocyte–stromal communication behind the chronic inflammation in IBD.

The OSM result may translate to human therapies. Notably, phase-1 and phase-2 trials for RA show that humanized anti-OSM monoclonal antibodies are well tolerated, and OSMR-deficient mice are healthy and viable. Additionally, large scale studies with functional genomics on many biopsies can help to identify different subsets of patients. One such study had 210 patients with Crohn's disease and 35 healthy controls. This study found that patients with Crohn's disease were clearly distinguished from healthy controls by biopsy gene expression of 29 genes enriched with genetic risk variants [35]. Further, the 29-gene signal was also able to identify which patients were more likely to progress to complicated disease. Taken together, functional genomics in stromal cells reveals how stromal cell interact with leukocytes, and it helps to define clinically relevant patient subsets in RA and Crohn's disease.

OPPORTUNITIES TO ADVANCE FIBROBLAST BIOLOGY WITH SINGLE-CELL GENOMICS

Transcriptomic profiling of single cells is a powerful technique to reveal cellular and molecular heterogeneity in tissues with complex and dynamic cellular compositions. These technologies are being applied at scale in efforts such as the human single-cell atlas, with the goal of providing a reference map of all common types of cells in the human body [36]. These types of large-scale studies will transform our understanding of cell identity and function. Single-cell RNA-seq can sometimes overcome the need for cell surface markers to sort different cell types, and can give a holistic perspective of how abundances and functions of different cell types relate to each other in patient tissues.

Recent advances in immunoprofiling and transcriptomic assay technologies has created an opportunity to assay sorted stromal cells directly from tissues [37]. Advances in sequencing library construction have lowered the requirements for genomics assays to tens or hundreds of cells per sample [38]. To apply single cell technologies successfully, it

is critical to carefully dissociate tissue into a single-cell suspension and preserve cell viability. Recently, advances in droplet-based platforms for single-cell RNA-seq have enabled analysis of large number of single cells from a single donor [41,42].

Single-cell genomics will include discover new cell types specific to anatomical sites or disease states. It will also reveal dysregulations of molecular pathways exclusive to particular cell types, and genetic effects on those pathways [39]. One recent study demonstrated that genetic effects on gene expression can be masked whenever assaying total peripheral blood mononuclear cells (PBMCs). This single-cell expression quantitative trait loci (eQTL) analysis showed that the same variant influences expression of TSPAN13 in CD4 T cells, but not in dendritic cells or other cell types [40].

Investigators have begun applying single-cell technologies to query tissues in pathological conditions.

Two independent studies have identified Thy-1-positive and Thy-1-negative fibroblasts in the synovium [41,43]. By applying cell sorting, low-input RNA-seq, and single-cell RNA-seq to synovial biopsies, they identified different functional subsets of fibroblasts [43]. Notably, the Thy-1 positive fibroblasts were found to be significantly overabundant in the synovial tissue of RA relative to osteoarthritis [43]. We checked if we could identify overabundance of Thy-1-positive fibroblasts in a recent study of synovial biopsies [44] and found that RA has higher relative expression of Thy-1-positive fibroblast genes than Thy-1-negative fibroblast genes (Fig. 2). Expression of Thy-1 influences multiple aspects of fibroblast biology such as propensity to differentiate into adipocytes [45], and this protein is also involved in neuronal development and oncogenesis [5].

Despite these exciting initial applications, little is known about the complexity of stromal populations, and single-cell genomics offers a powerful way to capture the heterogeneity and compare gene expression of membrane proteins, transcriptional regulators, and signaling pathways that might reveal phenotypic and functional subsets of fibroblasts. Building on the foundation of the human single-cell atlas, new studies will focus on relevant tissues from patients. In the coming years, each new study of single cells from chronic inflammatory diseases will give deeper insights about the commonalities and differences between them. For example, the Accelerating Medicines Partnership (AMP) consortium is focused on generating large scale datasets that include single-cell genomics from disease-affected tissues in RA and systemic lupus erythematosus (SLE) patients. These datasets will show how cell-type abundances and functions change over the course of disease and in response to therapy. We envision that many future studies will take advantage of the new clarity provided by single-cell technologies.

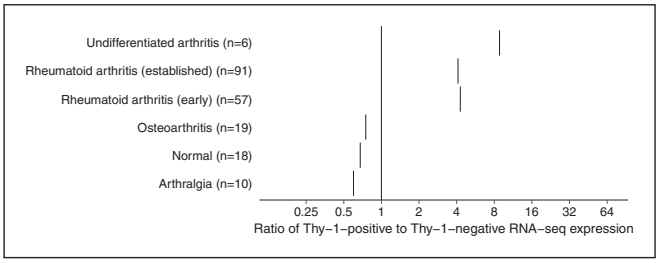

FIGURE 2. Thy-1-positive synovial fibroblast gene expression in RA. First, we selected four genes from single-cell RNA-seq data [43] that are highly expressed in sublining CD34-negative Thy-1-positive fibroblasts (*THY1*, *POSTN*, *TGFBI*, *CCL2*) and four genes highly expressed in lining CD34-negative Thy-1-negative fibroblasts (*CLIC5*, *TSPAN7*, *SLC2A12*, *SELP*). Next, we took the synovial biopsy RNA-seq expression data from a recent study (GSE89408), converted counts to counts per million (CPM), computed the sum of each gene set, and plotted the ratio of the gene sets. Vertical lines are medians. Early RA is significantly different than osteoarthritis (Wilcoxon rank sum test) ($P = 8.5 \times 10^{-4}$), and established RA is also significantly different than osteoarthritis ($P = 9.7 \times 10^{-5}$). This result is consistent with the recently reported results that synovial biopsies from RA tissues have an overabundance of the Thy-1-positive fibroblast population relative to tissue from normal or osteoarthritic patients.

CONCLUSION

In the past few years, oncologists have moved from general chemotherapy to personalized treatment based on genetic measurements. Treatment of rheumatic disease can follow a similar path as ongoing and future studies pave the way forward [46]. For example, measurement of the novel OSM gene expression biomarker in IBD patients may predict successful response to anti-TNF therapy [21▪▪]. As synovial biopsy tissue acquisition becomes more common, researchers will have more opportunities to use functional genomic technologies to uncover disease-relevant molecular phenotypes. Researchers will assay transcriptomics, DNA methylation, chromatin accessibility, histone modifications, and other molecular signals to find associations with disease activity and response to therapies. Armed with this information, biopsy data may be then used to predict optimal treatments for patients. The advancement of molecular phenotyping will help to deliver on the promise of precision medicine, wherever therapies are adjusted for maximum benefit to each individual patient.

Functional genomics has the potential to reveal common and differential sets of molecular pathways across multiple anatomical sites and diseases. Stromal biology has thus far been mostly focused on single systems like cancer-associated fibroblasts from prostate cancer, gut fibroblasts from IBD patients, or synovial fibroblasts from inflamed joints. Many pathological perturbations of stromal cells are likely to be different across diseases. However, careful analysis of the genome-wide measurements from functional genomic techniques can reveal the commonalities in molecular phenotypes across those diseases. For example, the cytokine gene expression profiles of prostate cancer-associated fibroblasts are similar to synovial fibroblasts from RA joints as well as fibroblasts from the gut.

We envision that synovial biopsy samples will be treated *in vitro* to study responses to known and novel therapies. We expect that hematopoietic and stromal cells from treatment responders might behave differently than cells from nonresponders, and future studies will find the molecular basis for these differences. These findings will give unprecedented resolution about pathogenic functions in the synovium, and will suggest new diagnostic assays and therapeutic targets that might be addressed by existing or future therapies [15].

Acknowledgements

We are very thankful to Ilya Korsunsky for valuable discussions.

Financial support and sponsorship

This work is supported in part by funding from the Ruth L. Kirschstein National Research Service Award (F31AR070582) from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (K.S.), the Rheumatology Research Foundation Scientist Development Award (K.W.), the National Institutes of Health (UH2AR067677, 1U01HG009088, and 1R01AR063759), and the Doris Duke Charitable Foundation Grant #2013097 (S.R.).

Conflicts of interest

There are no conflicts of interest.

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