Integrated Single Cell and Spatial Transcriptomics Analysis Reveals Distinct Senescent Cell Phenotypes that Regulate Musculoskeletal Fibrosis

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INTRODUCTION: Fibrosis from musculoskeletal injuries, diseases, or implant complications causes significant pain and immobility, but there remains a lack of effective therapeutics to halt and reverse fibrosis. Senescent cells (SnCs) are growth-arrested cells expressing a senescence-associated secretory phenotype (SASP) that have been recently linked to fibrosis in various diseases and pathological implant responses [1]. However, SnCs are heterogeneous *in vivo*, making it difficult to identify them using a single marker and to characterize fibrosis-associated phenotypes and functions (senotypes). As a result, the senotypes of SnCs in fibrosis remain poorly understood, hindering the development of senescence-targeting therapies. In this study, we establish a new computational pipeline that incorporates transfer learning and non-negative matrix factorization algorithms to identify and profile SnCs in single-cell and spatial transcriptomics datasets. Applying this pipeline to a murine muscle implant fibrosis model enriched in SnCs, we develop a spatial taxonomy of senotypes that are associated with distinct aspects of fibrotic tissue development and remodeling.

METHODS: To study fibrosis-associated senotypes, we employed a murine muscle injury implant model that induces fibrosis by creating a volumetric muscle loss injury in the quadriceps and implanting synthetic polycaprolactone particles into the injury site. This model reproducibly induces fibrosis with a high senescence burden at 6 weeks following injury [1], enabling in-depth profiling of SnC subpopulations. To conduct scRNA-seq, we isolated the cells from the fibrotic muscle implants, enriched for CD45+ immune cells, and sequenced them using 10x Chromium 3' (n=3 mice). To conduct spatial transcriptomics, we applied the VisiumHD platform to FFPE sections of the fibrotic muscle implants (n=2 mice) following manufacturer protocols. All datasets were processed using a standardized pipeline in Seurat. We then used a transfer learning approach, projectR [2], to label SnCs using an *in vivo*-derived senescence signature, SenSig, that can capture heterogeneous SnC subpopulations [3]. Cells (scRNA-seq) or pixels (VisiumHD, 8 µm bin) with a projection weight >0 and p_{adj}<0.01 (Wald Test) were considered senescent. We conducted differential expression analysis and non-negative matrix factorization, CoGAPS [2], on select subpopulations within the scRNA-seq dataset to identify biological processes associated with distinct senotypes. Select sequencing results were validated using protein-based multiplex immunofluorescent staining and colocalization analysis in HALO software (n=3-4 mice). Due to cost limitations associated with sequencing, only female mice were used in the analyses to minimize variability across biological replicates. All experiments were conducted under an approved JHU IACUC protocol.

RESULTS: Using scRNA-seq and transfer learning, we identified multiple clusters that were enriched in SnCs (>30% SnCs per cluster): activated fibroblasts (ActF), myofibroblasts (MyoF), and perivascular cells (PV) (**Fig. A**). We validated the presence of fibroblast (F) and PV SnCs by co-staining p16 (senescence marker) with CD31 (endothelial cells), CD11b (myeloid cells), and PDGFRb (F and PV cells). The majority of p16+ cells were positive for PDGFRb (62.0±16.6% PDGFRb+ of p16+; n=4) and negative for CD31 and CD11b (91.1±3.76% CD31- and 68.4±6.02% CD11b- of p16+; n=3). Using differential expression analysis, we identified distinct SASP genes associated with each SnC subtype (p_{adj}<0.05, Wilcoxon Rank Sum Test), illustrating their phenotypic differences. ActF SnCs upregulated diverse genes related to inflammation (*Cxcl12, Ccl8, Saa3*), ECM degradation (*Mmp2/3, Ctsk, Adamts7*), and fibrocartilage ECM (*Col11a1, Fmod, Cilp*), while MyoF SnCs upregulated fibrosis-associated collagens, glycoproteins, and regulators (*Col5/6/12/15a1*, *Tnc, Spp1, Timp1*). PV SnCs upregulated several vascular associated ligands and ECM molecules (*Angpt1/2, Col4a1/2, Col18a1, Igfbp7*).

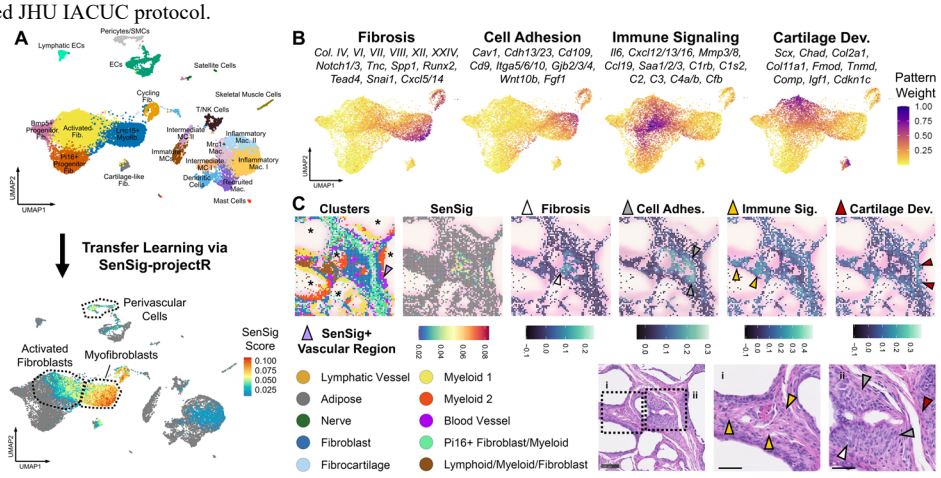

Fig. A. Application of transfer learning (projectR) to scRNA-seq of muscle implant fibrosis using SenSig. SenSig+ cells are classified as SnCs. **Fig. B.** CoGAPS patterns upregulated in fibroblast SnC subtypes. CoGAPS pattern weights in the fibroblast clusters from (**A**) are plotted via UMAP. Select marker genes in each pattern are listed above the plots. **Fig. C.** Application of projectR using SenSig and CoGAPS patterns to VisiumHD of muscle implant fibrosis. Spatial plots show cluster identity, SenSig weight, and CoGAPS pattern weights (in fibroblast clusters only) in each 8 µm bin (top). Serial section H&E images show cellularity and tissue structure in the same region as the spatial plots (bottom, scale bar: 100 µm, 50 µm for insets). Arrows in the spatial plots and H&E images point to regions containing different SnC subtypes. *Implant particles

As clustering was insufficient to fully capture the heterogeneity of fibroblast SnC gene expression patterns, we conducted CoGAPS on the fibroblast clusters to identify gene patterns related to specific senotypes that extended beyond discrete clusters. We identified four patterns upregulated in SnCs (p_{adj}<0.05, t-test between SnC and non-SnC with BH correction) that were expressed by distinct fibroblast subpopulations: Immune Signaling & ECM Degradation ActF (p_{adj}=2e-8), Cartilage Development ActF (p_{adj}=3e-305), Fibrosis MyoF (p_{adj}=1e-118) and Cell Adhesion MyoF (p_{adj}=4e-15) (**Fig. B**).

Next, we used transfer learning to spatially map SenSig and the CoGAPS patterns onto the paired VisiumHD and H&E datasets. Fibrosis and Cell Adhesion MyoF were restricted to regions around the implant particles in proximity to *Spp1*-high myeloid cell clusters (**Fig. C**). By contrast, Immune Signaling & ECM Degradation ActF resided in regions of loosely organized ECM, lymphocytes, and myeloid cells, and Cartilage Development ActF colocalized with aligned ECM fibrils surrounding the implant-adjacent zones. PV SnCs were detected in regions with blood vessels and were not spatially confined to specific zones relative to the implant particles.

DISCUSSION: Understanding the different senotypes that regulate fibrosis will allow us to develop targeted therapies to modulate the fibrotic response. Using a new transcriptomics pipeline, we unveiled heterogeneous fibrosis-associated SnCs that express different SASP profiles and reside in distinct functional spatial niches. MyoF SnCs express fibrosis-associated ECM and colocalize with implant-adjacent *Spp1*-high myeloid cells previously linked to fibrosis [4], suggesting these senotypes may drive fibrotic ECM deposition and maturation. By contrast, ActF SnCs resided outside of the implant interface and were either associated with immune-active and ECM remodeling zones or fibrocartilage-like regions. Lastly, PV SnCs were located throughout the implant and expressed genes related to vascular remodeling. These findings suggest that SnCs regulate diverse processes associated with immune signaling, vascular remodeling, and ECM deposition and form distinct functional niches that may differentially regulate fibrosis progression.

SIGNIFICANCE/CLINICAL RELEVANCE: Our findings provide a taxonomy of fibrosis-associated SnCs that will inform the development of cell-type specific senolytics to treat a wide range of musculoskeletal fibrotic conditions.

REFERENCES (PMID): [1] 32295900, [2] 31121116, [3] 37079217, [4] 40047497

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