A single cell atlas of knee arthrofibrosis reveals cellular heterogeneity in pro-fibrotic microenvironment

Jenny Magallanes[1], Nancy Q. Liu [1], Arijita Sarkar[1], Jiankang Zhang[1], Yuxin Ouyang[1], Tadiwanashe Mkaratigwa[1], Fangzhou Bian[1], Jade Tassey[1], Una Stevic[1], Tautis Skorka[1], Frank A. Petrigliano[1] and Denis Evseenko[1]

[1]University of Southern California (USC), Los Angeles, CA, USA

Disclosures: the authors declare that there is no conflict of interest.

INTRODUCTION. Knee arthrofibrosis is a fibrotic joint disorder where aberrant buildup of scar tissue and adhesions develop around the joint. Although diverse clinical treatment options are available to treat knee arthrofibrosis, there is an urgent need for safer and more effective approaches. Targeted pharmacological interventions may have the potential to alter the outcome of severe knee joint trauma and thereby prevent and treat joint contractures, but more mechanistic studies are needed to understand the molecular and cellular mechanisms of posttraumatic arthrofibrosis. During severe trauma, improper mesenchymal progenitor differentiation can be maladaptive, causing pathological healing. This altered programming of mesenchymal progenitors is manifested in the process of arthrofibrosis however, there is a lack of *in vivo* studies that demonstrate the contribution of various cell populations of multipotent progenitors to these post-traumatic knee joint pathologies. Here, we present an in-depth study profiling the initial changes that contribute to scarring of the joint after post-traumatic joint injury in a novel arthrofibrosis mouse model developed by us followed by single-cell RNA sequencing to understand the cellular heterogeneity of joint capsules 7 days post-injury.

METHODS. Induction of knee arthrofibrosis was performed as previously described (1) under a dissection microscope (Leica). Following wound closure, animals were allowed free cage activity. The right knee joint served as uninjured controls. We microdissected joint capsules, which included the infrapatellar fat pad, patella, synovium, and menisci after removing the skin and muscle from 7 days post-injury (dpi) (n = 3) or 7 days post-sham (dps) (n = 3) mice. Joint capsules were digested, and live, Ter 119- cells were sorted and single-cell RNA sequencing (scSEQ) was performed using 10X genomics 3'UTR gene expression protocol. All animals were on a C57BL/6 background. Genotyping was performed according to protocols provided by JAX. All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of USC. scSEQ data was analyzed using Cell Ranger (v7.0.1) and further filtered using Seurat (v4.3.0). Filtered cells were normalized with SCTransform, and dimensionality reduction was performed using the first 30 principal components (PCs) with UMAP (min_dist = 0.5, n_neighbors = 30). CellChat was used to determine cell-cell communication networks across clusters using a combination of social network analysis, pattern recognition and manifold learning approaches. The CellChatDB mouse was used for analysis. Functional annotation for marker genes for each cluster was performed using Enrichr.

RESULTS. To dissect the molecular landscape of arthrofibrosis we developed a novel arthrofibrosis mouse model (1) and profiled joint capsules from these mice after 7 dpi and 7 dps using scSEQ, constructing a single cell atlas of pro-fibrotic microenvironment. We generated a dataset yielding high quality profiles of 1,251 cells per sample (3222 and 4612 median genes per cell for dpi and dps respectively) to study cellular heterogeneity of this system. We initially performed graph-based clustering of the complex landscape and identified 13 cell clusters comprised of fibroblasts, neutrophils, pericytes, endothelial cells, macrophages, and chondrocytes. The various cell populations were annotated based on the expression of corresponding marker genes in a cluster. Fibroblast cell types were identified based on the expression of broad fibroblast markers (*Pdgfra, Lum, Loxl1, Fbln2, Col1a1, Twist1*). The identities of clusters outside the Fibroblast subset were resolved using previously reported markers, and included Neutrophils (*S100a9, Csf3r, Il1b*), Endothelial cells (*Tie1, Pecam, Cdh5*), Macrophages (*Lyz2, Cd68, Itga4*), and Chondrocytes (*Col2a1, Acan, Sox9*). All 13 clusters were present in 7 dpi and 7 dps. Neutrophils, endothelial cells, macrophages, and chondrocytes were all more abundant in 7 dpi. Fibroblasts were the predominant cell type, representing ~60% of the total population. This observation is consistent with previous studies that demonstrated that fibrotic scars are mostly composed of densely packed fibroblasts and myofibroblasts. Thus, we performed a detailed characterization of the Fibroblast cell types by reclustering them. Reclustering of the Fibroblast populations resulted in 9 clusters. Analysis of enriched genes for each cluster revealed a diversification of function with proliferation and extracellular matrix (ECM) remodeling pathways localizing to distinct cell types including myofibroblasts. In our dataset, myofibroblasts showed the strongest enrichment for core enrichment genes, including *Acta2* and *Tagln*. Among the myofibroblasts we identified cycling myofibroblasts which were previously identified and characterized in a scSEQ analysis of human Dupuytren's nodules, a localized fibrotic condition of the hand (1). These cycling myofibroblasts were more abundant in arthrofibrosis and expressed the highest levels of *Acta2* and proliferation-related genes (*mKi67, Top2a, Cenpa*). We also observed a cluster enriched for genes related to Wnt signaling, including Wnt2, which is involved in regenerative fibroblast competency in skin wound healing. This cluster was dominated by 7 dps cells suggesting that arthrofibrosis leads to a decrease in the frequency of Wnt2 expressing fibroblasts, which have an increased capacity to support regenerative wound resolution. CellChat was used to infer global cell-to-cell communication networks among the various fibroblast clusters. Comparative analysis revealed a total 5694 and 3032 interactions with high interaction strength in 7 dpi and 7 dps respectively. Of the several signaling pathways, Tgfβ and Wnt signaling networks were found to be playing a predominant role in arthrofibrosis.

DISCUSSION. The pro-fibrotic microenvironment consists of multiple cell subpopulations with diverse genetic and phenotypic characteristics. Although arthrofibrosis pose an immense clinical problem, the cellular origin and molecular mechanisms contributing to this disorder remains elusive. In addition, the current animal models of knee arthrofibrosis and stiffness are developed in rats and rabbits, limiting genetic experiments that would allow us to explore the contribution of specific cellular targets to these knee pathologies. Here we present a novel arthrofibrosis mouse model and build a single cell atlas of pro-fibrotic microenvironment. Our results delineate a unified molecular program emphasizing on distinct fibroblast and myofibroblast cell types. We show that myofibroblasts especially cycling myofibroblasts are the key drivers of dysregulated wound-healing process that defines fibrosis. Cell-to-cell communication analysis mediated by ligand receptor interactions predicted key incoming and outgoing signals e.g., Tgfβ and Wnt signaling for specific cell types indicating their critical role in wound healing.

SIGNIFICANCE. In summary, this study presents a novel clinically relevant injury model of posttraumatic knee arthrofibrosis in mice. The establishment of this mouse model of post-traumatic joint injury provides an opportunity to test the roles of various cell populations in knee arthrofibrosis. The data presented here will serve as a foundation to understand how these cell populations promote an abnormal wound healing response to injury and could improve strategies to reverse or halt arthrofibrosis.

ACKNOWLEDGEMENTS. This work is supported by NIH grants R01AG058624 (DE) and DoD grant HT9425-23-1-0718 (DE).

REFERENCES.

1. Magallanes J, Liu NQ, Zhang J, Ouyang Y, Mkaratigwa T, Bian F, Van Handel B, Skorka T, Petrigliano FA, Evseenko D. A new mouse model of post-traumatic joint injury allows to identify the contribution of Gli1+ mesenchymal progenitors in arthrofibrosis and acquired heterotopic endochondral ossification. Front Cell Dev Biol. 2022 Aug 24; 10:954028.
2. Layton TB, Williams L, McCann F, Zhang M, Fritzsche M, Colin-York H, Cabrita M, Ng MTH, Feldmann M, Sansom SN, Furniss D, Xie W, Nanchahal J. Cellular census of human fibrosis defines functionally distinct stromal cell types and states. Nat Commun. 2020 Jun 2;11(1):2768.