Cellular Drivers of Regeneration and Fibrosis in Connective Tissue Repair

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Introduction: Connective tissue wound healing can result in either regenerative repair or fibrotic scarring. Normal wound healing is a tightly regulated and organized process in which the inflammatory response is rapidly resolved, enabling tissues to return to homeostasis. In contrast, prolonged or chronic wound healing can lead to pathological fibrosis. This process involves a wide range of cell types working in a highly coordinated manner. Senescent cells have been shown to play dual roles in wound healing. While they can promote tissue repair, their accumulation along with the senescence-associated secretory phenotype (SASP), can exert both local and systemic effects, including alterations in immune cell function. Fibroblasts are central to connective tissue wound healing, and during tissue injury, they can differentiate into activated phenotypes such as myofibroblasts, which drive tissue contraction and extracellular matrix remodeling. These activated fibroblasts not only influence tissue structure but also modulate immune responses during the healing process. Macrophages are another essential cell type in wound repair. They contribute to debris clearance and facilitate the transition from inflammation to regeneration, playing a key role in the resolution and remodeling phases of healing. We hypothesize that regenerative healing results in activation of distinct fibroblast and macrophage populations, and that these populations could be potential regenerative wound healing targets.

Methods: Single-cell RNA GEO sequencing data (GSE141814) was analyzed using Seurat (5.0.1) in R studio[1]. Single, live cells were isolated from day 18 wound healing from mice[1]. Regenerative wound healing was generated in a de novo follicle healing model compared to a wound fibrotic healing model in young (6-8 week old) male and female mice C57Bl/6j background. Full-thickness skin excision was performed. These large full-thickness wounds can result in a regenerative (de novo hair follicles) or fibrotic (hairless scars). Wounds closed in all models within 48 hours. Wounds showed dermal remodeling and reepithelization within 14 days. The tissue was taken from regenerative and fibrotic models on day 18 post wound healing induction. Cells were isolated from 7 to 10 wounds from each model type. The regenerative model captured approximately 10,500 dermal cells with read depth of approximately 41,000 across 19,000 genes with 3200 unique identifiers, the fibrotic model captured approximately 5500 dermal cells with read depth of approximately 70,000 reads across 17,000 genes with 2,400 unique molecular identifiers[1]. Data normalized and clustered using principal component analysis (PCA). Single-gene and gene set expression profiles were mapped onto UMAP visualizations. The data were further interrogated using gene sets related to senescence, aging, regeneration, and macrophage function, including SenMayo, Reactome, GenAge, and KEGG pathways. Additionally, functional enrichment analysis was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery) and STRING, incorporating pathways from Gene Ontology (GO), KEGG, and Reactome.

Results: Single cell sequencing assessments found multiple cell populations in both the regenerative and fibrotic tissue, including fibroblast, macrophage, neutrophil, dendritic and endothelial populations (Figure 1). Upregulation of senescence markers using SenMayo, a database that is enriched for putative cellular senescence secretory phenotype, termed the Senescence Associate Secretory Phenotype (SASP) were found in both tissue healing systems (Figure 2). Reactome analysis showed an increase in macrophage function in both regenerative and fibrotic wound healing (Figure 3). Interestingly we found increased expression of tenascin-c and periostin in the regenerative healing as compared to fibrotic healing. Using DAVID functional analysis in fibroblast and macrophage populations we found that regenerative fibroblasts are enriched for collagen/matrix and acetylation pathways. Regenerative macrophage populations were also enriched for acetylation ans well as mitochondrial related expression. In contrast fibrotic wound healing was enriched for protein binding and ribonucleoprotein complex as were macrophages

Discussion: Senescence is an important regulator of wound repair, which can limit fibrosis and promote regeneration. Single-cell analysis revealed increased senescence markers in both the regenerative and the fibrotic wound healing models. Using gen sets including SenMayo, REACTOME and GenAge1 we identified a marked senescence-related expression profile in regenerative wounds. The markers were particularly elevated in fibroblasts and macrophages. Further functional enrichment analysis showed that regenerative wounds exhibited increased signatures of acetylation, mitochondrial activity and elevated expression of collagen and extracellular matrix components as compared to fibrotic wounds. Acetylation has been linked to regulation of proliferation and immune modulation, while hypoacetylation is associated with fibrotic healing. In contrast, fibroblasts and macrophages from fibrotic wounds were enriched in pathways related to protein binding, acetylation, and ribonucleoprotein complexes. These features are indicative of cellular stress response, excessive extracellular matric projection and activation of profibrotic signaling pathways. Notably, the expression of tenascin C and periostin, key extracellular matrix components, were significantly upregulated in regenerative wounds compared to fibrotic wounds. This pattern aligns with human connective tissue wound healing studies. Ongoing research is investigating the dual role of cellular senescence in both promoting regenerative wound healing and contributing to chronic senescence associated with fibrosis in connective tissue.

Significance: Connective tissue, matrix and the immune cells play a central role in wound healing. Understanding the in situ cellular matrix

References: 1. Gay, D. et al. *Sci Adv* 2020 20;6(12):eaay3704.

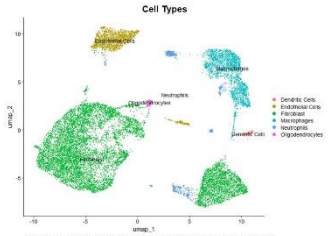

Figure 1: Cell-typing from single cell RNA-Seq analysis of regenerative and fibrotic dermal mouse wounds using existing mouse model data sets.

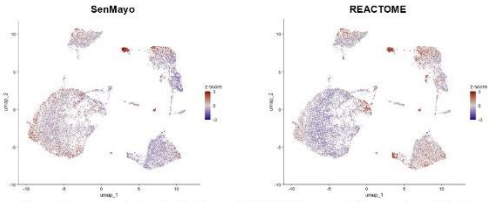

Figure 2: Gene-set analysis using SenMayo and REACTOME gene sets of regenerative and fibrotic dermal mouse wounds using existing mouse model data sets.