Proteome Composition and Tissue Structural Dynamics During Scar-free Ligament Regeneration in Zebrafish

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INTRODUCTION: Following injury, humans ligaments demonstrate limited regenerative capacity. When ruptured, some ligaments fail to bridge and cannot be repaired without surgical intervention. Other ligaments are capable of limited healing, but bridge to form fibrotic scar tissue with a disorganized extracellular matrix (ECM) that is biomechanically inferior to the uninjured tissue[1]. This limitation in ligament healing capacity is not shared by highly regenerative species like the zebrafish. Following total transection of the interopercular-mandibular (IOM) ligament in the jaw, zebrafish are capable of fully regenerating mechanically competent, scar free tissue[2]. Legumain, a cysteine protease that is known to regulate ECM components like fibronectin1, and activate matrix metalloprotease-2, is significantly upregulated in tissue macrophages during this regeneration process. Knockout of Legumain results in phenotypically normal zebrafish which heal with a scarring phenotype, and generate reduced and dysmorphic ligaments with poorly organized extracellular matrix following ligament transection. The objective of this study was to define the matrix environment associated with normal regenerative processes in a zebrafish model and identify differentially expressed proteins associated with pro-regenerative, scar free tissue remodeling. To understand the landscape of the normative and fibrotic ligament healing, we performed a global label-free proteomic screen on whole joint samples micro dissected from uninjured zebrafish and healing ligaments 3 days post ligament transection (dplt) in legumain mutant and wildtype fish. Using label-free Mass Spectroscopy, we assess the proteomic landscape proteins associated with normal and dysregulated ligament regeneration. We additionally captured the temporal dynamics of tissue organization using high resolution Fast Green imaging of the collagen network throughout healing. The objective of this study was to define the matrix environment associated with normal regenerative processes in a zebrafish model and identify differentially expressed proteins between scar-forming and scar-free ligament regeneration.

METHODS: With IACUC approval (AABF1555), complete transection of the IOM ligament was performed on 5-12 month-post-fertilization adult zebrafish as described previously[3]. Joint tissue was collected from uninjured (UI) and three days post-ligament transection (3 dplt) *legumain* wildtype ($lgmn^{WT}$), and *legumain* mutant ($lgmn^{MUT}$) fish. Triplicate pools of tissue from 15 uninjured fish ($lgmn^{WT}$ 20M, 25F. $lgmn^{MUT}$ 30M, 15F) and from 10 3 dplt fish ($lgmn^{WT}$ 14M, 16F. $lgmn^{MUT}$ 15M, 15F) were shipped on dry ice to MS Bioworks for label-free quantitative protein profiling of ligament tissue samples. 500ng of purified protein extracted from each pooled sample was analyzed by LC/MS with a ThermoFisher Vanquish Neo UPLC system interfaced to a ThermoFisher Orbitrap Astral operated in data-independent mode. Peptides were loaded on a trapping column and eluted over a 150µm analytical column heated at 55C at 1.5µL/min with a 30-minute gradient. Sequentially, full scan MS data (240,000 FWHM resolution) from m/z 380-980 was followed by 300 x 2m/z precursor isolation windows; products were acquired in the Astral at 40,000 FWHM resolution. DIA data were analyzed using DIA-NN (v1.9.2) with database search results filtered at 1% peptide and protein false discovery rates against the Uniprot Zebrafish library. Statistical analysis of captured proteins was conducted using R and Rstudio with the DEP, MatrisomeAnalyzeR, and GSEA packages. Additional $lgmn^{WT}$ and $lgmn^{MUT}$ fish (M13, F29) underwent IOM transection and were sacrificed at 0, 3, 7, 14, and 28 dplt. Samples were fixed for 24 H in paraformaledehyde, photobleached on a hydrogen peroxide solution, and permeabilized in a KOH solution. Samples were then dehydrated in MeOH to enable efficient anhydrous collagen staining with Fast Green FCF[4]. The tissue was cleared via the iDISCO+ protocol, and collagen architecture was imaged through the full ligament depth with a Leica SP8 confocal microscope.

RESULTS: Proteomic analysis resulted in the detection of 10,023 unique proteins across all samples, including 402 core matrisome and matrisome-associated proteins. In uninjured and 3dplt groups, Legumain was present in all $lgmn^{WT}$ samples and absent in all $lgmn^{MUT}$, confirming complete KO. Analyses of $lgmn^{MUT}$ and $lgmn^{WT}$ samples identify distinct proteomic profiles, with 549 proteins differentially expressed across two or more sample groups ($lgmn^{WT}$ UI, $lgmn^{WT}$ 3 dplt, $lgmn^{MUT}$ UI, $lgmn^{MUT}$ 3dplt). During normal healing processes, healing is driven by macromolecular and peptide biosynthetic processes as new matrix is deposited to bridge the injured ligament. Gene ontology analysis of biological processes comparing the $lgmn^{WT}$ and $lgmn^{MUT}$ samples at the early regenerative 3 dplt timepoint indicate that the greatest differential expression of proteins is found in protein families involved in heterooligomerization and proteolysis. No significant differences were found in fibrillar collagen or elastin content between $lgmn^{MUT}$ and $lgmn^{WT}$ samples in uninjured or 3 dplt samples. Full-tissue collagen imaging shows greater persistence of proximal ligament stub and in $lgmn^{MUT}$ fish and an apparent reduction in crimped collagen fibers following transection.

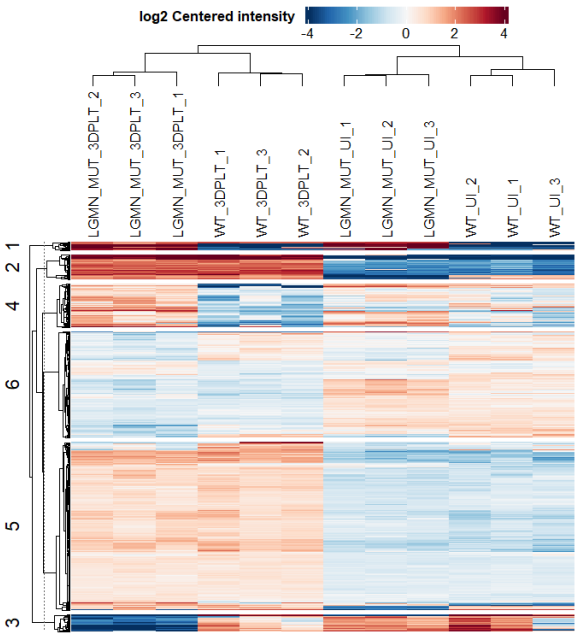

Figure 1: Heat map of proteins with significant ($p<0.05$) differential expression across two or more sample groups ($lgmn^{WT}$ UI, $lgmn^{WT}$ 3 dplt, $lgmn^{MUT}$ UI, $lgmn^{MUT}$ 3 dplt)

DISCUSSION: Differences between $lgmn^{WT}$, and $lgmn^{MUT}$ proteomic profiles indicate that while legumain is crucial for regenerating ligaments to organize the deposited extracellular matrix, it does not directly modulate the extracellular matrix composition through regulation of the quantity of major structural ECM components like fibrillar collagens during early ligament regeneration. These data indicate that Legumain is responsible for the differential activation of proteases in early regeneration that govern the crosslinking and clearance of matrix structural proteins. Dysregulation of proteases involved in clearance of damaged matrix and stabilizing deposited collagen via crosslinking results in a disorganized matrix that does not recapitulate uninjured tissue architecture.

SIGNIFICANCE/CLINICAL RELEVANCE: This work aims to elucidate the biological processes underlying organized and disorganized ECM regeneration throughout the healing of highly loaded tissues. Differentially expressed proteins between scarring and non-scarring ligaments may provide promising targets for promoting scar-free ligament regeneration in humans.

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