Probe-sequencing Applications Allows for Transcriptomic and Epigenetic Profiling of Fibroblast-Derived, Ossification-Resistant Cartilage in the Regenerating Green Anole Lizard Tail

Darian Gamble, Thomas Lozito 1 Department of Stem Cell Biology and Regenerative Medicine, Keck School of Medicine, University of Southern California, 1425 San Pablo St, Los Angeles, CA 90033, USA. 2 Department of Orthopedic Surgery, Keck School of Medicine, University of Southern California, 1540Alcazar, Los Angeles, CA 90089, USA. Email of presenting author Djgamble@usc.edu

Disclosures: None

Introduction: Enhancing healing outcomes in wounded patients, including new tissue formation and scar inhibition, is a fundamental goal of medicine. A branch of regenerative medicine studies cellular and molecular mechanisms regulating "super-human" healing abilities in various animal species toward improving rehabilitation in patients. Lizards are the only amniotes, and the closest relatives of mammals, capable of regenerating amputated appendages involving multiple tissue lineages as adults. Lizards undergo blastema-based regeneration characterized by the formation of a mass of multipotent mesenchyme derived from resident fibroblastic connective tissue cells (FCTCs) at the amputation site. These blastema cells differentiate to regrow all connective tissues present in regenerated appendages. Lizard tail regeneration is characterized as "imperfect" due to important differences between original and regrown tail tissues. Most notably, ossified vertebrae present in original tails are replaced by a hyaline cartilage tube that resists endochondral ossification for the lifetime of regenerates. The natural abilities of lizards to convert somatic fibroblasts to ossification-resistant hyaline cartilage represents an attractive pathway to treat human joint pathologies, such as osteoarthritis, which are marked by cartilage degeneration and fibrosis. However, the transcriptomic and epigenetic changes that support the conversion of FCTCs to ossification-resistant cartilage remain poorly characterized. Previous single-cell sequencing analyses of lizard blastemas from our lab indicated that fibroblast chondrogenesis followed a sequential gene expression pattern (Figure 1A-D). We propose leveraging this knowledge of lizard blastemas gene markers and utilizing probe-sequencing technology to isolate specific chondrogenic FCTC populations for deep transcriptomic and epigenetic analyses. We hypothesize that lizard blastema chondrogenesis and ossification resistance are supported by epigenetic reprogramming in tail fibroblasts; pro-chondrogenic gene enhancers become more accessible to Sox9 engagement, while pro-osteogenic enhancers become less accessible to RunX2 binding. We aim to identify and validate these specific gene regions associated with lizard blastema cartilage development. Recreating similar chromatin accessibility changes in mammalian fibroblasts will allow these cells to regenerate hyaline cartilage instead of forming scars.

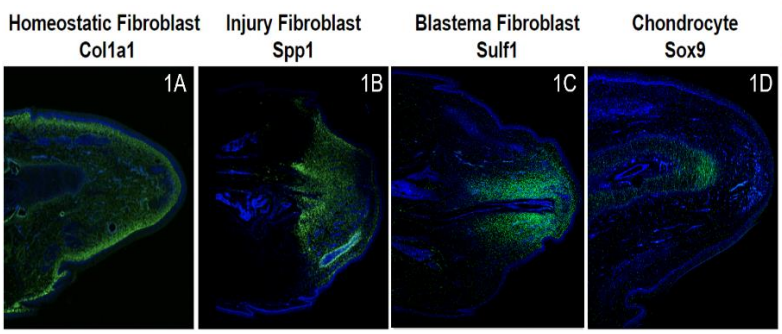
Methods: Dissociation: For each experiment, 10 green anole lizards undergo amputation with a sterile blade approximately two inches from the base of the tail. 14- or 28-days post amputation, regenerating tissue was collected for dissociation. Epidermal tissue was removed from all samples and placed into a digestion solution of 0.25% trypsin and 0.65% collagenase type 2 for an hour at 37 C. Cells were then fixed in 4% NFB for 15 mins at room temperature. Hybridization chain reaction fluorescence in situ hybridization (HCR-FISH): HCR-FISH was performed according to the most recent version of Molecular Instruments LLC protocol for FISH on cells in suspension. Briefly, cells underwent multiple incubation periods with mRNA probes and fluorophores allowing gene expression to be associated with fluorescence intensity. Cells were then sorted using an ARIA FACS and collected into RNA-protect from Qiagen for storage. RNA-isolation was performed using the Qiagen FFPE total RNA extraction kit. Library preparation was performed using the Illumina Total-RNA library prep kit with Ribo-zero plus rRNA depletion which included probes specific to green anole rRNA. Read alignments were performed using STAR and DEseq2 was used for bulk RNA-seq analysis of cell populations. ATAC-seq kits were purchased from Active Motif and used according to the included protocols. Galaxy was used to complete ATAC-seq analysis. All procedures involving animals have been performed in accordance with approved USC IACUC guidelines and protocols.

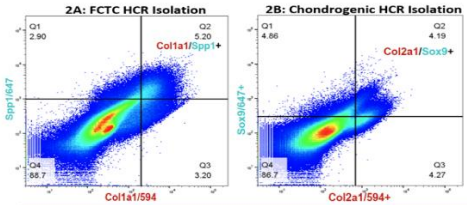
Results: HCR-FACS is a successful method to isolate cells based on expression of multiple genes in the regenerating lizard tail. Figure 2A shows a FACS plot of isolated FCTC cells from 10 blastema stage tails sorted by expression of Col1a1, a homeostatic fibroblast marker, and Spp1, an activated fibroblast marker denoting regeneration competence. Figure 2B shows a FACS plot of chondrogenic cells isolated from 10 fully regenerated tails sorted by expression of Sox9, a chondrogenic progenitor cell marker, and Col2a1, a mature chondrocyte marker. In both plots, cells from each population were collected and PCR amplification of marker genes showed successful isolation of pure populations. Transcriptome library construction from fixed, HCR sorted cells yields raw reads capable of differential gene expression analysis. Libraries were constructed from total RNA isolated from HCR sorted populations. Table 1 lists sequencing results showing high levels of reads generated comparable to unfixed samples. HCR sorted HbD positive red blood cells serve as a negative control for correlation analysis between samples to test effectiveness of our Bulk-sequencing analysis.

Discussion: In order to address the main hypothesis that lizard blastema chondrogenesis and ossification resistance are supported by epigenetic reprogramming in tail fibroblasts; pro-chondrogenic gene enhancers become more accessible to Sox9 engagement, while pro-osteogenic enhancers become less accessible to RunX2 binding, we first needed a way to isolate pure populations of chondrocytes and their precursors cells. HCR -FACS has been successful in isolating specific populations dependent on expression of multiple expression markers. Analysis of these populations using PCR analysis shows that sorted samples are pure and show strong expression of their sorted markers. Total RNA isolation and rRNA depletion yielded high quality RNA capable of being sequenced for transcriptomic analysis of cells along the FCTC to chondrocyte transition.

Significance and Clinical Relevance: Enhancing the regenerative capabilities of mammalian cells to regenerate ossification resistant cartilage could cure or alleviate symptoms of patients with arthritis diseases or are in need of repair. In determining the mechanism by which lizards regenerate ossification-resistant hyaline cartilage we aim to recreate these principles in mammalian cells and enhance chondrogenic regeneration while preventing scarring.

ACKNOWLEDGEMENTS: Funding by the NIH (R01 GM115444) and USC (Wright Foundation Pilot Award)





Sample ID	Conc.	Units	Sample	Data(million)
Col1a1 6/29	3.827	ng/ul	Col1_1	37.9
Col1a1 s001	1.035	ng/ul	Col1_2	40.3
Hbd s001	1.848	ng/ul	Hbd_1	43.9
Hbd s002	8.018	ng/ul	Hbd_2	47.2
Spp1 s001	1.113	ng/ul	Spp1_1	38.2
Spp1 004	2.089	ng/ul	Spp1_2	35.1
Spp1 005	1.049	ng/ul	Spp1_3	34.6
			Total	277.2