# Understanding Lineage Capacity of Blastema-Driven Craniofacial Joint Regeneration

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**INTRODUCTION:** Humans cannot naturally repair joint tissues after injury or aging-dependent tissue degradation. However, we find that Zebrafish have lubricated synovial joints in their jaws and can regenerate hyaline cartilage, supportive ligaments, as well as joint morphology and function after total joint resection[1–3]. We previously described that following a complete joint resection injury, adult zebrafish restore tissue morphology and joint function within 2 months[3]. This regeneration is driven by a transient population of cells that form at the site of injury known as a blastema. Previous models of blastema-based regeneration each involve a single blastema that grows and patterns to form tissues, organs, or limbs. In contrast, our model results in the formation of three individual blastema that later fuse together to form a single regenerated joint that may be interrogated both individually and collaboratively to reveal the regenerative program(s) capable of restoring a functional joint after complete loss. Currently, the cell populations that contribute to the blastema responsible for regenerating the zebrafish jaw joint remain unknown, as does how each of the 3 individual blastema may influence the composition of the regenerating joint. Using transgenic lines for whole-mount stereoscope and confocal imaging, as well as histological analyses and single cell sequencing, we define molecular markers of the early blastema and track the composition of regenerating joints *in vivo* up to 56 days post joint resection (dpjr). Furthermore, to test the role of cell extrinsic versus cell intrinsic cues in blastemal cell fate bias, we developed a new protocol for zebrafish craniofacial blastema isolation and joint tissue explant culture. The goal of this study was to test whether the anatomical origin of a blastema can influence the composition of the tissue and structure being regenerated.

**METHODS:** Unilateral Joint Resection Surgery: Adult zebrafish were anesthetized and spring scissors are used to make 3 cuts to completely resect the quadrate-articular jaw joint. The cuts are made through the anguloarticular bone, the interopercular bone, and through the quadrate bone. Next, the joint is removed using forceps and fish are revived in system water. Histological Analyses: For hematoxylin & eosin (H&E) staining, 5 µm paraffin sections were deparaffinized with Hemo-De Xylene Substitute before H&E staining and being mounted onto slides with cytoseal. Immunofluorescence and smFISH experiments were performed on 5µm formalin-fixed paraffin embedded (FFPE) tissue sections. For Single-molecule fluorescence *in situ* hybridization was performed using RNAScope Multiplex Fluorescent Reagent Kit v2 Assay or Hybridization Chain Reaction RNA-Fish. scRNAseq: Joints were collected from adult zebrafish at seven different timepoints to capture major stages of healing post-resection, including uninjured, 1, 3, 7, 14, 28, and 70 dpjr. Following FACS for all live cells, we used the 10X Genomics Chromium Controller to generate barcoded single cells. scRNAseq libraries were prepared for a minimum of two independent samples per timepoint. Downstream analyses were performed using Seurat in R and, after quality control filtering, a total of 63,305 high-quality cells were clustered into 10 broad superclusters. Skeletal cell clusters were subset and further analyzed. Tissue Clearing and Imaging: Transgenic *thbs4a_p1:eGFP;scxa:mCherry* fish were used to visualize changes in cartilage, ligaments, and tendon during regeneration. *sp7:GFP* transgenic or calcein blue stain were used to label osteoblasts or mineralized bone tissue. Following CUBIC tissue clearing[4], joints were imaged with a Leica SP8 confocal microscope with lightning deconvolution. *In Vitro* Culture: To test for cell-intrinsic bias in lineage fate commitment decoupled from spatial cues or mechanical signaling, individual blastema were surgically removed, sterilized using a mixture of DMEM, 20% FBS, 1% penicillin-streptomycin, and 2% amphotericin B, dissociated using accutase, and grown *in vitro* to discern their relative lineage fate capacity. Zebrafish cell cultures, as well as whole joint explants, were incubated at 28.5°C, 3% $O_2$, 2.5% $CO_2$, in low glucose DMEM containing 20% FBS and 1% penicillin-streptomycin. Statistical analysis was carried out using Prism 10 by One-Way ANOVA with Tukey's multiple comparison test. All experimental cohorts were pooled and sexes were equally represented within pools. This study was approved by CUIMC IACUC.

**RESULTS SECTION:** We find that following whole jaw joint resection, three blastema (anterior, posterior, and dorsal) are induced. Through single-cell RNA sequencing, single-molecule *in situ* hybridization, and PCNA immunofluorescence, we find that blastema cells are broadly marked by transient expression of *fn1a*, *tnfaip6*, and *hmga1a*, and that the 3 blastema proliferate at approximately similar rates. We first found expression of these genes in periosteal, stromal and bone-adjacent mesenchyme compartments at 1 dpjr, suggesting a potential tissue origin for these blastemal fibroblasts around the resected bones. Histological analysis shows that blastemal cells cap the cut bone ends by 3 dpjr. Blastemal cell proliferation peaks at 3 days-post-injury to fill in the injury space by 1 week following resection (n=6)[3]. Next, to isolate the relative contribution of individual blastema to the regenerating joint, repeat resections were used to prevent two of the three blastema from regenerating, allowing only a single blastema to contribute cells to the regenerate before analyzing regenerate composition at 56 dpjr. Through repeated resections, we found that the anterior blastema more readily forms osteogenic fates (n=5; p=0.0339), with associated reduced formation of *scxa*+ connective tissue cell fates (n=5; p=0.043). We find that hypoxic atmospheric conditions as low as 3% $O_2$ and elevated FBS concentrations as high as 20% promote healthy proliferation of zebrafish blastema cells *in vitro* (n=3), while standard human cell culture conditions of 20% $O_2$ and 10% FBS resulted in accelerated cell death of the blastemal cells within 7 days (n=8). Using the same cell culture techniques we refined for blastemal cells, explants of the quadrate-articular joint survived and maintained *scxa*+ ligament identity *ex vivo* for up to 14dpjr (n=3). After performing whole joint resections and allowing the 3 blastema to proliferate out to 4dpjr, we then surgically isolated the blastema and chemically dissociated them before growing the combined blastemal cells in culture. We then observed an *in vitro* bias to adopt *sp7*+ osteogenic fates by 21 days when all blastema are cultured together (n=3).

**DISCUSSION:** Previously we showed *de novo* articular joint formation in zebrafish following a complete joint resection, here we investigated how the three blastema function cooperatively or independently when restoring all mature joint tissues during regeneration. We observe that unlike single plane amputation models with unilateral growth, our avulsion-style injury model features multiple sites of blastema-driven regeneration that provide a unique patterning with the three blastema proliferating to meet near the center. Through repeat resections we observed differential tissue composition of the regenerating joint, with anterior blastemal cells biased towards osteogenic fate at the expense of connective tissue fate. This implies that while individual blastema are capable of forming the major joint cell lineages, there is lineage bias in their output *in vivo* depending on injury site differences in blastemal composition. Isolated cell cultures of each blastema suggest that tissue origin may play a pivotal role in regenerative lineage capacity based on etiological origin, potentially defining regenerate composition. By developing new culture conditions that allow for *ex vivo* joint explant culture and *in vitro* manipulation of zebrafish craniofacial blastemal cells this study opens possibilities for future assays to probe cell-intrinsic lineage fate commitment biases in regeneration and define the cues that drive articular cartilage and ligament fate from blastema cells. Taken together, our results define the molecular signature of joint blastema cells and probe the lineage biases of blastema-driven regeneration of joint tissues.

**SIGNIFICANCE/CLINICAL RELEVANCE:** Ligament and tendon injury in humans normally resolves in the formation of a fibrotic scar that is functionally inferior in terms of providing support and stability to its respective joints, however following a complete resection injury, we find that zebrafish possess endogenous pathways to completely reform the mature tissue types of a joint including ligament, bone, and lubricated articular cartilage, from progenitor populations derived from surrounding tissues. By enhancing our understanding of the regenerative program of zebrafish blastema-driven regeneration, including both intercellular signaling and the skeletal cell populations that contribute to this functional joint regeneration, we work towards translational medical approaches to improve clinical outcomes that restore mature tissue types and function.

**REFERENCES:** [1] Smeeton, J. *et al.* Regeneration of Jaw Joint Cartilage in Adult Zebrafish. *Front. Cell Dev. Biol.* **9**, (2022). [2] Anderson, T. *et al.* Ligament injury in adult zebrafish triggers ECM remodeling and cell dedifferentiation for scar-free regeneration. *Npj Regen. Med.* **8**, 1–15 (2023). [3] Blumenkrantz, M. *et al.* Dynamic cell fate plasticity and tissue integration drive functional synovial joint regeneration. 2024.12.12.628180 Preprint at https://doi.org/10.1101/2024.12.12.628180 (2024). [4] Susaki, E. A. *et al.* Whole-Brain Imaging with Single-Cell Resolution Using Chemical Cocktails and Computational Analysis. *Cell* **157**, 726–739 (2014).

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