Dynamic cell plasticity during synovial joint regeneration in adult zebrafish

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INTRODUCTION: Synovial joints are complex organs consisting of lubricated articular cartilage, specialized synovial cavities, and ligaments stabilizing the articulating bones. In humans and mammalian models, mature joint tissues have a limited ability to regenerate in their native forms. Specifically, repairing articular cartilage remains a major clinical problem. Despite the sizeable clinical burden of joint disease, we currently have a poor understanding of both the gene regulatory networks driving articular cartilage cell fate specification, as well as the cellular mechanisms through which joint progenitor cells regenerate and integrate into functional tissues after injury. Although mammalian models are informative in uncovering cells and pathways contributing to articular cartilage development, they repair joint injury with fibrous tissue, not native cell types. The highly regenerative zebrafish, however, can regenerate individual musculoskeletal tissues including bone, tendons, and ligaments as adults. As they also have lubricated synovial joints, zebrafish may be a useful model for dissecting the pathways that enable regeneration of native articular cartilage. Here, we have developed a new whole-joint regeneration model to test the capacity of adult zebrafish to perform coordinated regeneration of a synovial joint. We hypothesized that following full resection of the synovial joint, adult zebrafish would exhibit dynamic cell plasticity that resulted in the regeneration of all synovial joint tissue types into a new 3D structure. Specifically, we hypothesized that tissue-resident fibroblasts express pro-regenerative genes and reactivate developmental programs, with these progenitor cells ultimately differentiating into mature synovial joint cell types.

METHODS: Our lab has devised an adult zebrafish joint regeneration model involving complete resection of the synovial jaw joint. The surgery consists of 3 cuts (along the anguloarticular, interopercular, and quadrate bones in the jaw), and removes the synovium, articular cartilage, and interopercular-mandibular ligament. Microcomputed tomography was used to confirm joint removal and visualize 3D bone structure after regeneration. Histological analysis characterized the early injury response and confirmed the regeneration of a joint cavity. To assess the re-establishment of mature joint tissue types, we used a combination of live repeated stereoscopic imaging and 3D confocal imaging of tissue-cleared fluorescent transgenic zebrafish lines labeling bone, ligament, or articular cartilage. A cranial neural crest-specific Sox10 enhancer to drive Cre recombinase in a line of fish expressing a ubiquitous loxP-BFP-loxP-DsRed (*Sox10:Cre;β-actin:BFP→DsRed*) was used for preliminary lineage tracing of regenerating joint cells. To define the cell population and gene expression changes after joint removal, we performed single-cell RNA sequencing at 7 unique time points spanning the time course of joint regeneration. We followed the 10X Chromium Single Cell Gene Expression protocol to sequence all live cells from uninjured zebrafish jaw joints as well as regenerating jaw joints at 1, 3, 7, 14, 28, and 70 days-post-joint-resection (n=2 independent samples per timepoint). Using Seurat in R, we integrated the datasets and clustered the cells using the standard QC and analysis workflow. Fluorescence *in situ* hybridization for cell lineage markers was used to confirm the transcriptional changes observed *in silico*. All zebrafish experiments were approved by the Columbia University Institutional Animal Care and Use Committee.

RESULTS SECTION: Following whole-joint resection, we find that endogenous neural-crest-derived progenitor cells resident within the adult zebrafish skeleton respond to loss of the jaw joint by mesenchymal bridging driven from 3 blastema (anterior, posterior, and dorsal). The initial injury response involves epithelium capping the cut sites by 1 day-post-joint-resection (dpjr), with mesenchymal invasion into the wound initiating by 3 dpjr. By 7 dpjr, epithelial and mesenchymal tissue fully bridge the resected area (n=4 per timepoint). Immunofluorescence staining for proliferating cell nuclear antigen (PCNA) shows proliferation surrounding the resected bone stubs, with PCNA expression peaking by 3 dpjr and decreasing by 7 dpjr (n=2-3 per timepoint; ANOVA p=0.01; 1 dpjr vs 3 dpjr, p=0.01; 3 dpjr vs 7 dpjr, p=0.04). Live imaging showed that ligament and articular cartilage fate (*thbs4a_p1:eGFP+*) is re-established as early as 14 dpjr (n=21). 3D imaging of the tissue-cleared regenerate at a higher resolution revealed that *thbs4a_p1:eGFP+* cells with rounded, articular chondrocyte-like morphology are present by 2 months-post-joint-resection (mpjr) (n=13). Single-cell transcriptomics of regenerating joint tissues revealed the presence of several fibroblast populations that were unique to early regeneration (1 dpjr – 7 dpjr). The transcriptional signature of these fibroblasts includes both known genetic drivers of joint development, such as *sox9a* and *nkx3-2*, as well as regeneration-specific genes. As early as 14 dpjr, mature bone (*ifitm5+*, *bglap+*), ligament (*scxa+*, *thbs4a+*), cartilage (*mia+*, *col2a1a+*), articular cartilage (*thbs4a+*, *prg4b+)*, and synovium (*dub+*, *prg4b+*) clusters were present and transcriptionally similar to uninjured joint cell populations. By 1 year-post-joint-resection, new bone growth has bridged the resected area and a regenerated joint-like structure is visible via microcomputed tomography (n=7). Fluorescence *in situ* hybridization for *prg4b*, which encodes lubricin, the lubricating glycoprotein found in synovial fluid, showed that *prg4b* is strongly expressed in cells lining the new joint cavity at 14 dpjr, 21 dpjr, and 2 mpjr (n=3 per timepoint). Thus, adult zebrafish have the capacity to regenerate joints with lubricating articular cartilage.

DISCUSSION:
Our data show that after whole-joint resection in adult zebrafish, the injured site undergoes a wound healing response, mesenchymal bridging, lineage differentiation, and reintegration of all mature synovial joint cell types into a new 3D structure that includes articular cartilage. Our *in vivo* imaging data of both live and tissue-cleared transgenic animals showed that after all mature joint tissues were removed following resection injury, adult zebrafish were consistently able to regenerate all synovial joint cell types in a coordinated manner. Our single-cell RNA sequencing analysis confirmed this finding on a transcriptome-wide scale, first showing a loss of cell clusters expressing mature joint cell markers at early regeneration timepoints (1-3 dpjr) and then a re-establishment of mature joint cell transcriptional profiles during later regeneration (14-70 dpjr). The regeneration of every synovial joint tissue type after complete removal suggests a dynamic plasticity of tissue-resident fibroblasts. Indeed, our single-cell transcriptomics analysis points to a putative mesenchymal cell progenitor population for regenerating articular cartilage. One limitation of our model is that the tissue patterning of the regenerated joint does not fully recapitulate that of the uninjured joint, likely reflecting the severity of our injury. However, all joint cell types regenerate in correct orientation to each other (i.e., articular cartilage capping the ends of bone, ligaments connecting bones). Thus far, cellular contributors and pathways underpinning the ability to regenerate articular cartilage have remained elusive. By performing our joint resection injury in a model organism that naturally regenerates, we have shed light on cellular and genetic mechanisms that promote endogenous regeneration of articular cartilage. The dynamic plasticity and differentiation of mesenchymal progenitor cells into several mature joint cell lineages is a particular strength of our model, enabling the study of not only the regrowth of one tissue type, but the crosstalk involved in regenerating a whole joint organ containing multiple cell types.

SIGNIFICANCE/CLINICAL RELEVANCE: Our whole-joint resection injury provides a useful model for dissecting the pathways driving endogenous regeneration of synovial joint tissues and for defining key tissue crosstalk mechanisms deployed in rebuilding a complex 3D organ. Uncovering factors required for endogenous articular cartilage regeneration will aid future translational studies in improving *in vitro* articular chondrocyte differentiation protocols, advancing tissue-engineering approaches to cartilage repair, and increasing the efficacy of therapies for human degenerative joint diseases. Hence, our model is well-suited for studying the regeneration of joint cell fate and can be used to complement poorly-healing mammalian models towards the translational goal of improving the regenerative capacity of the human joint.