Single-Cell RNA Sequencing of Repair Tissue Formed After Growth Plate Injury Reveals a Potential Role for Macrophages and Mesenchymal Progenitor Cells in Bony Bar Formation

Aditya Mantha[1], Stacey M. Thomas[1], Ethan J. Beltrand[1], Karin A. Payne[1]
[1]University of Colorado Anschutz Medical Campus, Aurora, CO
aditya.mantha@cuanschutz.edu

Disclosures: The authors report no conflicts of interest in relation to this study.

INTRODUCTION: The growth plate is a region of cartilaginous tissue at the ends of children's long bones that when injured can heal with unwanted bony repair tissue, known as a bony bar. This can tether the epiphyseal and metaphyseal bone and lead to growth deformities. Bony bar formation has been shown to involve inflammatory and osteogenic processes, as well as potential recruitment and osteogenic differentiation of bone marrow derived mesenchymal stem cells (MSCs).[1] However, the precise molecular and cellular events leading to bony bar formation have not been fully elucidated. Uncovering the profile of potentially important inflammatory cells and pathways participating in healing and impacting MSC differentiation could inform growth plate regenerative medicine approaches. The objective of this study was to characterize the identity and transcriptomic profile of cells at the growth plate injury site using single-cell RNA sequencing (scRNA-seq) to assess their role in bony bar formation.
METHODS: Animal studies were approved by the IACUC. Female Sprague-Dawley rats (6 weeks-old) underwent bilateral drill-hole injuries to the proximal tibia growth plate. After 4 and 7 days, repair tissues were digested to isolate single cells. Live cells were submitted to the Genomics and Microarray Core for scRNA-seq. Sequences were aligned (CellRanger), then filtered, normalized, and scaled (Seurat). Clusters were identified with PCA reduced data (Seurat). Cell types for each cluster were identified by matching marker genes enriched for each cluster to known MSC and immune cell markers. Gene ontology (GO) and KEGG pathway analysis was performed on the marker genes (gprofiler2). Injured tibias were also harvested at 1, 3, 7, 10, and 14 days post-injury, fixed, decalcified, embedded, cryosectioned, and immunostained to confirm markers found through scRNA-seq.
RESULTS: Mesenchymal progenitor cells (MPs) were identified at days 4 and 7 after growth plate injury, with six MP populations identified on day 4 (Fig 1A). GO terms—cell surface, plasma membrane, extracellular—revealed CD109 and CD9 to be surface markers common to all six populations (Fig 1B). Immunostaining for CD109 confirmed the presence of these cells on days 1, 3, 7, 10, and 14 after injury (Fig 1C, Day 3). While all MP populations expressed osteogenic genes, *Bgn* was upregulated in MP 3, *Lmna* in MP 5, and *Ogn* in MP 6 (Fig 1B). On day 7, the MP population upregulated *Alpl* and *Runx2*. It was also observed that Day 4 MP 2 had high *FoxA2* expression. Foxa2+ cells were found in growth plate repair tissue at days 3, 7, 10, and 14 after injury (not shown). CD45 expressing clusters were analyzed to identify immune cells within the injury site. At day 4, immune cell clusters were identified as either B cells, T cells, neutrophils, macrophages, or osteoclasts (Fig 1A). Day 7 clusters were identified as B cells, T cells, neutrophils, monocytes, or macrophages (Fig 1A). Subclustering of the day 7 macrophages uncovered two distinct clusters (Fig 1D). Macs 1 is a pro-inflammatory phenotype expressing *Cd69*, *Tnf*, *Il1b*, *Ptgs2* (COX2), *Nos2* (iNOS) and *Vegfa*. Macs 1 also express *Vcan* and *Mmp14*, associated with intramembranous ossification. Macs 2 is a pro-reparative phenotype expressing *Mrc1* (CD206), *Pdgfa*, *Tgfb1*, *Igf1*, *Osm*, and *Bmp2*. They also express *Gpnmb*.
DISCUSSION: This study provides the first -omics level analysis of growth plate injury response and characterizes the identity and transcriptomic profile of cells at the injury site over time. *Ogn*, *Bgn*, *Alpl*, *Runx2*, and *Lmna* are genes that promote osteogenesis, and their upregulation at various time points following growth plate injury suggests that MSCs contribute to the formation of the bony bar. A population of skeletal stem cells expressing *Foxa2* has recently been implicated in promoting chondrogenesis and restoring cartilage in a growth plate injury.[2] The high expression of *Foxa2* in MP 2 and the presence of Foxa2+ cells suggests that this specific stem cell population may also be present in our model of growth plate injury and warrants further investigation. COX2, iNOS, and *Vegfa* have previously been linked to bony bar formation[3,4] and were shown in this study to be expressed by the pro-inflammatory macrophages found at day 7 post injury. Additionally, these macrophages expressed genes (e.x. *Vcan, Mmp14*) associated with intramembranous ossification. Pro-reparative macrophages expressed *Gpnmb*, which promotes MSC migration, proliferation, and survival.[5] They also expressed *Osm*, *Tgfb1*, *Igf1*, and *Bmp2*, which are associated with endochondral and intramembranous ossification. Although the pro-inflammatory macrophages primarily express genes and are associated with GO terms that contribute to bony bar formation, it is possible that the pro-reparative macrophages also have a role, presumably in the recruitment of MSCs to the injury site.
SIGNIFICANCE/CLINICAL RELEVANCE: By understanding which cell populations are expressing osteogenic genes, a more targeted approach can be utilized to prevent osteogenesis and potentially promote chondrogenesis after growth plate injury.
REFERENCES: 1. Chung R et al. J Mol Endocrinol. 2014;53(1):T45-61. **2.** Muruganandan S et al. Nat Commun. 2022;13(1):2515. **3.** Arasapam G et al. J Cell Biochem. 2006;99:450-461. **4**. Erickson CB et al. 2020. J Orthop Res 39:1658-1668. **5.** Yu B et al. J Cell Biochem. 2016;117(7):1511-21.

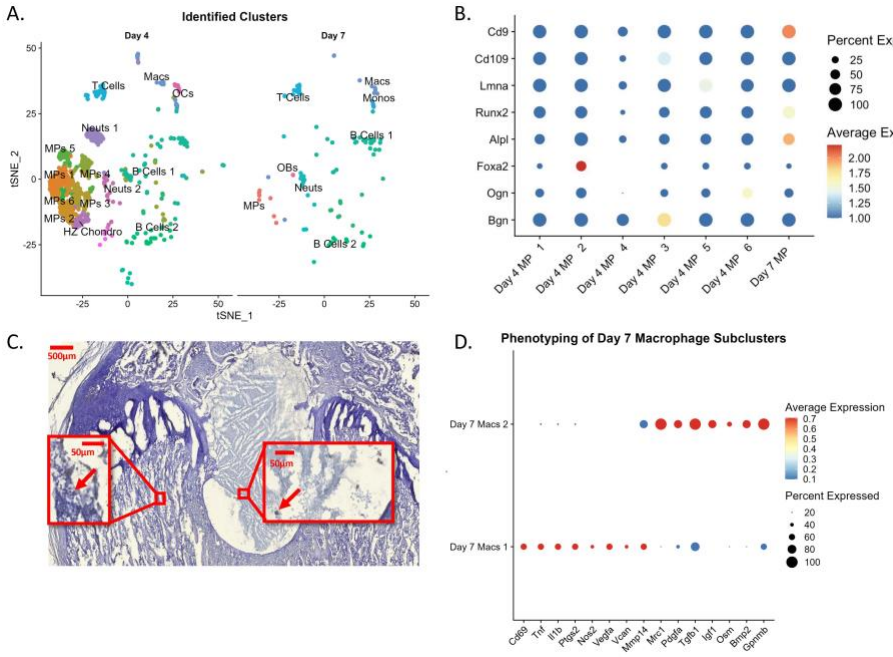

Figure 1. A) tSNE plot of all the identified cell clusters at Day 4 and Day 7 after growth plate injury. **B)** Phenotypic characterization of the MP populations at Day 4 and Day 7 after growth plate injury. **C)** CD109 immunostaining of growth plate repair tissue 3 days after injury. **D)** Phenotypic characterization of the Day 7 macrophage subclusters classified subcluster 1 (Macs 1) as pro-inflammatory and subcluster 2 (Macs 2) as pro-reparative.