# Spatial Transcriptomics Reveals Regulators of Mouse Joint Development

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**INTRODUCTION:** Synovial joints are essential for biomechanical articulation and locomotion, and their function relies on a complex integration of tissues, including ligaments, tendons, synovium, menisci, and articular cartilage. During early limb development, chondroprogenitors (CPs) at the presumptive joint site downregulate Col2a1 and Sox9 and form the interzone (IZ), a transient structure marked by Gdf5 expression at embryonic day 12.5 (E12.5). These Gdf5-lineage CPs ultimately differentiate into the cellular components of all joint-specific structures. However, the molecular regulators controlling IZ CP fate remain unclear.

**METHODS:** All experiments were conducted under IACUC approval at Johns Hopkins University. To characterize the cellular lineages involved in joint formation, we performed single-cell RNA sequencing (scRNA-seq) on hindlimbs from E11.5 to E14.5 mouse embryos. Following tissue dissociation, CD45+ (hematopoietic) and Ter119+ (erythroid) cells were removed via negative selection, and the remaining cells were processed using the PIPseq T100 3′ Single Cell and Chromium Single Cell 3′ platforms. The resulting single-cell transcriptomic data were analyzed to identify key genes involved in joint development. Additionally, spatial transcriptomic data were obtained from E11.5 to E14.5 hindlimb samples using Slide-seq (Curio Seeker; 10 µm resolution). Presumptive IZ regions were selectively extracted using Cellxgene, and genes specifically enriched in the IZ at E12.5 were screened. Expression of candidate genes was further validated via in situ hybridization. To evaluate the function of the screened genes, we conducted an explant culture using E12.5 hindlimbs from Sox9-GFP reporter mice and examined cartilage formation and joint development (Fig. 2A–C). Samples were divided into three groups: normal (untreated), siNeg (negative control siRNA), and siTrps1 (Trps1-specific siRNA), with n = 7 limb explants per group (mixed gender). siRNA was injected directly into the presumptive knee joint region twice (1 and 24 hrs after harvest), and explants were cultured under air–medium interface conditions for three days after harvest. Macroscopic morphology and Sox9-GFP fluorescence were monitored daily. Explants were collected on day 1 for siRNA efficacy testing and on day 3 for analysis of cartilage formation. Quantitative data are presented as mean ± standard deviation (SD). Parametric data were analyzed using one-way ANOVA followed by Tukey's HSD post hoc test. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant.

**RESULTS:** scRNA-seq revealed that Trps1 was highly expressed in the CP cluster across all developmental stages, with the highest expression observed at E12.5 (Fig. 1A–C). Furthermore, spatial transcriptomic analysis showed that Trps1 expression was localized to the IZ at E12.5 and E13.5—corresponding to the initial stages of joint formation—and colocalized with Sox9+ and Col2a1+ CP populations, as well as with the IZ-specific marker Gdf5, suggesting its involvement in early joint development (Fig. 1D–F). In the siTrps1 efficacy test, in situ hybridization performed on Day 1 of explant culture confirmed that Trps1 expression was significantly reduced in the siTrps1 group compared to the normal and siNegative (siNeg) controls (Fig. 2D). By Day 3, Macroscopy morphology revealed clear joint-like structures in both the normal and siNeg groups, whereas the siTrps1 group failed to develop a distinct joint structure. Sox9-GFP expression was clearly observed in the cartilage regions and delineated the presumptive joint space in the normal and siNeg groups, while it was markedly diminished in the siTrps1 group, indicating impaired joint formation and chondrogenesis (Fig. 3A, B). Additionally, in situ hybridization showed that Col2a1 expression was significantly reduced and distributed in the siTrps1 group, whereas it was strongly and distinctly expressed in the femur and tibia of the control groups (Fig. 3C, D). Safranin-O/Fast Green staining further demonstrated well-formed cartilage and joint spaces in the normal and siNeg groups, but markedly reduced cartilage formation and failed joint development in the siTrps1 group (Fig. 3E, F). These results demonstrate that Trps1 is expressed in the presumptive joint region during initial joint formation and plays an important role in this developmental process.

**DISCUSSION:** This study integrates single-cell and spatial transcriptomics with a spatially targeted explant culture system to identify regulators of IZ formation during joint development. We identified Trps1 as an IZ-enriched gene whose expression is important for normal joint formation. A key limitation is that gene knockdown was performed ex vivo, and additional in vivo validation could be needed to confirm Trps1's role under physiological conditions. Overall, this work provides a framework for discovering developmental regulators of joint formation with potential therapeutic relevance to osteoarthritis.

**SIGNIFICANCE/CLINICAL RELEVANCE:** Understanding the molecular regulators of IZ progenitor cell fate provides critical insight into the early steps of synovial joint formation. This knowledge lays the groundwork for identifying therapeutic targets to preserve or restore joint integrity in degenerative diseases including osteoarthritis.

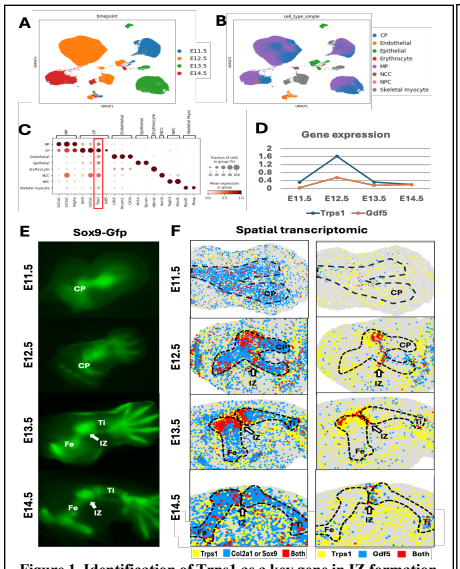


**Figure 1. Identification of Trps1 as a key gene in IZ formation during joint development using multi-omics. (A-C)** Single-cell RNA-seq UMAP and Dot plot showing cell populations and major cell types in E11.5 to E14.5. **(D)** Spatial transcriptomic data showing peak Trps1 and Gdf5 expression in the IZ at E12.5. **(E)** Sox9-GFP expression of normal hindlimbs. **(F)** Spatial colocalization of Trps1 with Sox9/Col2a1 and with Gdf5. CP; chondroprogenitor cells, IZ; Interzone, Ti; Tibia, Fe; Femur

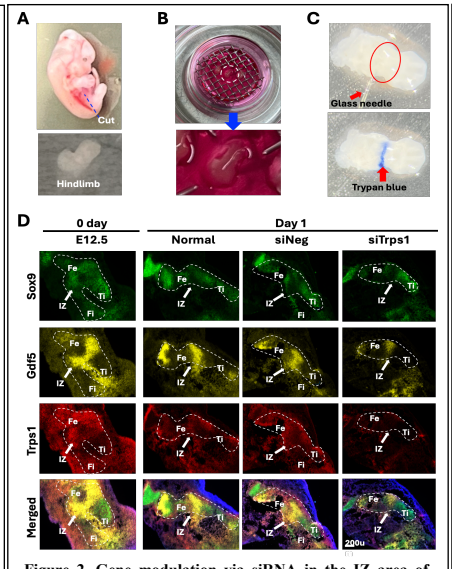


**Figure 2. Gene modulation via siRNA in the IZ area of hindlimb explants. (A)** Hindlimb dissection of E12.5 mouse embryos. (B) Explant culture at the air-medium interface dish for three days. (C) siRNA was injected into the presumptive IZ area using a micro-glass needle twice; Trypan blue (a blue dye) was used to visualize injection accuracy. (D) In situ hybridization of Sox9, Gdf5 and Trps1 expression.

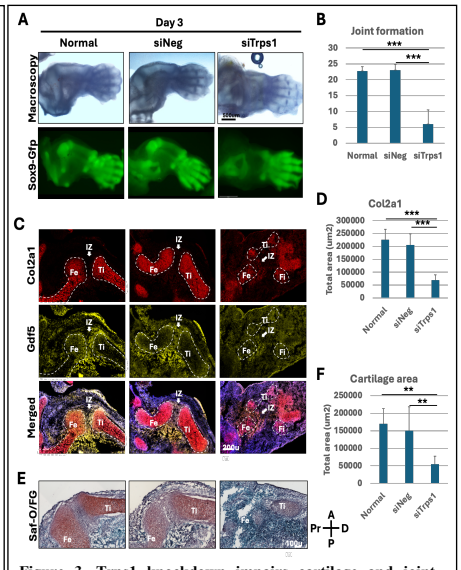


**Figure 3. Trps1 knockdown impairs cartilage and joint formation of explants.** (A) Macroscopic and Sox9-GFP images at Day 3 post-culture. (B) Semi-quantitative analysis of joint formation. (C) In situ hybridization of Col2a1 and Gdf5 expression in femur and tibia (D) Quantification of Col2a1 signal. (E) Safranin-O/Fast Green staining for cartilage matrix. (F) Quantification of GAG-rich area in femur and tibia. Pr; Proximal, A, Anterior, P; Posterior, D; distal