

Distal-less homeobox 5 (DLX5) regulates musculoskeletal wound healing pathway networks

Jay Trivedi¹, Salomi Desai¹, and Chathuraka T. Jayasuriya¹

¹Department of Orthopaedics, Brown University/Rhode Island Hospital, Providence, RI, USA

jay_trivedi@brown.edu

DISCLOSURES: Jay Trivedi (N), Salomi Desai (N), Chathuraka T. Jayasuriya (N)

INTRODUCTION: Knee Osteoarthritis (OA) is a leading cause of disability and functional impairment in the United States.¹⁻² As the articular cartilage is regularly exposed to biomechanical forces from joint impact and torsion, focal cartilage defects via traumatic injury and chronic degeneration are common. There is a need to develop effective strategies to restore damaged cartilage tissue. Bone-marrow-derived mesenchymal stromal cells (BM-MSCs) have been extensively researched in preclinical models of cartilage restoration. However, BM-MSCs have certain limitations. During late-stage chondrogenesis, BM-MSCs exhibit increased gene expression of common cartilage hypertrophy-ossification markers, which pose a significant challenge for cartilage tissue engineering.³⁻⁴ Our data from an *in-vivo* study revealed that intraarticular injection of cartilage-derived progenitor cells (CPCs) into a destabilized knee preserved cartilage tissue health, relative to animals injected with BM-MSCs (**Fig.1**). Interestingly, cell transcriptome characterization by RNA sequencing (RNA-Seq.) revealed that BM-MSCs have significantly higher expression of distal-less homeobox 5 (DLX5) compared to CPCs.⁵ Further, DLX5 was among the top differentially regulated musculoskeletal system related markers between these two cell types. This ubiquitous protein is a bone-morphogenic protein 2 (BMP-2) inducible transcription factor upstream of late-stage chondrogenesis and hypertrophy markers. It is also significantly upregulated in the chondrocytes isolated from the osteoarthritis (OA) patients.⁴ Further, we observed that knocking down DLX5 in BM-MSCs reduces the expression of common cell hypertrophy and cartilage catabolism markers while still allowing these cells to retain their chondrogenic capacity.⁵ Hence, we **hypothesized** that inhibiting DLX5 activity may promote chondroprotection. To test our hypothesis, we established both stable DLX5 knock-down (DLX5 KD) human BM-MSCs and human chondrocytes. Here, we describe our most recent findings from comparative transcriptome analysis of BM-MSCs and chondrocytes with reduced DLX5 activity.

METHODS: *DLX5 knock-down in cells:* DLX5 KD BM-MSCs and C28/12 chondrocytes were established using lentivirus bearing either DLX5 shRNA or non-targeting control shRNA (NTC). Cells were infected at different multiplicity of infection (MOI) followed by Puromycin selection. The knock-down was confirmed by the presence of GFP and western blot analysis. *RNA-Sequencing:* Total RNA was extracted from the cells and processed for transcriptome analysis using Genewiz platform. The resulting data was analyzed using Ingenuity Pathway Analysis (IPA) software. The bar graph of representative pathways relevant to musculoskeletal tissue that were significantly modulated based on z-scores where negative z-score (blue bars) represents overall pathway inhibition whereas a positive z-score (orange bars) indicates overall pathway activation. *Animal Studies:* Skeletally mature male athymic RNU rats (≥ 12 weeks of age) were anesthetized, and medial side parapatellar arthrotomy was performed (by blunt dissection to minimize bleeding) on the right knee joints. The medial meniscus was destabilized by partial tearing. After the meniscal destabilization injury, the synovial capsule was closed, followed by muscle and skin closure. Cells were injected at day 7 and 28, following surgery by intra-articular injection. Animals were euthanized, and hind limbs were isolated for histology.⁶ *Histology Analysis:* The knee joint was harvested and fixed in 10% Neutral Buffered Formalin, followed by decalcification and processing. Samples were sectioned and the femoral condyle and tibial plateau were stained with Saf-O/Fastgreen. Modified Mankin Scoring was used to evaluate articular cartilage surfaces. *Statistical analysis* was performed by student's *t*-test when comparing two groups.

RESULTS: Unlike animals that received intra-articular CPC injections, animals that received BM-MSC injections showed no sign of improved cartilage health following meniscal destabilization (**Fig 1A-1E**). We **hypothesized** that the difference in efficacy might, at least in part, be due to BM-MSCs having high DLX5 expression.⁴ Further, cartilage catabolism genes MMP-2, MMP-13, and ADAMTS4 are significantly upregulated in BM-MSCs (**Fig 1F**). DLX5 being upstream of these cartilage catabolism markers provided more rationale that DLX5 may play a central role in the cartilage catabolism that we observed in this *in vivo* model. To test our hypothesis, we created DLX5 knock-down BM-MSC and C28/12 chondrocyte cells and performed transcriptome analysis. Our results indicate that DLX5 knock-down inhibits OA pathway in BM-MSCs (**Fig.2**) which is unaltered in C28/12 chondrocytes (**Fig.3**). Additionally, we observed that the wound healing pathway is activated and interferon signaling pathway is inhibited in both the cell types. Additionally, HIF1 α signaling and VEGF signaling also show different profile in both cell types (**Fig.2-3**).

DISCUSSION: DLX5, an inducible transcription factor upstream of late-stage chondrogenesis, cartilage hypertrophy, and cartilage catabolism markers, is significantly upregulated in BM-MSCs but not in CPCs⁵. Knock-down of DLX5 helps reduce the expression of hypertrophy markers in BM-MSCs⁴. Based on these findings, we hypothesized that DLX5 may play a crucial role in regulating the cellular pathways associated with cartilage tissue homeostasis. DLX5 knockdown in BM-MSC and C28/12, followed by RNA Seq analysis demonstrate that DLX5 regulates wound healing pathways in human BM-MSCs and chondrocytes. Further detailed characterization of DLX5 as a potential therapeutic target may open novel avenues to develop treatments for cartilage wound repair.

SIGNIFICANCE/CLINICAL RELEVANCE: This study focuses on refining our understanding of the potential of DLX5 as a therapeutic target for articular cartilage repair and the prevention of PTOA.

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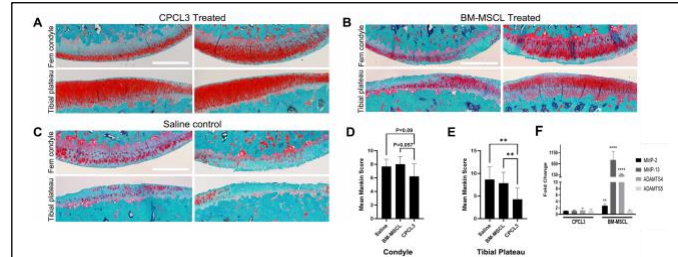


Fig-1: BM-MSCs demonstrate increased expression of hypertrophy and catabolic markers. Representative images of Rat knee cartilage histology of (A) CPC, (B) BM-MSC and (C) Saline treated animal are shown. Scale bar: 500 μ m. Average cartilage histological scoring using Modified Mankin method of (D) medial femoral condyle and (E) tibial plateau. N \geq 8 per treatment group. **, p \leq 0.01. (F) Hypertrophy biomarkers were analyzed by mRNA expression. ****, p \leq 0.0001.

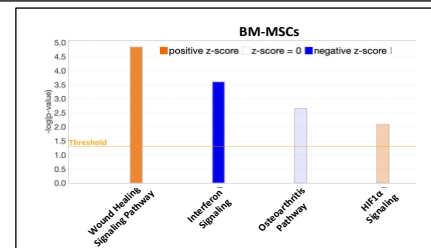


Figure – 2: RNA-Seq. analysis of DLX5 KD BM-MSCs reveals that OA pathway and interferon signaling is inhibited (blue bars) whereas the wound healing signaling pathway and HIF1 α signaling is activated (orange bars).

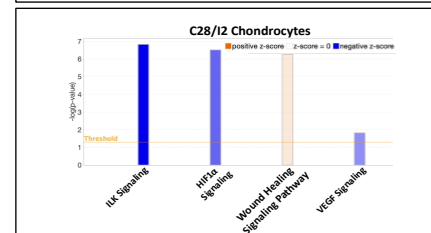


Figure – 3: RNA-Seq analysis of DLX5 KD C28/12 chondrocytes reveals that ILK signaling, HIF1 α signaling and VEGF signaling (blue bars) is inhibited and wound healing signaling pathway is activated (orange bars).