DLX5 Orchestrates Chondrogenic Stability, Hypertrophy, and Catabolic Signaling in BM-MSCs

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INTRODUCTION: Cartilage injuries induce catabolic alterations within the joint microenvironment, and if left untreated, they can progress to the development of posttraumatic osteoarthritis[1]. Stem/Progenitor cell based therapy have opened novel avenues in the field of tissue regeneration. Bone marrow–derived mesenchymal stem cells (BM-MSCs) are multipotent progenitors capable of differentiating into chondrogenic, osteogenic, and hypertrophic lineages, making them attractive candidates for regenerative therapy of fibrocartilaginous tissues. Precise regulation of lineage commitment is essential, as dysregulated differentiation can lead to aberrant hypertrophy, matrix catabolism, and cartilage degeneration. While extracellular cues such as BMP-2 influence MSC fate, the intrinsic transcriptional regulators coordinating chondrogenic stability, hypertrophy, osteogenesis, and catabolic remodeling remain poorly defined[3]. Distal-less homeobox 5 (DLX5) is a BMP-2–inducible transcription factor implicated in skeletal development, chondrocyte maturation, and osteogenesis. BM-MSCs exhibit high DLX5 expression, and its levels is elevated in chondrocytes from osteoarthritis (OA) patients[3-4]. Notably, DLX5 knockdown in MSCs reduces hypertrophy and catabolic markers while preserving chondrogenic capacity, suggesting a central role in modulating cartilage tissue homeostasis[4]. Here, we investigate DLX5 function in BM-MSCs using shRNA-mediated knockdown to assess its impact on chondrogenic markers (SOX9, COL2), hypertrophy (COLX), osteogenesis (RUNX2), and matrix catabolism (MMP13). Our results reveal that DLX5 orchestrates a coordinated program promoting hypertrophic and osteogenic differentiation while facilitating catabolic remodeling. Understanding DLX5's regulatory functions may inform strategies to optimize cartilage repair by limiting premature hypertrophy and matrix degradation without compromising chondrogenic potential. **METHODS**: *DLX5 knockdown in cells:* DLX5 KD was established using lentivirus bearing either DLX5 shRNA or non-targeting control shRNA (NTC). The knockdown was confirmed by the presence of GFP and Immunoblotting. *Immunoblotting*: Cells were lysed, and the protein lysate was resolved on a polyacrylamide gel and then transferred onto a PVDF membrane, followed by blocking with 5% skimmed milk. The proteins of interest were probed with respective antibodies, and the blots were analyzed using a chemiluminescent substrate. *MTT Assay*: Cells at passage 17 were seeded in 96 well plate. 10 uL MTT (5 mg/mL) was added, and cells were incubated in the dark for 4 h. The purple formazan crystals were dissolved with DMSO, and absorbance was measured at 540 nm. *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. *Osteogenesis assay*: Unmodified control BM-MSCs, vector control BM-MSCs, and DLX5 knockdown BM-MSCs were cultured in osteogenesis media. 48 hours later, the cells were harvested, followed by mRNA expression and marker analysis at the mRNA level by real-time PCR. *Statistical analysis* was performed by Student's *t*-test when comparing two groups. **RESULTS:** We first performed DLX5 knockdown in BM-MSCs using a lentivirus expressing GFP downstream of DLX5 shRNA. Knockdown efficiency was confirmed by GFP expression, with variant 1 (V1) showing maximal inhibition *(Fig. 1A)* and selected for all subsequent experiments. Immunoblotting further validated DLX5 suppression *(Fig. 1B)*, and cell proliferation was not significantly affected *(Fig. 1C)*. Transcriptome analysis identified 1,068 differentially regulated genes, including COL10, CXCL12, BMP-2, and MMP13, with upstream pathway analysis revealing significant modulation of wound healing, osteoarthritis, and interferon signaling pathways *(Fig. 2)*. DLX5 knockdown suppressed chondrogenic markers, significantly reducing SOX9 and COL2 expression. In contrast, hypertrophic marker COLX was significantly reduced in DLX5-deficient cells, demonstrating that DLX5 inhibits hypertrophic differentiation. Osteogenic and catabolic regulators were also impaired: RUNX2 and MMP13 expression were strongly downregulated, highlighting DLX5's role in osteogenic commitment and matrix remodeling *(Fig.3)*. Overall, DLX5 suppression diminishes chondrogenic stability (SOX9, COL2), blunts hypertrophy (COLX), and impairs osteogenic and catabolic pathways (RUNX2, MMP13), underscoring its central role in BM-MSC fate regulation. **DISCUSSION:** DLX5, an inducible transcription factor upstream of late-stage chondrogenesis, cartilage hypertrophy, and catabolic markers, is highly expressed in chondrocytes isolated from arthritis patients, underscoring its role in catabolism-related pathways. Knockdown of DLX5 reduces hypertrophy marker expression, suggesting its role in regulating cartilage tissue homeostasis. Using lentiviral-mediated DLX5 knockdown followed by RNASeq, we found that DLX5 modulates pathways associated with wound healing, interferon signaling, and osteoarthritis. Functionally, DLX5 orchestrates a balance between chondrogenic stability, hypertrophic progression, osteogenic commitment, and catabolic remodeling in BM-MSCs. DLX5 knockdown reduced SOX9 and COL2, indicating compromised chondrogenic stability, and partially rescued COL2 with BMP-2, suggesting DLX5 acts downstream or parallel to BMP signaling. Suppression of COLX demonstrated that DLX5 inhibits hypertrophic differentiation, with its loss preserving BM-MSCs in a less terminally differentiated state. Downregulation of RUNX2 and MMP13 further highlights DLX5's role in promoting osteogenic commitment and matrix remodeling, facilitating cartilage-to-bone transition. Collectively, DLX5 functions as a molecular switch that (i) inhibits hypertrophy and osteogenesis via RUNX2 and COLX and (ii) enables catabolic remodeling through MMP13 downregulation. Its suppression shifts BM-MSCs toward a less hypertrophic, less catabolic state. Therapeutically, targeting DLX5 may help limit premature hypertrophy and matrix breakdown while preserving cartilage integrity, addressing key challenges in stem cell–based cartilage regeneration and tissue engineering. **SIGNIFICANCE/CLINICAL RELEVANCE:** This study focuses on refining our understanding of the potential of DLX5 as a therapeutic target for hypertrophy, articular cartilage repair, and the prevention of PTOA. **REFERENCES**: **(1)** Trivedi J *et al*., *Front Bioeng Biotechnol.* 22;9:787330 (2022). **(2)** Newberry, J. *et al*. *Connect. Tissue Res.*, 1-11 (2019). **(3)** Mueller *et al., Arthritis Rheum.* 58:1377-1388 (2008) **(4)** Twomey-Kozak, J. *et al*. *Int. J. Mol. Sci.* 21, 4823 (2020). **ACKNOWLEDGEMENTS**: This research is supported by NIH NIAMS grant R21AR077326, a Pilot Project from NIH NIGMS parent grant P20GM104937, and ON/ORS Kick Starter grant 23-269.

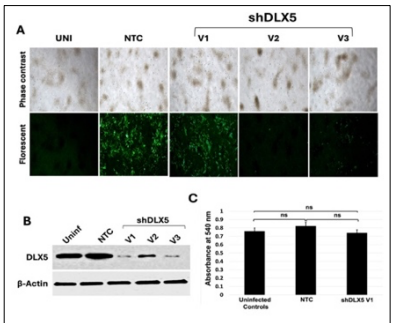

Fig-1: DLX5 knockdown in MSCs does not diminish cell proliferation. The different shDLX5 variants (V1, V2, V3) were tested. Nontargeting controls (NTCs) were used as negative controls. The knockdown was confirmed by fluorescent imaging **(A)** and western blot analysis **(B)**. Cell proliferation was determined by MTT assay after passage 17 post-infection. ns = not significant.

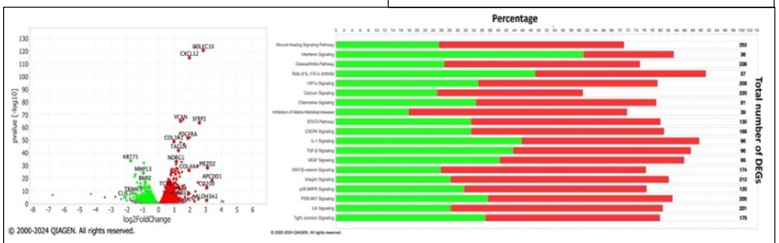

Fig.2: DLX5 knockdown modulates transcription landscape of mesenchymal lineage cells ***(A)*** Volcano plot indicates 1068 differentially expressed genes (DEGs) in MSCs with shDLX5 KD as compared to empty lentivirus infected control. ***(B)*** Upstream analysis shows modulated cellular pathways. Numbers on the Y-axis represent the total number of DEGs in each pathway and X-axis represents the percent change in pathways. Green represents downregulation, red is upregulation in both panels.

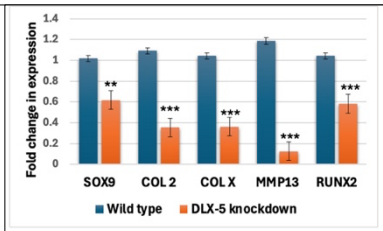

Fig.3 DLX5 regulates osteogenesis, hypertrophy, and catabolism-related pathways: Bar graph represents mRNA level expression analysis of SOX9, Collagen 2, Collagen X, MMP13, and RUNX2 in DLX5 knockdown BM-MSCs (Orange bars) compared with unmodified wild type control BM-MSCs (Blue bar). The y-axis represents fold change (n = >4) ** = $p \leq 0.001$, *** = $p \leq 0.001$.