A Transcriptome Analysis of the Role of Cartilage Progenitor Derived Extracellular Vesicles in Meniscal Repair

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INTRODUCTION: The menisci are crescent-shaped fibrocartilage structures in the knee that serve as key biomechanical stabilizers, helping to evenly distribute axial loads [1]. Avascular white-white zone (WWZ) meniscal lesions are a significant clinical challenge due to the area's weak intrinsic repair capabilities. Cartilage-derived progenitor cells are recognized for their strong proliferative ability, chondrogenic capacity, and resistance to undergoing hypertrophic differentiation [2]. Our group has previously demonstrated that CPCs and bone marrow–derived mesenchymal stromal cells (BM-MSCs) produce similarly sized extracellular vesicles (EVs) though in different quantities and with different levels of EV protein markers [3]. We have also determined that treating inner meniscal fibrochondrocytes with extracted CPC-EVs observably and consistently increased cell viability and stimulated cell proliferation and wound healing in culture, outperforming BM-MSC-EV treatments [3]. A representative scratch assay with four treatment groups at different time intervals is depicted **(Fig. 1A)**. In these 2D assays, CPC-EVs significantly accelerated wound closure compared to the 1% serum medium control, particularly after 28 hours **(Fig. 1B)**. This study investigates the individual biological mechanisms by which the CPC-EVs improve tissue repair. Through transcriptomic profiling of human meniscal fibrochondrocytes under conditions by which human CPCs drive repair, we analyze the distinctive patterns of genes and signal cascades upon which meniscal repair stimulation by CPCs is based.

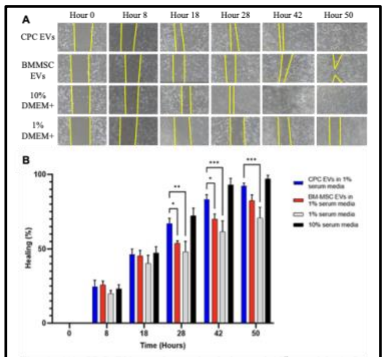

Fig. 1: (A) CPC EVs at the optimal dose of 10^7 particles/mL increased wound closure rates. Light microscope imaging at 10x magnification shows a representative scratch assay of all treatment groups. **(B) CPC-EVs significantly outperformed BM-MSC-EVs and 1% Serum Media by hour 28**. Quantification of percent recovery in a 2D wound healing assay following EV treatment of inner meniscal fibrochondrocytes. N = 7 in each experimental group.

METHODS: **EV Isolation and Quantification**: CPCs and BM-MSCs were grown in DMEM+ until 95% confluency, then incubated for 48 hours. For EV quantification, cells were incubated in serum-free media for 24 hours. The supernatant was collected, centrifuged at 2,500 RPM for 15 minutes, and ultracentrifuged at 23,200 RPM for 1.5 hours. The pellet was resuspended in 1,000 µL of PBS or 1x RIPA buffer with 1mM PMSF. **Characterization of EV Size and Distribution**: EVs were resuspended in phosphate buffered saline (PBS) and analyzed for size and concentration using a NanoSight device. **EV Treatment:** Human inner meniscal fibrochondrocytes (2.0×10^5) were plated in 6-well plates, cultured in DMEM+ for 48 hours, and treated with EVs (1×10^7 particles/mL) from CPCs or BM-MSCs and a PBS control for 28 hours. Total RNA was extracted and quantified, and cDNA reverse transcribed using standard kits. **RNA Sequencing**: Transcriptome analysis was performed using the Genewiz platform with 1 ug of RNA collected from fibrochondrocytes treated with CPC-EVs and a PBS control. The resulting RNA Seq. data was analyzed using Qiagen's Ingenuity Pathway Analysis (IPA) software. Differentially expressed genes (DEGs) in cells treated with CPC-EVs were considered for further analysis if they had a log2foldchange ≤ -0.5 or ≥ 0.5 and p-value ≤ 0.05. **Gene Expression Analysis**: Seventeen genes were selected from the remaining DEGs after cross checking with the subsequent RNA sequencing analysis set and previous literature regarding their effects on cell viability and proliferation. RT-qPCR was performed with a Beta-Actin reference to confirm expression levels of key genes. **Statistical Analysis**: GraphPad Prism 10 was used to perform statistical analysis using a two-way ANOVA, followed by a Dunnett's Multiple Comparison Test in 2D wound healing experiments. P-values ≤ 0.05 were considered significant. Assays were repeated until $N \geq 7$.

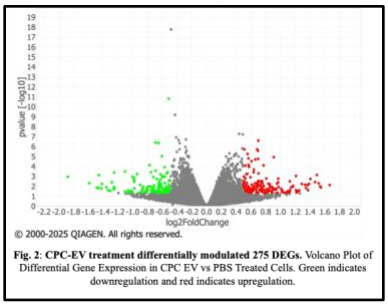

Fig. 2: CPC-EV treatment differentially modulated 275 DEGs. Volcano Plot of Differential Gene Expression in CPC EV vs PBS Treated Cells. Green indicates downregulation and red indicates upregulation.

RESULTS: EVs from both CPCs and BM-MSCs were characterized, showing a similar size distribution (100-600 nm), though CPCs produced 30% fewer EVs. The extracted RNA showed that on average, CPC-EV treated fibrochondrocytes had an RNA concentration of about 75.28 ng/ul, while BM-MSC-EV and PBS treatments yielded concentrations of 58.18 ng/ul and 58.5 ng/ul respectively. RNA Sequencing revealed 275 DEGs fitting our log2foldchange and p-value requirements, with 120 downregulated and 155 upregulated genes **(Fig. 2)**. Further pathway enrichment analysis highlighted nine signaling pathways that regulate cell proliferation and wound healing **(Fig. 3)**. In Qiagen figures **(Fig. 2, Fig. 3)** genes and signaling cascades of interest highlighted in green and red represent downregulation and upregulation respectively. Over 50% of all identified differentially expressed genes held regulatory roles in each identified relevant wound healing signaling pathway. Gene expression analysis narrowed down specific gene targets from the selected list of 17. Notably, MAML3, TSPAN1, and IL1RN all showed increased expression following treatment with CPC-EVs when compared to the PBS controls, and this trend was confirmed in the RT-qPCR. A scoping literature review suggested that MAML3 and TSPAN1 drive proliferation, migration, invasion, and tumorigenesis in various cancers [4,5], while IL1RN has an antagonistic role against the effects of Interleukin-1, a pro-inflammatory cytokine known to inhibit meniscal cell proliferation and impair meniscal repair [6].

Fig. 3: Enriched Pathway Analysis revealed 9 affected pathways relevant to cell proliferation and wound healing. Green indicates downregulation and red indicates upregulation.

DISCUSSION: This study identified 275 differentially expressed genes in CPC-EV-treated meniscal fibrochondrocytes and nine pathways involved in cell proliferation and wound healing. Specifically, MAML3, TSPAN1, and IL1RN are strong candidates as potential mediators of CPC-EV repair effects, based on the current scientific literature. Significantly affected pathways were the cell migration, proliferation, and ECM remodeling pathways, consistent with the faster rate of scratch closure and healing in CPC-EV–treated cultures. ERK/MAPK and p38 MAPK regulate proliferation, migration, and stress response, while Integrin Signaling and Integrin cell surface interactions regulate adhesion, migration, and ECM interaction. Ephrin Receptor and NCAM signaling guide directional migration and cytoskeletal rearrangements, and Actin Nucleation by the ARP-WASP Complex facilitates cytoskeletal remodeling. Together, these cascades offer a mechanistic explanation for the reparative effect of CPC-EVs on meniscal fibrochondrocytes. These potential DEGs may explain CPC-EV effects on migration, proliferation, and matrix remodeling. While this work identifies candidate genes and signaling cascades through in vitro transcriptome analysis, in vivo validation will be essential to confirm their relevance in the complex biological environment of meniscal repair. Future studies will focus on functional knockout and overexpression of these target genes and analyzing the induced effects.

SIGNIFICANCE/CLINICAL RELEVANCE: This project provides novel insight into the mechanism by which CPC-EVs instigate meniscus healing, identifying potential genes that are affected by differences in EV secretions. Because all experiments were performed in monolayer culture, subsequent in vivo studies will be essential to evaluate the effectiveness of CPC-EVs in musculoskeletal soft tissue repair. Building on these findings, future work will integrate CRISPR or RNAi approaches for select targets to define the specific molecular drivers of CPC-EV mediated repair.

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