**EXTRACELLULAR VESICLE MICRORNA CROSSTALK BETWEEN EQUINE CHONDROCYTES AND SYNOVIOCYTES – AN *IN VITRO* APPROACH**

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**Purpose**

Extracellular vesicles (EVs) are crucial in cell-to-cell communication influencing the joint microenvironment. EVs derived from diseased synovial tissue can contribute to osteoarthritis (OA) progression by inducing inflammation and cartilage degradation. Mesenchymal stem cell-derived EVs can enhance cartilage regeneration and prevent OA, suggesting the effect of EV exchange is highly dependent on EV-donor cell characteristics. Thus, the exact role of EVs in joint tissues warrants further investigation.

EVs contain microRNAs (miRs); powerful regulators of gene expression. To comprehend how they might regulate joint homeostasis, we explored the EV-mediated microRNA exchange in the joint. Our study investigated the EV microRNA crosstalk between equine chondrocytes and synoviocytes *in vitro,* using RNA tracking and sequencing.

**Methods**

Cartilage (n=8) and synovial membrane (n=9) were collected from grossly normal equine metacarpophalangeal joints obtained from an abattoir, and chondrocytes and synoviocytes isolated. Cells were incubated with a 5-Ethynyl Uridine tag that labeled newly synthesised RNA. Control EV-donor cells were cultured in parallel and processed in a similar way but without tag. EVs were isolated from culture media using size exclusion chromatography and characterised by nanoparticle tracking analysis. Recipient cells (chondrocytes, n=5; synoviocytes, n=4) were incubated with freshly isolated tagged EVs, ensuring that recipient chondrocytes were incubated with synoviocyte-derived EVs, and recipient synoviocytes were incubated with chondrocyte-derived EVs. Control recipient samples were incubated with control-derived EVs. Following incubation, all recipient cells were trypsinised, total RNA extracted and 5-EU labelled RNA recovered using an affinity recovery kit, then sequenced (Fig. 1).

Processed sequencing reads were mapped first against the genomic reference EquCab.3.0 and subsequently miRBase v22.1, filtered for miRs of horse only. Data variation was assessed through principal component analysis (PCA). Differential expression (DE) was analysed using edgeR v3.32 and the quasi-likelihood negative binomial generalized log-linear model function. Results were used for pathway analysis (IPA) and target prediction (Targetfilter) with data filtered to include only experimentally observed target mRNA in chondrocytes, osteoblasts and cartilage.

**Results**

PCA revealed separation between chondrocyte and synoviocyte-derived EV groups (Fig. 2A). 198 miRs were identified in synoviocytes (Fig.1c), corresponding to the 5-EU labelled RNA in chondrocyte-derived EVs (Fig.1b). 26 of these were unique to chondrocyte-derived EVs. A total of 213 miRs were identified in chondrocytes (Fig.1f), corresponding to 5-EU labelled RNA in synoviocyte-derived EVs (Fig. 1e) with 41 unique to synoviocyte-derived EVs. The top 5 abundant miRs were similar for both chondrocyte and synoviocyte-derived EVs: miR-21, miR-221, miR-222, miR-100 and miR-26a.

DE analysis revealed a total of 9 miRs, 4 of at FDR<0.05. miR-27b, miR-23b, miR-31, miR-191a and miR-199a-5p, were increased in synoviocyte-derived EVs, while miR-143, miR-21, miR-181a and miR-181b were increased in chondrocyte-derived EVs. A filtered list of 23 experimentally observed targets was obtained, and pathway analysis revealed significant links to migration of cells (p=3.53x10-19), angiogenesis (p=7.79x10-17) and apoptosis (p=1.11x10-14). Other relevant processes included cell viability of connective tissue cells, inflammation of joint and rheumatoid arthritis (Fig.2B).

**Conclusions**

There were a higher number of miRs uptaken by the chondrocytes from synoviocyte-derived EVs, as well as a higher number of synoviocyte-specific miRs. This could reflect the increased metabolic activity of synoviocytes when compared to chondrocytes. We also found different patterns of miR vesicle cargo depending on cell origin, as previously described in the literature. DE miRs appear to be related to cell viability and inflammation, and might reflect the specific contributions of the joint tissues to these processes. Some of these molecules have also been suggested as potential biomarkers for OA, indicating EVs may play an important role in disease progression. Still, the most abundant miRs were similar between both cell types and some were previously shown to regulate extracellular matrix maintenance and respond to mechanical loading. We hypothesise they may be implicated in preserving healthy joint homeostasis.



