Characterization of Human Follicular Fluid Extracellular Vesicle Subtypes and Their Impact on Human Granulosa-Like Tumor Cell Line KGN Transcriptome

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Extracellular vesicles (EVs) are lipid-bilayered nanoparticles released by all cell types. They carry biologically active cargo, including proteins, lipids and nucleic acids that influence recipient cell function. The presence of extracellular vesicles in follicular fluid (FF) has been established across various species, with indications that FF EVs may play a role in oocyte maturation and granulosa cell proliferation. While previous studies have focused on the collective pool of FF EVs, EV subtypes may serve distinct functions within the ovary. This study aimed to characterize the surface tetraspanin profile of FF EV subtypes and investigate their impact on the transcriptome of the human granulosa-like tumor cell line KGN.

Ethical approval was obtained from the Research Ethics Committee of the University of Tartu, Estonia. FF was collected from preovulatory follicles (>18 mm diameter) at Nova Vita Clinic in Tallinn, Estonia. Small and large EVs (SEVs and LEVs, respectively) were purified from FF pools using size exclusion chromatography followed by tangential flow filtration. EV characterization involved nanoparticle tracking analysis (NTA) with ZetaView PMX110 (Particle Metrix), transmission electron microscopy (TEM) with JEM-1400 (Jeol), and single vesicle analysis with the ExoView R100 platform (NanoView Biosciences). For RNA-sequencing, KGN cells were incubated with 10^9/ml SEVs (n=6), 10^8/ml LEVs (n=6) or DPBS (n=6) for 24 hours and then lysed. Total RNA was extracted using the Qiagen miRNeasy kit, and sequencing libraries were prepared from 200 ng of RNA using the Revvity NextFlex Rapid Directional RNA-seq kit. Single-end sequencing was performed with the Illumina NextSeq2000 instrument. Reads were mapped to and annotated according to the human genome version GRCh38 (Ensembl). Differentially expressed genes (DEGs) were identified with the R package DESeq2 version 1.42.0 and DEGs with an adjusted p-value ≤ 0.1 were selected for further analysis.

The mean diameter of isolated SEVs and LEVs was 93 nm and 299 nm, respectively ($p = 2.26 \cdot 10^{-11}$). In total, an average of $8.4 \cdot 10^{10}$ SEVs and $2.4 \cdot 10^9$ LEVs were obtained from 20 ml of FF. TEM confirmed the presence of EVs with round morphology. ExoView analysis revealed differences in the surface tetraspanin profile between the EV subtypes, with a higher proportion of $CD9^+CD63^+CD81^+$ particles in SEVs compared to LEVs ($p<0.05$). RNA-seq analysis of KGN cells treated with SEVs showed 1006 DEGs, with 642 downregulated and 364 upregulated genes. G:Profiler analysis categorized downregulated genes into pathways related to cholesterol biosynthesis, cell cycle and extracellular matrix organization, while upregulated genes were enriched into translation-related pathways. Treatment with LEVs resulted in 86 DEGs, with 63 downregulated genes enriched into extracellular matrix and

adhesion pathways, and 23 upregulated genes enriched into translation-related pathways similarly to SEVs.

This study sheds light on distinctive tetraspanin marker compositions among EV subtypes in human FF, suggesting that both subtypes possess unique molecular characteristics. Notably, both SEVs and LEVs alter the expression of genes related to extracellular matrix regulation and translation processes, both essential for ovarian follicle development and oocyte maturation. Furthermore, SEVs, but not LEVs, downregulate genes related to the biosynthesis of cholesterol, a precursor for the synthesis of steroid hormones. This suggests a potential mechanism through which SEVs contribute to the maintenance of the hormonal environment within the follicle. These findings provide novel insights into the functionality of FF EV subtypes and enhance our understanding of the intercellular communication within the ovarian follicle.