Towards the Optimization of Targeted Systems for the *In Vitro* Culture of Isolated Bovine Primordial Follicles

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To date, the production of live offspring through *in vitro* follicle culture from the ovarian reserve has only been achieved in mice, providing the proof-of-principle of the potential value of primordial follicles (PMF) as a source of fully grown oocytes. The ability to grow undifferentiated oocytes *in vitro* from PMF would increase the supply of fully grown oocytes destined for downstream applications in the livestock industry and fertility preservation programs. However, in large mammals, such as bovine, *in vitro* follicle culture systems to produce mature oocytes from PMF are still experimental due to high follicle mortality following isolation from the surrounding tissue. As evidenced in humans, isolated PMF undergo cell death after a short period in culture. While understanding the cause of follicle death is necessary to inhibit the induction of programmed cell death (PCD), it is also essential to elucidate the mechanisms responsible for activating PMF and steering them towards the next stage of development, i.e., primary follicles (PF). Mechanistically resolving both sides of the story - PMF death and development - would then provide insights into key factors responsible for deciding the fate of the PMF in culture. Condensation of this genetic repertoire would then optimize the current base culture system and improve the development of isolated bovine PMF to PF entirely *in vitro*.

Heifer ovaries were collected from the abattoir and transported on ice to the laboratory in saline. PMF and PF were mechanically isolated, and PMF were cultured in a defined system. Follicle viability of freshly isolated (PMF T0) and PMF cultured for 16 (PMF T16) and 24 hours (PMF T24) were assessed. PMF T0, PMF T16, and PF T0 were subjected to bulk RNA sequencing, generating 50bp paired-end reads. Raw data were trimmed with TrimGalore to remove artificial constructs and low-quality bases. Trimmed data were mapped to the Bos taurus ARS-UCD1.3 transcriptome, and reads were quantified with Salmon. Differentially expressed genes (DEGs) between PMF T0 and PMF T16 were then obtained with DESeq2 (Wald's Test). A chi-square test was performed to determine an association of the DEGs with PCD genes. DESeq2 (Likelihood Ratio Test) was further employed to perform an inter-follicle (PMF T0-PMF T16-PF T0) time course gene expression analysis followed by hierarchical clustering of significant genes (IFGEA) with the DEGreport package in R.

We observed a significant reduction in PMF viability after 16 hours of culture, while no significant differences were observed between 16 and 24 hours ($p<0.0001$ and $p=0.9753$, respectively, two-way ANOVA followed by Tukey's test). Furthermore, we report the transcriptome profiles of freshly isolated bovine PMF and PF and 16-hour cultured PMF for the first time. PCA showed clear clustering of all the samples. 1949

DEGs (adjusted p-value<0.1) were identified, which were determined to be associated with genes involved in ferroptosis and autophagy (chi-square test, p-value<0.05). IFGEA yielded 4 clusters of 1173, 1570, 162, and 22 genes (padj<0.05) with similar expression patterns in clusters 1-4, respectively.

Our transcriptome analyses indicate that ferroptosis and autophagy are the elected PCD mechanisms bringing about PMF death *in vitro* and can be inhibited to nurture current culture systems. Based on comparison with PF, systems may be further optimized to allow the holistic development of isolated PMF entirely *in vitro* in the future.

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