

# Mimicking the Human Endometrium: The Role of Stromal Signaling in Organoid Complex Models

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**Introduction:** The human endometrium can be replicated *in vitro* using 3D human endometrial epithelial organoids (hEEOs). However, the lack of stromal cells providing crucial biochemical cues avoid to mimic tissue complexity. Complex models combining both types of cells have been investigated in the last years. In this study, we have developed an *in vitro* complex model based on the co-culture of hEEOs with stromal cells from the same individual, and we have compared gene expression profile between single and complex models and with native tissue. To ensure reproducibility, we also tested a stromal stem cell line (ICE7) as an alternative. The objective was to assess whether the stromal part (primary cells or cell line) guides hEEOs toward a more native-like phenotype.

**Methods:** Based on previous protocols, epithelial and stromal cells were isolated to generate hEEOs and primary cultures from endometrial biopsies of healthy women (N=6). *Monolayer in vitro* culture of ICE7 cell line was also carried out. Then, hEEOs and both stromal cells were co-cultured in a Transwell system. All conditions were maintained for 7 days with an optimized expansion culture medium and cell viability was assessed by a specific viability assay. Co-cultures were characterized by immunofluorescence with epithelial (E-CAD) and stromal ( $\alpha$ -SMA) markers. RNA sequencing was carried out in all *in vitro* conditions and native tissues, and the media from both co-cultures were collected for secretome analysis by mass spectrometry-based proteomic. Descriptive analyses, including PCA and heatmaps, and functional analyses using GSEA were performed. Finally, common and differentially expressed genes were identified and validated by RT-qPCR.

**Results:** All co-culture conditions showed viability levels of  $\geq 99\%$  and the immunofluorescence confirmed epithelial expression (E-CAD) in hEEOs and stromal phenotype ( $\alpha$ -SMA) in stromal parts. Data from RNA-seq analyzed by PCA segregated into four discrete clusters—(i) hEEOs, (ii) hEEOs co-cultured with both stromal cells, (iii) native endometrial tissue, and (iv) stromal cells. Co-cultured hEEOs resembled native tissue more than hEEOs cultured alone, as evidenced by PCA, heatmap and correlation matrix. GSEA of KEGG, BP and Reactome pathways analyses (FDR < 0.05) highlighted significant enrichment of proliferation, metabolism, and homeostasis pathways in co-cultured conditions. Expression of specific genes like *CDK1*, *BUB1B* and *TOP2A* (proliferation); *SOX17* (receptivity and implantation); and, *ESR1* (hormonal regulation) were identified in co-cultured hEEOs and native tissue but not in monocultured ones. These genes were validated by RT-qPCR. Finally, secretome analyses revealed a similar proteomic profile in the media of both co-culture models.

**Conclusion:** Co-culturing hEEOs with stromal cells significantly increased their similarity to native endometrial tissue. Both stromal sources similarly influenced hEEOs allowing paracrine communication. These facts underscore the stromal role in maintaining endometrial functions and producing a more faithful *in vitro* model.