

Advancements in Mouse Testis Organoid Formation and Characterization: A 3D *In vitro* Model for Studying Spermatogenesis

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Testis is a complex organ where different cells interact to achieve healthy sperm production. Several factors can compromise this process, leading to fertility failure. However, studying these complexities *in vivo* can be challenging, necessitating the use of simpler systems to understand the dynamics between cells during spermatogenesis. Organoids offer a promising solution by providing an *in vitro* model that mimics *in vivo* processes. While many organs have been successfully replicated using organoids, creating functional testis organoids has remained elusive.

To address this gap, we have developed a novel protocol for generating mouse testis organoids *in vitro*. Our protocol involves forming and maintaining organoids from a testis cell suspension. Organoid formation was achieved by aggregating 1.2×10^6 cells isolated from a mix of 10-day post-partum mouse testes, enriched with 5×10^5 germ cells from a mix of 16-day mouse testes, in AggreWell™400 plates, yielding approximately 1200 organoids per well. We utilized Dazl-EGFP transgenic mice alongside wild-type mice in our experiments. These transgenics were produced by our research group and contain the promoter of Dazl (2 kb sequence in the 5' flanking region of the mouse Deleted in Azoospermia-Like –Dazl- gene) expressing EGFP. They exhibit high expression of EGFP in spermatocyte stages, with weaker expression in spermatogonia and round and elongating spermatids.

Following formation, organoids were collected and cultured for up to 35 days in small groups (approximately 15-20 organoids) in spermatogenesis culture medium at 34°C to foster the development and differentiation of testis cells. Over time, small organoids spontaneously aggregated to form larger structures. We conducted structural analysis of 5 µm waxed organoid sections using Hematoxylin/Eosin staining, identified apoptotic cells using the TUNEL assay, and determined the distribution of different testis cells through immunohistochemistry. Additionally, we analyzed the expression of specific spermatogenesis-related genes using qPCR at various points during *in vitro* culture and compared them with *in vivo* testis expression levels.

Our analyses revealed a higher concentration of cells in the periphery of the organoids, surrounded by isolated or small clusters of cells in the central region. TUNEL analysis showed a small number of apoptotic cells in small organoids, with an increased presence of compromised cells as the organoids grew. Immunohistochemistry indicated an internal localization of Leydig and peritubular cells, while Sertoli and germ cells were primarily located externally, with the testis barrier (Collagen IV) serving as a reference point. Additionally, acrosome reaction was observed on the exterior of the organoids. The use of Dazl-GFP transgenic mice facilitated monitoring the location and progression of germinal cells during the culture process through EGFP expression.

These results demonstrate the successful maintenance of testis cells *in vitro*, with cells aggregating and localizing strategically to preserve testis structure and function. Our 3D *in vitro* model presents

a valuable tool for studying cell-to-cell interactions and the spermatogenesis process, offering a simpler alternative to *in vivo* studies.