Leydig Cell Regeneration after Injury and MEK/ERK Signaling Dynamics in Mice Testes
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Abstract
Leydig cells are testosterone-producing cells in the interstitial compartment of mammalian testes that serve a critical role in supporting spermatogenesis. Previous research in rats has shown that Leydig cells are capable of regenerating after chemical ablation. However, the precursors of regenerating Leydig cells remain unknown. Using lineage tracing in mice models, we have determined that Gli1-positive peritubular myoid cells and perivascular cells are the origin of these regenerating Leydig cells. Furthermore, we have assessed MEK/ERK signaling of Leydig and surrounding cells in the tests using Kinase Translocation Reporter mice. This pathway, a potential indicator of growth and proliferation, can show us the mechanisms by which these cells grow and divide. By investigating the precursors of Leydig cell regeneration and the signaling pathways of these cells, this project paves the way for biological therapies for patients suffering from low testosterone.

Background
The mouse testis is comprised of seminiferous tubules that contain Sertoli cells and spermatagonia/other germ cells. Between the seminiferous tubules is the interstitial space which contains Leydig cells, macrophages, and perivascular cells, among others. A Kinase Translocation Reporter mouse was gifted from the Regot lab to assess dynamic changes of MEK/ERK. When MEK/ERK signaling is active, signal will fluoresce mostly in the cytoplasm. When signal is inactive, it will fluoresce predominantly in the nucleus.

Methodology
The purpose of this experiment is to mark Leydig cell precursors, ablate Leydig cells, and subsequently trace the regeneration of these Leydig cells. The marking was done by injecting a Gli1-CreERT2^1 or mTmG^2 mouse with tamoxifen to activate the CreER recombinase. Gli1-positive cells will be marked in green. Leydig cells would then be ablated using Ethane dimethane sulfonate (EDS) and their regeneration would be traced. For our Kinase Translocation Reporter mice, testes were dissected, de-terminated, stained, and then imaged.

Results
Initial marking using Gli1-CreERT2 marks peritubular cells and perivascular cells
Transgenic mice from our cross were injected with tamoxifen to activate CreER recombinase. (A) No induction was observed in our negative corn oil control. (B) In our experimental conditions, no marking of Leydig cells (3BHSD) was observed after tamoxifen injection. Furthermore, peritubular cells also appeared to be marked. (C) The GFP signal colocalized with ACTA2 staining, suggesting that these cells are peritubular myoid cells.

Leydig Cells regenerate after EDS ablation from Gli1-expressing cells
(D) Three weeks after EDS ablation, GFP-positive cells (arrowhead) are present in the interstitial space of the testes. This suggests that they are progeny of Gli1 marked peritubular myoid cells. These positively marked cells colocalize with 3BHSD which indicates that they are Leydig cells.

Interpretation & Conclusions
According to our results, our lineage tracing experiment works and marks the cells we hypothesized to be marked. Notably, peritubular myoid cells are Gli1-positive as suggested by the GFP positive signal colocalizing with ACTA2 staining. After EDS ablation, regeneration of Leydig cells was also observed. To better visualize this process, we evaluated live-imaging and whole mount methods. To determine how long the tissue remains viable, we conducted a cell viability assay using Acridine Orange and Ethidium Bromide and found that the tissue can remain viable for several hours before completely dying. Staining with Nile Red and Concanavalin A-488 was used to confirm morphological landmarks in the tissue. Finally, our KTR results showed data that recapitulate what is known in the literature. Spermatids and Sertoli cells ( Sox9) do express active ERK signaling in the testis, and as anticipated, certain cells in the tests were shown by the KTR to be active while others were inactive. Future directions include quantifying our Gli1 tracing results to determine how many cells regenerate after EDS ablation and using our live imaging results to visualize MEK/ERK dynamics in Gli1+ cells.

Sources and Acknowledgements

Landmark structures of the tests can be identified in live conditions
(R,C) Tissue was stained in live, whole mount conditions. (B) Nile Red stains for lipid droplets which are expected to be abundant in Leydig cells. (C) To distinguish the structures within the testis, Concanavalin A-488 was used to visualize the basement membrane. The seminiferous tubules, blood vessels, and interstitial space are all visible.

Our KTR recapitulates what is known in the literature
To observe what the KTR looked like in the testis, a KTR mouse was sacrificed and tests were stained in (D) fixed and in (E) live conditions. (D) The KTR mouse in fixed conditions marks the nuclei of spermatids. The cytoplasm of Sertoli cells are also somewhat marked. (E) In whole mount and live-imaging conditions, KTR signaling colocalizes with the nuclei of some cells and the cytoplasm of others.