Summer 2020 Continuing Researchers Support Program - Final Report

Confirming and Evaluating the Immunoexpression of Sperm Protein-Reactive Antisperm Antibody (SPRASA) in Feline and Canine Ovarian Tissue

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Original Objectives

1. To confirm expression of SPRASA in normal canine ovarian tissue, based upon previously published research.
2. To compare SPRASA expression between normal and abnormal canine ovarian tissue, to determine if SPRASA can be used as a diagnostic marker of pathology.

Deviation from Original Objectives

The original objectives were modified based on initial experimental discoveries, available resources and the limited duration of the summer research period. The first immunohistochemistry experiment that we performed utilized normal and abnormal canine ovarian tissue. This experiment yielded weak tissue staining that confirmed the immunoexpression of SPRASA in canine ovarian tissue but incentivised further protocol development in order to increase the intensity and clarity of staining. Feral cat ovarian tissue slides became available after this first experiment and were included in the second experiment due to their high follicular density and representation of pre- and post-pubescent follicular development. The focus of this research shifted away from qualitatively comparing SPRASA immunoexpression in normal and abnormal canine ovarian tissue to pursue quantification of immunoexpression in feline ovarian tissue. This summer's research centered on the improvement of the immunohistochemistry protocol to achieve superior immunohistochemical staining that would better support imaging. The revised summer objectives reflecting these shifts in research are listed below:

1. To confirm expression of SPRASA in normal canine and feline ovarian tissue and to compare expression to previously published research.
2. To determine the optimal dilution and antibody brand in order to achieve clear and measurable immunohistochemical staining for pre- and post-pubescent feline ovarian tissue.

Methods

Antibody Dilution Experiment. One canine ovarian tissue sample was obtained from the Oregon State University Veterinary Diagnostic Laboratory archives while one equine and one feline sample were cut from paraffin blocks retained by Dr. Kutzler from previous projects. Equine

tissue was included as a model for comparison as another student had obtained successful results using this sample in a previous immunohistochemistry experiment. Seven slides for each sample were cut in order to compare two different antibody brands at three different dilutions each.

Briefly, serial 5-µm sections were cut from each paraffin block containing tissue of interest and mounted on charged slides. Slides were deparaffinized in xylene and rehydrated in a graded ethanol series (100%, 75%, 50%). Antigen retrieval was conducted by incubating slides in a citrate buffer (Target Retrieval Solution #S1699, Dako North America Inc., Carpinteria, CA) placed in a Nordicware® tender cooker and heated in a microwave until the pressure valve rose before being cooled for 20 minutes. Slides were then washed in buffer (Wash Buffer #S3006, Dako North America Inc.) and endogenous peroxidases were inhibited by incubating slides in 30% hydrogen peroxide. Slides were washed again in buffer, and blocked for 20 minutes at room temperature with serum-free protein (Protein Block Serum-Free ready to use #X0909, Dako North America Inc.). Slides were then tapped off before the primary antibody was applied.

Two antibody brands (SPACA3 Rabbit Polyclonal antibody #21137-1-AP, Proteintech, Rosemont, CA and Anti-SPACA3 Polyclonal antibody #HPA023633, Atlas Antibodies, Stockholm, Sweden) were employed in order to evaluate any differences in staining pattern or intensity between them. The Proteintech brand of antibody was diluted in antibody diluent (Background Reducing Components #S3022, Dako North America Inc.) in ratios of 1:20, 1:100 and 1:200. The Atlas Antibodies brand of antibody was diluted using the aforementioned diluent in ratios of 1:50, 1:200 and 1:500. Slides were incubated at room temperature for 105 minutes. Specificity of immunostaining was verified by replacing the primary antibody with negative control rabbit antibody (#NC495H, Biocare Medical, Pacheco, CA) on adjacent sections.

Slides were then washed in buffer several times and a secondary antibody (One Step Horse Radish Peroxidase-Conjugated Polymer Anti-Rabbit IgG, #IH-8064-OSU-15, Immuno BiosCience, Mukilteo, WA) was applied to each slide. Slides were incubated at room temperature for 30 minutes, washed several times, and then VECTOR NovaRED (#SK4800, Vector Laboratories Inc., Burlingame, CA) was applied to each slide for about 5 minutes at room temperature. Slides were counterstained in hematoxylin, dehydrated in a graded series of ethanol (50%, 75%, 100%), moved through a series of three xylene baths, and cover slipped. Slides were evaluated by a single observer at 20X and 40X magnification with a Leica DM4000B microscope using bright field microscopy. Several representative images from each slide were digitally captured using a QImaging camera (QICAM 12-BIT, #QIC-F-M-12-C, QImaging, Surrey, British Columbia) and QCapturePro image capture software.

Key Findings to Date

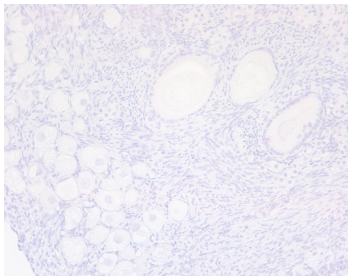
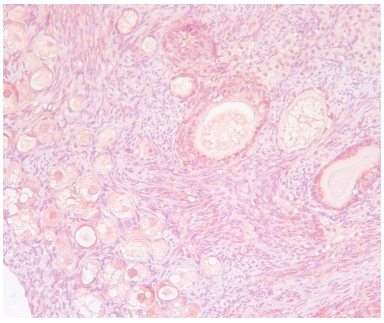
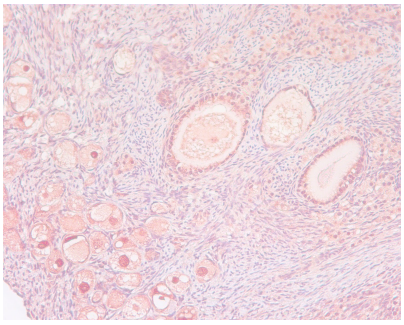
Expression of SPRASA in canine and feline ovarian tissue was localized to the oocyte nucleus, granulosa cells, ooplasm and theca cells. Oocyte nuclei were not evaluated for the equine tissue. Staining intensity per area of localization for each sample species was ranked through subjective visual analysis, displayed in Table 1. The antibody dilution experiment suggests that the

Proteintech brand of antibody is less specific to SPRASA as those slides yielded more background staining when visually compared to the slides with the Atlas Antibodies brand of antibody, demonstrated in Table 2.

Table 1. Comparing Staining Intensity Per Localization Based on Subjective Visual Analysis

	Most to Least Intense SPRASA Staining Per Localization
Canine	Oocyte Nucleus ≥ Granulosa Cells > Ooplasm > Theca Cells
Feline	Oocyte Nucleus > Granulosa Cells > Ooplasm > Theca Cells
Equine	Oolemma > Granulosa Cells > Theca Cells

Table 2. Proteintech Versus Atlas Antibodies Brands of SPRASA Antibody for Feline Sample

Negative *Feline*	Proteintech, 1:200 Dilution *Feline*	Atlas Antibodies, 1:200 Dilution *Feline*
		

Next Steps

This research intends to develop a reliable method of immunoexpression quantification, likely using Image J analysis software, in order to measure the intensity of staining localized in the corpora lutea as well as theca cells and granulosa cells of primordial, primary, secondary and tertiary feline ovarian follicles. Images of feline slides captured from the antibody dilution experiment will be analyzed to quantitatively determine the best antibody brand and dilution for future experiments. Tentatively, the next immunohistochemistry experiment will involve sixteen feline ovarian tissue samples and eight feline testicular samples from two to 24 months of age, followed by microscopy, image capture and subsequent image analysis. This research may lead to further investigation of SPRASA's potential as a feline immunocontraceptive.

No additional funding was obtained during this period.
No presentations were given during this period.