

THESIS

IDENTIFICATION OF CLATHRIN AND DYNAMIN II IN THE PORCINE OVARY
SUPPORTS THE PRESENCE OF CLATHRIN-MEDIATED ENDOCYTOSIS

Submitted by

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ABSTRACT

IDENTIFICATION OF CLATHRIN AND DYNAMIN II IN THE PORCINE OVARY SUPPORTS THE PRESENCE OF CLATHRIN-MEDIATED ENDOCYTOSIS

The feral swine population in the United States has grown to 6 million animals located in 41 states, and causing an estimated \$1.5 billion annually in damages and control. Feral swine are well-known for spoiling crops, preying on smaller wildlife, spreading disease, and damaging the land's ecology. In 2014, the federal government initiated the Animal Plant and Health Inspection Services (APHIS) National Feral Swine Damage Management Program to combat this overabundant wildlife population. One of five research areas identified as a key component in the advancement and improvement of tools and methods to manage feral swine is the development of reproductive inhibitors that can cause permanent sterility. Successful reproduction in mammals depends on an adequate number of healthy oocytes present in primordial follicles within the ovaries. Maintenance of the primordial follicular pool requires the coordinated actions of both oocyte survival factors and factors that maintain the follicles in a non-growing state until they are activated to grow. There is a finite number of primordial follicles in the ovaries of mammals, which if destroyed would leave the animal permanently sterile. Relatively little is known about the cellular communication mechanisms utilized by primordial follicles. The purpose of this study was to investigate whether primordial follicles express components of clathrin-mediated endocytosis (CME), the most common form of receptor-mediated endocytosis used in eukaryotes.

This process, if present, could be exploited as a method to deliver chemosterilants to the primordial follicle pool. This study focused on determining the expression and localization of two key components of this CME, clathrin and dynamin II. Ovaries from 6 piglets and 6 gilts were bisected longitudinally, fixed in formalin, embedded in paraffin, and cut into 5µm thick sections which were mounted on microscope slides. Fluorescent immunohistochemistry using specific antibodies labeled with fluorescein isothiocyanate was performed to determine the expression and localization of clathrin and dynamin II on the mounted tissue sections. Expression of clathrin and dynamin II was revealed in the cytoplasm of oocytes of all follicular stages examined, suggesting that CME could be a mechanism of cell signaling in porcine oocytes.

A second aim of this study was to establish methods to visualize and characterize the internalization process in pig oocytes. The isolation of primordial follicles and oocytes and the live cell imaging of FM1-43 membrane probe uptake was completed. Primordial follicle isolation was attempted in piglet ovaries using a combination of chemical and mechanical methods. This process used enzymatic digestion and filtration of chopped cortical tissue from porcine ovaries. Mature oocytes were imaged over time after the addition of FM1-43. This established a protocol for live cell handling and imaging that would be useful in future studies. In addition, several ligands and their receptors that may utilize CME were investigated in porcine oocytes. The development of tools and methods to characterize cellular communication mechanisms in oocytes can contribute to the formulation of a chemosterilant to be used to cause non-lethal permanent sterility in feral swine.

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LIST OF ACRONYMS

AMH: Anti-Mullerian Hormone, also known as MIS
AMHRII: Anti-Mullerian Hormone Receptor type II, also known as MISRII
ARBL: Animal Reproduction and Biotechnology Laboratories
BLAST: Basic Local Alignment Search Tool
CCP: Clathrin coated pits
c-KIT: CD117 receptor, also called Stem Cell Factor Receptor, that binds to SCF
CME: Clathrin-mediated endocytosis
COC: Cumulous oocyte complexes
CSU: Colorado State University
DAB: 3, 3'-diaminobenzidine
DPBS: Dulbecco's phosphate buffered saline
FITC: Fluorescein isothiocyanate
FM1-43: (N-(3-Triethylammoniumpropyl)-4-(4-(Dibutylamino) Styryl) Pyridinium Dibromide)
GRAVY: Grand average of hydropathy
HBS: Hank's Buffered Saline
H-CDM-M: oocyte handling medium
HIER: heat induced epitope retrieval
HRP: Horseradish peroxidase
IDRC: Infectious Disease Research Center
IHC: Immunohistochemistry
IVM: *in vitro* maturation
MIS: Mullerian Inhibiting Substance, also known as AMH
MISRII: Mullerian Inhibiting Substance Receptor type II, also known as AMHRII
NWRC: National Wildlife Research Center
PAS: Periodic acid-Schiff stain
PBS: phosphate buffered saline
RME: Receptor-mediated endocytosis
SCF: Stem Cell Factor, also known as KIT-ligand, KL, steel factor
SCFR: Stem Cell Factor Receptor, also called c-KIT, CD117, KIT
TBS: Tris buffered saline
TBST: Tris-Buffered Saline with Tween-20

DEFINITION OF TERMS

Antigen retrieval: a method in immunohistochemical staining used to breakdown the methylene bridges that can form in formalin-fixed tissue which can mask antigenic sites; breakdown of these methylene bridges by either heat induced epitope retrieval (HIER) or enzymatic retrieval exposes the masked antigenic sites, which can result in stronger signal expression.

Basic Local Alignment Search Tool (BLAST): a tool used to find homologies between amino acids sequences that can be used to associate functional relationships between the sequences; this is especially useful when trying to identify similarities between the sequences for a protein found between different species.

Clathrin-mediated Endocytosis (CME): a specific type of receptor-mediated endocytosis where a ligand interacts with a receptor at the cell surface causing a signal cascade leading to the endocytosis of materials via a clathrin-coated pit.

Endocytosis: the process of internalization of material from the environment by vesicles formed from the plasma membrane

Feral: the return of animal to a wild-type state

Fluorescein isothiocyanate (FITC): A commonly used fluorescent marker that appears green in color.

Granulosa cell: a somatic cell associated with the oocyte in the ovary of mammals

GRAVY: Grand average of hydropathy; measure of hydrophobicity or hydrophilicity ranging from -2 to 2 in most proteins, with positive numbers relating to being more hydrophobic

Immunizing peptide: a peptide used to create an antibody that will target that specific peptide; for peptide blocking studies, a peptide that corresponds to an epitope recognized by an antibody that is used to neutralize that antibody by being incubated in excess during experimentation; this makes the antibody no longer available to bind to the epitope present in the protein found on the cells.

Immunohistochemistry: a chemical process used to detect antigens in cells or tissue using antibodies specified against the antigen of interest and then subsequent antibodies used to visualize the antigens using fluorescent or chromogenic markers.

Ligand: material that binds to a receptor

Oocyte: unfertilized egg

Porcine: relating to pigs

Primordial follicle: characterized as the oocytes that are arrested in prophase I of meiosis that is surrounded by a single flat layer of granulosa cells, and is then enclosed by a basement membrane [Hunter, 2000].

ProtParam: a tool that computes the characteristics, both physical and chemical, for a protein or specific protein sequence; the output information includes information such as molecular weight, amino acid composition, atomic composition, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY).

Wild: not tame or domesticated; a natural state

CHAPTER 1: INTRODUCTION

1.1. Summary

An estimated 6 million feral hogs were located in the United States in 2014. Although these animals are a focal point in the commercial hunting industry in some states, their population is growing at such an alarming rate that they cost an estimated \$1.5 billion annually in damages and management (APHIS, 2013 and 2015). Feral swine are well-known for spoiling crops, preying on smaller wildlife, spreading disease, and damaging the land's ecology (Seward, 2004). When swine numbers were fewer, hunting was considered a reasonable management technique to keep the population at a level that kept damages minimal. The lack of natural predators and the high reproductive capacity of swine have proven that hunting alone cannot manage the population size. A toxicant in the form of a bait could be a viable tool in aiding population control, but none are currently approved in the United States [Lapidge, 2012]. The development of a permanent sterilant could also help to mitigate this overly abundant wildlife population. As with other wildlife, an integrated management approach, including fertility control, is a growing area of interest that could meet demands for ethical wildlife control (Fagerstone, 2002; West, 2009).

One way to permanently sterilize an animal is to target the primordial follicles. Primordial follicles form a finite population of oocytes. While some primordial follicles will undergo atresia, most will be maintained in a non-growing state until they receive the appropriate controlled signals that will commit them to grow into primary follicles. Primary follicles can continue follicular growth to more advanced follicle stages with the

potential for ovulation or undergo atresia. The death or committed growth of primordial follicles all at once would cause permanent sterility. A non-surgical technique to target primordial follicles would be to exploit their naturally occurring cellular communication mechanisms to deliver a chemosterilant. Little is known about the mechanisms of cellular communication utilized by primordial follicles. Identifying whether clathrin-mediated endocytosis is utilized in primordial follicles, could be a first step in determining a mechanism for tissue specific drug delivery.

1.2 Purpose of the Study

The purpose of this study was to determine whether primordial follicles found in the porcine ovary express of receptor-mediated endocytosis factors, specifically those found in clathrin-mediated endocytosis. This study was part of the underpinning research necessary in the development of a sterilant formulated to target the female feral swine population. The ideal sterilant for wildlife would be highly specific to reproductive tissue, and would only need to be administered one time to reduce the amount of effort and money needed for distribution. There is finite population of oocytes in mammalian ovaries. In follicular growth, primordial follicles are the structures containing a primary oocyte surrounded by a single layer of flattened granulosa cells contained by a basement membrane [Hunter, 2000]. These follicles represent the entire reproductive potential of a female. In an effort to destroy this ovarian reserve via a sterilant, the physiology of these follicles must be understood. To date, none have identified or visualized the cellular communication mechanisms that exist in primordial follicles in swine.

Exploiting a cellular communication pathway to deliver a drug or toxicant would be one way to deliver a sterilant. One of the most common mechanisms of cellular communication in eukaryotes is clathrin-mediated endocytosis (CME). This study aimed to identify clathrin and dynamin II in the porcine ovary using immunohistochemistry (IHC); these are two of the key components in the machinery utilized in CME. By identifying CME in primordial follicles, researchers can begin experimentation to find out what materials are being internalized by the follicles, which could be potentially used for drug conjugates. Once the internalization process is better characterized, the focus can turn to developing a chemosterilant that targets primordial follicles causing their demise.

CHAPTER 2: LITERATURE REVIEW

2.1 Summary:

A thorough review of the current literature surrounding the feral pig population, female reproductive physiology, endocytosis, possible ligands or receptors to target for drug delivery, toxicants, and possible methods of drug delivery are the necessary framework for this study. Background on why the feral swine population is so important to focus on and what current practices are proving inadequate in population control is provided to show the need for these novel studies in reproductive physiology. The lack of information regarding primordial follicles and their communication mechanisms made it necessary to evaluate a wide variety of studies to develop scientific protocols used in experimentation. The foundational aspects of this research to further future studies is outlined.

2.2 Introduction:

This literature review provides information on the purpose of evaluating primordial follicle communication mechanisms in swine, why this study is focused to feral swine, the process of clathrin-mediated endocytosis, and why elucidating this process is significant for future studies.

2.3 Primordial follicles and reproductive potential

Primordial follicles consist of oocytes that are arrested in prophase I of meiosis surrounded by a single flat layer of granulosa cells, and enclosed in a basement

membrane (Figure 1) [Hunter, 2000]. No such finite population exists in males, which is one of the reasons that females are a better candidate for reproductive control. These structures have been the focus of much research across many species since the sum total of primordial follicles are considered the representation of the reproductive potential of a female [McLaughlin and Mclver, 2009; Skinner, 2005; Fortune, 2003]. In swine, the number of these follicles at birth were thought to be around 500,000 per pair of ovaries [Black and Erickson, 1968]. Over the lifetime of a female, highly controlled signaling in primordial follicles allows them to initiate growth to become primary follicles. Maturation is a committed step and occurs in groups of follicles called cohorts. Initiation of growth is carefully controlled by various local growth factors to provide an adequate supply of follicles throughout reproductive life [Reddy, 2010; Skinner, 2005; Fortune, 2003]. Follicular growth advances and the follicles enlarge as the layers of granulosa cells increase, the theca cells form, and a fluid filled antrum develops. All follicles will become atretic (>99.9%) except for those that will become dominant and ovulate, which can be one or more depending on the species (Figure 2) [Hunter, 2009; McLaughlin and Mclver, 2009].

Even with copious research efforts on mechanisms that mediate the dormancy or awakening of primordial follicles, the actual mechanism of communication of particular factors during primordial follicle recruitment remains elusive [McLaughlin and Mclver, 2009; Reddy et al., 2009; Wang et al., 2014; Adhikari and Liu, 2009; Fortune, 2003]. By identifying the cellular communication mechanism in primordial follicles, a series of

projects that aim to develop a non-lethal method of controlling feral swine overpopulation with the use of a sterilant can be initiated.

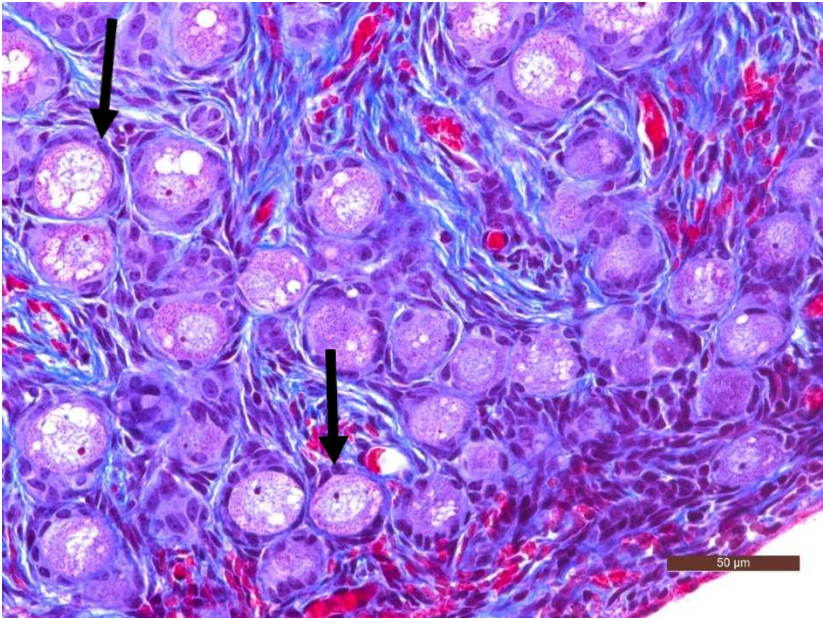


Figure 1. Primordial follicles (arrows) in the cortical region of a porcine ovary. Histology image using Masson's trichrome stain.

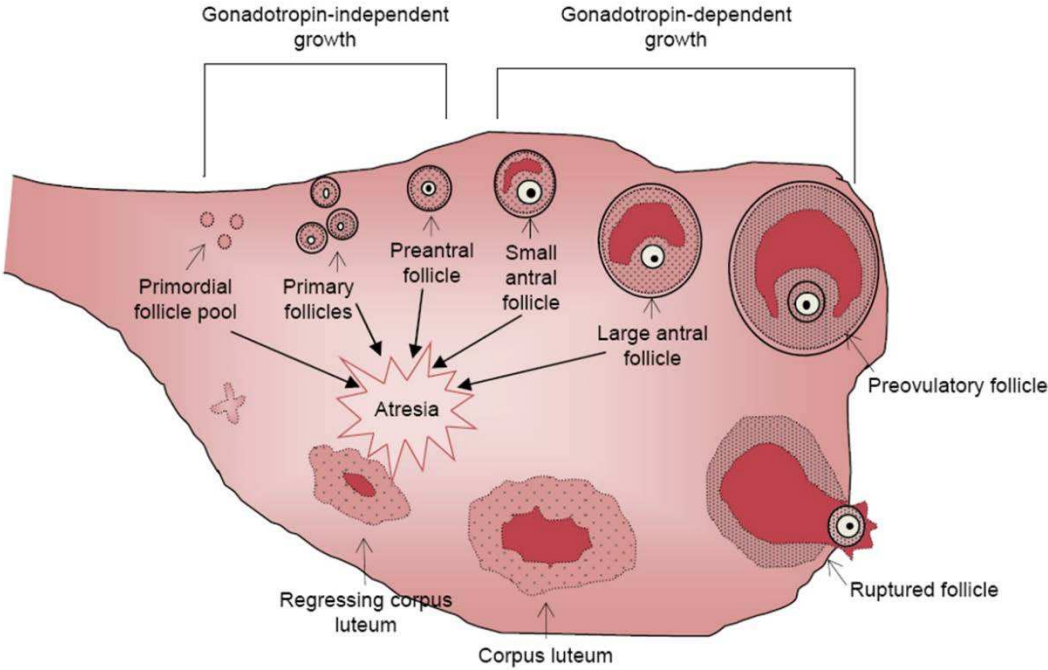


Figure 2. Representation of an ovary and follicular growth. [Chapman, 2015]

2.4 The Feral Swine Problem

The feral swine population of the United States includes both domesticated pigs and Eurasian wild boars, which have been present even prior to 1981. These hogs are all of the *Sus scrofa* species, including hybrids between the population, [Hellgren, E. 1999]. Domestic animals were first introduced in 1539 by explorers coming to the New World. The National Feral Swine Mapping System maintained by Animal and Plant Health Inspection Service (APHIS) has maps with reported feral swine locations from 1982, 1988, 2004, and 2014. The map from 1982 shows feral swine populations isolated to 17 mostly southern states (Figure 3). In the most recently released 2014 map, swine populations are seen in at least 41 states, with an estimated 6 million animals (Figure 4) [APHIS, 2013 and 2015]. The uncontrolled feral swine population originates from the combination of domesticated hogs getting loose, the release of Eurasian wild boars for commercial hunting operations, the lack of natural predators, and the high reproductive capacity.

Feral swine are not only dispersing more throughout the country, they are leaving a wake of environmental and economic damages as their population expands. These animals reproduce year-round at an unmatched rate compared to other ungulate families; feral swine can have one to two litters a year averaging from 4.2 to 7.5 piglets per litter, [Hellgren 1999, Taylor 1998]. A high plane of nutrition and favorable weather conditions influence their reproductive rate. These animals are extremely resourceful and consume an omnivorous diet that ranges from grasses and seeds to frogs and white-tailed deer fawns. Documentation of natural predators for feral swine, including

mountain lions and alligators, are described as limited and opportunistic in nature [Seward, 2004; Hellgren, 1993].

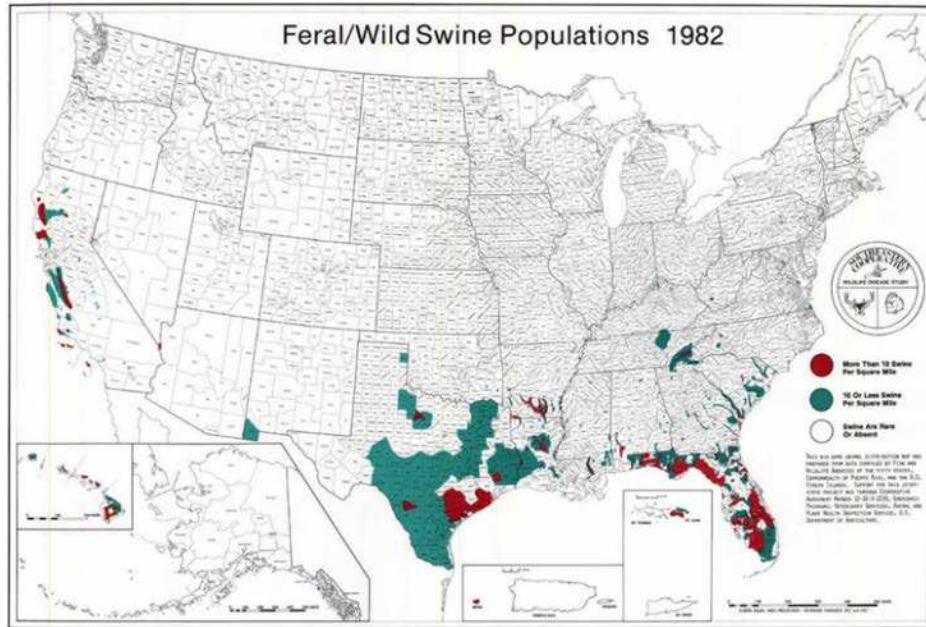


Figure 3. National Feral Swine Mapping System 1982 Map showing the approximate locations of feral swine.

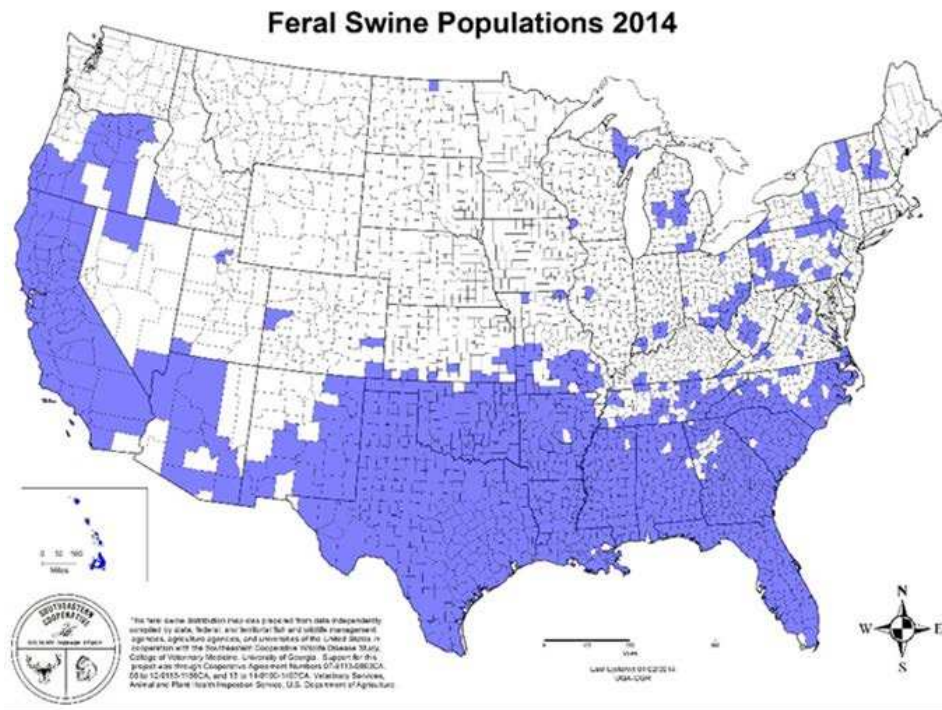


Figure 4. National Feral Swine Mapping System 2014 showing the approximate locations of feral swine.

A growing population of any kind will start to affect the surrounding ecology, and this can lead to damage, disease and financial burden. Feral swine cause environmental damage and wildlife depredation in their search for food [Seward, 2004]. Their rooting, wallowing and trampling behaviors disrupt natural nutrient cycles by compacting and disrupting soils. Soil disruption helps spread invasive plant species, which can colonize these damaged areas more quickly than some native plant species [Mississippi State University Extension Service, 2014]. As this damage spills over from wilderness into developed agricultural areas, crops are ruined, stock is killed and zoonotic diseases are spread. Damage costs related to feral swine is over \$1.5 billion annually [APHIS, 2013]. Swine also carry viral and bacterial diseases such as foot-and-mouth disease virus, pseudorabies, and brucellosis [Seward, 2004; Cooper, 2010; Meng, 2009]. Therefore, population mitigation is necessary.

2.5 Feral Swine Control

Current methods of feral swine damage management include fencing, cages or corral traps, snares, aerial shooting, ground shooting, and hunting with dogs [Campbell, 2009; Massei, 2011]. Although these methods can reduce some damage to particular areas, they cannot be used in all environments, specifically urban areas. The total feral swine population continues to grow regardless of these techniques.

The advantages and disadvantages of the various methods mentioned above have been evaluated previously, only the pertinent methods are discussed here [Campbell, 2009; Massei, 2011]. Some techniques, such as aerial shooting and baiting animals to traps, appear most effective when population numbers are large. The cost of

using aerial shooting and traps does not outweigh the benefits of damage reduction as a population becomes smaller. However, due to the commercial and recreational hunting industry, baiting and aerial shooting remain popular. Aerial shooting and other forms of hunting are considered legally unacceptable in urban areas. Currently there are no approved toxicants in the United States to manage the feral swine population [Campbell, 2009; Campbell, 2006]. With the number of feral swine still growing, it is apparent that commercial capture, shooting, and consumption of hogs is not reducing numbers enough to curb growth [Massei, 2011]. Combination efforts of fencing, trapping and hunting were done in a study in Pinnacles National Monument. It took an estimated 67.5 hours to remove each pig and there were 200 pigs. In summary, the project spent 13,489 hours, \$623, 601 (United States currency), and 3 years to eradicate the feral swine. Before pig removal could begin, it took 18 years and \$2 million to build the fence to enclose the 57km² area [McCann, 2008]. With a 6 million feral swine a, costs would exceed \$18 billion to manage feral swine using these methods that costed \$3,118 per pig, not accounting for increases in the population size during the time it takes to build the fencing or the cost of the fencing.

Non-lethal methods for reducing feral swine damages over time include fertility control, fencing, repellants, diversionary feeding, and translocation [Massei, 2011]. Currently fertility control studies in overabundant wildlife have focused on gonadotropin releasing hormone (GnRH) inhibition. GnRH inhibitors focus on the gonadotrophin dependent phase of the folliculogenesis, meaning that the ovarian reserve of primordial follicles is intact. Given that the potential for viable oocytes is still present, the efforts in fertility control using GnRH inhibitors should not be considered a permanent solution, as

it is acting as a contraceptive instead of a means of permanent sterilization. Reversible contraceptive drugs used in various species are in forms such as GnRH agonist implants (Deslorelin), oral contraceptives (Melengestrol acetate) or immunocontraceptives (porcine zona pellucida (PZP) vaccine, and GonaCon™). These drugs currently require boosters, ranging from daily to every few years, and none are currently being employed to control feral swine [Massei, 2014; Naz, 2005; Miller, 1999, Massei and Cowan 2014, Barfield 2006]. Initially, research studies in feral swine fertility control appear promising, but have not shown long term abilities and have high contraceptive variability between groups [Quy, 2014; Herbert, 2005]. In addition, the quantity of drug used and method of administration are still considered financially and environmentally questionable. [Hampton, 2015; Killian et al., 2008; Campbell, 2006; West et al., 2009]. Immunocontraception in feral swine could be a viable option if compounds with higher efficiency, greater longevity and permanent effects were developed [West et al., 2009].

As previous wildlife studies have modeled, targeting females would be considered most effective; the polyandry nature of males and the skewed sex ratio towards males in feral swine would make targeting boars less efficient [Hobbs, 2000; Merrill, 2003]. Other non-lethal control methods have become futile (fencing and diversionary feeding) or illegal (translocation) due to the size and spread of the feral swine population.

2.6 Focusing on primordial follicles and their isolation

Primordial follicles represent the total reproductive potential of a female mammal. These structures consist of an oocyte surrounded by a single layer of flattened granulosa cells, and are enclosed in a basement membrane [Hunter, 2000]. Although some work suggests oocyte regeneration in mammalian ovaries is possible, these studies have not been repeatable [Johnson, J., et al. 2005, Pan, Z., et al. 2015, Telfer, E. E., et al. 2005]. Given the lack of repeatability or additional support of such findings, it is logical to continue to focus on the destruction of primordial follicles as an effective and permanent form of sterilization for the feral swine population. A large part of this literature investigation focuses on probable communication mechanisms within the mammalian ovary.

A procedure for the viable isolation of primordial follicles is important for work targeting these cells. Literature from the 1980's describe isolating primordial follicles from prepubertal gilt ovaries using combinations of mechanical dissection and chemical isolation using collagenase 1A, or a mixture of collagenase, DNase and pronase [Roy, 1985; Greenwald, 1989]. Those studies looked at the ability of the follicles to synthesize proteins for 12 hours post isolation to assess viability. Another study involved complex procedures involving mechanical and an enzymatic component, collagenase type I, combined with multiple centrifugations using Percoll gradient separation, and cell sorting to increase the number and purity of primordial follicles retrieved [Shi, 2007]. Shi Kerong [2007] obtained 779 000 primordial follicles per prepubertal ovary, a much higher number than previously shown. The difference in primordial follicles numbers reiterate the high variability and lack of knowledge regarding these follicles in swine.

Based on how few studies have been completed, and how inconsistent the numbers of isolated primordial follicles are, it would be beneficial to look into developing a consistent and efficient procedure to isolate primordial follicles from pig ovaries.

2.7 Receptor-mediated endocytosis

For this thesis, I will focus on supporting the presence of receptor-mediated endocytosis, specifically CME in porcine ovaries, as this pathway could be targeted for drug delivery (Figure 5). CME is one of the most abundant cellular transport mechanisms in eukaryotic cells, and it is both efficient and selective, utilizing the protein clathrin to form membrane pits that subsequently undergo endocytosis, [Johannes and Lamaze, 2002]. Using CME, ligands that link to receptors on the cell surface that become entrapped in clathrin-coated pits can be internalized by the cell [Pelkmans and Helenius, 2002; Doherty and McMahon, 2009; Tarragó-Trani and Storrie, 2007].

Endocytic signaling at the cell surface by an adaptor protein, AP2, gives rise to the creation of a clathrin coated pit, which later undergoes scission from the internal membrane surface with the help of a large GTPase, dynamin [Tarragó-Trani and Storrie, 2007; McMahon and Boucrot, 2011]. Although there are three isoforms of dynamin found in mammals, dynamin-2 is the only one ubiquitously expressed [Parker et al., 2009; Ferguson and Camilli, 2012]. Once fission occurs, clathrin coated vesicles and their cargo travel intracellularly to interact with other cellular membranes for transport, disassembly and possible recycling of receptors and ligands [Brodsky et al., 2001]. It is this signaling pathway that could be targeted to allow entry of a

chemosterilant into primordial follicles that could lead to follicular destruction or altered function.

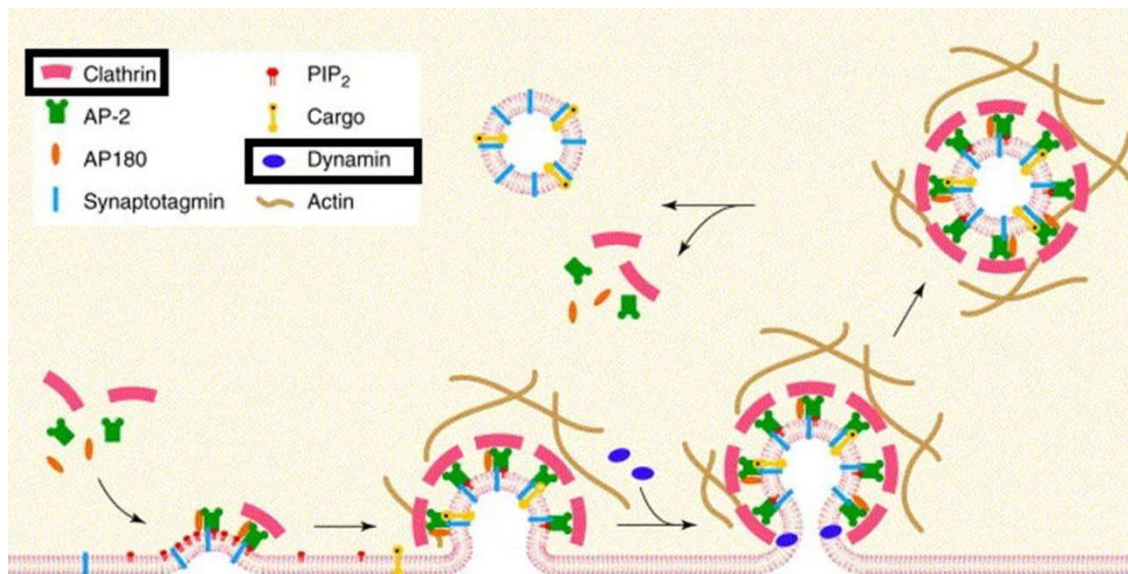


Figure 5. Schematic of Clathrin-Mediated Endocytosis. In this process, a ligand binds to receptors at the cell surface causing a signal cascade that causes a clathrin-coated pit to form. This pit forms into a vesicle that is internalized with the help of the large GTPase called dynamin. (Takei 2001)

2.8 Target Ligands in the Ovary

Assuming that CME is utilized by primordial follicles, imaging receptor-mediated endocytosis in primordial follicles by finding ideally oocyte specific ligands would be the next step in developing a permanent sterilant that exploits this delivery mechanism. Based on literature review, there is at least one oocyte specific ligand and two other non-specific ligand that could be good candidates for drug conjugation for oocyte delivery [Holt, 2006; Zuccarello, 2011; Driancort, 2000; Hutt, 2006; Jones, 2013].

Stromal derived factor 1 (SDF1), also known as CXCL12, is a chemoattractive cytokine that attaches most commonly to the seven trans-membrane G-protein-coupled receptor CXCR4. SDF1 is constitutively expressed in many mammalian tissues,

including neonatal ovarian tissue; oocyte expression of both ligand and its receptor has been assessed using confocal scanning microscopy and immunohistochemistry in human and mouse tissue [Zuccarello, 2011; Holt, 2006]. Studies have shown that SDF1 and CXCR4 inhibit follicular activation, but exact signaling pathways are still unknown [Holt, 2006]. Other studies in zebrafish and mouse ovaries have shown that CXCR4 is not only expressed in primordial germ cells, but is necessary for normal migration of primordial germ cells [Molyneaux, 2003]. Since this ligand and receptor are located and produced in a broad range of tissues for immunological purposes, these cells could be replaced or replenished in other areas of the body [Bleul et al., 1996]. Even though SDF1 and CXCR4 are not oocyte specific, the ubiquitous nature and confirmed presence of both ligand and receptor in the pre-ovulatory oocyte make this receptor-ligand pair worth further investigation.

Another ligand and receptor combination previously found in the ovary is Kit ligand (KL), also known as Stem Cell Factor (SCF), and its type III transmembrane tyrosine kinase receptor, c-Kit (stem cell growth factor receptor, CD117, tyrosine-protein kinase Kit, KIT) [Driancort, 2000; Hutt, 2006; Jones, 2013]. Not only are there multiple studies on this receptor-ligand complex in mammalian ovaries, but there has been work in porcine oocytes, including primordial follicles [Moniruzzaman, 2007]. Expression of c-Kit was detected in primordial oocytes of neonatal and prepubertal pigs using immunohistochemistry [Moniruzzaman, 2007]. KL is known to have roles specifically in primordial follicle formation and primordial to primary follicle transition [Hutt, 2006; Driancort 2000; Parrott, 1999]. It is thought that c-Kit signaling, much like SDF1 and CXCR4, could work synergistically with other pathways such as hematopoiesis that

have high rates of replenishment, which also makes them appealing for further investigation [Jones, 2013].

Lastly, Anti-mullerian Hormone (AMH) is a member of the transforming growth factor- β (TGF β) superfamily, which includes factors for growth and differentiation, is found in reproductive tissue. It is made by fetal sertoli cells and postnatal granulosa cells [Baarends, 1995; Durlinger, 2002]. The receptor for AMH is a type II receptor called AMHRII. This ligand-receptor complex is thought to regulate primordial follicle growth by inhibiting primordial follicle recruitment [Gruijters, 2003]. In multiple studies, AMH and AMHRII were expressed in granulosa cells of follicles from the primary stage up through the antral stage. These studies also support that AMH plays an important role in inhibiting primordial follicle recruitment [Nilsson, 2011; Durlinger, 1999; Weenen, 2004; Nilsson, 2007; Rice, 2007; Visser, 2006; Gruijters, 2003]. The role of both components of this receptor-ligand relationship should be studied in regards to mammalian reproductive physiology and follicular recruitment.

Varying results from previous AMH studies suggest that there may be differences in expression of AMHRII and AMH between species [Pellatt, 2010]. Research suggests that AMHRII is likely expressed in primordial follicles in mice since AMHRII mRNA expression is found even before birth, but exact localization was still not found [Durlinger, 2002; Baarends, 1995]. One abstract from Proceedings of the 17th International Congress on Animal Reproduction described the use of immunohistochemistry (IHC) to find minimal expression of AMH in primordial follicles in porcine oocytes, but this abstract had no additional or supportive studies that could be found [Almeida, 2012]. Most recent studies suggest that the inhibitory action of AMH in

piglets in utero is highest around parturition time based on AMH mRNA levels that are detectable at day 50 of gestation and increase at day 108 [Knapczyk-Stwora, 2014]. In oogenesis in the pig, days 90 to 100 post coitum have higher numbers of oocytes in the diplotene stage, the non-growing stage of the primordial follicle, than days 20 to 80. This information would support that the expression changes of AMH mRNA levels could be specifically associated with primordial follicles [Black and Erickson, 1968; Bielańska-Osuchowska, 2006]. If AMH does undergo endocytosis in primordial follicle pool in pigs, a chemosterilant could be designed for reproductive tissue targeting.

2.9 Toxicants and Nanomedicines

Toxic substances including chemotherapy agents, polycyclic aromatic hydrocarbons, and basic occupational hazardous chemicals have effects on female reproduction, and so they could be considered in chemosterilant development. Chemotherapeutic agents including cyclophosphamide, chlorambucil and nitrogen mustard can cause ovarian failure, although little is known regarding how ovotoxicity occurs [Hoyer, 2005; Verp, 1986]. Polycyclic aromatic hydrocarbons, a toxicant group found in cigarette smoke, including benzo[a]pyrene (BaP), 3-methylcholanthrene (MC), and 9:10-dimethyl-1:2-benzanthracene (DMBA) reduce fertility by destroying follicles and causing sterility of female offspring [Hoyer, 2005; Mattison, 1979; Mattison, 1980]. 4-vinylcyclohexene (VCH) and the chemically similar diepoxide metabolites are created as a by-product during the manufacturing of synthetic rubbers such as tires, and have raised questions regarding fertility in women. These chemicals target primordial and

primary follicles [Hoyer, 2001; Hoyer, 1996]. The toxic substances target biological systems, but are not specifically used as ovotoxins.

Nanomaterials are being studied and used to deliver materials to specific areas of the mammalian body, which minimizes negative effects to non-target areas. There have already been successes in the manipulation of receptor-mediated endocytosis with creation of antibody drug conjugates and targeted prodrugs (Figure 6) including Mylotarg, Ontak, and Vintafolide [Akinc, 2013; Nabhan and Tallman, 2002; Olsen et al., 2001; Lorusso et al., 2012]. In reproduction studies, ribosome inhibiting proteins have been conjugated to analogs of gonadotropin-releasing hormone to form an immunotoxin or hormonotoxins. These conjugates specifically target and appear to destroy gonadotrophs; these may be able to render an animal sterile as well as target any other cell types with GnRH receptors [Nett, 2005; Yang, 2002]. A GnRH analogue conjugated to pokeweed antiviral protein has been shown to disrupt reproduction in male dogs effectively with a booster for up to 37 weeks, [Sabeur, 2003]. Success in those studies indicates that it could be possible to create other conjugates that could target receptors, if found, in primordial follicles in the porcine ovary.

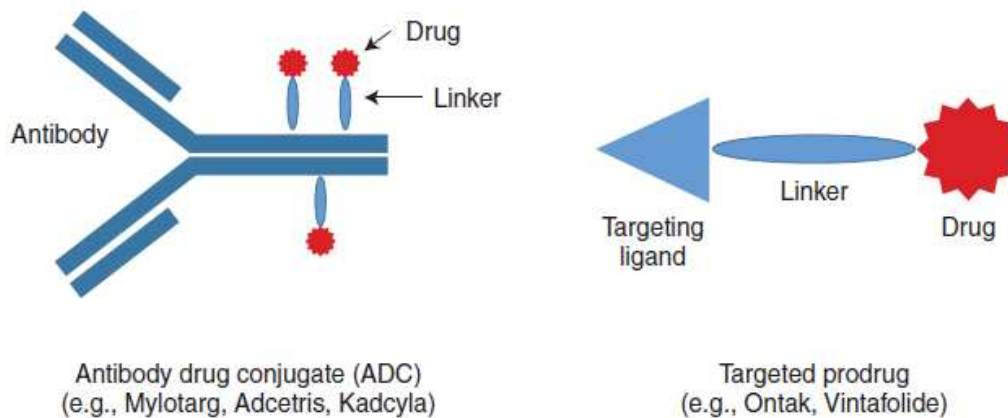


Figure 6: General structure of antibody drug conjugates (ADC) and targeted prodrugs. Image Credit: Akinc, A. and G. Battaglia (2013). Modified 4/25/16.

2.10 Conclusions

One conclusion from this review is that despite current efforts to control the feral swine population, the population is still growing. Although recreational hunting addresses a lack of natural predators for feral swine and can reduce the population, there is currently no long-term method for targeting the high reproductive capacity of this species. By turning scientific studies towards developing a permanent reproductive control method, a contributing factor to this abundant population growth can be addressed. A method to deliver a drug or toxicant that will cause the primordial follicles to all die or mature at one time could be used in combination with current control efforts to better regulate feral swine populations resulting in reduced damages.

The ligands and their respective receptors used in follicular CME are unknown. With the help of advanced imaging, ligands may be identified in live cell culture. Several possible ligands that are expressed in the mammalian ovary, such as SDF1, c-Kit, and AMH, are potential targets. Once ligands and receptors are identified, they can be

tested for their use in pharmacodelivery to the primordial follicles. Chemosterilant delivery to these follicles could then result in a permanent method of reproductive control for the feral swine population.

CHAPTER 3: IDENTIFICATION AND LOCALIZATION OF DYNAMIN II AND CLATHRIN IN PORCINE OVARIES

3.1 Background

The mechanisms by which primordial follicles communicate are still unknown, but it is likely to involve CME. The purpose of this study was to determine if porcine primordial follicles express components of clathrin-mediated endocytosis (CME). This common cellular communication process is considered both efficient and selective [Johannes and Lamaze, 2002]. CME, if present, could be exploited as a method to deliver tissue specific chemosterilants. Targeting the primordial follicle pool causing total committed growth or atresia would leave an animal permanently sterile. Identifying major components of clathrin-mediated endocytosis, clathrin and dynamin II, is a first step to understanding and exploiting CME for this purpose.

3.2 Materials and Methods

3.2.1 *Tissue Samples*

Ovaries were obtained from gilts of various breeds from a local slaughterhouse and from piglets averaging around 40lbs from Colorado State University's Veterinary Teaching Hospital.

3.2.2 *Tissue preparation for immunohistochemistry*

Ovaries were bisected longitudinally with a #20 scalpel blade and fixed in 10% formalin. Tissue samples from gilts (n=6) and piglets (n=6) were imbedded in paraffin blocks, cut into 5µm sections and mounted on slides for ovarian histology and

immunohistochemistry. Slides had various stains applied for ovarian histology: hematoxylin and eosin, Periodic acid-Schiff, and Masson's trichrome (Colorado State University Diagnostic Laboratory).

3.2.3 *Antibodies*

Primary antibodies were: rabbit polyclonal Dynamin 2 (dilution 1:50, #PA5-29658, Thermo Fisher), goat polyclonal anti-Clathrin HC (C-20) (1:50, sc-6579, Santa Cruz Biotech). Secondary antibodies were: rabbit anti-goat IgG (whole molecule) FITC (1:100, Sigma Aldrich, F7367), goat anti-rabbit IgG-FITC (1:100, sc-2012, Santa Cruz Biotech). Clathrin HC (C-20) (200µg/ml, sc-6579P, Santa Cruz Biotech) was used to block the clathrin primary antibody. Antibodies used to optimize conditions are listed in APPENDIX A.

3.2.4 *BLAST Analysis*

BLAST (Basic Local Alignment Search Tool) analysis between human and *Sus scrofa* (pig) amino acid sequences representing several antigens of interest were used. Dynamin II, clathrin light chain, clathrin heavy chain and those antigens associated with receptors and ligands of future interest went through BLAST analysis. BLAST analysis for CXCL12, CXCR4, c-Kit, Kit Ligand, AMH and AMHRII can be found in APPENDIX B. The amino acid sequence of each antigen of interest was entered into the query sequence for comparison. Entry DYN2_HUMAN (P50570) was compared to F1S593_PIG (F1S593) for dynamin II. Entry CLCA_HUMAN (P09496) was compared to

B5ATG0_PIG (B5ATG0) for clathrin light chain (CLTA) protein. Entry CLH1_HUMAN (Q00610) was compared to C0MHR2_PIG (C0MHR2) for clathrin heavy chain.

3.2.5 Immunohistochemistry

The IHC procedure was optimized using different antigens prior to focusing on the antigens of interest. Protocols were developed from the Pierce® Peroxidase Detection kit and literature review [Thermo Scientific, #3600; Abcam, 2016; Moniruzzaman, 2007; Shi, 2007; Ramos-Vara, 2005; Qian, 2005; Rattanapinyopituk, 2014]. Initial IHC attempts and their results can be found in APPENDIX A. Slides containing tissue sections imbedded in paraffin were heated using FisherBiotech™ MicroProbe™ Manual Staining System for 15 minutes at 65°C and then cooled at room temperature for 5 minutes before paraffin removal. Tissue was deparaffinized in xylene, and then rehydrated gradually through serial dilutions of ethanol and rinsed in deionized water. Then slides were placed into wash buffer (0.05M Tris-buffered saline with 0.05% Tween-20, pH 7.0, TBST, Sigma, T9039) two times for five minutes each. Then slides underwent antigen retrieval using heat induced epitope retrieval methods (HIER) to unmask antigenic sites [Ramos-Vara, 2005]. Slides were submerged in 0.01M citrate buffer (pH 6.0) and heated using a microwave (Magic Chef™, 900W, 2450MHz, cook power level 10) in 10 minute intervals totaling 30 minutes; every 10 minutes any evaporated solution was replaced with warmed deionized water. Slides were then washed in TBST before the primary antibody was added. A hydrophobic barrier creating pen (ImmEdge Pen, Cat No H-4000, Vector Laboratories) was used to encircle the tissue sections to minimize the amount of liquid needed for incubations, which were all

done in a humidified chamber (100%). Non-specific binding sites were blocked using 10% (v/v) normal serum from the species associated with the secondary antibody host diluted in TBST (goat serum - SLBM5884V and rabbit serum - SLBL5150V, Sigma-Aldrich) for 1 hour at room temperature. The appropriate primary antibody was applied after blocking; it should be noted that all antibody concentrations were previously optimized (APPENDIX A) and all primary antibodies were diluted in 1% (v/v) normal serum from the species associated with the secondary antibody host. Sections were washed with TBST after incubating for 2 hours at room temperature. Antigens were visualized using fluorescently-tagged secondary antibodies diluted in TBST, which were incubated for 45 minutes at room temperature. Negative control samples were made by incubating sections in 1% (v/v) normal serum instead of a primary antibody. All sections were washed in TBST three times for three minutes each and the ImmEdge Pen lines were removed. Slides were rinsed in deionized water and then mounted in PermaFluor™ aqueous mounting media (TA-030-FM, Thermo Scientific). All microscopy was completed using a Leica microscope, and images were obtained using LASV4.5 imaging software.

Primary antibody specificity was confirmed using a blocking peptide [Abcam, 1998-2016]. In this experiment, the pre-absorbed primary antibody was applied to the tissue for the treatment slide, and the control slide underwent the regular primary antibody application. To create this pre-absorbed primary antibody, the antigen peptide, either dynamin or clathrin, was added at five times excess to the primary antibody to be applied to the treatment slide. Blocking immunizing peptide studies conducted with dynamin II antibodies are described in APPENDIX A (results not shown). Here, the

regular primary antibody was applied to the control slide with an equivalent amount of 1% v/v normal serum instead of the peptide (anti-Clathrin HC (C-20), sc-6579, Santa Cruz Biotech; Clathrin HC (C-20), sc-6579P, Santa Cruz Biotech). Both antibody solutions were incubated with gentle agitation at room temperature for 30 minutes before application and then subsequent IHC steps continued as previously described. Images were again captured and evaluated.

3.2.6 Image Analysis

IHC images were evaluated after capture with Leica Application Suite (LAS) software. Appropriate fluorescent binding was determined qualitatively looking at signal to noise ratio, consistency of staining appearance in tissues between multiple subjects, and the use of controls. Areas of bright green fluorescence that were uniform in the treatment slides but were eliminated in the peptide blocking treatment identified binding specificity for an antigen.

3.3 Results

3.3.1 Ovarian Histology

Porcine ovaries contained primordial follicles (Figures 7 – A and B and 8 – A) through tertiary follicles (Figure 8 – B). Images at lower magnifications showed that primordial follicles were concentrated in the outer cortical tissue regions in the porcine ovary (Figure 7 – A). These follicles were easily distinguished by an oocyte surrounded by a single or incomplete layer of squamous granulosa cells (Figure 7 – B). Periodic acid-Schiff stain (PAS) allowed visualization of basement membranes (Figure 8 – A).

More mature follicles, including secondary and tertiary follicles, were seen in gilt ovaries (Figure 8 – B).

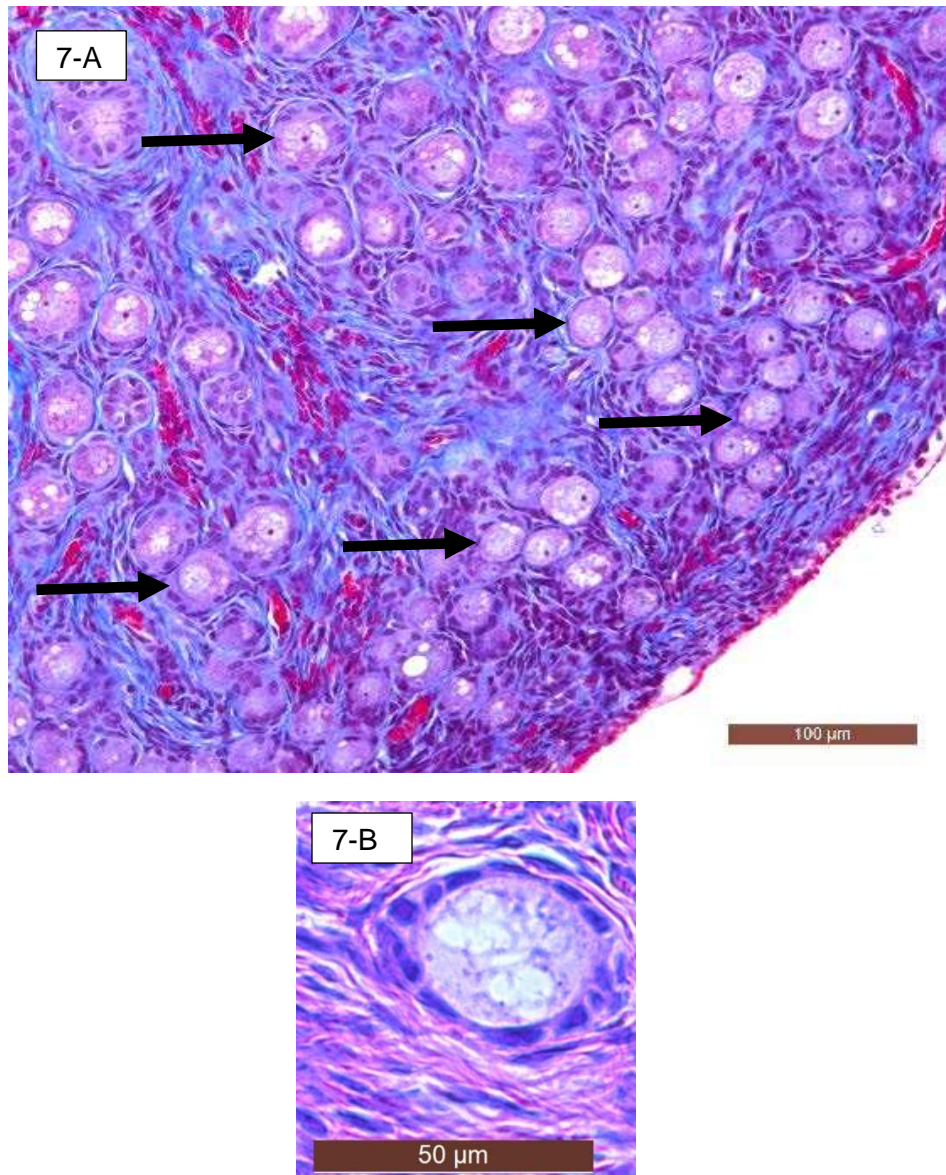


Figure 7 (A-B): Histological sections of porcine ovaries stained with Masson's trichrome. Primordial follicles are visible in the cortical region of the ovary (A, arrows). Increased magnification allows a close up view of a primordial follicle (B).

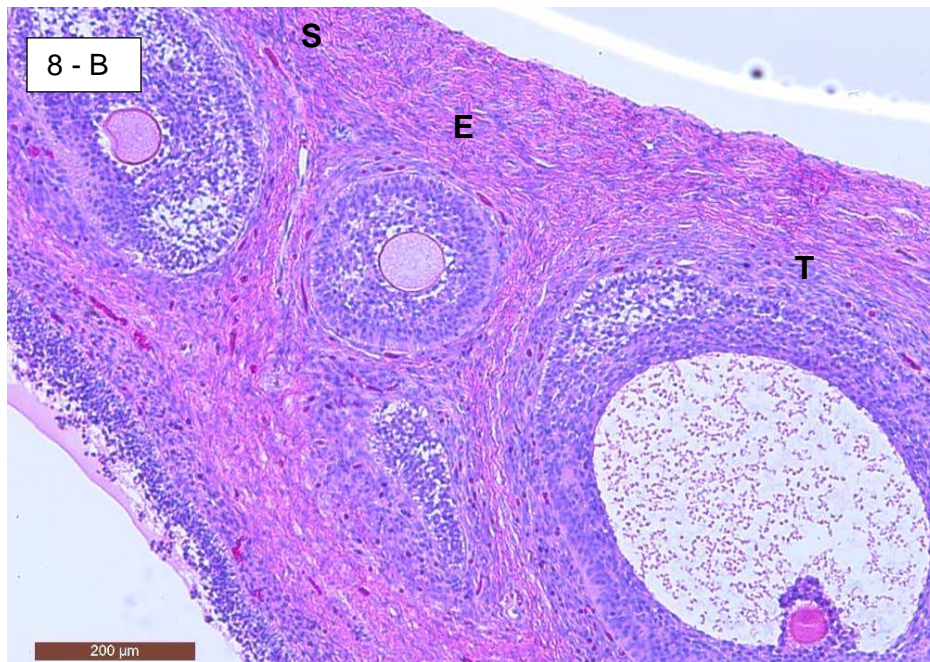
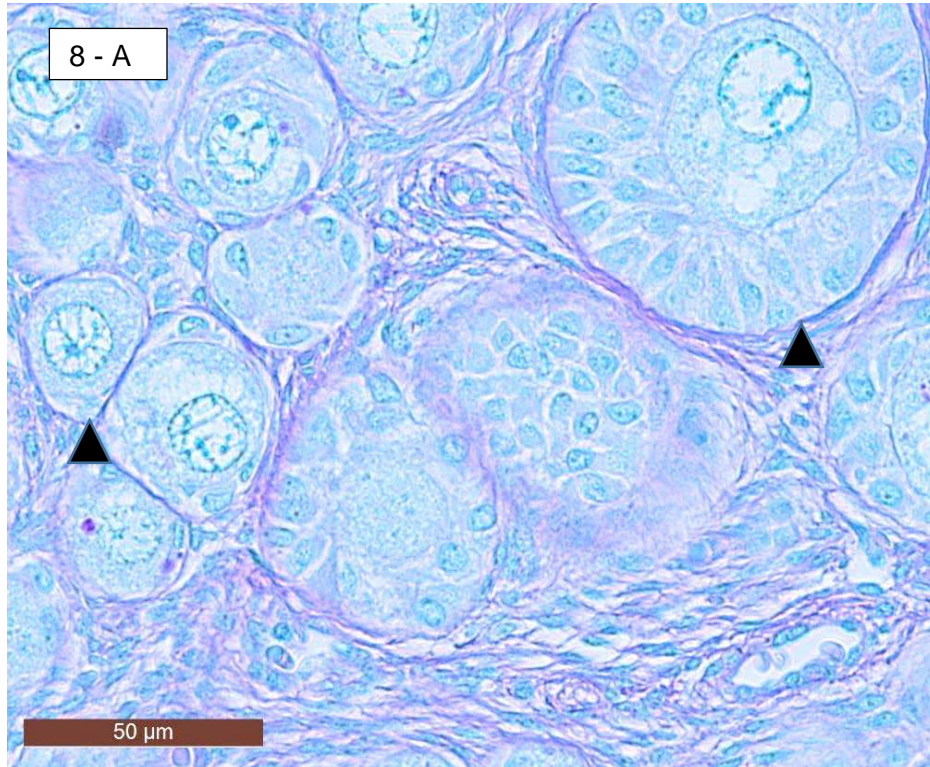


Figure 8 (A-B): Histological sections of porcine ovaries stained with PAS and hematoxylin with eosin. Basement membranes enclosing primordially follicles and granulosa cells (A, arrowheads). Secondary (S), early secondary (E) and tertiary (T) follicles were observed in gilt ovaries (B).

3.3.2 BLAST analysis

Basic Local Alignment Search Tool (BLAST) analysis showed that the amino acid sequences of three antigens of interest are >80% identical for human and *Sus scrofa* proteins (Table 1). Alignment identity is 95.0% for dynamin II (824 amino acids out of 870 being identical), 81.2% for clathrin light chain (131 amino acids out of 161 being identical), and 100% for clathrin heavy chain (all 1675 amino acids are identical).

Table 1. BLAST results showing alignments between the amino acid sequences of humans and *Sus scrofa* for dynamin II and clathrin

Antigen	Percent alignment
Dynamin II	95
Clathrin light chain	81.2
Clathrin heavy chain	100

3.3.3 Immunohistochemistry

Dynamin II and clathrin were detected in the cytosol of oocytes from both piglet and gilt ovaries. Figure 9 (A and B) and Figure 10 (A – C) show the resulting fluorescent IHC images. Protocol development IHC results for c-Kit, dynamin II, and clathrin, and MSY2 can be found in APPENDIX A. The signal for dynamin II was weaker than the signal seen in clathrin experimentation. No staining was seen in negative controls (Figure 9 – B and Figure 10 – B). Clathrin antibody specificity was confirmed by having no observed staining in the oocytes that underwent peptide blocking (Figure 10 – C

compared to Figure 10 – A). Some non-specific staining was seen in red blood cells in all treatments (Figure 9 A and B and Figures 10 A – C).

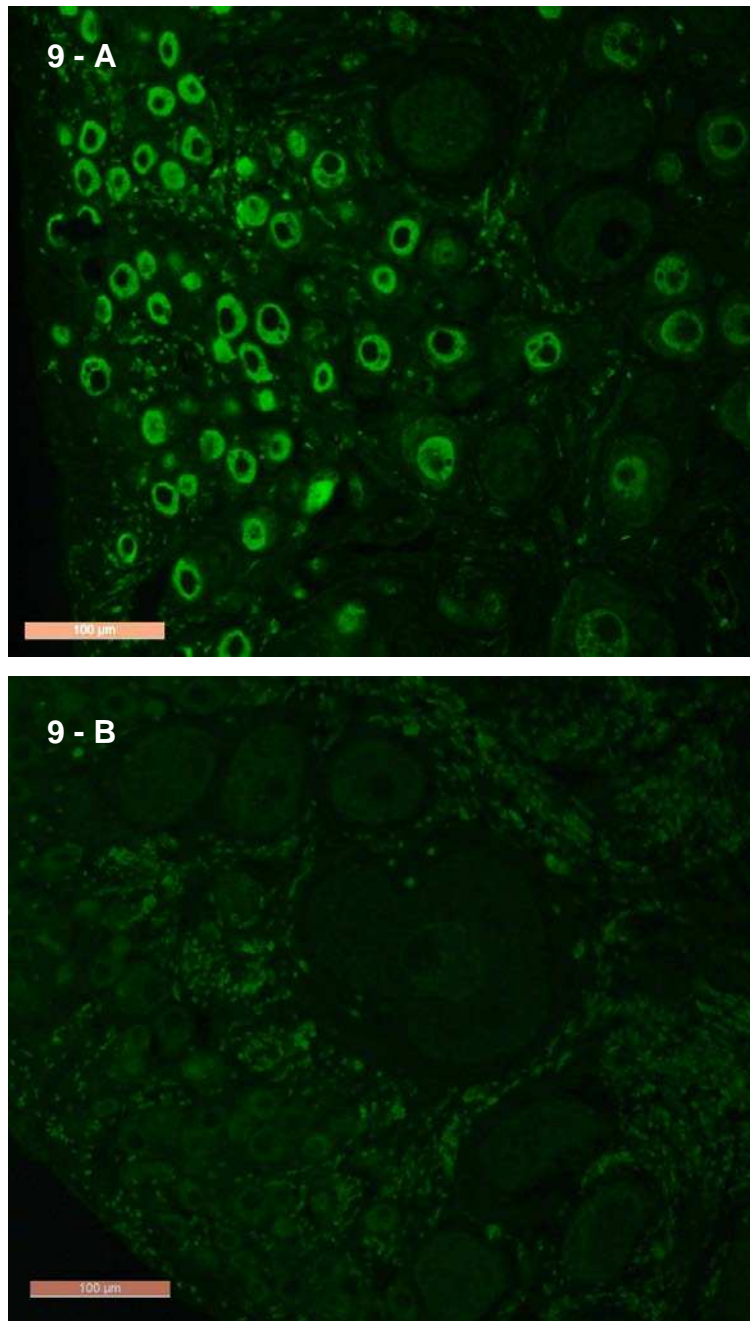


Figure 9 (A and B): Expression of dynamin II in a porcine ovary. A) Positive staining in oocytes of all follicles observed. B) Negative control—no staining. Scale bars represent 100µm. Non-specific staining of red blood cells was present (A and B).

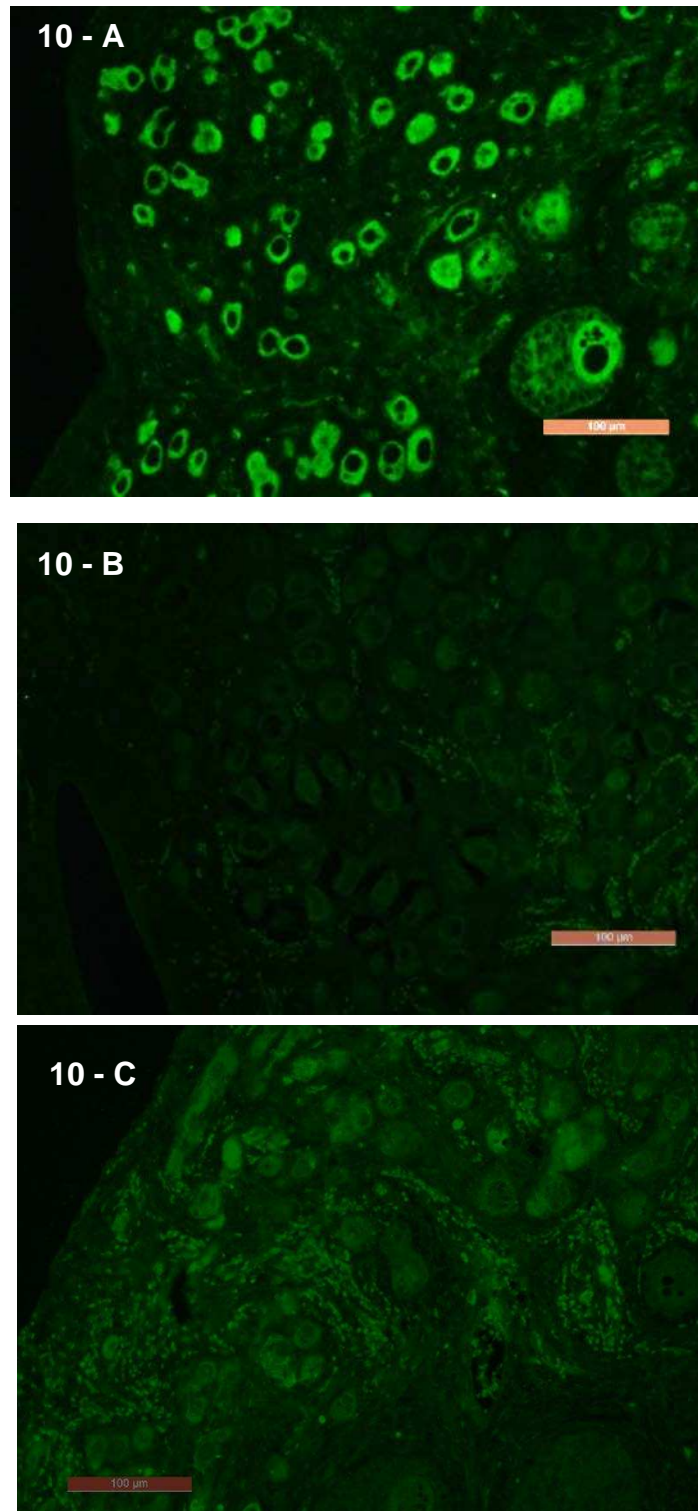


Figure 10: Expression of clathrin in a porcine ovary. A) Positive staining observed in oocytes of all follicles observed. B) Negative control—no staining. C) Primary antibody pre-adsorbed with clathrin peptide— no staining observed in oocytes. Scale bar represents 100µm. Non-specific staining of red blood cells was present (A, B and C).

3.4 Discussion

3.4.1 Summary of Findings

The expression of dynamin II and clathrin in porcine oocytes at all follicular stages, using fluorescent IHC, supports the hypothesis that the molecules necessary for clathrin-mediated endocytosis are present in the porcine ovary. This is a first step towards determining if CME can potentially be used for chemosterilant delivery targeting oocytes/primordial follicles.

3.4.2 BLAST

BLAST was able to determine that the amino acid sequences of three antigens of interest are very similar for human and pig proteins; therefore, the antibodies for these proteins already developed against the human protein should be able to detect the porcine protein as well. Results indicate that this is the case.

3.4.3 Localization of dynamin II and clathrin

Identifying and locating two of the main components of CME in primordial follicles is a major step in determining if this type of cellular communication mechanism is taking place inside pig primordial follicles. Strong positive staining was seen in the cytoplasm of oocytes at all observed follicular stages, including primordial follicles, of both piglets and gilts for dynamin II and clathrin. Dynamin II staining proved more difficult than clathrin and the first dynamin II antibody had a poor signal to non-specific binding ratio, making it difficult to detect dynamin II. The immunizing peptide amino acid sequence was proprietary for this antibody, results not shown. A different dynamin II antibody

raised against a known amino acid sequence, determined using BLAST, was better, but a matching blocking peptide was not available. Peptide blocking IHC eliminated clathrin staining in oocytes, confirming the clathrin antibody specificity.

CHAPTER 4: ISOLATION OF PORCINE PRIMORDIAL FOLLICLES

4.1 Background

Porcine primordial follicles needed to be isolated in order to determine whether they utilize CME. Few studies have isolated primordial follicles from porcine ovaries, and variability exists in the number and quality of primordial follicles obtained. Therefore, better methods need to be developed for isolating large numbers of quality primordial follicles.

4.2 Materials and Methods

4.2.1 Tissue Obtainment

Ovaries were obtained from gilts of various breeds from a local slaughterhouse and from piglets averaging around 40lbs from Colorado State University's Veterinary Teaching Hospital.

4.2.2 Tissue preparation

Tissue was prepared as described previously, [Shi, 2007; Roy, 1985; Greenwald, 1989]. Gilt and piglet tissues were processed separately. Cortical tissues were sliced from 2 faces of each prepubertal ovary to approximately 0.5mm thickness, using a Stadie Riggs apparatus. All follicles >2mm in diameter were pierced with a 19g needle and corpora lutea and medullary regions were mechanically removed by hand or with a surgical blade in gilt ovaries. Cortical slices, either from gilts or piglets, were cut into

0.5mmX0.5mmX0.5mm sections using a scalpel blade. This tissue was weighed into masses of 0.2-0.4g and placed into individual 50ml falcon tubes.

4.2.3 Tissue digest

Tissues digests had 9.54mL Dulbecco's phosphate buffered saline (DPBS) with calcium and magnesium (HyClone™, #SH30264) added to each of the 50ml tubes containing cortical tissue. A 300 µl volume of collagenase IA (13860U/mg, Sigma, C9891) in Hank's buffered saline solution (HBSS, Hyclone™, SH30588.01), 100µl collagenase II (1281 U/mg, Sigma, C6885) in HBSS and 60µl DNase (3213KU/mg, Sigma, D5025) in DPBS was added. The tubes were transferred to a 37°C shaking water bath for a total of 40 minutes for piglet tissue and 60 minutes for gilt tissue. The incubation time difference is due to the piglet tissue becoming friable and wispy in appearance during mechanical digestion much faster compared to gilt tissue. The solution was mixed 20 times every 10 minutes by loading and expelling a Pasteur pipette, which was made jagged by breaking the tip of the pipette. The digest was terminated by adding 10mL of 4°C DPBS with 10% fetal bovine serum (FBS, Benchmark™ fetal bovine serum, Gemini BioProducts, #100-106) to each 50ml tube.

4.2.4 Tissue filtration

Each digest was passed sequentially through 70µm, 40µm, and 20µm cell strainers, rinsing with 1mL of DPBS for each strainer (Fisher Brand Nylon Mesh Sterile Cell Strainer, Cat No. 22363548, 22363547 and Pluriselect Life Science PET Mesh, #43-50020-50). The follicles were collected by inverting the 20µm strainer over a new

50mL Falcon tube and rinsing the strainer with 10mL DPBS. The falcon tubes containing the 20-40 μ m cell suspension were centrifuged for 12 minutes at 300xg at 4 $^{\circ}$ C. Following centrifugation, the supernatant was removed and the cell pellet was placed into a 1.5mL microcentrifuge tube coated with bovine serum albumin (BSA, AKRON, cat#AK8909). The volume was then brought up to about 1mL using DPBS and mixed. The cell suspension was then centrifuged at 600xg for 10 minutes at room temperature. The supernatant was again removed and DPBS was added to make 500 μ l. The cells were re-suspended and placed into a petri dish or well plate for evaluation.

4.2.5 Primordial follicle evaluation

Primordial follicles were evaluated qualitatively for size (20-40 μ m), morphology, and viability after staining with neutral red viability stain for 30 minutes (15.15 μ l/ml of 0.33% neutral red, Sigma, N2889).

4.3 Results

Primordial follicles isolated with chemical digests of 40 or 60 minutes with mechanical disruption every 10 minutes resulted in primordial follicles and similarly sized somatic cells from piglet ovaries. The primordial follicles were round in shape, ranged from 25-40 μ m (Figure 11 A and B) and were mostly viable. The suspension also contained various sizes of cellular debris made up of somatic cell conglomerates. Primordial follicles were not isolated from gilt ovaries (results not shown).

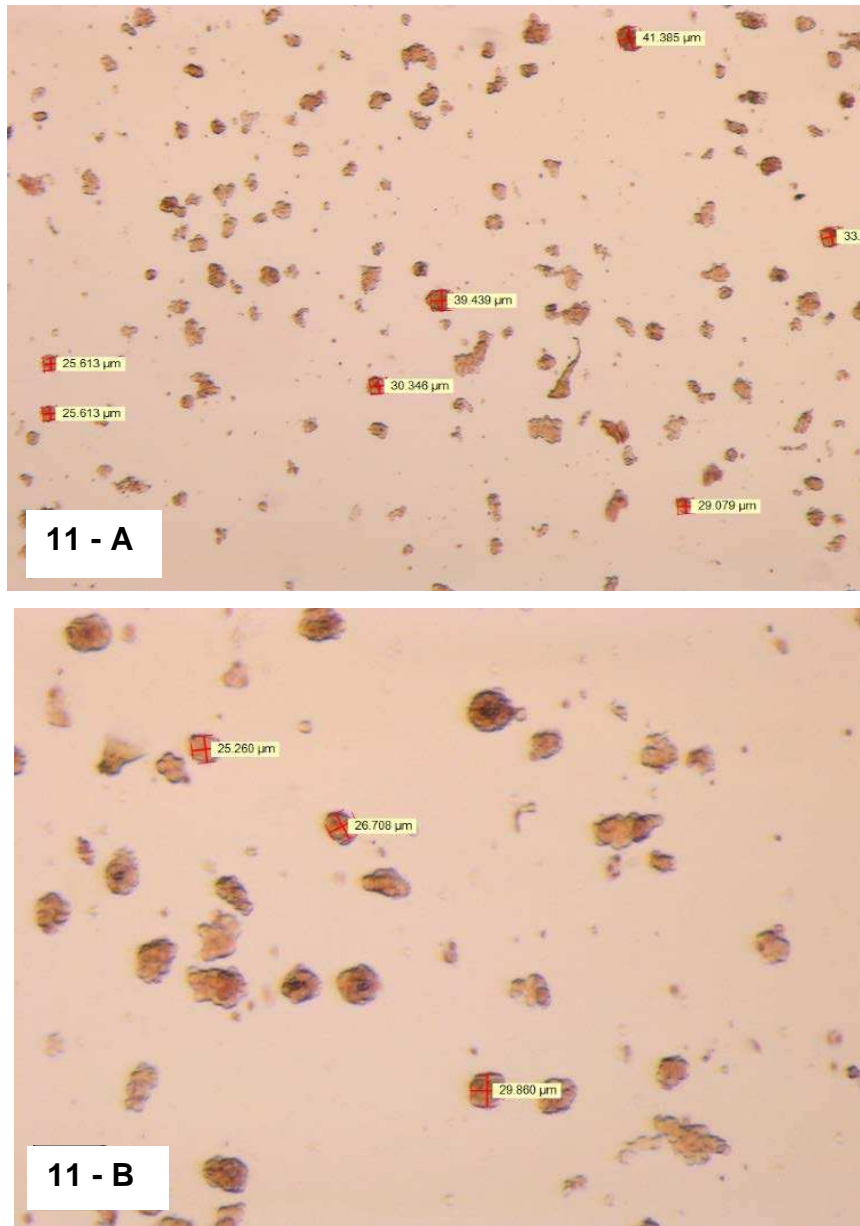


Figure 11 (A and B): Piglet ovary digest containing cells 20-40 μ m in size at 8X (A) and 16X (B) magnifications.

4.4 Discussion

The optimal digest time varied from 40 minutes to 60 minutes depending on the tissue source, piglets or gilts, with tissue from gilts taking longer. This time difference could be due to morphological and structural changes that occur with aging in the ovary.

Digest times exceeding 60 minutes could reduce cell viability, but longer digest times might be considered in future work. In addition, increasing enzyme concentration could permit decreased incubation time, as well as increase the number of primordial follicles isolated, but may decrease follicle viability.

CHAPTER 5: LIVE CELL HANDLING AND IMAGING

5.1 Background

The ability to maintain viable and stable primordial follicles is important for the long term study of CME in primordial follicles. However, previous studies dealing with follicular culture, cultured mature oocytes aspirated from antral pig follicles. Culture conditions included phosphodiesterase inhibitors such as Dipyramidole and Milnirone at 0.1% to maintain those oocytes in meiotic arrest, which could be pertinent to future work with primordial follicles [Mayers and Sirard, 2002; Sasseville, 2009; Yuan, 2012; Marques, 2007; Wongsrikeao, 2005].

5.2 Materials and methods

5.2.1 Media for live cell handling

Media used for cell culture are delineated in APPENDIX C. The basic handling medium was called HCDM-M (Barfield laboratory, Colorado State University). Oocytes were initially collected in HCDM-M [De La Torre-Sanchez, 2006] and oocytes were incubated in an *in vitro* maturation media (IVM) supplemented with either 0.1% Dipyramidole (Sigma, D9766) dissolved in dimethylsulfoxide (DMSO, AMRESCO, #0231), 0.1% Milnirone (Sigma, M4659) dissolved in DMSO, or DMSO (final concentration of DMSO was 0.1% in IVM). Denuding medium consisted of M199 (Sigma, M7528) supplemented with 5 mg/mL hyaluronidase (Sigma, H3506) and 0.01% BSA (AKRON, cat#AK8909). Washing medium was M199 without the added hyaluronidase. Zona thinning, suggested by Lowther (2011), was completed using

200 μ L of M199 with 0.3%BSA to which a 0.1% protease from *Streptomyces griseus* (Sigma, P8811) was added.

5.2.2 Collection and handling of oocytes

Ovaries were rinsed in 0.01M Dulbecco's phosphate-buffered saline (Sigma, SLBJ5110V) and transported to the laboratory within 3 hours of slaughter. Follicular fluid and cumulus-oocyte complexes (COCs) were aspirated by hand using an 18-gauge needle and a 6mL syringe from 3-5mm follicles. COCs were collected in a warmed 50mL Falcon tube, washed in HCDM-M four times and placed in IVM overnight in a 37C incubator (5% CO₂, 85% relative humidity.) Oocytes in some trials were sorted into two grades after overnight incubation: grade 1 oocytes had two or more complete layers of compact cumulus cells surrounding the zona pellucida, and high fat content in the ooplasm (denoted by dark brown ooplasm) and grade 2 oocytes had partially expanded cumulus cells or only one layer of cumulus cells, and low fat content seen in the ooplasm (denoted by light brown to yellow ooplasm). Oocytes were discarded before the denuding process if they lacked all cumulous cells, had a broken ooplasm membrane, or had already expanded cumulous cells.

5.2.3 Denuding oocytes and zona thinning

Oocytes were denuded using M199 containing 0.01%BSA and 5mg/mL hyaluronidase in a 15mL conical tube [Krisher and Yuan, 2012]. Sorted oocytes were added to the tube and mixed using a vortex machine placed on a medium to high setting for 4 minutes. After mixing, 5mL of M199 was added directly to the tube and

mixed to halt enzymatic activity. Oocytes were then washed several times in M199 prior to zona thinning.

Denuded oocytes in some trials were placed in a protease solution (200µL of M199 containing 0.3%BSA and 0.1% protease from *Streptomyces griseus*) and observed using a stereomicroscope for structural changes in the zona. Oocytes remained in the protease solution for a minute and a half before seeing puckering and expansion of the zona. Protease activity was terminated by transferring oocytes into M199 washes. Gentle pipetting was used to loosen/remove any zona pellucida not completely removed by protease exposure.

5.2.4 Oocyte staining

Initially, cell viability was assessed using neutral red. Neutral red is taken up by viable cells by active transport and incorporated into lysosomes, thereby staining the cells red. Non-viable cells do not take up the dye.

In subsequent experiments, FM1-43FX membrane probe (5µg/ml, Life Technologies, F35355) in DPBS containing no calcium and magnesium (ATCC®, #30-2200) was used as described by Lowther (2011). Oocytes were collected as described in section 5.2.2 and washed three times with HCDM-M. The oocytes were then sorted into two groups depending on oocyte grade as described in section 5.2.2, and incubated overnight in IVM containing 0.1% milnirone or 0.1% DMSO at 37°C (5% CO₂, 85% relative humidity). After incubation, all oocytes were denuded as described in section 5.2.3. After the last wash in M199, 10-100 oocytes per group were washed in 4°C DPBS containing no magnesium or calcium, and were transferred in 190µl of the final wash

into separate wells of a 96 well plate on ice and 10µl of FM1-43FX solution was added just prior to imaging with Operetta High-Content Imaging System (PerkinElmer).

5.2.5 Imaging

Cell images were taken using an Operetta High-Content Imaging System with Harmony Software, an inverted Leica microscope using Q-Capture software, a Leica stereomicroscope with Leica Application Suite (LAS) software or an Olympus IX81 (FV1000) scanning confocal microscope with Olympus Fluoview software. To increase image quality using the Operetta system, images were taken with z stacking every 5µm, starting at 45µm above the plate for 7 sections. A time series was taken as quickly as possible for 7 intervals taking up to 13 minutes and 42 seconds to complete. Confocal microscopy imaging was accomplished at the Infectious Disease Research Center (IDRC) with the help of Kevin Martin (results not shown).

5.3 Results

5.3.1 Live cell handling

Cells were imaged after initial sorting and after the denuding process, with and without neutral red to determine cell viability (Figure 12 A and B, Figure 13 A and B). Oocytes stained deep red (Figures 12 – B and 13 – B) after the collection, incubation, sorting, and the denuding process, indicating that the oocytes survived the process. Incubating oocytes in DPS with 0.1% Trypsin did not remove the zona pellucida, however, treating oocytes with DPBS and 0.1%BSA with 0.01% protease did cause

zona thinning (Figure 14 A and B), similar to protocols described by Tanihara(2013).

Zona removal was deemed unnecessary for our purposes.

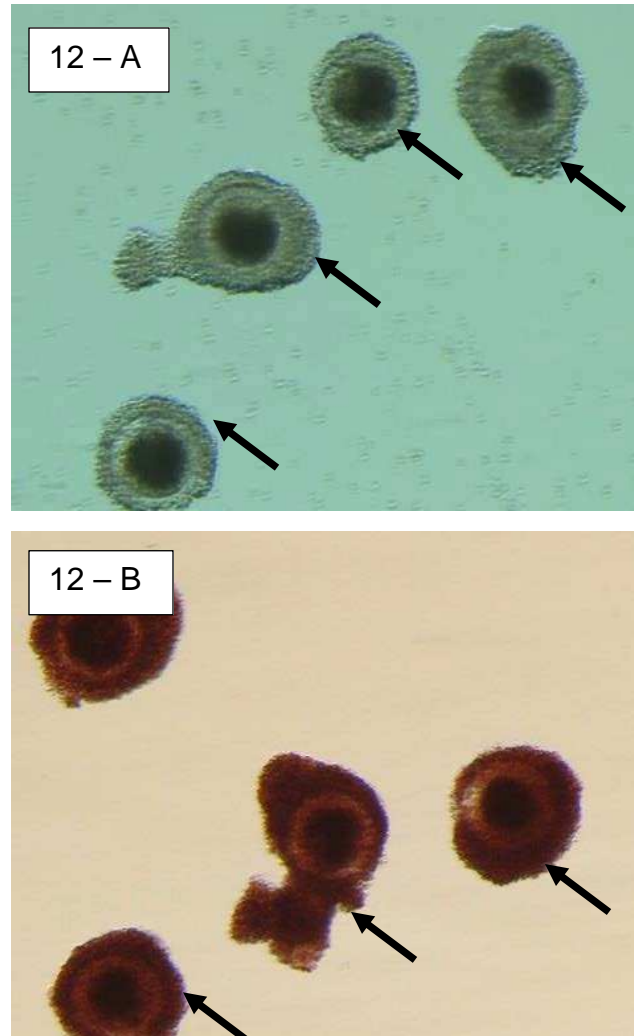


Figure 12 (A and B). Oocytes prior to denuding procedures, with and without neutral red viability dye application. Sorted oocytes with compact cumulous cells (arrows) seen surrounding the zona pellucida (A and B). Neutral red shows viability with dark red staining (B). Oocytes are around 120 μ m in diameter.

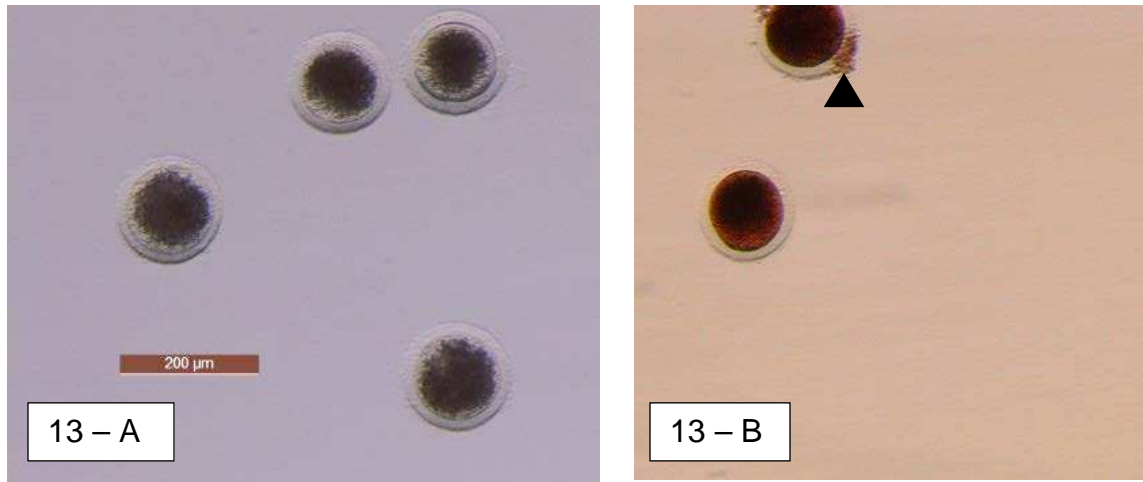


Figure 13 (A and B). Oocytes that have gone through the denuding process using 0.5% hyaluronidase and mixing using a vortex machine (A and B). Some remnant cumulous cells present on one oocyte post denuding (arrowhead, B). Neutral red shows viability with dark red staining (B.) Scale bar can be applied to both images; 200μm.

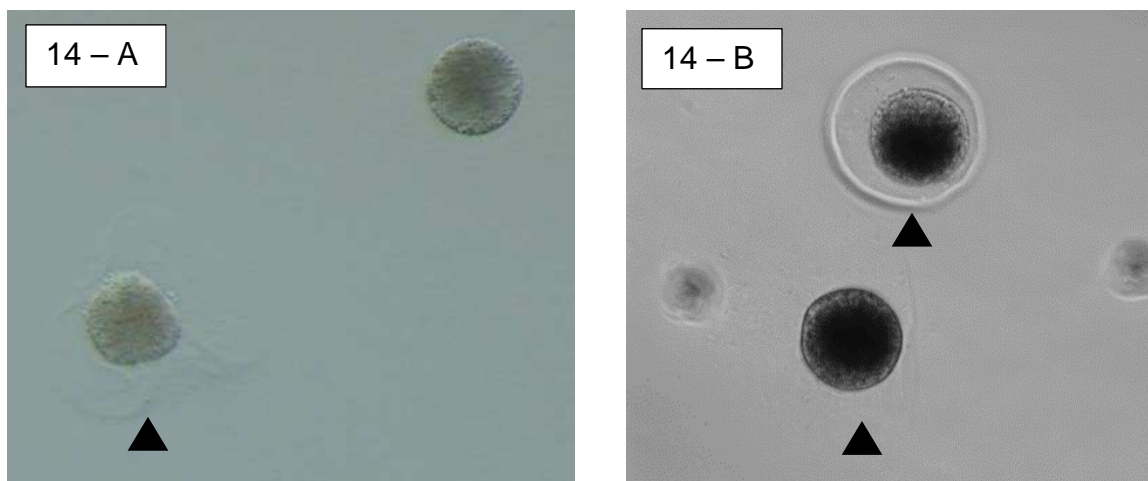


Figure 14 (A and B). Oocytes with zona pellucidae incompletely removed (arrowhead) and removed. Images taken using a stereomicroscope (A) and inverted microscope (B). Oocytes are around 120μm in diameter.

5.3.2 Live cell imaging

The Operetta imaging system was used for time-series images to monitor FM1-43 uptake into oocytes. The FM1-43 was incorporated into oocytes with and without a zona pellucida (Figure 15 and Figure 16), although zona pellucida intact oocytes incorporated FM1-43 at a slower rate, eventually the entire ooplasm was stained (Figure 16). In

addition, grade 1 oocytes incorporated FM1-43 at a faster rate than oocytes of lower grades.

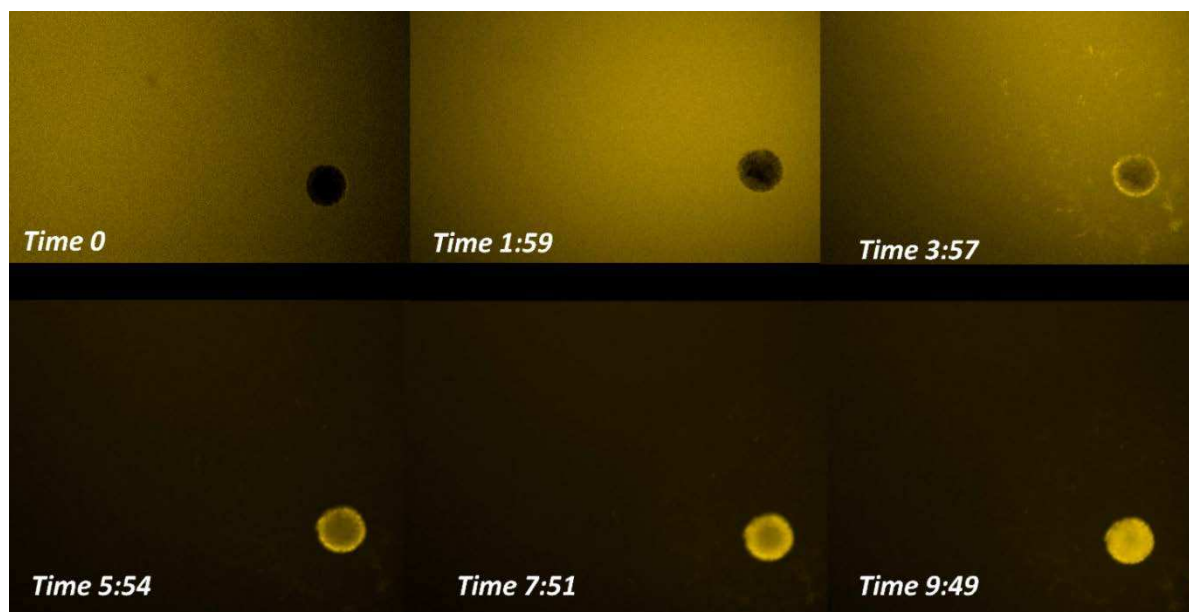


Figure 15. Time series of FM1-43 uptake in a zona free porcine oocyte. Time is listed in minutes and seconds relative to administration of FM1-43 to the well. Images taken with an Operetta system at +65 μ m z-stack and processed with Harmony software.

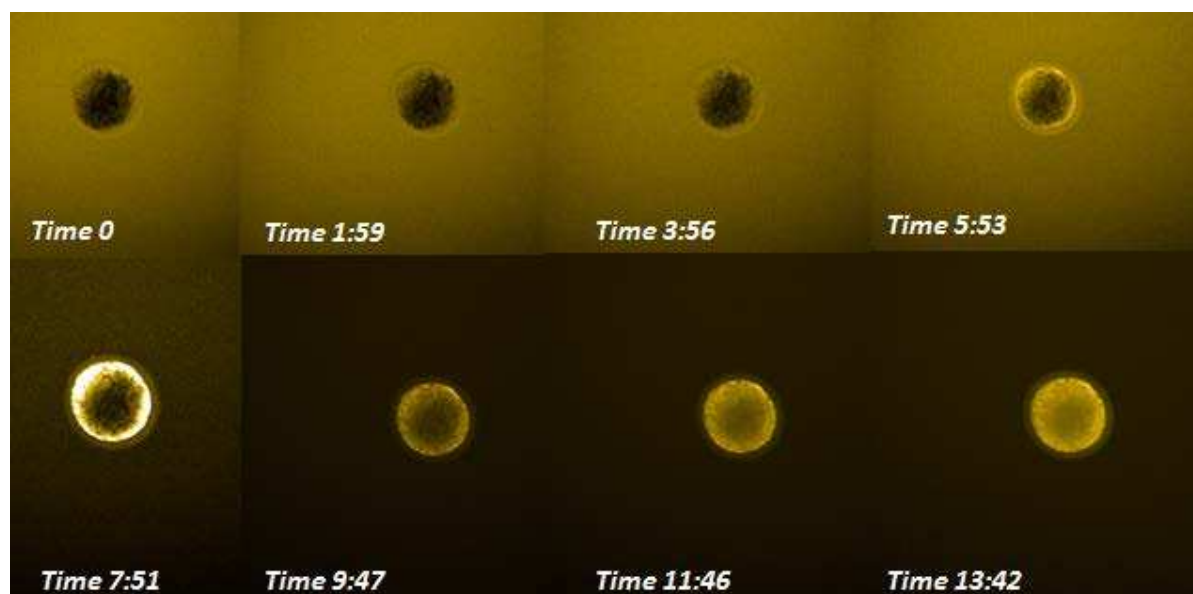


Figure 16: Time series of FM1-43 uptake in a zona intact porcine oocyte. Time is listed in minutes and seconds relative to administration of FM1-43 to the well. Images taken with an Operetta system at +65 μ m z-stack and processed with Harmony software.

5.4 Discussion

Real-time imaging of the receptor-mediated endocytosis process in mature oocytes would provide definitive support that oocytes utilize CME. Ideally these studies would be completed with primordial follicles to support the previously described IHC results, however, mature oocytes were used in this experiment as they are easy to isolate and culture [Yuan, 2012; Marques, 2007; Hyun, 2003]. Confocal microscopy is one way the internalization process of CME could be visualized and should be used in future studies with primordial follicles. Multiple attempts were made to visualize FM1-43 internalization in mature oocytes using confocal microscopy, however, no images could be attained during this study.

CHAPTER 6: OVERALL CONCLUSIONS

6.1 Summary

The overall goal of these studies was to develop new tools and methods that could be used to manage feral swine populations. Since CME could be a natural method for the delivery of a chemosterilant to primordial follicles, the aims were to determine expression and localization of clathrin-mediated endocytosis in primordial follicles, and to characterize the internalization process using microscopy. This project identified components of CME in the porcine ovary, developed methods to isolate primordial follicles, and established microscopy techniques for future studies.

The demand for permanent fertility inhibition is currently underutilized or not available in certain wildlife populations, including feral swine. Lethal efforts may be important for decreasing the overall population size of feral swine, but fertility control can reduce or halt population growth. It can be used to target animals in areas that are not conducive to lethal efforts and it can address some of the ethical considerations of lethal methods. Reproductive inhibition in feral swine in the United States can reduce the damage caused by these animals. Although no single method is likely to reduce feral swine populations, a combination of methods might.

6.2 Recommendations for Further Research

The information gathered during these studies could be foundational to reproductive inhibition research in feral swine. To move forward, primordial follicles need to be isolated and maintained in culture, receptors and/or ligands need to be

localized in primordial follicles, and the internalization process of these receptors and ligands using CME need to be visualized. Isolating and maintaining primordial follicles in culture will allow for experiments involving real-time imaging of the endocytic process. The identification of ligands and receptors that are likely used in CME by primordial follicles is preliminary work necessary for developing a chemosterilant that targets follicles; some of this preliminary work has been completed (APPENDIX B). Visualizing CME with a specific ligand or receptor will further support the presence of CME in primordial follicles and lead to the next stages of chemosterilant development.

REFERENCES

1. Abcam (1998-2016). "Blocking with immunizing peptide protocol (peptide competition)."
 - a. <http://www.abcam.com/protocols/blocking-with-immunizing-peptide-protocol-peptide-competition>
2. Abcam (2016). "Immunohistochemistry Application Guide."
 - a. <http://www.abcam.com/kits/introduction-to-immunohistochemistry-ihc>
3. Adhikari, D. and K. Liu (2009). "Molecular Mechanisms Underlying the Activation of Mammalian Primordial Follicles." Endocrine Reviews **30**(5): 438-464.
4. Akinc, A. and G. Battaglia (2013). "Exploiting endocytosis for nanomedicines." Cold Spring Harbor perspectives in biology **5**(11): a016980.
5. Almeida, F., Teerds, K.J., Auler, P., Soede, N.M. (2012). "Anti-Mullerian hormone: expression and possible roles in the porcine ovary." In: Proceedings of the 17th International Congress on Animal Reproduction (ICAR), Vancouver, Canada. P.496-497
6. APHIS. (2013). "Feral Swine: - APHIS – US Department of Agriculture."
7. APHIS. (2015). "Environmental Impact Statement: APHIS; Feral Swine Damage Management: A National Approach."
 - a. https://www.aphis.usda.gov/wps/portal/aphis/ourfocus/wildlifedamage?1dmy&urile=wcm%3apath%3a%2Faphis_content_library%2Fsa_our_focus%2Fsa_wildlife_damage%2Fsa_operational_activities%2Fsa_feral_swine%2Fct_eis
8. Baarends, W. M., J. T. Uilenbroek, P. Kramer, J. W. Hoogerbrugge, E. C. v. Leeuwen, A. P. Themmen and J. A. Grootegoed (1995). "Anti-müllerian hormone and anti-müllerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth." Endocrinology **136**(11): 4951-4962.
9. Barfield, J. P., E. Nieschlag and T. G. Cooper (2006). "Fertility control in wildlife: humans as a model." Contraception **73**(1): 6-22.
10. Bielańska-Osuchowska, Z. (2006). "Oogenesis in pig ovaries during the prenatal period: ultrastructure and morphometry." Reprod Biol **6**: 161-193.
11. Black, J. and B. Erickson (1968). "Oogenesis and ovarian development in the prenatal pig." The Anatomical Record **161**: 45-55.

12. Bleul, C. C., R. C. Fuhlbrigge, J. M. Casasnovas, A. Aiuti and T. A. Springer (1996). "A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1)." The Journal of Experimental Medicine 184(3): 1101-1109.
13. Brodsky, F. M., C. Y. Chen, C. Knuehl, M. C. Towler and D. E. Wakeham (2001). "Biological basket weaving: formation and function of clathrin-coated vesicles." Annu Rev Cell Dev Biol 17: 517-568.
14. Campbell, T. A., S. J. Lapidge and D. B. Long (2006). "Using Baits to Deliver Pharmaceuticals to Feral Swine in Southern Texas." Wildlife Society Bulletin 34(4): 1184-1189.
15. Campbell, T. A. and D. B. Long (2009). "Feral swine damage and damage management in forested ecosystems." Forest Ecology and Management 257(12): 2319-2326.
16. Chapman, C., L. Cree and A. N. Shelling (2015). "The genetics of premature ovarian failure: current perspectives." International journal of women's health 7: 799.
17. Cooper, S. M., H. M. Scott, G. R. d. I. Garza, A. L. Deck and J. C. Cathey (2010). "DISTRIBUTION AND INTERSPECIES CONTACT OF FERAL SWINE AND CATTLE ON RANGELAND IN SOUTH TEXAS: IMPLICATIONS FOR DISEASE TRANSMISSION." Journal of Wildlife Diseases 46(1): 152-164.
18. De La Torre-Sanchez, J. F., K. Preis and G. E. Seidel (2006). "Metabolic regulation of in-vitro-produced bovine embryos. I. Effects of metabolic regulators at different glucose concentrations with embryos produced by semen from different bulls." Reproduction, Fertility and Development 18(5): 585-596.
19. Doherty, G. J. and H. T. McMahon (2009). "Mechanisms of endocytosis." Annu Rev Biochem 78: 857-902.
20. Driancourt, M., K. Reynaud, R. Cortvrindt and J. Smitz (2000). "Roles of KIT and KIT LIGAND in ovarian function." Rev Reprod 5(3): 143-152.
21. Durlinger, A., J. Visser and A. Themmen (2002). "Regulation of ovarian function: the role of anti-Mullerian hormone." Reproduction 124(5): 601-609.
22. Durlinger, A. L. L., M. J. G. Gruijters, P. Kramer, B. Karels, H. A. Ingraham, M. W. Nachtigal, J. T. J. Uilenbroek, J. A. Grootegoed and A. P. N. Themmen (2002). "Anti-Müllerian Hormone Inhibits Initiation of Primordial Follicle Growth in the Mouse Ovary." Endocrinology 143(3): 1076-1084.
23. Durlinger, A. L. L., P. Kramer, B. Karels, F. H. d. Jong, J. T. J. Uilenbroek, J. A. Grootegoed and A. P. N. Themmen (1999). "Control of Primordial Follicle

- Recruitment by Anti-Müllerian Hormone in the Mouse Ovary." Endocrinology **140**(12): 5789-5796.
24. Engeman, R. M., B. U. Constantin, S. A. Shwiff, H. T. Smith and J. Woolard (2007). "Adaptive and economic management methods for feral hog control in Florida." Human-Wildlife Interactions **1**(2): 178-185.
 25. Fagerstone, K. (2002). "Wildlife fertility control." USDA National Wildlife Research Center - Staff Publications. Paper 489.
 26. Ferguson, S. M. and P. De Camilli (2012). "Dynamin, a membrane-remodelling GTPase." Nat Rev Mol Cell Biol **13**(2): 75-88.
 27. Fortune, J. E. (2003). "The early stages of follicular development: activation of primordial follicles and growth of preantral follicles." Animal Reproduction Science **78**(3-4): 135-163.
 28. Greenwald, G. S. and R. M. Moor (1989). "Isolation and preliminary characterization of pig primordial follicles." Journal of Reproduction and Fertility **87**(2): 561-571.
 29. Gruijters, M. J. G., J. A. Visser, A. L. L. Durlinger and A. P. N. Themmen (2003). "Anti-Müllerian hormone and its role in ovarian function." Molecular and Cellular Endocrinology **211**(1-2): 85-90.
 30. Hampton, J. O., T. H. Hyndman, A. Barnes and T. Collins (2015). "Is Wildlife Fertility Control Always Humane?" Animals **5**(4): 1047-1071.
 31. Hellgren, E. C. (1993). Biology of feral hogs (*Sus scrofa*) in Texas. Pages 50- 58 in C. W. Hanselka and J. F. Cadenhead, editors. Feral swine: A compendium for resource managers. Texas Agricultural Extension Service, Kerrville, TX, USA.
 32. Hellgren, E. (1999). "Reproduction of feral swine." Proceedings of the First National Feral Swine Conference **5**; 67-68.
 33. Herbert, C. A. and T. E. Trigg (2005). "Applications of GnRH in the control and management of fertility in female animals." Animal Reproduction Science **88**(1-2): 141-153.
 34. Hobbs, N. T., D. C. Bowden and D. L. Baker (2000). "Effects of Fertility Control on Populations of Ungulates: General, Stage-Structured Models." The Journal of Wildlife Management **64**(2): 473-491.
 35. Holt, J. E., A. Jackson, S. D. Roman, R. J. Aitken, P. Koopman and E. A. McLaughlin (2006). "CXCR4/SDF1 interaction inhibits the primordial to primary follicle transition in the neonatal mouse ovary." Developmental Biology **293**(2): 449-460.

36. Hoyer, P. B. (2005). "Damage to ovarian development and function." Cell and Tissue Research **322**(1): 99-106
37. Hoyer, P. B., P. J. Devine, Xiaoming Hu, K. E. Thompson and I. G. Sipes (2001). "Ovarian Toxicity of 4-Vinylcyclohexene Diepoxide: A Mechanistic Model." Toxicologic Pathology **29**(1): 91-99.
38. Hoyer, P. B. and I. G. Sipes (1996). "Assessment of follicle destruction in chemical-induced ovarian toxicity." Annual review of pharmacology and toxicology **36**(1): 307-331.
39. Hunter, M. (2000). "Oocyte maturation and ovum quality in pigs." Reviews of Reproduction **5**(2): 122-130.
40. Hunter, M. and F. Paradis (2009). "Intra-follicular regulatory mechanisms in the porcine ovary." Control of Pig Reproduction VIII: 149-164.
41. Hutt, K. J., E. A. McLaughlin and M. K. Holland (2006). "Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis." Molecular Human Reproduction **12**(2): 61-69.
42. Hutton, T., T. DeLiberto, S. Owen and B. Morrison (2006). "Disease Risks Associated with Increasing Feral Swine Numbers and Distribution in the United States." Michigan Bovine Tuberculosis Bibliography and Database **59**.
43. Hyun, S. H., G. S. Lee, D. Y. Kim, H. S. Kim, S. H. Lee, S. Kim, E. S. Lee, J. M. Lim, S. K. Kang, B. C. Lee and W. S. Hwang (2003). "Effect of maturation media and oocytes derived from sows or gilts on the development of cloned pig embryos." Theriogenology **59**(7): 1641-1649.
44. Johannes, L. and C. Lamaze (2002). "Clathrin-dependent or not: is it still the question?" Traffic **3**(7): 443-451.
45. Johnson, J., J. Bagley, M. Skaznik-Wikiel, H.-J. Lee, G. B. Adams, Y. Niikura, K. S. Tschudy, J. C. Tilly, M. L. Cortes, R. Forkert, T. Spitzer, J. Iacomini, D. T. Scadden and J. L. Tilly (2005). "Oocyte Generation in Adult Mammalian Ovaries by Putative Germ Cells in Bone Marrow and Peripheral Blood." Cell **122**(2): 303-315.
46. Jones, R. L. and M. E. Pepling (2013). "KIT signaling regulates primordial follicle formation in the neonatal mouse ovary." Developmental Biology **382**(1): 186-197.
47. Killian, G., L. Miller, J. Rhyhan and H. Doten (2006). "Immunocontraception of Florida Feral Swine with a Single-dose GnRH Vaccine." American Journal of Reproductive Immunology **55**(5): 378-384.

48. Knapczyk-Stwora, K., M. Grzesiak, M. Duda, M. Koziorowski and M. Slomczynska (2013). "Effect of flutamide on folliculogenesis in the fetal porcine ovary—regulation by Kit Ligand/c-Kit and IGF1/IGF1R systems." Animal reproduction science **142**(3): 160-167.
49. Lapidge, S., J. Wishart, L. Staples, K. Fagerstone, T. Campbell and J. Eisemann (2012). "Development of a feral swine toxic bait (Hog-Gone®) and bait hopper (Hog-Hopper™) in Australia and the USA." Proceedings of the 14th WDM Conference: 19-24.
50. LoRusso, P. M., M. J. Edelman, S. L. Bever, K. M. Forman, M. Pilat, M. F. Quinn, J. Li, E. I. Heath, L. M. Malburg and P. J. Klein (2012). "Phase I study of folate conjugate EC145 (Vintafolide) in patients with refractory solid tumors." Journal of Clinical Oncology **30**(32): 4011-4016.
51. Lowther, K. M., V. O. Nikolaev and L. M. Mehlmann (2011). "Endocytosis in the mouse oocyte and its contribution to cAMP signaling during meiotic arrest." Reproduction **141**(6): 737-747.
52. Marques, M. G., A. C. Nicacio, V. P. d. Oliveira, A. B. Nascimento, H. V. A. Caetano, C. M. Mendes, M. R. B. Mello, M. P. Milazzotto, M. E. O. D. A. Assumpção and J. A. Visintin (2007). "In vitro maturation of pig oocytes with different media, hormone and meiosis inhibitors." Animal Reproduction Science **97**(3–4): 375-381.
53. Massei, G. and D. Cowan (2014). "Fertility control to mitigate human–wildlife conflicts: a review." Wildlife Research **41**(1): 1-21
54. Massei, G., D. Cowan and D. Eckery (2014). "Novel Management Methods: Immunocontraception and Other Fertility Control Tools." Behaviour and Management of European Ungulates.
55. Massei, G., D. P. Cowan, J. Coats, F. Gladwell, J. E. Lane and L. A. Miller (2008). "Effect of the GnRH vaccine GonaCon on the fertility, physiology and behaviour of wild boar." Wildlife Research **35**(6): 540-547.
56. Massei, G., S. Roy and R. Bunting (2011). "Too many hogs? A review of methods to mitigate impact by wild boar and feral hogs." Human-Wildlife Interactions **5**(1): 79-99.
57. Mattison, D. R. (1980). "Morphology of oocyte and follicle destruction by polycyclic aromatic hydrocarbons in mice." Toxicology and Applied Pharmacology **53**(2): 249-259
58. Mattison, D. R., M. S. Nightingale and K. Shiromizu (1983). "Effects of toxic substances on female reproduction." Environmental Health Perspectives **48**: 43-52.

59. Mattison, D. R. and S. S. Thorgeirsson (1979). "Ovarian Aryl Hydrocarbon Hydroxylase Activity and Primordial Oocyte Toxicity of Polycyclic Aromatic Hydrocarbons in Mice." Cancer Research **39**(9): 3471-3475.
60. Mayes, M. A. and M.-A. Sirard (2002). "Effect of Type 3 and Type 4 Phosphodiesterase Inhibitors on the Maintenance of Bovine Oocytes in Meiotic Arrest." Biology of Reproduction **66**(1): 180-184.
61. McCann, B. E. and D. K. Garcelon (2008). "Eradication of Feral Pigs From Pinnacles National Monument." The Journal of Wildlife Management **72**(6): 1287-1295.
62. McLaughlin, E. A. and S. C. McIver (First published online 30 September 2008). "Awakening the oocyte: controlling primordial follicle development." Reproduction: The Journal of the Society for Reproduction and Fertility **137**(1): 1-11.
63. McMahon, H. T. and E. Boucrot (2011). "Molecular mechanism and physiological functions of clathrin-mediated endocytosis." Nat Rev Mol Cell Biol **12**(8): 517-533.
64. Meng, X., D. Lindsay and N. Sriranganathan (2009). "Wild boars as sources for infectious diseases in livestock and humans." Philosophical Transactions of the Royal Society of London B: Biological Sciences **364**(1530): 2697-2707
65. Merrill, J. A., E. G. Cooch and P. D. Curtis (2003). "Time to reduction: factors influencing management efficacy in sterilizing overabundant white-tailed deer." The Journal of wildlife management: 267-279.
66. Miller, L. A., B. E. Johns, D. J. Elias and G. J. Killian (1999). "Oral Vaccination of White-Tailed Deer Using a Recombinant Bacillus Calmette-Guérin Vaccine Expressing the Borrelia burgdorferi Outer Surface Protein A: Prospects for Immunocontraception." American Journal of Reproductive Immunology **41**(4): 279-285.
67. Miller, R. T. (2001). "Technical immunohistochemistry: achieving reliability and reproducibility of immunostains." Soc. Appl. Immunohistochem: 56.
68. Mississippi State University Extension Service. (2014) "wild pig info." "Damage to the Environment." <http://wildpiginfo.msstate.edu/damage-environment-wild-pigs.html>
69. Molyneaux, K. A., H. Zinszner, P. S. Kunwar, K. Schaible, J. Stebler, M. J. Sunshine, W. O'Brien, E. Raz, D. Littman, C. Wylie and R. Lehmann (2003). "The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival." Development **130**(18): 4279-4286.

70. Moniruzzaman, M. and T. Miyano (2007). "KIT-KIT ligand in the growth of porcine oocytes in primordial follicles." Journal of Reproduction and Development **53**(6): 1273-1281.
71. Nabhan, C. and M. S. Tallman (2002). "Early phase I/II trials with gemtuzumab ozogamicin (Mylotarg®) in acute myeloid leukemia." Clinical Lymphoma **2**: S19-S23.
72. Naz, R. K., S. K. Gupta, J. C. Gupta, H. K. Vyas, Talwar and GP (2005). "Recent advances in contraceptive vaccine development: a mini-review." Human Reproduction **20**(12): 3271-3283.
73. Nett, T., L. Glode, M. Wieczorek and P. Jarosz (2005). Method for inactivating gonadotrophs, Google Patents.
74. Nilsson, E., N. Rogers and M. K. Skinner (2007). "Actions of anti-Müllerian hormone on the ovarian transcriptome to inhibit primordial to primary follicle transition." Reproduction **134**(2): 209-221.
75. Nilsson, E. E., R. Schindler, M. I. Savenkova and M. K. Skinner (2011). "Inhibitory Actions of Anti-Müllerian Hormone (AMH) on Ovarian Primordial Follicle Assembly." PLoS ONE **6**(5): e20087.
76. Oh, N. and J.-H. Park (2014). "Endocytosis and exocytosis of nanoparticles in mammalian cells." Int J Nanomedicine **9**(Suppl 1): 51-63.
77. Olsen, E., M. Duvic, A. Frankel, Y. Kim, A. Martin, E. Vonderheid, B. Jegasothy, G. Wood, M. Gordon and P. Heald (2001). "Pivotal phase III trial of two dose levels of denileukin diftitox for the treatment of cutaneous T-cell lymphoma." Journal of Clinical Oncology **19**(2): 376-388.
78. Pan, Z., M. Sun, X. Liang, J. Li, F. Zhou, Z. Zhong and Y. Zheng (2015). "The Controversy, Challenges, and Potential Benefits of Putative Female Germline Stem Cells Research in Mammals." Stem Cells International.
79. Parkar, N. S., B. S. Akpa, L. C. Nitsche, L. E. Wedgewood, A. T. Place, M. S. Sverdlov, O. Chaga and R. D. Minshall (2009). "Vesicle formation and endocytosis: function, machinery, mechanisms, and modeling." Antioxid Redox Signal **11**(6): 1301-1312.
80. Parrott, J. A. and M. K. Skinner (1999). "Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis 1." Endocrinology **140**(9): 4262-4271.
81. Pelkmans, L. and A. Helenius (2002). "Endocytosis via caveolae." Traffic **3**(5): 311-320.

82. Pellatt, L., S. Rice and H. D. Mason (2010). "Anti-Müllerian hormone and polycystic ovary syndrome: a mountain too high?" Reproduction **139**(5): 825-833.
83. Pires, E. S., P. P. Parte, P. K. Meherji, S. A. Khan and V. V. Khole (2006). "Naturally Occurring Anti-albumin Antibodies Are Responsible for False Positivity in Diagnosis of Autoimmune Premature Ovarian Failure." Journal of Histochemistry & Cytochemistry **54**(4): 397-405.
84. Qian, J., J. Yang, A. F. Dragovic, E. Abu-Isa, T. S. Lawrence and M. Zhang (2005). "Ionizing radiation-induced adenovirus infection is mediated by Dynamin 2." Cancer research **65**(13): 5493-5497.
85. Quy, R. J., G. Massei, M. S. Lambert, J. Coats, L. A. Miller and D. P. Cowan (2014). "Effects of a GnRH vaccine on the movement and activity of free-living wild boar (*Sus scrofa*)." Wildlife Research **41**(3): 185-193.
86. Ramos-Vara, J. A. (2005). "Technical Aspects of Immunohistochemistry." Veterinary Pathology Online **42**(4): 405-426.
87. Rattanapinyopituk, K., A. Shimada, T. Morita, M. Sakurai, A. Asano, T. Hasegawa, K. Inoue and H. Takano (2014). "Demonstration of the clathrin-and caveolin-mediated endocytosis at the maternal–fetal barrier in mouse placenta after intravenous administration of gold nanoparticles." Journal of Veterinary Medical Science **76**(3): 377-387.
88. Reddy, P., W. Zheng and K. Liu (2010). "Mechanisms maintaining the dormancy and survival of mammalian primordial follicles." Trends in Endocrinology & Metabolism **21**(2): 96-103.
89. Rice, S., K. Ojha, S. Whitehead and H. Mason (2007). "Stage-Specific Expression of Androgen Receptor, Follicle-Stimulating Hormone Receptor, and Anti-Müllerian Hormone Type II Receptor in Single, Isolated, Human Preantral Follicles: Relevance to Polycystic Ovaries." The Journal of Clinical Endocrinology & Metabolism **92**(3): 1034-1040.
90. Roy, S. K. and G. S. Greenwald (1985). "An enzymatic method for dissociation of intact follicles from the hamster ovary: histological and quantitative aspects." Biology of Reproduction **32**(1): 203-215.
91. Sabeur, K., B. Ball, T. Nett, H. Ball and I. Liu (2003). "Effect of GnRH conjugated to pokeweed antiviral protein on reproductive function in adult male dogs." Reproduction **125**(6): 801-806.
92. Sahay, G., D. Y. Alakhova and A. V. Kabanov (2010). "Endocytosis of nanomedicines." Journal of controlled release **145**(3): 182-195.

93. Sasseville, M., F. K. Albuz, N. Côté, C. Guillemette, R. B. Gilchrist and F. J. Richard (2009). "Characterization of Novel Phosphodiesterases in the Bovine Ovarian Follicle." Biology of Reproduction **81**(2): 415-425.
94. Seward, N. W., K. C. VerCauteren, G. W. Witmer and R. M. Engeman (2004). "Feral Swine Impacts on Agriculture and the Environment." Sheep & Goat Research Journal **12**.
95. Shi, K., X. Yuan, L. Du, D. Pan, Y. Zhang, Y. Zhao, X. Deng, X. Hu, C. Wu and N. Li "Advanced methods of isolation and identification of porcine primordial follicles." Animal Reproduction Science **101**(1): 163-171.
96. Skinner, M. K. (2005). "Regulation of primordial follicle assembly and development." Human Reproduction Update **11**(5): 461-471.
97. Takei, K. and V. Haucke (2001). "Clathrin-mediated endocytosis: membrane factors pull the trigger." Trends Cell Biol **11**(9): 385-391. Modified 11/2015.
98. Tanihara, F., M. Nakai, H. Kaneko, J. Noguchi, T. Otoi and K. Kikuchi (2013). "Evaluation of Zona Pellucida Function for Sperm Penetration During In Vitro Fertilization in Pigs." The Journal of Reproduction and Development **59**(4): 385-392.
99. Tarragó-Trani, M. T. and B. Storrie (2007). "ALTERNATE ROUTES FOR DRUG DELIVERY TO THE CELL INTERIOR: PATHWAYS TO THE GOLGI APPARATUS AND ENDOPLASMIC RETICULUM." Adv Drug Deliv Rev **59**(8): 782-797.
100. Taylor, R. B., E. C. Hellgren, T. M. Gabor and L. M. Ilse (1998). "Reproduction of Feral Pigs in Southern Texas." Journal of Mammalogy **79**(4): 1325-1331.
101. Telfer, E. E., R. G. Gosden, A. G. Byskov, N. Spears, D. Albertini, C. Y. Andersen, R. Anderson, R. Braw-Tal, H. Clarke, A. Gougeon, E. McLaughlin, A. McLaren, K. McNatty, G. Schatten, S. Silber and A. Tsafiriri (2005). "On Regenerating the Ovary and Generating Controversy." Cell **122**(6): 821-822.
102. Thermo Fisher Scientific (2010). "The Molecular Probes ® Handbook: A Guide to Fluorescent Probes and Labeling Technologies 11th Edition." 15-20.
103. Verp, M. S. (1986). "Environmental factors causing ovarian failure in humans." Obstetrics and gynecology. Philadelphia: Harper and Row: 1-11.
104. Visser, J. A., F. H. de Jong, J. S. E. Laven and A. P. N. Themmen (2006). "Anti-Müllerian hormone: a new marker for ovarian function." Reproduction **131**(1): 1-9.
105. Wang, Z.-P., X.-Y. Mu, M. Guo, Y.-J. Wang, Z. Teng, G.-P. Mao, W.-B. Niu, L.-Z. Feng, L.-H. Zhao and G.-L. Xia (2014). "Transforming Growth Factor-β Signaling

Participates in the Maintenance of the Primordial Follicle Pool in the Mouse Ovary." The Journal of Biological Chemistry **289**(12): 8299-8311.

106. Weenen, C., J. S. E. Laven, A. R. M. von Bergh, M. Cranfield, N. P. Groome, J. A. Visser, P. Kramer, B. C. J. M. Fauser and A. P. N. Themmen (2004). "Anti-Müllerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment." Molecular Human Reproduction **10**(2): 77-83.
107. West, B. C., A. L. Cooper and J. B. Armstrong (2009). Managing wild pigs: a technical guide, Jack H. Berryman Institute, Department of Wildland Resources Utah State University Logan UT 84322-5230 USA.
108. Wongsrikeao, P., Y. Kaneshige, R. Ooki, M. Taniguchi, B. Agung, M. Nii and T. Otoi (2005). "Effect of the Removal of Cumulus Cells on the Nuclear Maturation, Fertilization and Development of Porcine Oocytes." Reproduction in Domestic Animals **40**(2): 166-170.
109. Yang, W.-H., M. Wiczorck, M. C. Allen and T. M. Nett (2003). "Cytotoxic Activity of Gonadotropin-Releasing Hormone (GnRH)-Pokeweed Antiviral Protein Conjugates in Cell Lines Expressing GnRH Receptors." Endocrinology **144**(4): 1456-1463.
110. Yuan, Y. and R. L. Krisher (2012). In Vitro Maturation (IVM) of Porcine Oocytes. Germline Development: Methods and Protocols. W.-Y. Chan and A. L. Blomberg. New York, NY, Springer New York: 183-198.
111. Zuccarello, D., A. Ferlin, A. Garolla, M. Menegazzo, L. Perilli, G. Ambrosini and C. Foresta (2011). "How the human spermatozoa sense the oocyte: a new role of SDF1-CXCR4 signalling." International Journal of Andrology **34**(6pt2): e554-e565.

APPENDIX A
IMMUNOHISTOCHEMISTRY OPTIMIZATION

A – 1 Materials and methods

A – 1.1 Tissues

All tissues were collected and prepared in the same fashion as described in CHAPTER 3.

A – 1.2 Antibodies

Antibodies were received from either Santa Cruz Biotechnology, Thermo Fisher, Sigma Aldrich or Abcam. Tables 2 and 3 contain the primary antibodies, peptides, and secondary antibodies used.

Table 2: Primary antibodies and any peptides used for IHC optimization.

Primary Antibodies or Peptides	Product number	Dilutions tried	Final dilution
goat polyclonal anti- c-Kit	sc-1494	1:50, 1:250	1:50
goat polyclonal anti-MSY2 (C-15)	sc-21316	1:100	1:100
goat polyclonal dynamin I/II (N-19)	sc-6401	1:50, 1:100, 1:200	None acceptable
rabbit polyclonal Dynamin 2	PA5-29658	1:50	1:50
mouse monoclonal anti-Clathrin HC	sc-58714	1:50, 1:200	None acceptable
goat polyclonal anti-Clathrin HC (C-20)	sc-6579	1:50	1:50
polyclonal rabbit dynamin II antibody	PA1-661	1:50	1:50
dynamin I/II peptide (N-19)	sc6401P	200µg/ml	200µg/ml
human Dyn2 (residues 758-777) synthetic peptide	PEP-056	200µg/ml	200µg/ml

Table 3: Secondary antibodies used for IHC optimization

Secondary Antibodies	Product number	Dilutions tried	Final dilution
rabbit anti-goat HRP	A5420	1:500	1:500
goat anti-mouse IgG-HRP	sc-2005	1:500	1:500
goat anti-rabbit HRP	A6154	1:500	1:500
rabbit anti-goat IgG-HRP	A50-100P	1:500	1:500
rabbit anti-goat IgG (whole molecule) FITC	F7367	1:100, 1:200, 1:500	1:100
goat anti-rabbit IgG-FITC	sc-2012	1:100	1:100
goat anti-mouse IgG-FITC	sc-2010	1:100, 1:400	None acceptable

A – 1.3 Slide preparation: deparaffinization, signal amplification, and antigen retrieval

Initial IHC studies with MSY2, c-Kit, dynamin I/II and clathrin used Citrisolv as an alternative to xylene for deparaffinization process. After consulting with Colorado State University Diagnostics Laboratory, the switch to xylene was made, as well as including heating slides to 65°C for 15 minutes using the MicroProbe Systems to aid in paraffin removal and tissue drying.

During c-Kit IHC, a tyramide signal amplification technique was compared to heated antigen retrieval with citrate buffer in an effort to increase the signal of the desired antigen. Tyramide signal amplification was completed after the secondary antibody incubation using a Tyramide Signal Amplification Kit according to manufacturer's instructions (Molecular Probes, stand-alone kit, Biotin-XX T20947.)

A – 1.4 Immunohistochemistry

Protocols were developed from the Pierce® Peroxidase Detection kit and literature review [Thermo Scientific, #3600; Abcam, 2016; Moniruzzaman, 2007; Shi,

2007; Ramos-Vara, 2005; Qian, 2005; Rattanapinyopituk, 2014]. Slides were heated using FisherBiotech™ MicroProbe™ Manual Staining System for 15 minutes at 65°C and then cooled at room temperature for 5 minutes before paraffin removal. Tissue was deparaffinized in xylene, and then rehydrated gradually through serial dilutions of ethanol and rinsed in deionized water. Slides were placed into wash buffer (0.05M Tris-buffered saline with 0.05% Tween-20, TBST, Sigma, T9039) two times totaling 10 minutes. Then slides underwent antigen retrieval using heat induced epitope retrieval methods (HIER) to unmask antigenic sites [Ramos-Vara, 2005]. Slides submerged in 0.01M citrate buffer were heated using a microwave (Magic Chef™, 900W, 2450MHz, cook power level 10) in 10 minute intervals totaling 30 minutes; every 10 minutes any evaporated solution was replaced with warmed deionized water. A hydrophobic barrier creating pen (ImmEdge Pen, Cat No H-4000, Vector Laboratories) was used to minimize amount of liquid needed for incubations, which were all done in 100% humidity chambers. Slides were quenched for endogenous peroxidase activity with the Peroxidase Suppressor from the Pierce Peroxidase IHC Detection Kit (#3600, Thermo Scientific) to reduce non-specific binding. Slides were washed in TBST before primary antibody incubations. Other non-specific binding sites were blocked for 1 hour at room temperature using 10% (v/v) normal serum associated with the secondary antibody host diluted in TBST (goat serum - SLBM5884V and rabbit serum - SLBL5150V, Sigma-Aldrich). After blocking, the appropriate primary antibody was applied; it should be noted that primary antibodies were diluted in 1% (v/v) normal serum associated with the secondary antibody host. After incubation for 2 hours at room temperature, sections were washed with TBST. Antigens were visualized using fluorescently-tagged

secondary antibodies or horseradish peroxidase (HRP) secondary antibodies with enhanced DAB substrate application. Soluble DAB binds to HRP oxidizing into a brown precipitate. Sections were incubated for 45 minutes at room temperature with secondary antibodies. Negative controls slides were incubated in 1% (v/v) normal serum instead of the primary antibody. All sections were washed in TBST three times for three minutes and had ImmEdge Pen lines removed. Slides in chromogenic studies went through additional incubation with DAB/metal concentrate for 15 minutes, followed by washes in TBST (Product#1856090, Thermo Scientific). These slides were counterstained with hematoxylin prior to dehydration using serial washes of distilled water, 70% ethyl alcohol, 100% ethyl alcohol and 100% xylene. Fluorescent procedures did not require dehydration steps. Chromogenic slides were mounted in organic mounting medium provided in the detection kit, while slides in fluorescent procedures were mounted in PermaFluor aqueous mounting media (TA-030-FM, Thermo Scientific). All microscopy was completed using a Leica microscope, and images were obtained using LASV4.5 imaging software.

Primary antibody specificity was attempted for dynamin II using a blocking immunizing peptide (results not shown). These attempts were executed as previously described for clathrin in Chapter 3.

A – 2 Results

A – 2.1 Immunohistochemistry

Chromogenic IHC results for c-Kit, clathrin and dynamin II can be found in Figures 17, 18 and 19. DAB staining highlighted c-Kit and clathrin with brown staining in

the cytoplasm of oocytes of pig ovaries (Figure 17 – A and 18 – A). The brown color of the DAB in tissues tested for dynamin II (Figure 19 – A) was not as dark as IHC using c-Kit or clathrin (Figures 17 – A and 18 – A). Control slides showed no DAB expression (Figures 17 – B, 18 – B and 19 – B). Tyramide signal amplification did not improve signal during experimentation (results not shown). Antigen retrieval using citrate buffer and microwave heat did make a difference in signal strength for MSY2 studies (all images shown used antigen retrieval). Staining appeared in the cytoplasm of all observed oocytes for MSY2 IHC (Figure 20 – A). No staining was seen in the control for MSY2 (Figure 20 – B). A primary antibody with matching peptide for a dynamin II immunizing peptide block IHC was not found (results not shown).

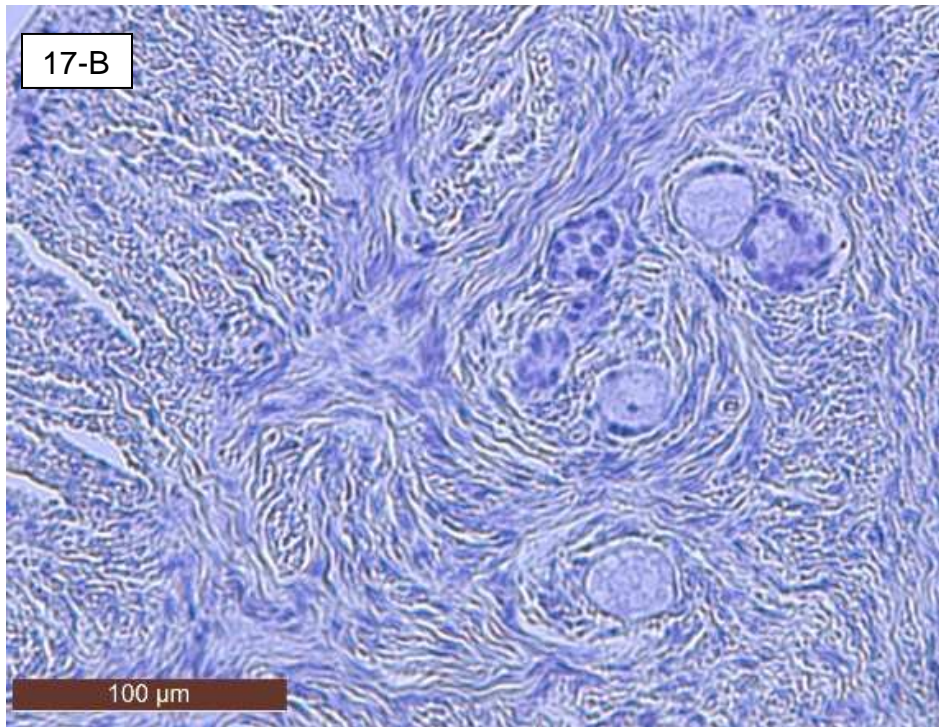
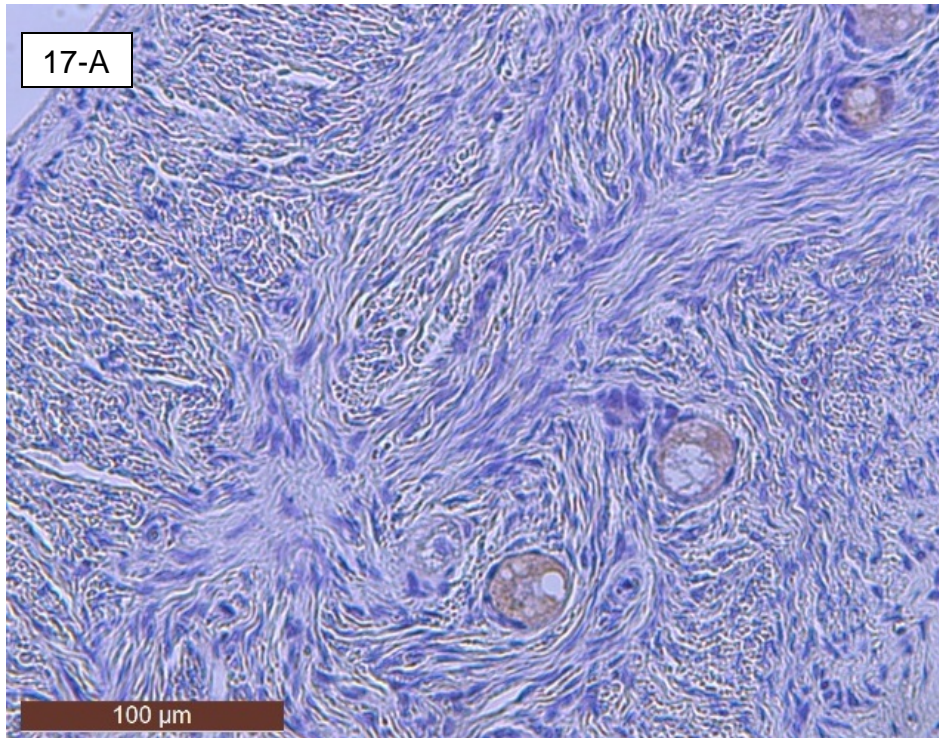


Figure 17. Chromogenic IHC results for c-Kit. A) Positive staining, brown precipitate from oxidized DAB, was seen in oocytes of all follicles observed. B) Negative control—no staining.

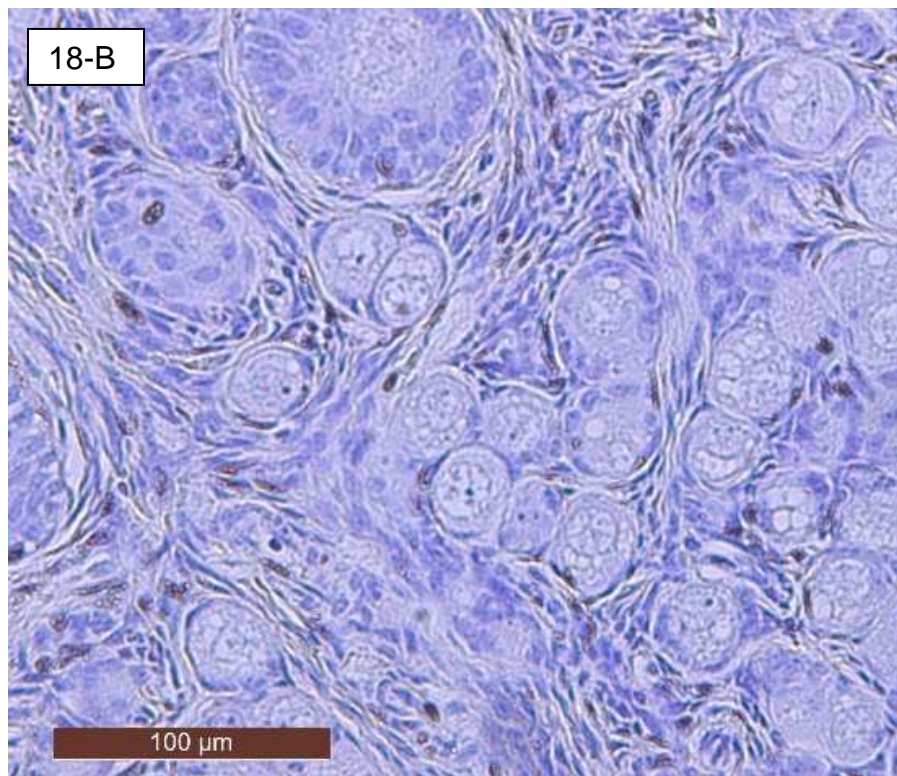
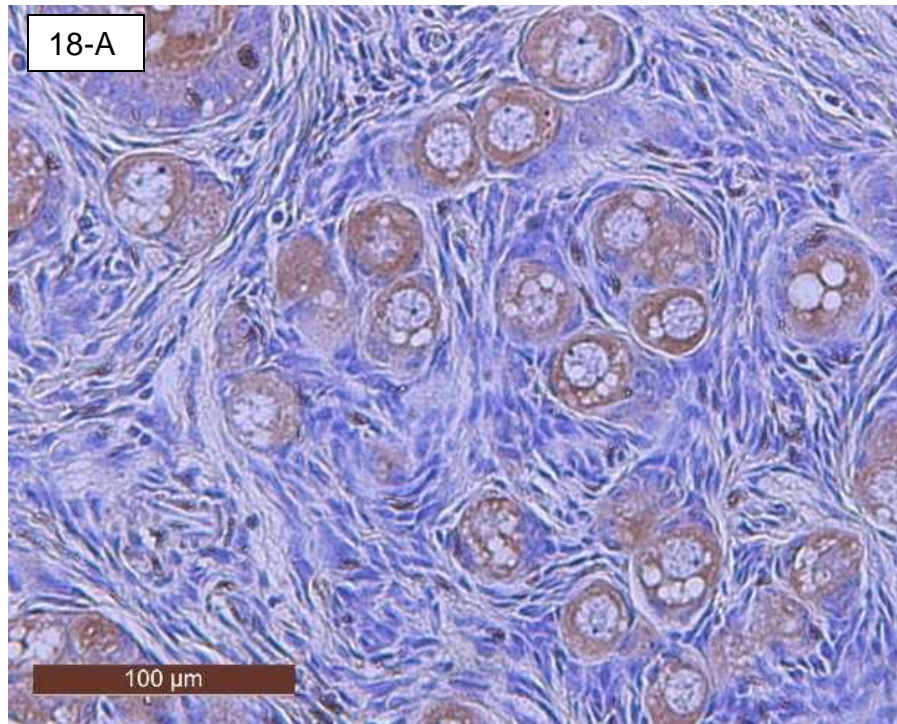


Figure 18. Chromogenic IHC results for clathrin. A) Positive staining, brown precipitate from oxidized DAB, observed in oocytes of all follicles observed. B) Negative control—no staining. Non-specific staining seen in certain cells.

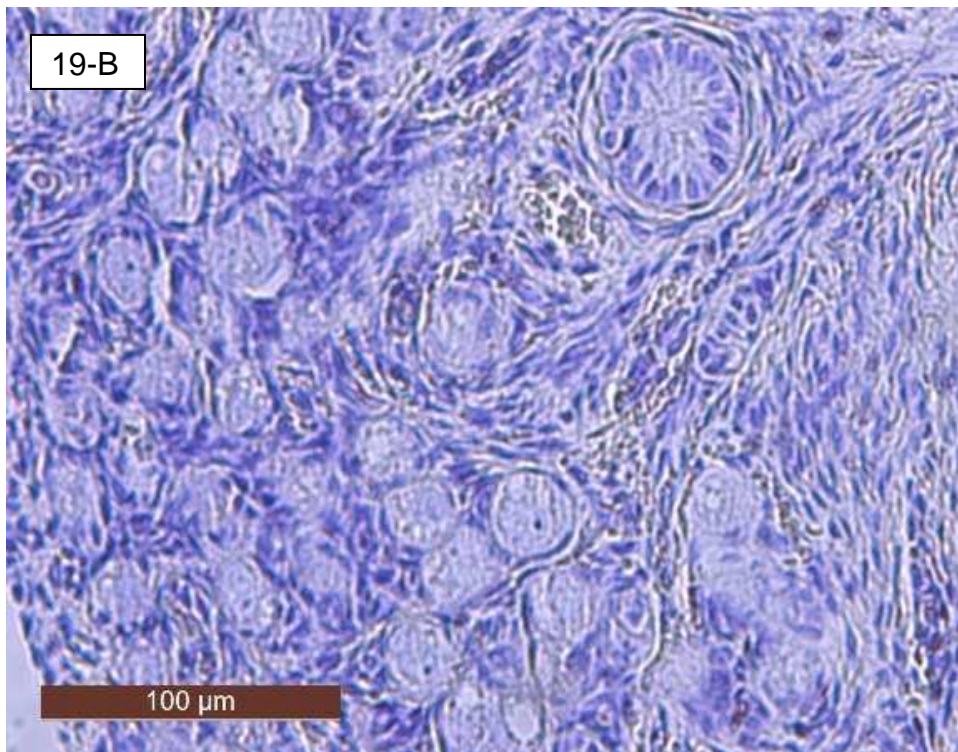
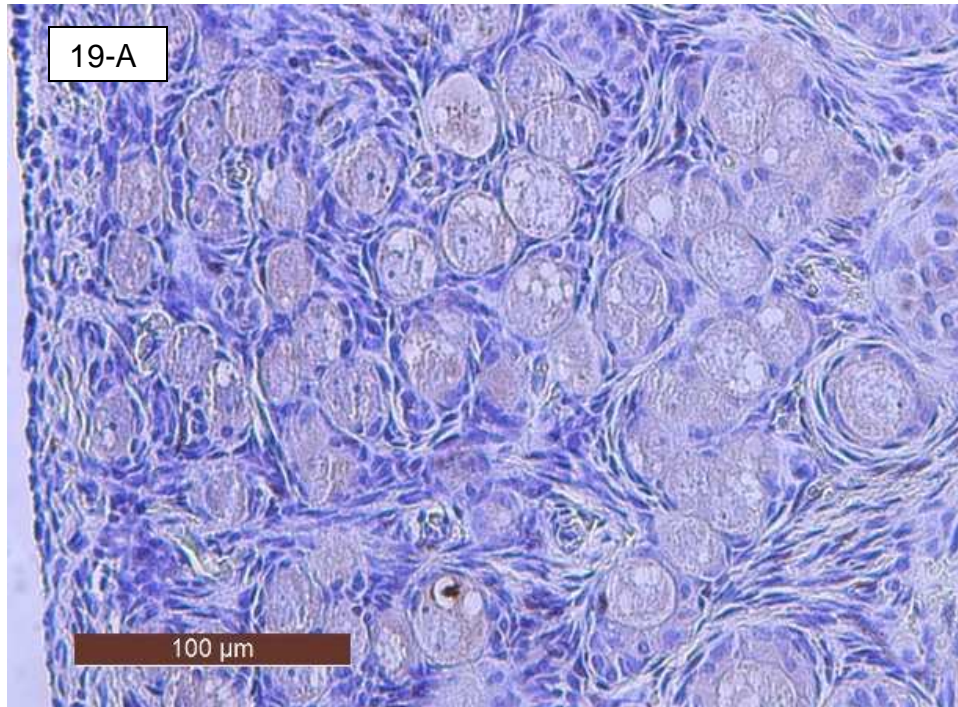


Figure 19. Chromogenic IHC results for dynamin II. A) Positive staining, brown precipitate from oxidized DAB, observed in oocytes of all follicles observed. B) Negative control—no staining.

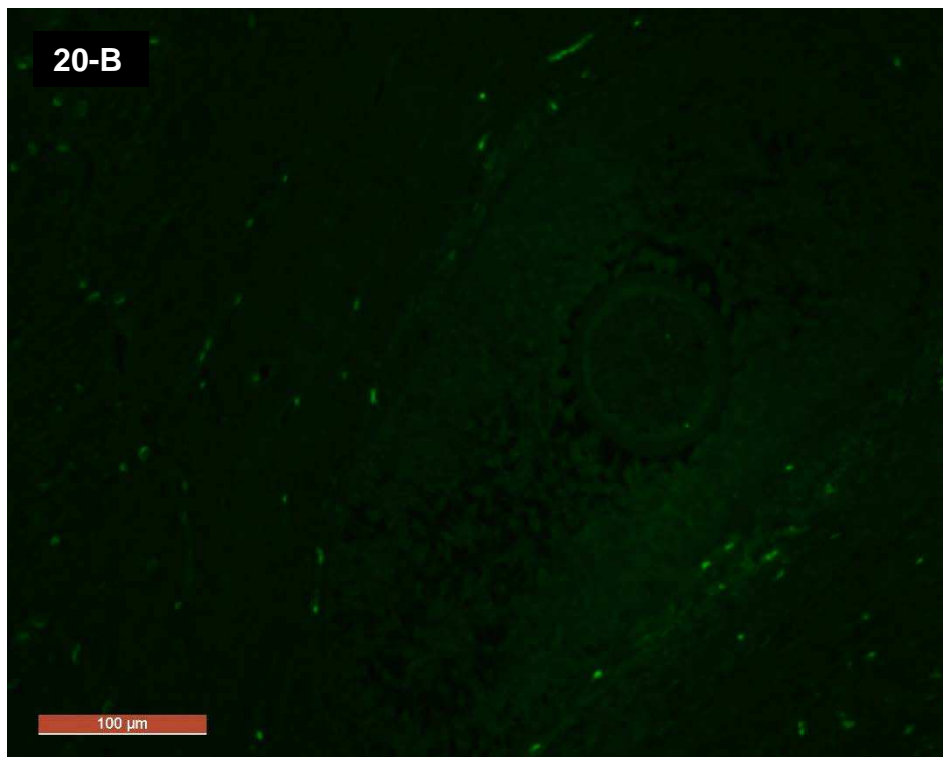
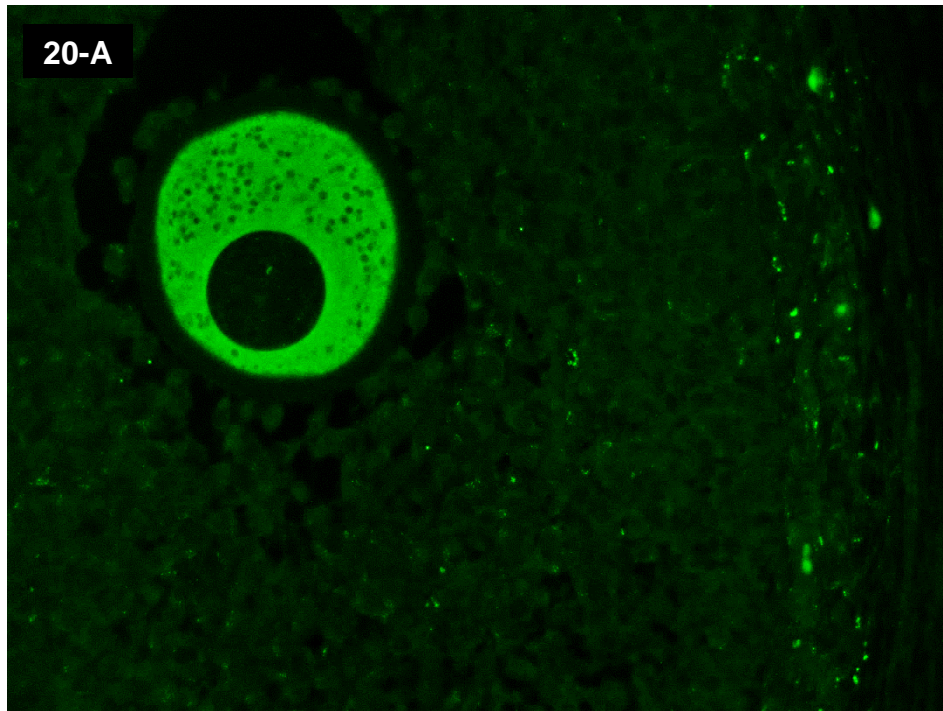


Figure 20. Fluorescent IHC results for MSY2. A) Positive green FITC staining in the cytoplasm of oocytes of all follicles observed. B) Negative control—no staining.

A – 4 Conclusions

The technical aspects of IHC, including antigens, antibodies, fixation, antigen retrieval, incubation buffers, blocking, and detection systems were carefully considered for all procedures, using previous studies and IHC technical guides [Ramos-Vara 2005, Miller 2001, Abcam 2016]. Each of the completed IHC studies lead to the optimization of the finalized reference protocol for antigens of interest.

C-kit is known to be expressed in pig ovaries and it is a receptor of interest for future project goals [Moniruzzaman, 2007; Jones, 2013]. C-kit was identified in the cytoplasm of all observed oocytes by dark brown color changes from DAB oxidation. Similarly a baseline for fluorescent IHC using MSY2; MSY2 was expressed in the cytoplasm of all observed oocytes.

Optimal antibodies for clathrin and dynamin II were not the ones tested in the first IHC experiments. BLAST results (Chapter 3) helped identify the correct antibody choices that were used in subsequent experiments. The differences in signal strength between dynamin II IHC and clathrin IHC could be due to the lack of homology between human dynamin II and *Sus scrofa* dynamin II compared to clathrin.

APPENDIX B PRELIMINARY EVALUATION OF LIGANDS AND RECEPTORS

B – 1 Background

The evaluation of ligands and receptors of interest is a key component to future studies to develop a chemosterilant. BLAST analysis of specific ligands and receptors helped determine antibody purchases for IHC that could localize these ligands and receptors in porcine ovaries. Additionally, the ability to visualize the internalization of ligands and receptors using FITC conjugation was analyzed through literature review and ProtParam analysis. FITC antibody labeling allows FITC to target amine groups on proteins, and lysine residues are the primary targets for modification [Thermo Fisher Scientific, 2010]. The ProtParam tool gave output on the characteristics of the receptors and ligands of interest, including the percentage of lysine residues in the molecule.

B – 2 Materials and methods

B – 2.1 BLAST analysis

BLAST analysis between human and *Sus scrofa* (pig) sequences for several antigens of interest was run. Kit ligand, also known as KITLG, steel factor, and stem cell factor (SCF), is a cytokine that exists as a transmembrane protein and a soluble protein. C-kit, also called KIT, stem cell factor receptor, and CD117, is a tyrosine-protein kinase cell-surface receptor for kit ligand. Anti-Mullerian hormone (AMH), also known as MIS (Mullerian inhibiting substance) is the ligand that binds to the transmembrane serine/threonine kinase receptor complex called Anti-Muellerian hormone type-2 receptor (AMHRII), also called Mullerian inhibiting substance type-2 receptor (MISRII).

Stromal cell-derived factor 1 (SDF1A, SDF1B, SDF1), also called CXCL12, is a C-X-C chemokine. CXCR4, also called fusin and CD184, is the receptor for CXCL12.

Table 4. Entry names and accession numbers for receptors and ligands of interest for human and *Sus scrofa* entered into BLAST for alignment analysis.

Name of receptor/ligand	Entry Name and Accession Number (Human)	Entry Name and Accession Number (Pig)
Kit Ligand/KITL/MGF/SCF	SCF_HUMAN, P21583	SCF_PIG, Q29030
AMH/MIS	MIS_HUMAN, P03971	MIS_PIG, P79295
SDF1/CXCL12	SDF1_HUMAN, P48061	Q6EKW4_PIG, Q6EKW4
CXCR4/fusin/CD184	CXCR4_HUMAN, P61073	CXCR4_PIG, Q764M9
AMHRII	AMHR2_HUMAN, Q16671	F1SFQ8_PIG, F1SFQ8
SCFR/c-Kit/CD117 antigen/KIT	KIT_HUMAN, P10721	KIT_PIG, Q2HWD6

B – 2.2 Amino Acid Sequence Analysis

Using the Swiss-Prot/TrEMBL accession number, a ProtParam tool was used to compute parameters of AMH, AMHRII, c-Kit, Kit Ligand, SDF1 and CXCR4 including molecular weight, number of amino acids, amino acid composition, and GRAVY (grand average of hydropathy).

B – 3 Results

B – 3.1 BLAST analysis

BLAST results showed amino acid overlaps in alignment of antigens of interest between human and *Sus scrofa* (Table 5). Alignment identity is 69.828% for CXCL12 (81 amino acids out of 116 being identical), 94.334% for CXCR4 (333 amino acids out

of 353 being identical), 85.766% for Kit ligand (235 amino acids out of 274 being identical), 89.857% for c-Kit (877 amino acids out of 972 being identical), 72.27% for AMH (417 amino acids out of 575 being identical), and 71.626% for AMHRII (414 amino acids out of 500 being identical).

Table 5. BLAST results showing percentage of matching amino acid alignments between *Sus scrofa* and humans for ligands and receptors of interest.

Ligand or Receptor	Percent alignment
CXCL12/SDF-1	69.828
CXCR4	94.334
SCF/Kit ligand/ Kit L/ MGF	85.766
SCFR/c-Kit/CD117 antigen/Kit	89.857
AMH/MIS	72.27
AMHRII/MISRII	71.626

B – 3.2 ProtParam tool analysis

ProtParam tool analysis resulted in a large amount of computed parameters for each of the ligands and receptors of interest, the most important of which are summarized (Tables 6 – A and B, 7 – A and B, 8 – A and B). Beyond just the percentage of Lysine which may prove helpful in FITC conjugation, the physical and chemical characteristics listed may be valuable in the conjugation of other substances to these potential receptors and ligands during chemosterilant development.

Table 6 (A and B). ProtParam tool summaries important for future internalization studies. Analyses of CXCL12 and associated receptor, CXCR4, with Lysine content highlighted that will be helpful for FITC conjugation (A, left, B, right).

Table 6 – A. CXCL12	
Number of amino acids	97
Molecular weight	10725.4
Amino Acid Composition	
Ala	7.2%
Arg	6.2%
Asn	6.2%
Asp	2.1%
Cys	4.1%
Gln	3.1%
Glu	4.1%
Gly	3.1%
His	2.1%
Ile	6.2%
Leu	12.4%
Lys	8.2%
Met	0.0%
Phe	2.1%
Pro	6.2%
Ser	12.4%
Thr	6.2%
Trp	1.0%
Tyr	2.1%
Val	5.2%
Pyl	0.0%
Sec	0.0%
Total number of negatively charged residues (Asp + Glu)	6
Total number of positively charged residues (Arg + Lys)	14
Grand average of hydropathicity (GRAVY)	-0.241

Table 6 – B. CXCR4	
Number of amino acids	353
Molecular weight	39944.7
Amino Acid Composition	
Ala	5.9%
Arg	4.0%
Asn	2.8%
Asp	4.2%
Cys	2.5%
Gln	2.3%
Glu	3.4%
Gly	5.9%
His	3.1%
Ile	9.6%
Leu	11.9%
Lys	5.4%
Met	1.1%
Phe	7.1%
Pro	2.8%
Ser	9.3%
Thr	4.5%
Trp	1.7%
Tyr	4.8%
Val	7.4%
Pyl	0.0%
Sec	0.0%
Total number of negatively charged residues (Asp + Glu)	27
Total number of positively charged residues (Arg + Lys)	33
Grand average of hydropathicity (GRAVY)	0.398

Table 7 (A and B). ProtParam tool summaries important for future internalization studies. Analyses of Kit ligand and it's associated receptor, c-Kit, with Lysine content highlighted, which will be helpful for FITC conjugation (A, left, B, right).

Table 7 – A. SCF/KitL/MGF	
Number of amino acids	249
Molecular weight	28099.0
Amino Acid Composition	
Ala	4.8%
Arg	3.6%
Asn	4.8%
Asp	6.8%
Cys	1.6%
Gln	2.8%
Glu	8.4%
Gly	2.4%
His	0.8%
Ile	6.0%
Leu	8.8%
Lys	8.4%
Met	2.4%
Phe	5.2%
Pro	5.2%
Ser	12.0%
Thr	4.4%
Trp	1.2%
Tyr	1.6%
Val	8.4%
Pyl	0.0%
Sec	0.0%
Total number of negatively charged residues (Asp + Glu)	38
Total number of positively charged residues (Arg + Lys)	30
Grand average of hydropathicity (GRAVY)	-0.291

Table 7 – B. SCFR/c-Kit/CD117 antigen	
Number of amino acids	947
Molecular weight	106231.2
Amino Acid Composition	
Ala	6.5%
Arg	4.0%
Asn	5.4%
Asp	5.3%
Cys	2.4%
Gln	2.3%
Glu	6.5%
Gly	5.5%
His	2.5%
Ile	5.4%
Leu	8.1%
Lys	6.2%
Met	2.9%
Phe	4.3%
Pro	5.1%
Ser	8.3%
Thr	5.7%
Trp	1.4%
Tyr	4.1%
Val	7.9%
Pyl	0.0%
Sec	0.0%
Total number of negatively charged residues (Asp + Glu)	112
Total number of positively charged residues (Arg + Lys)	97
Grand average of hydropathicity (GRAVY)	-0.226

Table 8 (A and B). ProtParam tool summaries important for future internalization studies. Analyses of AMH and its associated receptor, AMHRII, with Lysine content highlighted, which will be helpful in FITC conjugation (A, left, B, right).

Table 8 – A. AMH/MIS	
Number of amino acids	552
Molecular weight	59197.1
Amino Acid Composition	
Ala	10.7%
Arg	8.2%
Asn	2.4%
Asp	2.5%
Cys	2.2%
Gln	4.3%
Glu	6.0%
Gly	8.9%
His	1.3%
Ile	0.9%
Leu	15.8%
Lys	0.7%
Met	1.1%
Phe	2.4%
Pro	13.0%
Ser	6.0%
Thr	5.3%
Trp	2.4%
Tyr	0.9%
Val	5.3%
Pyl	0.0%
Sec	0.0%
Total number of negatively charged residues (Asp + Glu)	47
Total number of positively charged residues (Arg + Lys)	49
Grand average of hydropathicity (GRAVY)	-0.136

Table 8 – B. AMHRII	
Number of amino acids	482
Molecular weight	52987.8
Amino Acid Composition	
Ala	9.3%
Arg	6.8%
Asn	0.6%
Asp	4.6%
Cys	3.5%
Gln	5.4%
Glu	6.8%
Gly	7.9%
His	2.9%
Ile	3.3%
Leu	14.9%
Lys	1.9%
Met	1.0%
Phe	2.7%
Pro	10.2%
Ser	6.0%
Thr	3.9%
Trp	2.5%
Tyr	1.7%
Val	3.9%
Pyl	0.0%
Sec	0.0%
Total number of negatively charged residues (Asp + Glu)	55
Total number of positively charged residues (Arg + Lys)	42
Grand average of hydropathicity (GRAVY)	-0.164

B – 4 Conclusions

BLAST analysis can help in the selection of antibodies for future IHC. The ProtParam tool provided valuable data on the characteristics of each of the ligands and receptors of interest, which will aid in future internalization studies. The hydrophobicity and the overall charge of the ligands, may have implications on whether a conjugate will act similarly to the unaltered receptor or ligand. Any changes in the chemical nature could change the cellular pathway used for particle uptake or the eventually intracellular pathways utilized [Sahay, 2010]. Both BLAST and ProtParam analyses will provide information necessary for future studies.

APPENDIX C
STOCK SOLUTIONS AND MEDIA FORMULATIONS

Stock Solutions (Table 9)

Dissolve stock component of choice in 17.4MΩ purified water. Sterile filter components into new containers and store at 4°C. Aliquot solutions using sterile technique as necessary. Date and label with appropriate expiration.

Table 9: Stock solutions for culture media preparation including the component short-hand, the formula for the component, the respective catalog volume and mM stock amount (Barfield laboratory, 2015).

Stock Solutions for Culture Media Preparation			
Stock Component	Formula	Catalog #	Stock (mM or w/v)
ALA-GLU	Alanyl-glutamine	A-8185	100
B	NaHCO ₃	S-5761	250
BSA FAF	Bovine Serum Albumin (Fatty Acid Free)	AK8909	10%
C	CaCl ₂ 2H ₂ O	C-3881	200
CDM with phosphate	NaCl	S-9625	710
	KCl	P-5405	60
	KH ₂ PO ₄	P-5655	10
	Na-Citrate 2H ₂ O	C8532	5
EAA's	BME Amino Acid solution 50X	B-6766	73.56
G	D-Glucose	0188	350
GENTAMICIN	Gentamicin		Variable
GLYCN	Glycine	G7126	490
H(1:1)	HEPES free ac.	H-4034	100
	HEPES NA salt	H-3784	100
L-L	Sodium L-lactate	L-7022	1000
MI	Myo-inositol	I-7508	277
MS	MgSO ₄	M-2643	50
NaCl	NaCl	S-9625	5000
NEEAA'S	MEM Amino Acid sol 100X	M-7145	67.04
P	Na-Pyruvate	P2256	50
T	Taurine	T-8691	10

Media:

Mix media except for Gentamicin, test and adjust pH to 7.3-7.4. Once adjusted, sterile filter media into new containers and then add gentamicin, date and label. Store at 4°C for up to 3 weeks.

Tables 10 and 11: Media used for cell culture including H-CDM-M (Table 10) and IVM (Table 11). Components and respective molarities listed (Barfield laboratory, 2015).

Table 10: H-CDM-M	
Stock Component	Molarity (mM)
H2O	0.00
ALA-GLU	1.00
B	5.00
BSA	0.25
C	2.00
CDM	78.50
G	0.88
GLYCN	4.90
H (1:1)	2.00
L-L	10.00
MS	0.50
NaCl	22.50
NEEAA's	0.67
P	0.50
Gentamicin	25µg/mL

Table 11: Standard IVM	
Stock Component	Molarity (mM)
H2O	0.00
ALA-GLU	1.00
B	25.00
BSA	0.50
C	2.00
CDM	78.50
EAA's	n/a
G	3.50
GLYCN	4.90
H (1:1)	20.00
L-L	10.00
MI	2.77
MS	0.50
NEEAA's	n/a
P	0.50
T	0.10
Gentamicin	25µg/mL