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THE HORMONAL REGULATION OF CLAUDIN GENES IN THE OVARY

A Thesis

Presented to

The Graduate Faculty

Central Washington University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

Biological Sciences

by

Sean Dwayne Gadson

November 2016

CENTRAL WASHINGTON UNIVERSITY

Graduate Studies

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ABSTRACT

The Hormonal Regulation of the Claudin Genes in the Ovary

by

Sean Gadson

The ovary is a dynamic organ that responds to many hormonal signals. When these hormonal signals are disrupted, ovarian dysfunction can occur. One such example is Polycystic Ovarian Syndrome (PCOS). PCOS patients suffer from high levels of testosterone. Excess testosterone may misregulate genes in the ovary and disrupt ovarian function. The Claudin (*Cldn*) 3 and *Cldn11* genes have been shown to be regulated by androgens in the testis, while studies in ovarian cancer cells suggests a coregulatory mechanism for the expressions of *Cldn3* and *Cldn4* in the ovary. The objective of this study was to characterize the hormonal regulation of *Claudin* gene expression in the ovary. The ovaries of estrogen receptor alpha knockout (α ERKO) mice have high serum testosterone concentrations, therefore *Claudin* expression was measured in these ovaries. Experiments were conducted using Quantitative Real Time PCR (QRT-PCR) to monitor the expression of *Cldn3*, *4*, and *11* in wild-type (WT) and α ERKO mouse ovaries. These experiments indicated that *Cldn3*, *4*, and *11* were more highly expressed in α ERKO mice than their wild-type counterparts ($p < 0.05$, $n = 5$). Further experiments characterized Claudin expression in the ovaries of mice treated with Dihydrotestosterone (DHT) for 90 days which serve as a common mouse model of PCOS. DHT treated mice were found to express *Cldn3* and *Cldn11* significantly higher than control mice. *Cldn4* expression decreased in DHT treated mice when compared to the controls ($p < 0.05$, $n = 4$ for control and DHT groups). These findings indicated that *Cldn3* and *Cldn11* are upregulated by testosterone in the ovary.

The data also indicates *Cldn4* is regulated via different mechanisms than the other Claudin genes in the mouse ovary. DHT reduces expression of *Cldn4*, while increases are observed in the absence of ER α . Claudin expression was also evaluated in the ovaries of mice that were treated with testosterone propionate (TP) for three or six days. No expression of *Cldn3* or *Cldn11* was found, however *Cldn4* steadily increased in conjunction with the duration of the testosterone propionate treatment. Western blot analysis for the presence of CLDN3 in the ovaries of control and TP treated mice yielded no detectable signal for either group. Studies done in cell lines found that *CLDN4* expression decreased when BG-1 ovarian epithelial cells were treated with testosterone. These findings provide a first consider the regulation of the Claudin genes in the ovary, while providing a basis for future research to explore how they may contribute to PCOS.

Keywords: PCOS, Claudin, Ovary, *Cldn3*, *Cldn4*, Expression.

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CHAPTER I

INTRODUCTION (AND LITERATURE REVIEW)

Reproduction is the process where offspring are produced and is necessary for the survival of any species. Humans, like all mammals, reproduce sexually. This sexual reproduction requires genetic input from both a male and a female. These contributions are provided via specialized sex cells called gametes. The male gametes are sperm, while the female gametes are oocytes [1]. The processes by which these gametes are produced is tightly regulated since the quality of the gametes used in reproduction directly affects the development of the resulting offspring. Males produce their gametes by a process called spermatogenesis while females produce oocytes via oogenesis [1] [2]. The quality control of spermatogenesis and oogenesis are both extremely important. This calls for an environment that is tightly controlled, and one that receives and provides the various hormonal signals that drive these processes forward. The testes provide this environment in the male reproductive system. The ovary is the organ that provides this highly-optimized environment in the female reproductive system.

The Ovary

While signals from several organs in the body affect oogenesis, the direct care and maturation of a developing oocyte takes place in the female ovary [3], where certain gametes are selected, develop to maturity, and are eventually released into the fallopian tubes for fertilization. Given its role in an important process in biology, research has focused on how a healthy ovary functions, and how the ovary responds to various diseases and conditions which are not optimal to its normal activity. To provide

a solid knowledge base with which to explore ovarian diseases, we first turn to understand the healthy ovary. We start with the functional unit of the ovary known as the ovarian follicle, then transition into hormonal signals and their role in follicular development. Finally, we explore Polycystic Ovarian Syndrome, a model used to study the disease, and some genes of interest and how they relate to PCOS.

The Ovarian Follicle

As the oocyte within the ovary develops into maturity, hormonal signals and nutrients to be successful. The ovarian follicle is the structure that fulfills these requirements. The follicle itself is composed of three main cell types: the theca cells, granulosa cells, and the developing oocyte [4]. The follicle adopts a roughly spherical shape. The outermost cellular layer is composed of theca cells. While a main function of the theca cells is the production of androgens from cholesterol, these cells also have a number of other functions including the production of LH receptors and Vascular Endothelial Growth Factor (VEGF) [4]. The nurse cells of the ovarian follicle that come into direct contact with the oocyte are called granulosa cells. These cells express the enzyme aromatase which catalyzes the conversion of testosterone into estradiol [5]. In mammals, as oogenesis progresses, the follicle and the developing egg progress through several stages and grow. This development of the ovarian follicle is called folliculogenesis [6] and is necessary to produce a mature oocyte. After the oocyte has been ovulated, the granulosa cells of the follicle luteinize and become the corpus luteum, which provides the progesterone required for the successful implantation of the blastocyst and the establishment of a healthy pregnancy [1]. If no pregnancy is

established, the corpus luteum regresses and is reabsorbed into the ovary [7]. This event marks the end of one cycle of folliculogenesis. At any given time, an ovary will have multiple follicles at different stages of folliculogenesis.

Hormonal Signaling

The hypothalamic-pituitary-gonadal (HPG) axis regulates the normal function of a healthy ovary. This stepwise cascade ultimately provides the ovaries with the hormonal signals required to successfully complete folliculogenesis [1]. At puberty, the hypothalamus of the brain releases pulses of Gonadotropin Releasing Hormone (GnRH), a peptide hormone, directly to the pituitary. This GnRH signal acts on gonadotrope cells in the pituitary gland to secrete both Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) into the bloodstream [8]. During the early stages of folliculogenesis, FSH stimulates the ovarian follicle to grow. As the theca and granulosa cells propagate, low concentrations of LH act on the thecal cells which convert cholesterol into testosterone. During these same stages, FSH signals to the granulosa cells of the follicle, causing them to convert testosterone into estradiol using the enzyme aromatase [9]. These initial low levels of estradiol work through a negative feedback mechanism on the pituitary gland thereby suppressing the amount of LH that is released [1]. As follicular development progresses and the follicle grows, the amount of estradiol being produced increases due to higher numbers of granulosa cells expressing aromatase. When the follicle is prepared to ovulate the developed oocyte, estradiol production peaks. At this higher concentration, estradiol works as a positive feedback signal that results in more frequent GnRH pulses and a spike in the production and release of LH. This LH surge

pushes the follicle from the follicular stage of folliculogenesis into the luteal phase. During this phase, the mature oocyte is ovulated, and the granulosa cells of the remaining follicle becomes a structure known as the corpus luteum [8]. The corpus luteum produces the steroid hormone progesterone, which is essential for maintenance of pregnancy through actions on the uterus [1]. Progesterone also slows the GnRH pulsing of the hypothalamus, thereby reducing the levels of FSH and LH back to a baseline from which the next cycle can begin [10]. The corpus luteum also produces estrogen which is important in the regrowth of uterine endometrium [11]. Figure 1 highlights just a few of the hormones and feedback pathways of the HPG-axis.

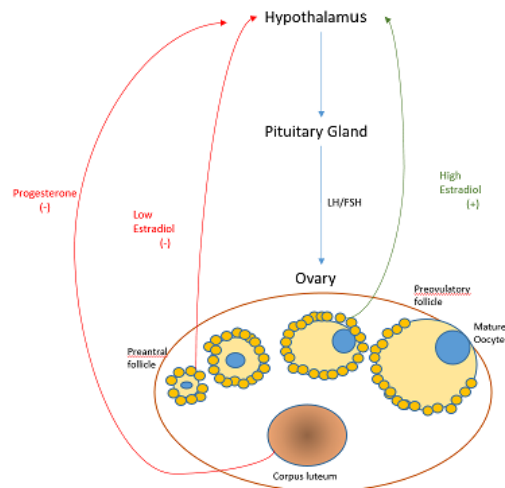


Figure 1: Hormonal Control of Positive and Negative Feedback on the HPG-Axis . The hypothalamus releases pulses of GnRH that act on the pituitary gland. The pituitary releases LH and FSH which acts on ovarian follicles that produce estradiol in response. Early stage preantral follicles release low levels of estradiol which inhibits LH/FSH secretion. Late stage preovulatory follicles release high levels of estradiol. This increases the frequency of GnRH pulses from the hypothalamus and causes a large amount of LH to be released which triggers ovulation of the mature oocyte.

Steroid Hormone Receptors

Sex steroid hormones and their receptors are fundamental to the function and maintenance of the reproductive systems of both males and females in a wide number of species. The receptors or hormones can work through a number of mechanisms that can depend on the presence of their ligand, or other non-genomic pathways that don't require the direct ligand-receptor interaction [12]. Direct signaling by steroid receptors occurs when the ligand bound receptor binds directly to a DNA sequence in order to affect transcription of one or multiple genes [13]. Ligand bound steroid receptors can also bind to and activate other transcription factors which then bind directly to DNA to alter gene transcription. Steroid receptor signaling can also initiate a signal cascade rather than causing the receptor to directly translocate into the nucleus [14]. One example would be an estrogen activated protein kinase signaling cascades[15]. Finally, steroid receptors can act as second messengers in signal cascades that can be activated by modifications such as phosphorylation [16]. Estrogen receptor alpha ($ER\alpha$, officially *Esr1*), estrogen receptor beta ($ER\beta$, officially *Esr2*) and the androgen receptor (*Ar*) are just a few of the receptors for steroid hormones that have been characterized in the ovary.

Estrogen receptor alpha binds estrogenic signaling hormones, the most widely recognized being estradiol. While $ER\alpha$ is important for sexual reproduction and the development of secondary sex characteristics, the receptor can affect a wide number of other physiological processes as well. Tissue differentiation [17], cell proliferation [18], and even the persistence of breast cancer have all been found to be affected by or

dependent upon signaling through ER α [19]. ER α signaling in part regulates the activity of the hypothalamic-pituitary-gonadal axis that controls the human menstrual cycle and rodent estrous cycle. Varying levels of estradiol act to communicate the status of a developing follicle to the hypothalamus, which in turn initiates ovulation when levels of estradiol pass a certain threshold [1]. Since estradiol can work through both positive and negative feedback mechanisms to affect the HPG axis, organisms that have their signaling via ER α disrupted develop a wide number of abnormal phenotypes. By using the Cre-Lox recombinase system of gene editing, mice have been generated that possess an allele of ER α that lacks the DNA-binding domain [20]. This renders the resulting truncated protein non-functional. This does not however, render heterozygous mice infertile. When heterozygotes are bred, homozygous individuals can be born that can mature to adulthood, but are sterile due to the lack of functional ER α . In addition to being sterile, homozygous estrogen receptor alpha knockout mice (α ERKO) have large ovaries that have cystic follicles and increased levels of serum testosterone [21]. These females also have elevated levels of estradiol and LH [22] since ER α can no longer inhibit the production of LH in the pituitary gland. The elevated levels of LH in the bloodstream of female α ERKO mice is considered to be the main cause of their ovarian phenotype [23]; a similar ovarian phenotype was observed in an elevated LH mouse model [24].

ER α and ER β share a high degree of structural similarity, and as a result can bind to the same ligands and DNA sequences [25]. In tissues where both receptors are expressed, ER β has been found to alter the transcriptional activity of ER α in both a permissive and an inhibitory manner. ER β can act as a competitive inhibitor of ER α by

binding to target DNA sequences and sterically blocking ER α from binding [26]. These two receptors can also form heterodimers with one another before translocating into the nucleus of a cell and activating gene transcription [27]. ER β is the predominant ER gene in the ovaries of mammals and is found almost exclusively in the granulosa cells [28],[29]. Mice lacking functional ER β (β ERKO) do not show ovarian gross morphologies like those lacking ER α [30]. In addition, these β ERKO mice do not show significantly altered levels of hormones, including testosterone and LH. However, β ERKO mice have been shown to form fewer corpora lutea and preovulatory follicles than wild-type mice which makes them sub-fertile[29],[31]. Mice lacking functional ER α and ER β ($\alpha\beta$ ERKO) share some characteristics with those of α ERKO mice such as hyperandrogenism and granulosa cell deficient antral ovarian follicles [25]. The double knockout mice do develop some novel ovarian phenotypes. One characteristic of the double knockout mice is the presence of Sertoli-like cells that develop post-pubertally [32],[21]. Sertoli cells are normally found in male testis and facilitate the progression of immature spermatids through spermatogenesis [33]. Also, $\alpha\beta$ ERKO mice do not develop preovulatory follicles [21]. The extent of the irregularities seen in any of the estrogen receptor knockout models highlights the importance of estrogenic signaling in the development and function of the ovary.

The androgen receptor (AR) binds to androgenic steroid hormones such as testosterone and dihydrotestosterone (DHT) [34]. While AR signaling is required for the development of masculine secondary sex characteristics, and reproduction in males; it is less commonly known that androgenic signaling also plays a role in female reproduction

as well [1]. AR protein in rodents has been found in the granulosa cells and theca cells of the ovarian follicle, as well as other cell types outside of the follicle such as the stroma and epithelium [35]. Female mice lacking AR are impaired in their ability to ovulate normally developed follicles as indicated by a substantially lower number corpora lutea in their ovaries [36]. This observation highlights the role of androgens in ovarian function as being more than just precursors for the synthesis of estrogenic hormones.

As these models show, steroid hormones are essential to the maintenance of a healthy reproductive phenotype. Many common fertility issues are treated with hormone therapies, and reproductive diseases often have an etiology rooted in hormonal imbalances. Therefore, by studying sex hormones as they relate to reproductive diseases, it is possible to identify a cause of the disease as well as the best avenues of treatment. Endocrinologists and reproductive biologists are actively studying the hormonal imbalances associated with the leading cause of infertility in women, Polycystic Ovarian Syndrome.

Introduction to Polycystic Ovarian Syndrome

Polycystic ovarian syndrome (PCOS) is a disease affecting 5-10% of women entering their reproductive ages [37]. PCOS can manifest as several symptoms that can vary from one patient to another. In order to minimize incorrectly diagnosing the disease and standardizing diagnostic criteria for PCOS, the Rotterdam European Society for Human Reproduction proposed the Rotterdam Criteria 2003 [38]. These criteria considered the most consistent symptoms of PCOS. The Rotterdam criteria defines PCOS as the development of two of the following three symptoms: (1) oligo-anovulation, (2)

polycystic ovaries, and (3) biochemical or clinical hyperandrogenism [39]. Oligovulation in humans is defined as menstrual cycles lasting longer than 35 days or occurring fewer than eight times a year [38]. The presence of polycystic ovaries is routinely evaluated via ultrasound, although the relative size and number of follicles that must be present to diagnose a woman with polycystic ovaries is under debate [40]. Typically, the first sign that a woman may have high circulating testosterone is hirsutism, the development of coarse hairs that grow in a male pattern on the body [41]. Other signs of excess serum androgens include obesity and clitoromegaly [40]. PCOS has also been attributed to the onset of other detrimental health conditions such as type II diabetes [37]. The hyperandrogenism that PCOS patients experience exposes the ovaries to more testosterone than is typically present in the ovary. Normally, testosterone in the ovary is converted into estradiol by the enzyme aromatase for use as a steroid hormone signaling molecule [42]. The excess testosterone in PCOS acts as an inappropriate signaling hormone that can bind the AR. This may upregulate the expression of genes that are normally silenced in ovarian tissue and thereby alter normal ovarian function, contributing to the symptoms that are characteristic of this disease.

There are many physiological effects of PCOS besides the symptoms that are used in its diagnosis. While these other symptoms are not ubiquitous in all PCOS afflicted women, they largely impact the lives of PCOS patients and make it difficult to maintain a healthy lifestyle. For instance, the infertility brought on by PCOS is often the first reason that a woman will seek medical care. To overcome this infertility, many people turn to hormonal therapies or *in vitro* fertilization (IVF). While women can find

success in restoring fertility with these methods, they can also be financially and emotionally draining given that it may require multiple attempts before a pregnancy is successfully established. Fifty percent of women with PCOS are either obese or overweight [43]. Unhealthy weight gain can adversely affect menstrual cycling which makes it more difficult to become pregnant [44]. The problem becomes two-fold when the difficulties of having children while overweight are compounded with an altered hormonal milieu brought on by PCOS. This is why healthy weight management practices are often effective in alleviating some PCOS symptoms [40].

PCOS has also been associated with several psychological disorders. Depression and anxiety have both been shown to be more prevalent in women with PCOS [45]. The exact etiology of these disorders is still debated, the persistence of these disorders can be exacerbated by the mental strain that infertility, hirsutism, issues with sense of self, and obesity that women with PCOS may face [40]. Prior studies have also associated specific treatments of bipolar disorder with the development of PCOS-like symptoms. Valproate™ is a common drug used as a treatment for bipolar disorder. It has been associated with hyperandrogenism, weight gain, polycystic ovaries, and irregular menstrual cycling [40]. Long term studies measuring the persistence of depression in PCOS afflicted women found a consistently high rate of depression over the two year period [46]. The relationship between PCOS and mental health disorders is complex with more clinical manifestations directly feeding into the psychological problems. To ensure the best quality of life possible for PCOS afflicted women, it is necessary to study, and

eventually be able to alleviate both the physiological and psychological complications associated with PCOS.

Dihydrotestosterone Treatment as a PCOS Model

Studying the etiology of this disease creates a need for a reliable animal model that can closely replicate the symptoms of PCOS. Mice are a common model used to study PCOS due to their stable genetic background, ease of maintenance, short reproductive cycle, feasibility of generating genetic mutations, and affordability [37]. PCOS-like phenotypes have been produced in mice by a number of methods including postnatal treatment with dihydrotestosterone (DHT). DHT is a form of testosterone that utilizes the same classic androgenic signaling mechanisms [47], but cannot be converted into estradiol by the enzyme aromatase. Given this property, it is possible to separate androgenic and estrogenic effects on gene expression experimentally. Treating prepubertal female mice with 10 mg of DHT over 90 days via subcutaneous implants has been shown to produce phenotypes that mimic PCOS symptoms. These include irregular estrous cycling, cystic ovaries, and obesity [48]. PCOS-like phenotypes have also been produced in mice given a lower dosage of daily DHT. Metabolic disorders and ovarian morphologies resembling those of PCOS women can be induced by treating prepubertal mice with DHT doses as low as 3 mg over 90 days [49]. By inducing these phenotypes by treating mice with DHT, we can study how PCOS symptoms develop in a mouse model.

Tight Junctions and Claudin Proteins

Tight Junctions (TJ) are large multi-protein complexes found on the plasma membrane that adheres neighboring cells together. These junctions have a number of

functions including the adherence of neighboring cells, the maintenance of cell polarity, control of paracellular ion flow, and the establishment of distinct tissue barriers [50]. Improper TJ formation or other loss of TJ function is associated with increased metastasis and a poor prognosis for cancers originating from tissues all over the body [51]. It does not take a complete ablation of TJ formation to increase cancer metastasis.

The Claudin genes are a large family of genes consisting of 24 members [52]. The Claudin proteins are localized to the plasma membrane and have four membrane spanning regions [52]. Some Claudin genes have been shown by numerous studies to have altered regulation in ovarian cancers [53], [54]. Prior studies suggest that the overexpression of *CLDN3* and *CLDN4* increases the invasiveness and motility of ovarian epithelial cells [55]. This suggests that the misregulation of the Claudin genes may have a pathogenic effect. Real-time RT-PCR analysis of Claudin gene expression in numerous neoplastic tissue samples indicates that the expressions of *CLDN3* and *CLDN4* are tightly correlated across multiple tissue types, which suggests a coordinated regulation of these genes [50]. Although most notably documented in ovarian cancer, this correlation has also been found in both ovarian and colon cancers [56], as well as pancreatic, prostate, and breast cancers [57]. Epidemiological evidence has been presented suggesting that women with PCOS are more likely to develop ovarian cancer [58]. Despite the association between the misregulation of the Claudin genes and these diseases, there has been no research to date that characterizes the regulation of the Claudin genes in the ovary. Alterations in the regulation of even one protein constituent of TJs like the Claudin proteins (*CLDN*) have been shown to have negative effects [51].

The Claudin genes also play an important role in male spermatogenesis. Both *CLDN3* and *CLDN11* are expressed in the Sertoli cells of the male testis. These Claudins are part of the tight junctions between neighboring Sertoli cells that form the blood-testis barrier [59]. This barrier separates the seminiferous epithelium into two distinct compartments [59]. Immature sperm develop as they move from one compartment to the other. Testosterone has been shown to increase the expression of *CLDN3* and *CLDN11* in the male testis [59], [60]. We hypothesize that the same androgenic hormone would have a similar effect on Claudin expression in the female ovary.

Conclusions

PCOS patients also have high levels of testosterone which could cause the overexpression of any androgenically regulated Claudin genes in the ovary. Should this overexpression occur in the ovarian epithelium, the excess protein could inhibit ovulation by not allowing the oocyte to erupt from the ovary. For this reason, we hypothesize that PCOS women overexpress *CLDN3* and *CLDN11* which further inhibits ovulation and contributes to the anovulation and infertility of PCOS. There have been studies that indicate that women who have PCOS are more likely to develop ovarian cancer later in life [61]. When these studies are coupled with evidence indicating that the overexpression of the Claudin genes leads to increased ovarian epithelial cell motility and invasiveness [55] [62]; the overexpression of the Claudin genes due to hyperandrogenism could cause these cells to adopt a cancerous phenotype. It is necessary to accurately evaluate the risk that PCOS women have of having increased expression of the ovarian Claudin genes. Doing so facilitates a deeper understanding of

the ovary, and the complications that are associated with ovarian diseases like PCOS.

The results from this study will lay the groundwork for future research to further explore the role of the Claudin genes in the ovary.

CHAPTER II
JOURNAL ARTICLE

Elucidating the Hormonal Regulation of the Claudin Genes in the Ovary

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Abstract

When the hormonal signals of the ovary are disrupted, it is possible to develop ovarian disease states such as Polycystic Ovarian Syndrome (PCOS). PCOS patients suffer from high levels of testosterone which misregulates genes in the ovary. The tight junction genes *Claudin (Cldn) 3* and *Cldn11* are regulated by testosterone in the testis. Studies suggests a coregulatory mechanism could exist between *CLDN3* and *CLDN4*, since they share a regulatory pattern in cancers derived from multiple tissues. This study sought to characterize the hormonal regulation of *Claudin* genes expression in the ovary. Estrogen receptor alpha knockout (α ERKO) mice have high serum testosterone concentrations which mimics the excess testosterone of PCOS, therefore *Claudin* expression was measured in these ovaries. Experiments were conducted using QRT-PCR to monitor the expression of *Cldn3*, *4*, and *11* in wild-type and α ERKO mouse ovaries. These experiments indicated that *Cldn3*, *4*, and *11* were expressed at significantly higher levels in α ERKO mice than their wild-type counterparts. Further experiments characterized *Claudin* expression in the ovaries of mice treated with dihydrotestosterone (DHT) for 90 days. DHT treated mice were found to express *Cldn3*

and *Cldn11* significantly more than controls. *Cldn4* expression significantly decreased in DHT treated mice. Claudin expression was also evaluated in the ovaries of mice that were treated with testosterone propionate for three or six days. No expression of *Cldn3* or *Cldn11* was found, however *Cldn4* steadily increased in conjunction with the duration of the testosterone propionate treatment. These findings demonstrate that *Cldn3* and *Cldn11* are upregulated by testosterone in the ovary, while *Cldn4* is regulated via a different mechanism.

Introduction

Polycystic Ovarian Syndrome (PCOS) is the leading cause of female infertility worldwide and is characterized by a few classic symptoms including cystic ovaries, metabolic disorders, and high serum levels of testosterone (hyperandrogenism) [37]. The hyperandrogenism associated with PCOS has been extensively studied as the presence of excess androgens has been shown to have multiple effects on the normal functioning of the ovary as well as the Hypothalamic-Pituitary-Gonadal axis as a whole[40]. This could significantly alter gene expression in the ovaries of PCOS affected women. This excess androgen could in turn contribute to the anovulation, cystic ovaries, and overall infertility that these women experience. PCOS afflicted women have also been shown to have an increased risk of developing ovarian epithelial cancers[58].

To study PCOS, reproductive biologists have turned to several mouse models that mimic PCOS symptoms. Treating mice with androgens such as dihydrotestosterone (DHT) or testosterone propionate (TP) both prenatally and prepubertally, have been shown to result in the development of PCOS-like ovarian phenotypes [37]. Treating mice

postnatally with aromatase inhibitors like letrozole also produces similar symptoms [48]. Genetic alterations can also lead to the development of PCOS-like phenotypes. Estrogen receptor alpha knockout mice (α ERKO) have share characteristics with PCOS mouse models, such as cystic ovaries and hyperandrogenism [21]. By using these models, it is possible to study the etiology of PCOS whether it be due to abnormal testosterone levels, exposure to an endocrine disrupting chemical like letrozole, or a genetic predisposition.

Two tight junction proteins Claudin 3 (CLDN3) and Claudin 4 (CLDN4) have been shown to be consistently over expressed in ovarian cancers [62]. CLDN3 and Claudin 11 (CLDN11) are normally not expressed in the female ovary but, have been shown to be upregulated by testosterone in Sertoli cells of the male testis [63]. *CLDN3* and *CLDN4* have also seem to be upregulated by the same signaling mechanism [50]. Our study sought to characterize the expression of the *Cldn3*, *Cldn4*, and *Cldn11* in the ovaries with hyperandrogenism in hopes of characterizing how the excess testosterone observed in PCOS can impact the expression of the Claudin genes in the ovary. Ovaries from three hyperandrogenous mouse models were used: estrogen receptor alpha knockout (α ERKO) mice, testosterone treated mice, and mice treated with dihydrotestosterone (DHT) for 90 days.

Materials and Methods

Estrogen Receptor Alpha Knockout Mice

Adult age matched wild type (n = 5) and estrogen receptor alpha knockout (n = 5) mouse ovaries were isolated from adult animals (5-6 months old), snap frozen in

liquid nitrogen, shipped on dry ice and stored at -80°C prior to RNA extraction. The ovaries were generously donated by Dr. Kenneth Korach from the National Institute of Environmental Health Sciences from animals under a protocol approved by NIEHS Institutional Care and Use Committee (ASP#01-30).

DHT Treated mice

Prepubertal (postnatal day 19) mice were randomly sorted into two groups and surgically implanted with a subcutaneous pellet that releases placebo or dihydrotestosterone (DHT) for 90 days (Innovative Research of America, Sarasota, FL). Pellets contained placebo or 2.5mg of DHT and administered a daily dosage of 27.5 µg. Mice were fed *ad libitum* and maintained on a 12-hour light/dark cycle. At the duration of the 90-day treatment, mice were euthanized and ovaries were snap frozen in liquid nitrogen and stored at -80°C. Treatment of the animals was done at National Institute of Environmental Health Sciences (NIEHS) under a protocol approved by NIEHS Institutional Care and Use Committee (ASP#01-30). Five ovaries from each treatment group were provided for this study.

Testosterone Propionate Treatments

C57BL/6J mice were used from the Binder lab breeding colony at Central Washington University. Mice were fed *ad libitum* and maintained on a 12-hour light/dark cycle. Fifteen female mice were sorted into either control, 3-day Testosterone Propionate (TP) (Sigma-Aldrich, St. Louis, MO) treatment, or 6-day TP treatment groups. Treatments began at 21 days old. Mice were given 3mg/kg TP (for either 3 or 6 days) subcutaneously in a sesame oil vehicle. Control mice were given 50ul of sesame oil and ovaries were

collected on day six. At the time of ovary collection, mice were euthanized via carbon dioxide inhalation followed by cervical dislocation to confirm death. Ovaries were collected and stored at -80°C. The protocol for maintaining, treating, and sacrificing the mice was approved by the Central Washington University Institutional Animal Care and Use Committee (Protocol#: A111509).

RNA Extraction and cDNA Synthesis

Tissue samples were homogenized in 1mL of Trizol Reagent™ (Invitrogen, Waltham, MA) prior to RNA extraction. RNA was isolated as previously described [64]. All cDNA syntheses were carried out using 1µg of RNA. First, samples were treated with DnaseI™ (Invitrogen, Carlsbad, CA) to ensure no genomic DNA remained. This was done in a master mix containing 1µl 10x DnaseI buffer™ (Invitrogen, Carlsbad, CA), 1 unit of DNaseI and 8µl of DEPC water for 1µg of RNA (Invitrogen, Carlsbad, CA). RNA was then reverse transcribed for 50 minutes at 42°C in a 20µl master mix containing 50ng of random hexamers (Applied Biosystems, Foster City, CA), 10nM dNTP mix, 5x First Strand Buffer, 0.1M DTT, 40 units of RnaseOut, and 200 units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA was then treated with 0.5µl RNaseH (Applied Biosystems, Foster City, CA) at 37°C for 20 minutes to ensure no mRNA transcripts remain after reverse transcription. RNaseH was then denatured by adding 0.5µl of 25mM EDTA (Invitrogen, Carlsbad, CA). Finally, cDNA was diluted 1:5 prior to PCR by adding 80µl of DEPC treated water.

QRT-PCR

Quantitation of cDNA transcripts was done using a quantitative real-time polymerase chain reaction method. Measurements were taken using the IQ5 Multicolor Real Time PCR Detection System (Biorad, Hercules, CA). PCR was conducted in 20 μ l master mixes containing 10 μ l SYBR Select Master Mix (Thermofisher, Waltham, MA), 4 μ l DEPC water, 1 μ l of 10mM primers, and 5 μ l cDNA. PCR was run with an initial 30 second cycle at 95 $^{\circ}$ C to separate DNA strands. The next cycle was set to 95 $^{\circ}$ C for 10 seconds to separate strands, and then 55 $^{\circ}$ C for primer binding and transcript extension. Readings of fluorescence were taken at this step. This cycle was repeated 40 times. Experiments were run in triplicate and the average Ct of each triplicate set was calculated. These averages were converted into standardized ratios using the Livak [65] method and *mPl7* as the control standard. The primer sets utilized are as follows:

Gene Name	Primer Sequences 5'-3'	Melting Temp	Source
<i>mPl7</i>	F: AGCTGGCCTTTGTCATCAGAA R: GACGAAGGAGCTGCAGAACCT	F: 59.93 $^{\circ}$ C R: 62.37 $^{\circ}$ C	Diez-roux, 2011
<i>mCldn3</i>	F: GGAAGGCCTGTGGATGAACT R: CCTTACGGTCATAGGCGGTG	F: 59.67 $^{\circ}$ C R: 60.25 $^{\circ}$ C	NCBI Primer Blast
<i>mCldn4</i>	F: ATGGCGTCTATGGGACTACA R: TTACACATAGTTGCTGGCGG	F: 57.92 $^{\circ}$ C R: 58.27 $^{\circ}$ C	NCBI Primer Blast
<i>mCldn11</i>	F: TCGTCACAACGTCCACCAAT R: GGATGCAGGGGAGAACTGTC	F: 59.89 $^{\circ}$ C R: 60.11 $^{\circ}$ C	NCBI Primer Blast
<i>mERα</i>	F: CCACCAACCAAGTGCACCAT R: GGTCTTTTCGTATCCCACCTTTC	F: 60.53 $^{\circ}$ C R: 59.31 $^{\circ}$ C	Latil, 2001

Table1: cDNA primers for *in vivo* QRT-PCR experiments.

Statistical Analysis

All comparisons between treatment groups were made using R version 3.2.3 with the RStudio plug-in. Statistical analyses were run using at least three average ratios for each treatment group. Student's *t*-tests were used compare average *Cldn*/mPL7 Livak ratios between treatment groups. Significance was determined using a significance level of 0.05.

RESULTS

Esr1 and Claudin Expression

Mice lacking estrogen receptor alpha (α ERKO) have been shown to develop a number of irregular morphological and hormonal characteristics including cystic ovaries, elevated androgen secretion, and anovulation [22]. These characteristics are also seen in mouse models of PCOS [37]. The expressions of *Cldn3*, *Cldn4*, and *Cldn11* were measured in the ovaries of female α ERKO mice. First, it was necessary to confirm that Estrogen Receptor alpha ($ER\alpha$) was knocked out in the α ERKO ovaries. To do this, Quantitative Real-Time PCR (QRT-PCR) was first used to measure the expression of $ER\alpha$ in wild-type (WT) and knockout (KO) mouse ovaries. As seen in figure 1, WT mice did express $ER\alpha$ with some variation between individuals, this expression contrasts the KO mice that showed no expression of *Esr1* in the ovary.

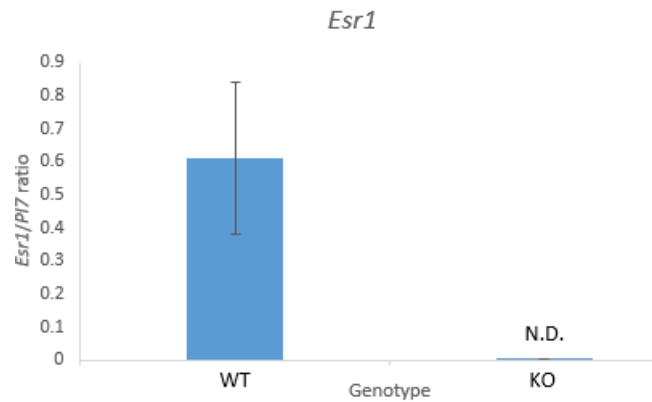


Figure 2: EsR1 Expression in Wild-Type and α ERKO Mouse Ovaries . *Esr1* expression was quantitated via QRT-PCR to confirm the presence of *Esr1* in the WT group, and the absence of *Esr1* in the KO group. Expression is reported as the average *Esr1*/*pl7* Livak ratio \pm SEM (n = 5 for both WT and KO groups). Non-detectable = N.D.

To gain a better understanding of how Claudin regulation may be altered in a hyperandrogenous model, gene expression was measured in the ovaries of both WT and α ERKO mice. There were significant differences in the expressions of *Cldn3*, *4*, and *11* between the two groups. Amplification of cDNA transcripts by Quantitative RT-PCR indicated that α ERKO mice expressed *Cldn3* at a significantly higher level than their WT counterparts (Fig.3, $p < 0.05$). This result indicates that a loss of *ER α* resulted in an increase in transcription of the *Cldn3* gene. Analysis of *Cldn4* expression yielded results similar to *Cldn3*. α ERKO mice expressed *Cldn4* significantly higher than WT individuals (Fig.3, $p < 0.05$). *Cldn11* expression was also found to be elevated in the α ERKO mice when compared to wild-type controls (Fig.3, $p < 0.05$). Each gene of interest was found to be transcribed roughly five –fold higher in the α ERKO than in WT. This data demonstrates that a loss of *ER α* in the ovary results in an increase in the ovarian expression of *Cldn3*, *Cldn4*, and *Cldn11*.

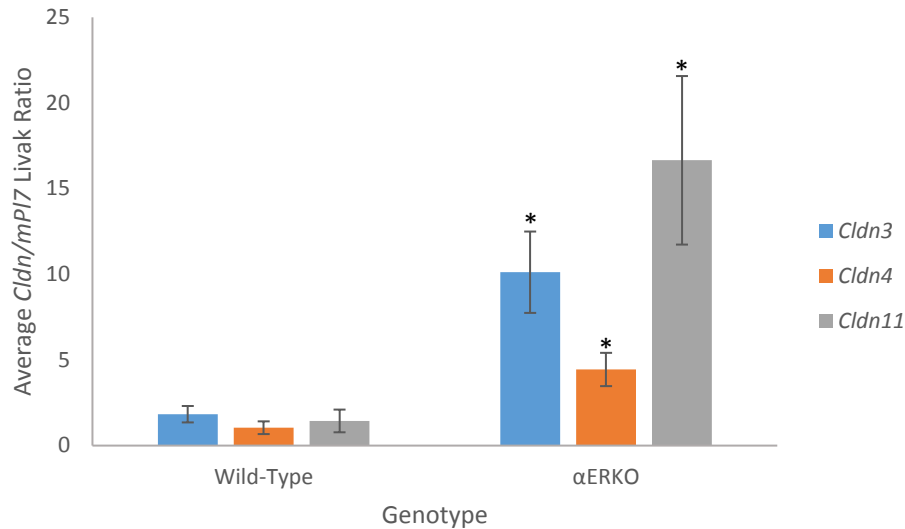


Figure 3: *Cldn3*, *Cldn4*, and *Cldn11* ovarian expression in wild-type (WT) and α ERKO (KO) mouse ovaries. Expression is reported as the average *Cldn/pl7* Livak ratio \pm SEM ($n = 5$ for both WT and KO groups). Students *t*-test was conducted for comparison of expression between genotypes between WT and KO mice (* $p < 0.05$). Non-detectable = N.D.

Excess DHT Alters Ovarian Claudin Expression

Given that α ERKO mice also have elevated levels of LH and estradiol levels in addition to excess testosterone [23], we investigated if a PCOS model with excess testosterone alone would show similar differences in Claudin gene regulation. Claudin expression was measured from ovaries of mice treated with dihydrotestosterone (DHT) for 90 days and compared to control mice treated with placebo. DHT treated individuals showed significant alteration in the expressions of *Cldn3*, *4*, and *11*. *Cldn3* and *Cldn11* were significantly elevated in the ovaries mice treated with DHT for 90 days (Figure 3A, $p < 0.05$), while *Cldn4* transcription was found to be decreased in the DHT mice when compared to controls (Figure 5, $p < 0.05$). Taken together, this data indicates that *Cldn3* and *Cldn11* have increased expression in response to excess DHT, while *Cldn4*

expression decreased in response to the excess DHT. When coupled with the expressions of *Cldn3*, *Cldn4*, and *Cldn11* in α ERKO mice (figure 3); the findings indicate that *Cldn3* and *Cldn11* are upregulated by testosterone signaling in the ovaries while *Cldn4* expression is regulated separately.

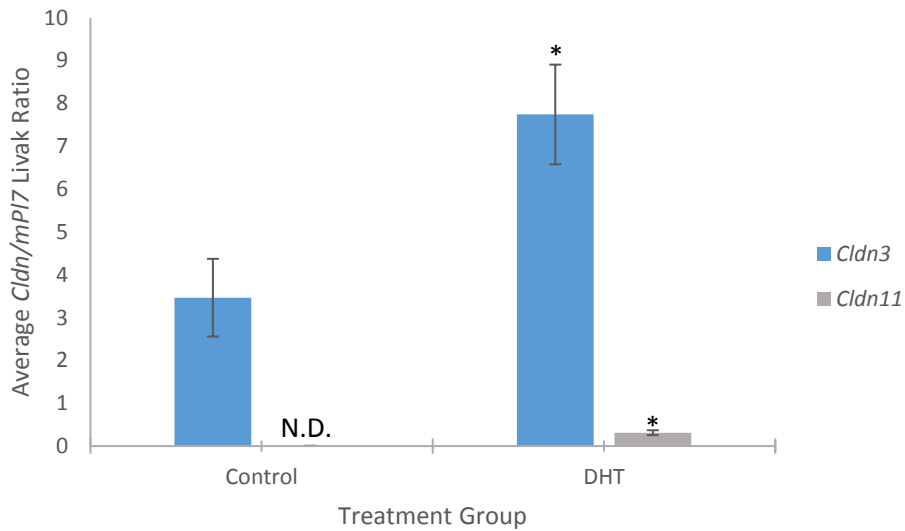


Figure 4: *Cldn3* and *Cldn11* in Control and 90d DHT Treated Mouse Ovaries. Expression is reported as the average *Cldn/pl7* Livak ratio \pm SEM (n = 4 for both control and DHT groups). T-test was conducted for comparison of expression between treatment groups. (*p < 0.05). Non-detectable = N.D.

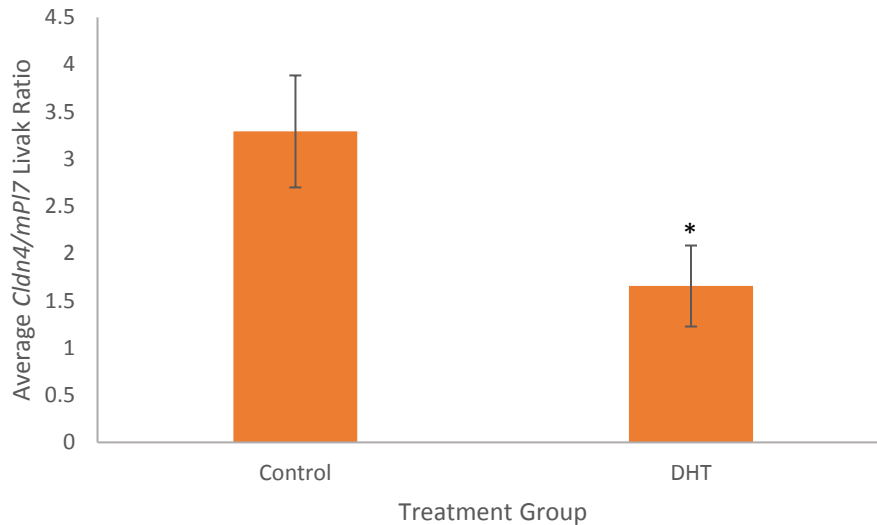


Figure 5: *Cldn4* Expression in Control and 90d DHT Treated Mouse Ovaries . Expression is reported as the average *Cldn/pl7* Livak ratio \pm SEM (n = 4) T-test was conducted for comparison of expression between treatment groups. (*p < 0.05).

Short Term Testosterone Propionate Treated Mice Have Increased *Cldn4* Expression

Treating mice with Testosterone Propionate (TP) postnatally has been shown to induce the development of PCOS like characteristics [37]. Disrupted ovulatory cycling, cystic ovaries, and hyperandrogenism have all been observed in mice treated with varying doses of TP for as little as one week to as long as sixteen weeks [37]. Given its use as a model of PCOS, the expressions of *Cldn3*, *4*, and *11* were measured by QRT-PCR from the ovaries of prepubertal mice treated with testosterone propionate. 3mg/kg of TP in an oil vehicle was administered subcutaneously for either three or six days to avoid the onset of puberty with extended treatment periods. Control mice were given just the oil vehicle and ovaries were collected on day six.

QRT-PCR analysis showed no detectable signal for the expression of either *Cldn3*

or *Cldn11* in the vehicle, 3-day, or 6-day treatment groups (data not shown). Ovarian *Cldn4* expression was found to steadily increase over the duration of the TP treatment. Expression of *Cldn4* in the 3-day treated group was significantly higher than the vehicle treated group (Figure 6, $p < 0.05$). Similarly, *Cldn4* expression in six day treated group was significantly higher than the vehicle treated group ($p < 0.05$).

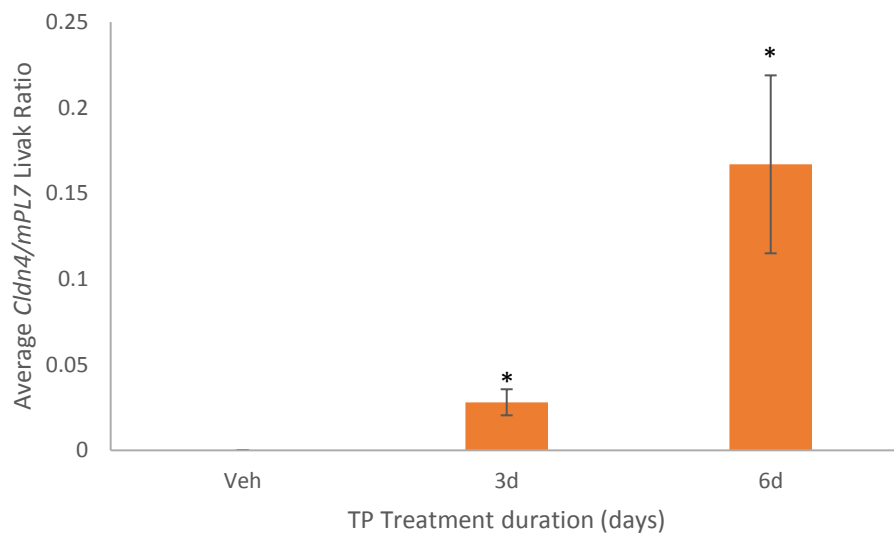


Figure 6: *Cldn4* Expression in the Ovaries of Control, 3d, and 6d TP Treated Mice. Expression is reported as the average *Cldn/p17* Livak ratio \pm SEM ($n = 4$ for 3d and 6d treatment groups, $n = 6$ for vehicle group). Significance shown as determined by a Student's *t*-test ($*p < 0.05$) Non-detectable = N.D.








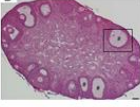




<u>αERKO Mice</u>	<u>DHT Treated Mice</u>	<u>Testosterone Treated Mice</u>
<ul style="list-style-type: none"> • Claudin 3  • Claudin 4  • Claudin 11  	<ul style="list-style-type: none"> • Claudin 3  • Claudin 4  • Claudin 11  	<ul style="list-style-type: none"> • Claudin 3  • Claudin 4  • Claudin 11  

Figure 7: Overview of *In Vitro* models and their expression of *Cldn3*, *Cldn4*, and *Cldn11*.

DISCUSSION

Claudin Genes and loss of functional ER α

Loss of ER α inhibits the negative feedback that low levels of estradiol has on the hypothalamus [23]. Left uninhibited, excess LH and FSH is released from the pituitary. In the ovary, high levels of LH inhibits aromatase activity [66], which in turn inhibits the conversion of testosterone into estradiol [67] leading to hyperandrogenism. In this same manner, the excess LH in the α ERKO mice contributes to their hyperandrogenism [44]. Prior research indicates *Cldn3* and *Cldn11* are upregulated by testosterone in the male testis [59, 60, 67]. CLDN4 has also been hypothesized to share a regulatory mechanism with CLDN3 in that they may both be upregulated by testosterone [50]. We hypothesized that testosterone could upregulate the expression of *Cldn3*, *Cldn4*, and *Cldn11* in the ovary. α ERKO mice were found to express ovarian *Cldn3*, *Cldn4*, and *Cldn11* significantly more than their wild-type counterparts. This indicates that a loss of ER α functionality either directly or indirectly results in the increased expression of these *Claudin* genes although the exact signaling mechanism remains unknown.

The Claudins: Regulation and Ovarian Cancer

If testosterone causes an increased expression of the Claudin genes in normal mice, it could also be contributing to the increased expression of the Claudin genes in the α ERKO mice. To test this idea, it was necessary to test the effect that excess testosterone has on Claudin expression in the ovary. In order to isolate the effect that excess testosterone has on the ovary, as well as characterize Claudin expression in a mouse model of PCOS; the expressions of *Cldn3*, *Cldn4*, and *Cldn11* were measured in the ovaries of mice treated with DHT for 90 days. Mice that were treated with DHT showed significantly elevated expression of *Cldn3* and *Cldn11* while *Cldn4* significantly decreased (Figure 3). This indicates that long term treatment with DHT alters the expression of these genes in the ovary.

Overexpression of CLDN3 has been found in ovarian cancers and the altered expression of Claudin proteins is continuing to be investigated as a possible marker for multiple types of cancer [53, 57]. In addition, prior studies have explored the expression of the Claudin genes in ovarian cancer in humans[69]. Altered expression of CLDN3 and CLDN4 has also been found to increase the metastatic properties of ovarian cancer cell lines [55] and increased *CLDN3* expression has been identified as an early marker in the development of ovarian epithelial cancer in humans [62]. Given the upregulation of *Cldn3* in our DHT model and the prior research exploring its role and end expression in ovarian cancer; increased expression of ovarian *Cldn3* due to PCOS attributed hyperandrogenism may be one of the links that connects PCOS and their increased likelihood to develop ovarian cancer.

It is possible that the lack of *Cldn3* and *Cldn11* expression seen in TP treated mice is due to their age. The TP treatments and ovary extractions were done prepubertally. In male mice *Cldn3* and *Cldn11* expression are not present until after puberty as the blood-testis barrier develops [70]. It is possible that the lack of *Cldn3* or *Cldn11* expression in the ovaries of TP treated mice is also due to their prepubescent age. These genes may be unresponsive to testosterone signaling prior to adulthood.

Cldn4 expression decreased in the DHT treated mice (Figure 3). Given that *Cldn3* increased, it stands to reason that a coregulatory mechanism that alters the expression of *Cldn3* and *Cldn4* in the same manner in response to testosterone does not come into effect in the mouse ovary. This contrasts prior research indicating a coregulatory mechanism may exist between *CLDN3* and *CLDN4* [50] in cancerous tissues. It is also possible that DHT may work through a different regulatory mechanism for each Claudin gene. This would account for the differences in the response of *Cldn3*, *Cldn11* and *Cldn4* to DHT treatment compared to Testosterone treated and α ERKO mice.

Ovarian Claudin Regulation and PCOS

The overexpression of ovarian Claudin genes in response to excess testosterone may help contribute to the anovulation that can accompany PCOS. Ovulation requires that the ovarian epithelium be able to rupture in order to release a mature oocyte [71]. It is possible that this rupturing is physically hindered by an increased titer of cell-adhesion proteins such as the Claudin proteins in the ovary due to the excess testosterone. As our data, has shown, ovarian *Cldn3* and *Cldn11* can be upregulated by excess androgenic signaling in the ovary. Excess androgens have also been shown to

correlate with an increased number of developing follicles within the ovaries of primates [72, 73]. These follicles become the cysts that give PCOS its name [38]. In this manner, the hyperandrogenism of PCOS affected women could culminate in both an increased number of ovarian follicles, and the inability to ovulate them.

Ovarian *Cldn3* and *Cldn11* expression remained unexpressed despite three or six days of subcutaneous testosterone propionate treatment. This is consistent with prior work showing that these genes are expressed to an extremely low degree in healthy human ovarian tissue [50]. Coupled with the results from the 90 day DHT-treated mice, the experiment indicates that significant changes in the expression of *Cldn3* and *Cldn11* in response to androgens takes place on a time scale longer than six days. This result suggests that a small transient exposure to testosterone may not alter the Claudin expression in mouse ovaries. It is possible that a long term endogenous dose of androgen like PCOS patients experience is required to significantly affect the levels of ovarian *CLDN3* and *CLDN11*. Another study measured the expression levels of *Cldn3* and *Cldn11* in the testis of hypogonadal mice[70]. It was found that both genes increased at the mRNA and protein levels after 10 days of DHT treatment forming fully functional tight junctions, and also that when DHT and FSH were administered together, these functional tight junctions formed in as little as 2 days[70]. This shows a dynamic interplay between androgens and FSH can affect the timescale on which the expressions of *Cldn3* and *Cldn11* are altered. Our study found no ovarian expression of either gene in response to three or six days of TP treatment. We hypothesize that in the ovary, where these genes are normally expressed to a much lower degree than the male testis,

upregulation of *Cldn3* and *Cldn11* takes longer than six days. The mice used in the TP experiments were 21 days old when they received the first treatment. This is too young for estrous cycling and so no FSH would be present in these mice. Nevertheless, the increase in the expression of both genes in response to the 90 day DHT treatment does help define the bounds of a time scale that can be further refined by future studies.

Ovarian *Cldn4* was found to steadily increase in response to the short-term testosterone propionate treatments. This result contrasts the decrease in *Cldn4* that was seen in the mice treated with DHT for 90 days. Differences in the regulation of this gene between these models could be attributed to different regulatory mechanisms controlling the expression of *Cldn4* in response to testosterone. Early in life, excess androgens could upregulate *Cldn4* while later in life the same hormonal excess could downregulate its expression. *Cldn4* has been previously documented to decrease when testosterone levels in the male mouse prostate are suppressed [59]. Also, *in vitro* studies have identified *Cldn4* to be a target of AR regulation in cells derived from male prostate cancers [74]. When coupled with our own study, it seems that *Cldn4* can be downregulated or upregulated by androgens in the female mouse ovary as well.

The α ERKO mice developed cystic ovaries, hyperandrogenism [20] and increased expression of *Cldn3*, *Cldn4*, and *Cldn11*. While these effects were due to a complete silencing of ER α signaling, future studies should elucidate the effect that a short-term treatment with ER α antagonists would have on the ovarian expression of the Claudin genes. This will allow us to understand if a complete silencing of ER α is required for the development of these characteristics, or if a transient exposure to an environmental

antagonist is sufficient. We hypothesize that the increase in *Cldn3*, *Cldn4*, and *Cldn11* expression is more directly caused by the high levels of testosterone in the serum of the α ERKO mice based on the expression of these genes in the DHT and TP treated mouse ovaries.

Future research should also seek to more definitively characterize the time scale on which the expression of ovarian *Cldn3* and *Cldn11* is significantly altered in response to testosterone. As our ability to diagnose PCOS progresses and earlier detection becomes available, understanding this time scale will allow us to better characterize what changes and complications women at different ages with PCOS might experience. The regulatory differences of *Cldn4* at different life stages should also be further explored. Given that *Cldn4* overexpression has been shown to increase cell invasiveness, we have an opportunity to better understand at what age a PCOS afflicted women may be at the highest risk of developing ovarian cancer. To fully understand how the Claudin genes affected by PCOS, the expression of the *Cldn3*, *Cldn4*, and *Cldn11* in the ovaries of women diagnosed with PCOS should be measured. Finally, future studies should further investigate the ramifications that altered Claudin expression has on the general health of the ovary. It's possible that the overexpression of *Cldn3* and *Cldn4* may have more deleterious health effects than just increasing cell invasiveness, and the health effects of the overexpression of *Cldn11* in the ovary has yet to be investigated. Through continued research, advancements in the treatment and understanding of PCOS will allow for more effective treatment of the disease and improved quality of life for those afflicted.

CHAPTER III

CELL CULTURE *IN VITRO* EXPERIMENTS

Introduction

The anovulation that PCOS women experience prevents the release of oocytes from ovarian follicles which results in the infertility. This inability to ovulate is the culmination of a number of normal ovarian functions being disrupted by PCOS such as aromatase and LH/FSH signalling [40]. In addition to a failure in hormonal signaling, a physical inhibition of ovulation could prevent the release of fully developed oocytes. Given that the *CLDN* proteins function to maintain tight junctions between cells, we hypothesized that an overexpression of these genes in the ovarian epithelium could physically block the ovulation of the ovarian follicles thereby contributing to the anovulation. This would contribute to both the infertility and ovarian cysts that characterize PCOS. In order to test this hypothesis, we measured the expression of *CLDN3*, *CLDN4* and *CLDN11* in ovarian epithelial cells in response to various testosterone treatments. Two different human ovarian epithelial carcinoma cell lines, BG-1 [75] and SKOV3 [76], were treated with different concentrations of testosterone and the effect on Claudin gene expression was measured.

Materials and Methods

Cell Culture and Plating

SKOV3 and BG-1 ovarian epithelial cells were cultured in Dulbecco's modified Eagles media F-12 (DMEM-F12); (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum. Cells were trypsinized into suspension and 500,000 cells were plated into each well of a six well plate for testosterone treatment. Twenty-four hours after

plating, and 24 hours before treatment cells were washed with PBS and the media replaced with DMEM F-12 lacking any FBS additive. Cells that were beyond passage 20 not used due to irregular cellular morphology.

RNA/Protein Isolation

RNA and Protein were extracted and isolated from as previously described [64][77]. All cDNA syntheses were carried out using 1µg of RNA. First, samples were treated with DnaseI™ (Invitrogen, Carlsbad, CA) to ensure no genomic DNA remained. This was done in a master mix containing 1µl 10x DnaseI buffer™, 1 unit of DNaseI, and 8µl of DEPC water (Invitrogen, Carlsbad, CA) for 1µg of RNA. RNA was then reverse transcribed for 50 minutes at 42° C in a 20µl master mix containing 50ng of random hexamers (Applied Biosystems, Foster City, CA), 10nM dNTP mix, 5x First Strand Buffer, 0.1M DTT, 40 units of RnaseOut, and 200 units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA was then treated with 0.5µl RNaseH (Applied Biosystems, Foster City, CA) at 37° C for 20 minutes to ensure no mRNA transcripts remain after reverse transcription. RNaseH was then denatured by adding 0.5µl of 25mM EDTA (Invitrogen, Carlsbad, CA). Finally, cDNA was diluted 1:5 prior to PCR by adding 80µl of DEPC treated water.

QRT-PCR

Quantitation of cDNA transcripts was done using a quantitative real-time polymerase chain reaction method. Measurements were taken using the IQ5 Multicolor Real Time PCR Detection System (Biorad, Hercules, CA). PCR was conducted in 20µl

master mixes containing 10 μ L SYBR Select Master Mix (Thermofisher, Waltham, MA), 4 μ L DEPC water, 1 μ L of 10mM primers, and 5 μ L cDNA. PCR was run with an initial 30 second cycle at 95 $^{\circ}$ C to separate DNA strands. The next cycle was set to 95 $^{\circ}$ C for 10 seconds to separate strands, and then 55 $^{\circ}$ C for primer binding and transcript extension. Readings of fluorescence were taken at this step. Experiments were run in triplicate and the average Ct of each triplicate set was calculated. These averages were converted into standardized ratios using the Livak method and *GADPH* as the control standard. Statistical analyses were run using at least three ratios for each experimental group. The primer sets utilized are as follows:

Gene Name	Primer Sequences 5'-3'	Melting Temp	Source
<i>hCLDN3</i>	F: AACACCATTATCCGGGACTTCT R: GCGGAGTAGACGACCTTGG	F: 59.16 $^{\circ}$ C R: 59.86 $^{\circ}$ C	NCBI Primer Blast
<i>hCLDN4</i>	F: GGGGCAAGTGACCAACTG R: GACACCGGCACTATCACCA	F: 58.24 $^{\circ}$ C R: 59.41 $^{\circ}$ C	NCBI Primer Blast
<i>hCLDN11</i>	F: CGGTGTGGCTAAGTACAGGC R: CGCAGTGTAGTAGAAACGTTTT	F: 59.16 $^{\circ}$ C R: 59.86 $^{\circ}$ C	NCBI Primer Blast
<i>hGAPDH</i>	F: AATGGGCAGCCGTTAGGAAA R: GCGCCCAATACGACCAAATC	F: 59.96 $^{\circ}$ C R: 59.97 $^{\circ}$ C	NCBI Primer Blast

Table 2: Primer sequences used for in vitro claudin studies .

Western Blotting

Protein from wild-type and α ERKO mouse ovaries was isolated using Trizol Reagent[™] (Invitrogen, Waltham, MA). 20ug of protein from each sample was denatured at 95 $^{\circ}$ C in 1x Laemmli sample buffer (Biorad, Hercules CA). Proteins were then separated by SDS PAGE gel electrophoresis in Mini-Protean[®] TGX[™] Precast Gels (Biorad, Hercules

CA) at 100V for 1 hour and transferred to a 0.2 μ m pore nitrocellulose membrane (Biorad, Hercules CA) at 100V for 50 minutes. Membranes were stained in Ponceau red stain for 5 minutes while shaking to confirm successful protein transfer. Membranes were then rinsed in Tris-Buffered Saline (TBS) supplemented with 0.1% Tween prior to viewing. After confirming the protein transfer was successful, membranes were incubated in a blocking solution of 5% powdered milk in TBST overnight. Primary CLDN3 antibody diluted 1:1000 in 5% powdered milk in TBS was then applied to the membrane and allowed to incubate overnight. Membrane was then rinsed in TBST and a 1:500 dilution of secondary antibody in milk-TBST solution was applied and allowed to incubate at room temperature for an hour. After another wash in TBST, 1x DAB substrate (Thermo Scientific, Waltham MA) was applied and the resulting staining was photographed. The same protocol was followed with anti-actin antibodies (Invitrogen, Carlsbad, CA) which served as a loading control.

Results

Preliminary experiments sought to evaluate the Claudin expression in ovarian epithelial cells given 24-hour testosterone treatment. Treating SKOV3 ovarian epithelial cells with 10nm, 100nm, or 1000nm testosterone yielded no significant alterations in the expressions of *Cldn3*, *Cldn4*, or *Cldn11* (Figure 6, $p > 0.05$). Given that the enzyme aromatase converts testosterone into estradiol within the ovary, it was also necessary to evaluate estradiol for an ability to alter Claudin expression. Given 24-hour treatments of 10nm, 100nm, or 1000nm estradiol, *CLDN4* expression trended toward decreasing as the concentration of estradiol increased but this trend failed to reach statistical significance given a Student's *t*-test (Figure 7, $p > 0.05$).

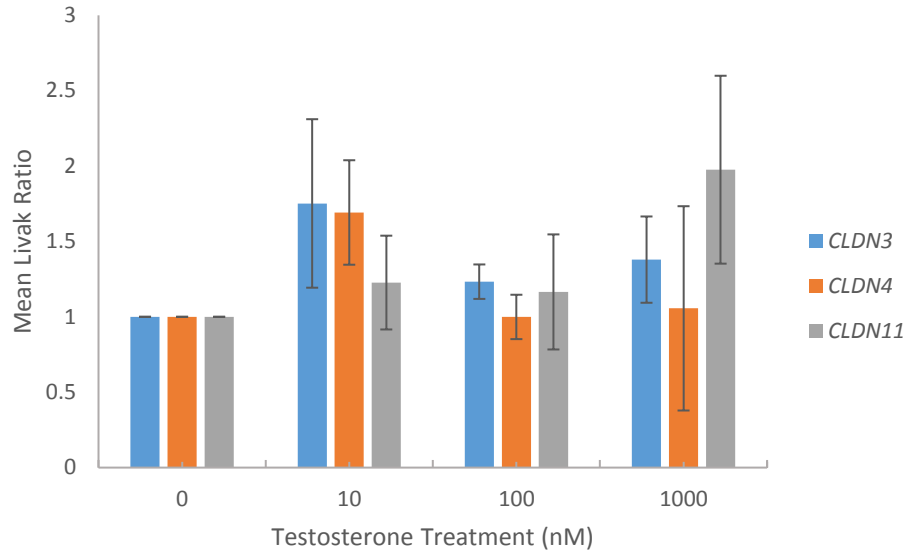


Figure 8: *CLDN* expression in SKOV3 cells treated with testosterone. Claudin expression was measured by QRT-PCR. Expressions are reported as mean *Cldn* livak ratio of testosterone treated groups to untreated (0nM) controls \pm standard deviation ($n = 2$ for all groups).

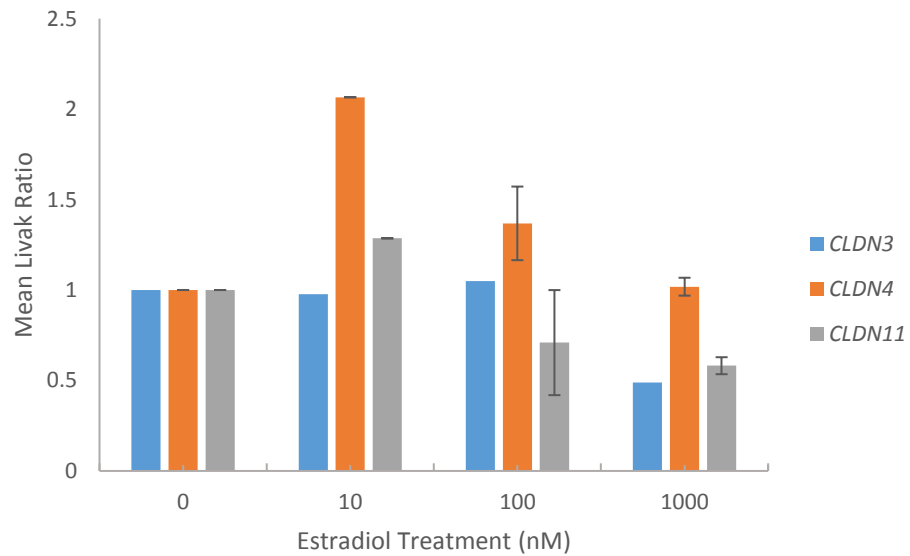


Figure 9: *CLDN* expression in SKOV3 cells treated with estradiol. Claudin expression was measured by QRT-PCR. Values are reported as mean *Cldn* livak ratio of testosterone treated groups to untreated (0nM) controls \pm standard deviation ($n = 2$ for *CLDN4* and *CLDN11*, $n = 1$ for *CLDN3*).

Another ovarian epithelial cell line BG-1 ovarian epithelial cells were also treated with vehicle, 0nM, 10nM 100nM, or 1000nM concentrations testosterone. QRT-PCR yielded no detectable signal for the expression of *Cldn3* or *Cldn11*. *CLDN4* expression was detected, and was found to decrease significantly in response to 10nM and 100nM treatments (Figure 10, $p < 0.05$). Although 1000nM testosterone treated cells showed a decrease in *Cldn4*, it was significantly different from untreated controls (Figure 10, $p < 0.05$).

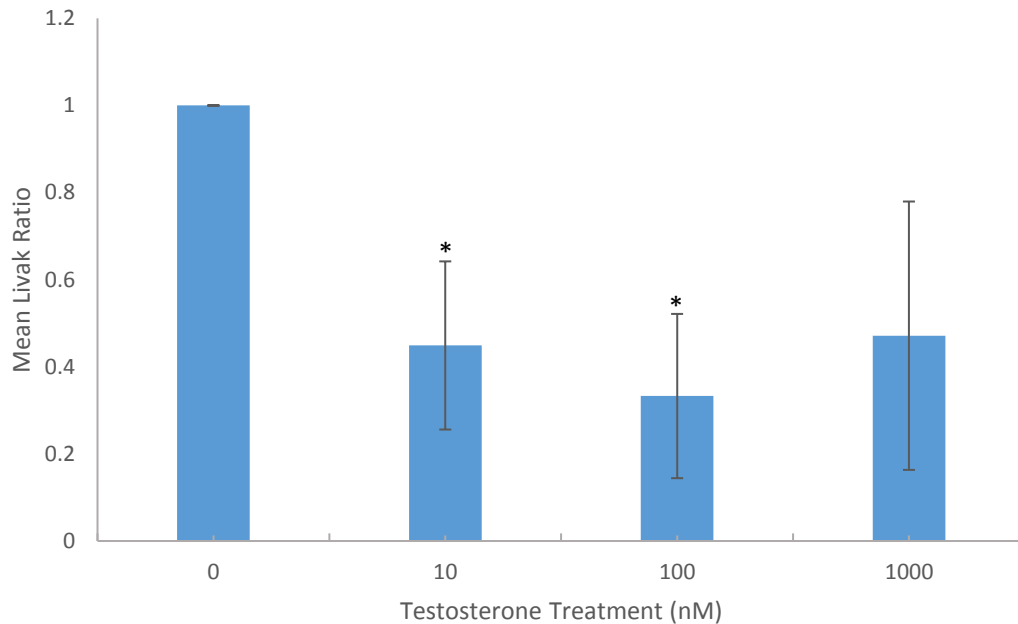


Figure 10: Decreasing *Cldn4* expression in BG-1 ovarian epithelial cells given testosterone treatment. *CLDN4* expression was measured by QRT-PCR. Expression is reported as mean *Cldn4* Livak ratio of testosterone treated groups to untreated control (0nM) group \pm SEM ($n = 4$ for all groups).

Given the upregulation of the Claudin genes at the mRNA level that the prior experiments indicated, a western blot was performed to measure the amount of *CLDN3* protein being produced in the ovaries of α ERKO mice. Conducting a western blot would determine if the possible upregulation of *CLDN3* seen at the level of the transcriptome translates to an increase

at the level of the proteome. Although Ponceau staining showed the protein transfer to the nitrocellulose membrane was successful (Figure 4S), no detectable signal for the presence of *CLDN3* was found (data not shown). Ovarian expression of *CLDN3* may be too low in both wild-type and α ERKO ovaries to be detected by western analysis. More sensitive methods of protein detection would benefit subsequent studies.

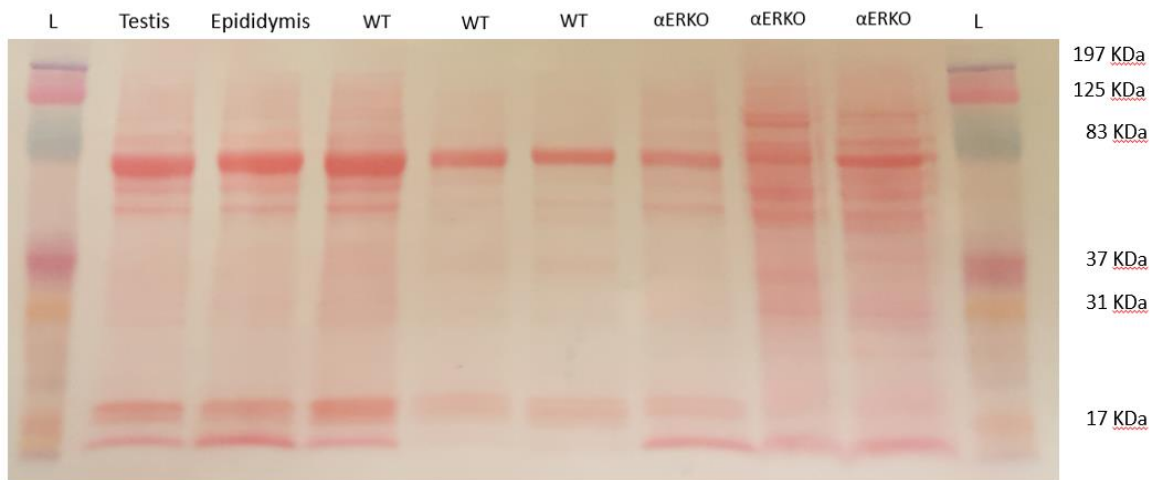


Figure 11: Ponceau Stain for Mouse Ovarian *Cldn3* Western Blot . Protein was isolated from the TRizol fraction of WT and α ERKO ovaries and subjected to SDS-PAGE followed by transfer to nitrocellulose membrane. Ponceau staining demonstrates that protein was transferred successfully.

Discussion

Preliminary work in the SKOV3 ovarian epithelial cells indicated no significant effect of testosterone or estradiol treatment on the expression of *Cldn3*, *Cldn4*, or *Cldn11*. This result lead us to consider whether this indicated that these genes were not regulated by either hormone, or if this issue was a cell line specific insensitivity. A previous study showed that the SKOV3 cell line does not express a functional androgen

receptor and has a frameshift mutation that renders its ER α non-functional [76]. The lack of a functioning ER α could explain why the cells were unresponsive to estradiol. In addition, ER β is still expressed and functional in this cell line [76], therefore it can be hypothesized that the stimulation of ER β by estradiol does not alter the expression of *CLDN3*, *CLDN4*, or *CLDN11* in this cell line. The lack of a functioning androgen receptor in the SKOV3 cell line indicates that an alternate model should be used to study CLDN responsiveness to testosterone. Future studies could introduce an AR or ER α expression vector and measure Claudin expression in response to the appropriate hormone.

Further work in the BG-1 Ovarian epithelial cell line showed that while there was no detectable expression of *CLDN3* or *CLDN11*; *CLDN4* significantly decreased given a 10nM or 100nM testosterone treatment. This data complements the trend of *Cldn4* expression in the DHT treated mice where we also saw a decrease in response to excess androgen supplementation. The lack of Claudin expression in the granulosa cells in response to testosterone suggests that hyperandrogenism does not alter the expression of *CLDN3*, *CLDN4*, or *CLDN11* in granulosa cells, the treatment duration was not long enough, or that a higher concentration of hormone is required. Future studies could further increase the concentration of testosterone that the cells are given, or use an *in vitro* model of hyperandrogenism to evaluate the response of these genes to excess testosterone.

In vitro studies do not perfectly mimic the results obtained from *in vivo* experiments. A new model may need to be developed to better study PCOS in these

ovarian cell lines. Therefore, transforming SKOV3 cells with a functional *Esr1* gene using plasmid DNA would make the cells sensitive to estradiol so its effect on gene expression in the ovarian epithelium could be studied. Another ovarian epithelial cell line would need to be used to study testosterone's effect on Claudin expression in the ovarian epithelium. This would further corroborate the trend seen in *CLDN4* expression in response to testosterone treatment of the BG-1 cells. By exploring additional models of PCOS, we can further understand the etiology of the disease, the long-term effects, and eventually what the most effective mode of treatment may be for those afflicted.

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