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Mutations in the Human Follicle Stimulating Hormone Receptor Caveolin Interaction Motif Cause Increased Basal Activation

Elizabeth Altman
Union College - Schenectady, NY

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By Elizabeth Altman

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Abstract

Over twelve percent of women aged fifteen to forty-five in America suffer from infertility and/or impaired fecundity and over seven million women have used infertility services, such as intrauterine insemination and *in vitro* fertilization. Some cases of infertility may be due to dysfunctional human follicle stimulating hormone (hFSH) signaling. hFSH plays a role in spermatogenesis in males, as well as follicle maturation and estrogen production in females. Problems with either hFSH or the hFSH receptor (hFSHR) decrease fertility in males and cause complete infertility in females. As part of the hypothalamic-pituitary-gonadal axis, hFSH is released from the pituitary gland and binds to hFSHR in the ovaries and testes.

It has been shown that hFSHR interacts with lipid rafts, stiffer regions of the plasma membrane with higher sphingolipid and cholesterol concentrations, and with caveolin, a protein found in certain lipid rafts. This interaction may occur through a specific sequence of amino acids in the 4th transmembrane domain of the hFSHR consistent with a caveolin interaction motif (CIM). Previous work from our lab also suggested that hFSHR signaling is regulated through residency in lipid rafts, such that hFSHR signaling is inhibited as a result of recruitment into lipid rafts.

The current study investigated the effect of mutations of the CBM and of MBCD treatment on hFSHR signaling. It was hypothesized that complex mutation of the CBM would cause increased hFSHR signaling by disrupting interactions with caveolin, preventing inhibition via residency in lipid rafts. Mutants were created using site-directed mutagenesis and expression vectors were transiently transfected into HEK293 cells and hFSHR signaling was qualitatively compared between mutants via western blot. Mutation of two sites in the CBM resulted in increased basal and induced hFSHR signaling. It was also hypothesized that MBCD treatment would lead to increased signaling, but an EPAC-based FRET assay found that cholesterol depletion decreased hFSHR signaling to near nothing. Further understanding of hFSHR signaling regulation would allow for development of new fertility treatments for men and women.

Introduction

About six percent of married American women aged 15 to 44 suffer from infertility, while about 12 percent of all American women in the same age group suffer from impaired fecundity (1). Infertility is defined as the inability to conceive after trying for one year or longer, while impaired fecundity is defined as difficulty getting and staying pregnant to carry a fetus to term (1). Infertility affects men, as well, with the male playing a role in about 35 percent of infertile couples' struggles and being solely responsible for the infertility of about eight percent of couples (1).

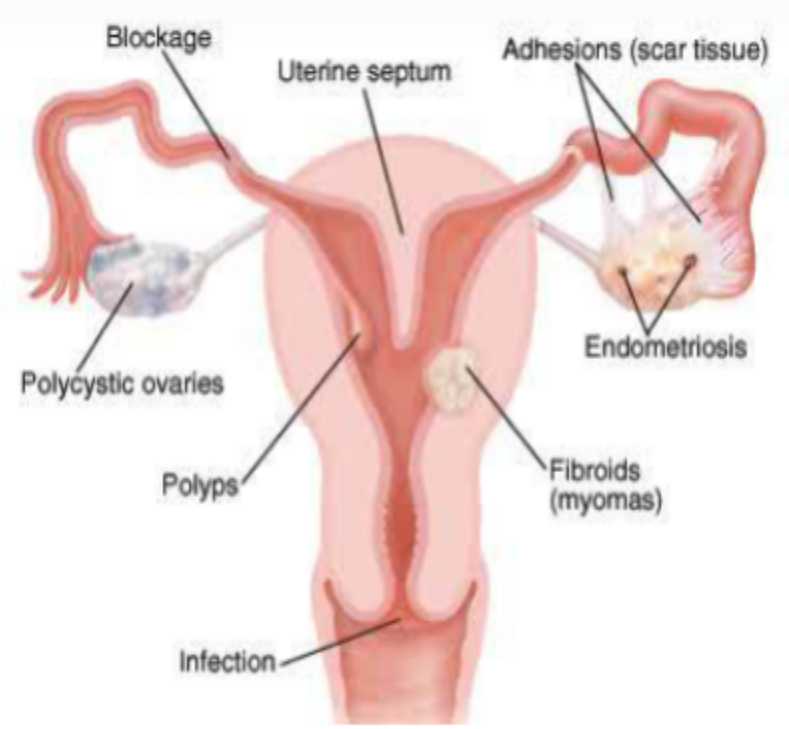

Figure 1: Diagram of possible structural and other health issues that can cause infertility or impaired fecundity. Image taken from IUI treatment in Mumbai. Gestation surrogacy. Web. https://gestationalsurrogacyindia.com/iui-cost-mumbai/.

The process of conception is complicated, leaving a plethora of places where the process can halt or occur improperly. For example, sperm can be immobile or generated with the incorrect proteins needed for recognition by the egg (1). Both of these problems alone can render a couple infertile, even if the woman's reproductive system is functioning perfectly. In women, follicles can fail to correctly mature, the ovaries can fail to release a mature oocyte during ovulation, there can be a structural barrier to the travel of the egg and sperm through the fallopian tubes, or the

endometrial lining can be poorly made and unsuitable for implantation of an embryo (Figure 1) (1). Again, all of these issues can render a woman completely infertile or result in impaired fecundity, and this is not an exhaustive list of complications when trying to conceive.

Infertility treatments include medications, surgery, and artificial reproductive technology (ART), or direct manipulation of the egg and/or sperm to achieve conception. Medications for infertility typically act to balance out abnormal hormone levels that may be causing infertility. In men, these medications normally aim to increase the number and quality of sperm produced, while medications for women typically try to regulate or induce ovulation (2). For example, medications such as Letrozole, hMG, and Follistim act directly on the ovaries, while Clomid acts on the pituitary, all with the same effect of prompting ovulation by correcting issues within the female hormone signaling pathways (2). While not as common as medications and ART, some women require surgery to remove a uterine septum, endometrial polyps, damaged or malformed fallopian tubes, or endometrial tissue that grew abnormally due to endometriosis (2,3).

Intrauterine insemination (IUI) involves the collection of a sperm sample and the direct injection of the sperm into the uterus around the time of ovulation (3). This increases the chances of conception because the sperm are directly placed in the uterus and thus have a shorter distance to travel, making a more direct pathway to conception than during intercourse. IUI, along with hormone treatments, can help a couple conceive in light of low sperm count or low sperm motility (3).

ART represents the most profitable side of the infertility field. ART techniques include egg/sperm donation, *in vitro* fertilization, the use of a gestational surrogate, and assisted hatching (2). ART techniques have the highest success rates, but they are very expensive and are not

always covered by insurance plans (3). *In vitro* fertilization (IVF) is the most common ART used and involves the removal of eggs from the ovaries, fertilization with sperm from the desired donor in the laboratory, and subsequent placement of embryos into the uterus for implantation (Figure 2) (3). Like natural conception, the percentage of women who are able to conceive using IVF decreases with age, with a national success rate of 31% for women under age 35 and 3% for women over age 44 (1).

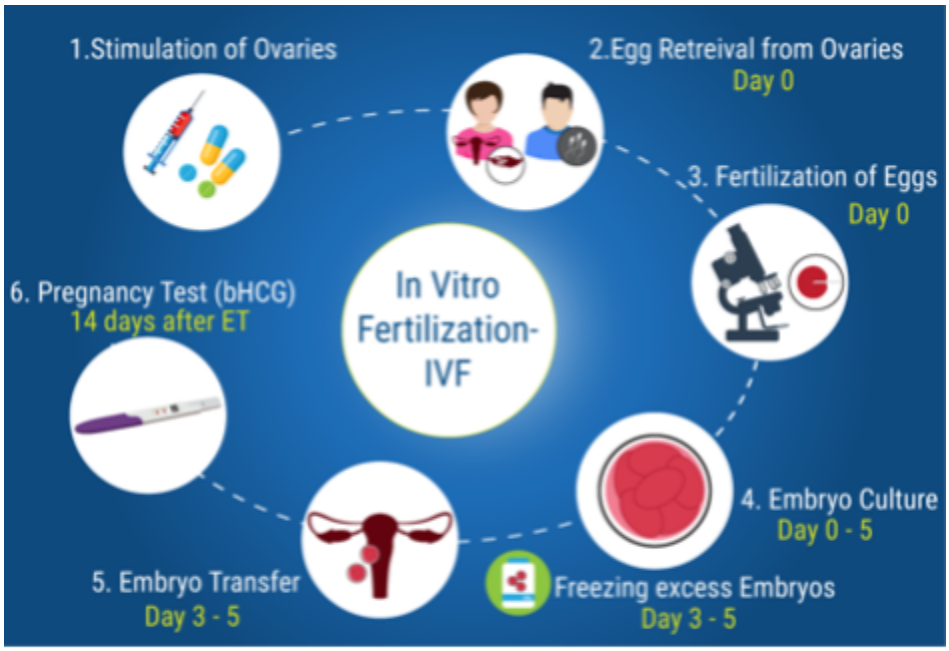

Figure 2: Diagram of steps in *in vitro* fertilization. Image taken from IVF. Indore Fertility Clinic. Web. http://www.indoreinfertilityclinic.com/our-services/infertility-treatment-services/female-partner/in-vitro-fertilization-ivf/

A study from 2010 found that the average annual cost of medication for women undergoing infertility treatments was $1,182, while the average total cost for a woman to successfully conceive in 18 months or less using IVF was over $61,000 (4). Still, not every patient will successfully conceive in 18 months and not every conception via IVF leads to a live birth. In addition to the financial burdens that infertility treatments can place on a patient, infertility can also cause additional stress and poor mental health. Studies between 1992 and 2004 found higher prevalence rates of major depression in infertile couples as compared to their fertile counterparts, with at least one member of 15-54% of infertile couples reporting symptoms of major depression (5). Studies between 2000 and 2004 found similar results for symptoms of

clinical anxiety, with 8-28% of infertile couples reporting that at least one member of the couple was experiencing anxiety (5). Together, these results suggest that infertility puts couples at higher risk of mental health problems on top of the financial burdens that can result from infertility.

Between one and two percent of American women undergo IVF treatment annually, suggesting that the field of infertility is both incredibly profitable and relevant to the American population, justifying further study of human reproduction (4). Moving forward, research regarding human reproduction should aim to increase the percent of patients who successfully conceive while lowering the costs of these treatments.

All infertility treatments are based on the normal functions of the human reproductive system, attempting to emulate those normal functions in patients where the reproductive system does not work correctly on its own. The human reproductive system is regulated by hormones- chemical messengers created in one cell to act on another cell. Most hormones involved in reproduction are endocrine hormones, which are made in one organ, introduced into the bloodstream, and act on target tissues around the body. Endocrine hormones are used to regulate many systems in the body in large axes that include multiple organs and levels of control to allow for the up- and down-regulation of certain organ functions to maintain homeostasis. Such axes include the hypothalamic-pituitary-adrenal axis, or the HPA axis, and the hypothalamic-pituitary-thyroid axis, or the HPT axis (6).

The hypothalamic-pituitary-gonadal, or HPG, axis represents the hormonal pathway that regulates the reproductive system in both males and females. The hypothalamus is a type of neural control center in the forebrain that helps regulate many aspects of homeostasis within the

body, such as temperature control, hunger, thirst, and circadian rhythms. Hanging directly below the hypothalamus in the midbrain is the pituitary gland, which is composed of many different cell types. When stimulated by hypothalamic hormones, these cells secrete various hormones that are named in correlation with the cell types that produce them. In the HPG axis, the hypothalamus releases gonadotropin-releasing hormone, GnRH, in waves. GnRH targets the gonadotrophs in the anterior pituitary, which release gonadotropin hormones, including human luteinizing hormone, hLH, and human follicle stimulating hormone, hFSH. These hormones bind to G protein-coupled receptors on gonadal cells, activating signaling pathways that lead to correct reproductive function (6).

In males, hLH targets the Leydig cells in the testes to stimulate testosterone production. hFSH and testosterone together target the Sertoli cells of the testes to stimulate spermatogenesis, or the formation of sperm cells. Spermatogenesis prompts the Sertoli cells to begin producing inhibin, and inhibin and testosterone act together on the pituitary gland to inhibit the production of more hFSH, finishing the feedback loop that governs male reproduction.

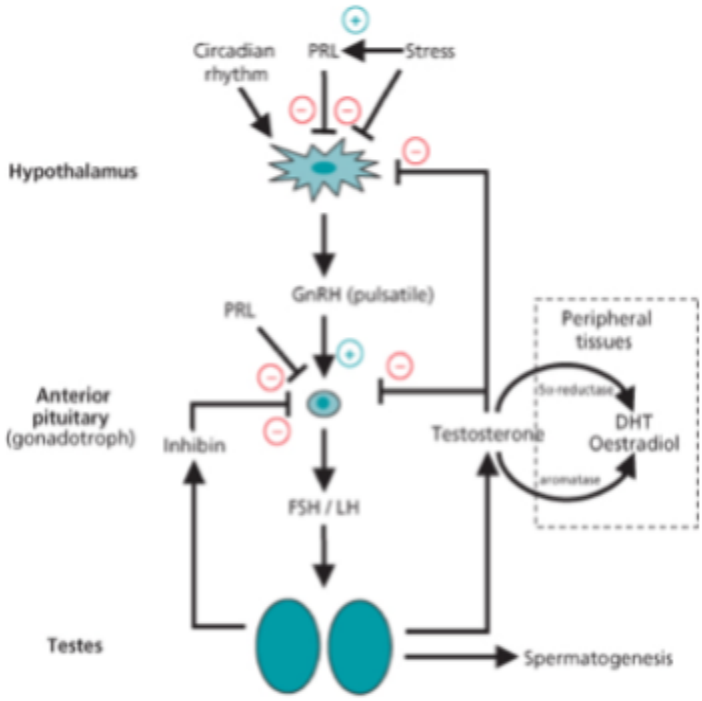

Figure 3: Hormonal control of the male reproductive system. Red minus signs indicate inhibitory effects. Blue plus signs indicate stimulatory effects. Image taken from Holt RI, Hanley NA. Essential Endocrinology and Diabetes. 6th ed. West Sussex, UK: Wiley-Blackwell; 2012.

Testosterone also inhibits GnRH production from the hypothalamus to shut down the signaling pathway earlier and stimulates the development and maintenance of male secondary sex characteristics (Figure 3) (7).

In women, hFSH and hLH both act on the follicular cells of the ovaries, leading to estradiol and progesterone production, as well as follicle maturation. Follicles are small sacs within the ovaries that nurture the developing egg cells. Progesterone signals for growth of the endometrium to prepare for pregnancy, and estradiols support development and maintenance of female secondary sex characteristics. As the follicles mature, they produce inhibin, similar to the Sertoli cells of the testes in men, which acts on the pituitary to decrease hFSH production. A surge of hLH from the pituitary in the middle of the menstrual cycle causes the follicle to burst, releasing the egg in an ovulation event. After ovulation, the ruptured follicle cell is called a corpus luteum and produces progesterone that inhibits hFSH and hLH production from the gonadotrophs of the anterior pituitary and GnRH production from the hypothalamus, shutting down hFSH production (Figure 4). The

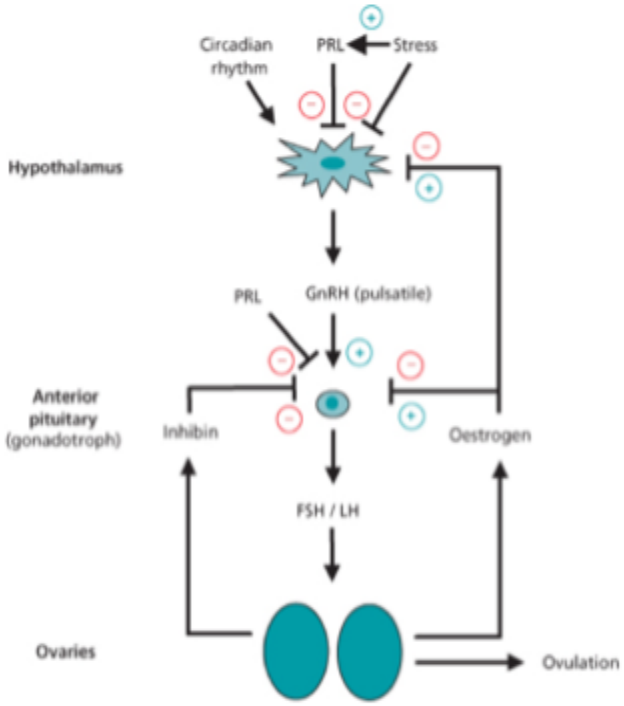

Figure 4: Hormonal control of the female reproductive system. Red minus signs indicate inhibitory effects. Blue plus signs indicate stimulatory effects. Image taken from Holt RI, Hanley NA. Essential Endocrinology and Diabetes. 6th ed. West Sussex, UK: Wiley-Blackwell; 2012.

endometrial lining will either be shed and re-formed or maintained to support a pregnancy, depending on whether or not the ovulated egg becomes fertilized and successfully implants (7).

Yet, hormones are only as useful as their receptors, so a functioning reproductive system relies heavily on working receptors for hFSH and hLH in the gonads. The hFSH receptor, hFSHR, is a G protein-coupled receptor. G protein-coupled receptors are membrane proteins with 7-transmembrane domains, an extracellular domain that receives signals from the cell's environment, and an intracellular domain that allows for the propagation of the extracellular signal through to the interior of the cell (Figure 5). In typical G protein-coupled receptors, the intracellular domain interacts with a heterotrimeric G protein. These G proteins consist of alpha, beta, and gamma subunits. When the extracellular domain of a G protein coupled receptor binds a hormone from the cell's environment, the intracellular domain catalyzes the exchange of a guanosine diphosphate (GDP) molecule for a guanosine triphosphate (GTP) molecule bound to the alpha subunit of the heterotrimeric G protein. This causes the alpha subunit of the G protein to separate from the beta/gamma joint-subunit.

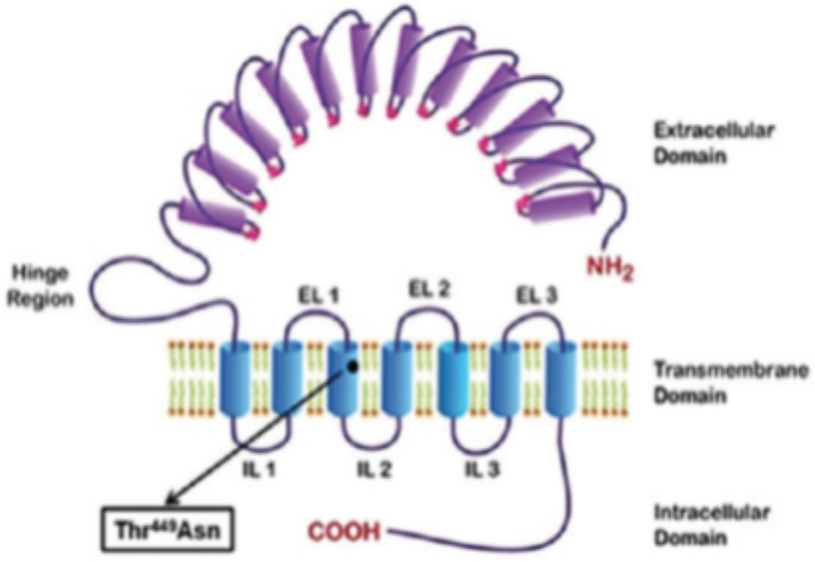

Figure 5: FSHR structure. Image taken from Chauhan, et al. (2015).

Both the alpha subunit and the beta/gamma subunit can signal inside the cell by activating

secondary signaling molecules, allowing for complex intracellular responses to one extracellular signal. This signaling will continue until the alpha subunit of the G protein catalyzes the hydrolysis of GTP to GDP and Pi, when the beta/gamma subunit re-joins the alpha subunit. The G protein remains in its heterotrimeric state, bound to GDP, until the G protein-coupled receptor is activated again to catalyze the exchange of GDP for GTP once again (9).

The intracellular signaling pathways activated by hFSHR are complex and not completely understood, but there are three secondary signaling molecules that are of importance in both the ovaries and the testes for research purposes. Through activation of adenylyl cyclase, adenosine triphosphate (ATP) is converted to cyclic adenosine monophosphate (cAMP) which then allows for the phosphorylation of cAMP response element binding protein (CREB) to phospho-CREB by protein kinase A (8). hFSHR signaling also yields the activation of beta-arrestin, allowing for activation of the MAPK pathway, resulting in the phosphorylation of p44 to phospho-p44 (8). cAMP, phospho-CREB, and phospho-p44 can be detected in biochemical assays and, therefore, used to identify hFSHR signaling based on the activation of these intracellular pathways.

Since human reproduction is a complex process, and hFSH, along with other hormones, has multiple roles in the process, there must be a way to regulate hFSH signaling within the cells to allow for complex regulation of downstream effects. The signals need to be timed correctly so that hFSHR signaling is only active when necessary, implying that a regulation mechanism exists to halt hFSHR signaling when the downstream effects are no longer needed. Previous studies in our lab have shown that hFSHR is present in lipid rafts fractions of a discontinuous sucrose gradient, suggesting an interaction between the two (9). This interaction may provide a

mechanism for signaling regulation, given that lipid rafts have been found to play a role in signaling (10). Lipid rafts are areas within the plasma membrane that have higher cholesterol and sphingolipid concentrations than the rest of the membrane, making them stiffer than the membrane around them (9). The difference in membrane composition between lipid rafts and the other regions of the membrane is thought to allow for colocalization or de-localization of signaling components, allowing for increased or decreased signaling capabilities, respectively (10). It has also been found that lipid rafts recruit signaling molecules into them, further suggesting that they allow for spatially-coordinated approaches to signaling, bringing together molecules that, until now, were thought to float freely through the plasma membrane (11). For example, IgE signaling in basophil cells seems to occur more efficiently when coordinated via lipid rafts (10).

Caveolae are a specific type of lipid raft that contain at least one of the caveolin proteins, a type of scaffolding protein, and take on a cone-like shape within the membrane, which is structured mostly from caveolin-1 and caveolin-3 (Figure 6) (12). Like other

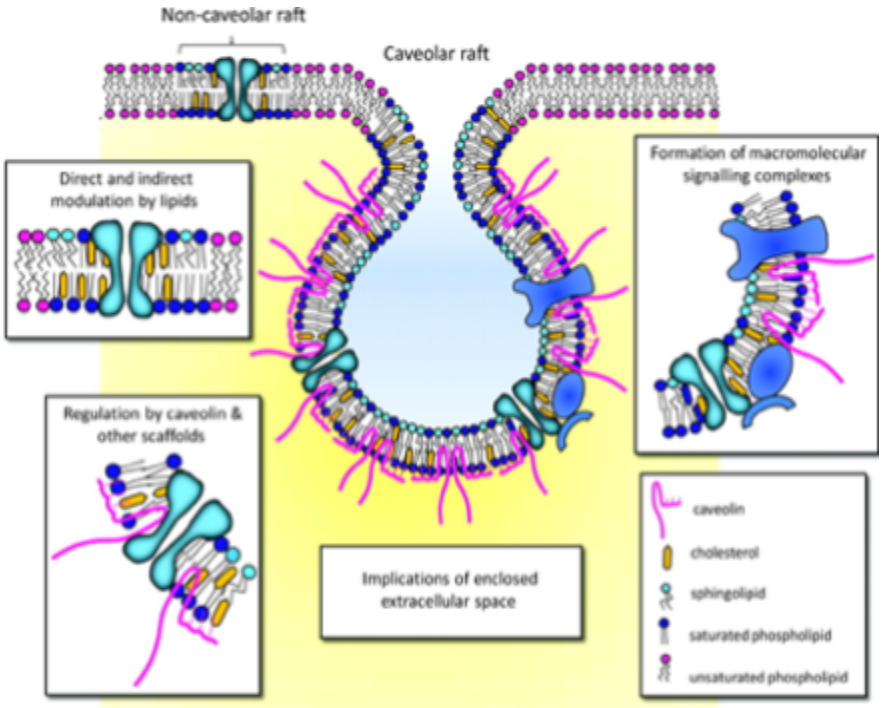

Figure 6: Structure of general lipid rafts and caveolaelipid rafts. Image taken from Dart C. Lipid microdomains and the regulation of ion channel function. J Physiol. 2010 Sep 1;588(Pt 17):3169-78.

types of lipid rafts, caveolae are known to have high concentrations of signaling molecules, making them interesting in hormone signaling regulation (12). Interactions between caveolin and G protein-coupled receptors have been investigated broadly with results suggesting that caveolin plays a role in inhibiting G protein-coupled receptor signaling pathways by trafficking proteins into lipid rafts (13). A study of caveolin knockout mice further supports the idea that caveolin plays a role in regulating signal transduction. Knockout mice lines were generated for caveolin-1, -2, and -3, and demonstrated pathologies related to signaling molecules and ion channels in the cardiovascular, pulmonary, urogenital, skeletal, neural, and endocrine systems, as well as increased susceptibility to cancer (14).

Scientists have identified a specific sequence of ten amino acids that is found in many proteins that interact with caveolin, also called the caveolin binding motif or caveolin interaction motif (15). This sequence of ten amino acids follows a φXφXXXXφXXφ pattern, where φ represents an aromatic amino acid residue, which are considered to be the functional units of this motif, and X represents any other amino acid residue (15). This motif is found in the hFHSR protein sequence, specifically in the fourth transmembrane domain. In order, the aromatic residues in this motif when found in the hFSHR sequence are colloquially referred to as the A, B, C, and D sites, which lie at residue positions 479, 481, 486, and 489, respectively. When viewed in a three-dimensional model, the B site is oriented opposite from the A, C, and D sites, suggesting that the B site may play a smaller role in the function of this motif because it is separated from the other functional units of the binding motif (Figure 7). Sequences of this pattern have been found in up to thirty percent of human proteins with known sequences, but not all of them appear to interact with caveolin (15). However, previous evidence from our lab also

suggests that hFSHR co-immunoprecipitates with caveolin-1 (9). Since lipid rafts are thought to play a role in signaling regulation, caveolin has been found to move proteins into lipid rafts, the hFSHR sequence includes a caveolin binding motif, and hFSHR has been found to co-immunoprecipitate with both lipid rafts and caveolin-1, this motif is of interest in further understanding hFSHR signaling regulation.

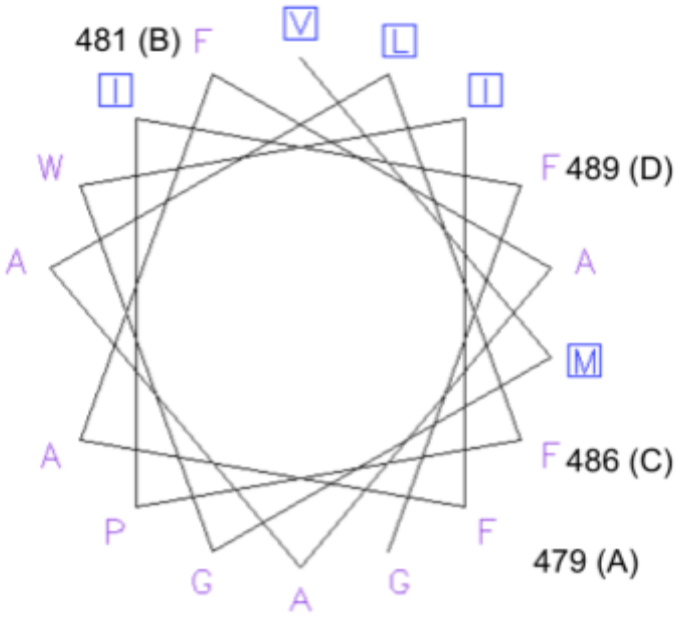

Figure 7: Helical wheel diagram of the CBM of hFSHR suspected caveolin binding motif with the four aromatic residues labeled. Note the orientation of B compared to that of A, C, and D.

Additionally, the role of lipid rafts should be investigated independently of caveolin, as the lipid rafts may play a role in signaling regulation separate from that of caveolin. One way to investigate lipid rafts directly is to disrupt their structure by removing the cholesterol from them. Methyl-beta-cyclodextrin, MBCD, has been shown to successfully lower cholesterol levels in cells, and established protocols have been suggested on how to do this without killing the cells (16). MBCD and other cyclodextrins have a large hydrophobic cavity which causes cholesterol to leave the cell membrane and enter the cavity, thus lowering the effective cholesterol concentration of the cell (15). Given the higher concentration of cholesterol in lipid rafts versus the rest of the plasma membrane, the lipid rafts would be affected more severely by MBCD treatment, causing them to lose their unique composition and become more like the rest of the plasma membrane.

Human thyroid stimulating hormone, hTSH, is an endocrine hormone in the hypothalamic-pituitary-thyroid axis with a structure very similar to that of hFSH (6). The hTSH receptor (hTSHR) has been found to interact with caveolin during signaling regulation (17). Lipid raft depletion using MBCD and caveolin knockdown both resulted in increased hTSH signaling, identified by looking for increasing cAMP concentrations, as cAMP is also produced in the intracellular pathways controlled by hTSHR activation (17). After MBCD treatment, hTSH signaling could not be regulated by lipid rafts because there were no lipid rafts present. Similarly, caveolin knockdown depleted the number of caveolae the cell could produce, making it unlikely that hTSH signaling could be regulated by caveolae, specifically. The increased hTSH signaling identified here after MBCD treatment and after caveolin knockdown suggests that interactions with lipid rafts in general and/or caveolae, specifically, are regulating hTSH signaling pathways in the cells (17).

A similar study to that performed with hTSH and hTSHR was performed in our lab using hFSH and hFSHR, which showed that depletion of the cholesterol content of a cell using MBCD is correlated with an increase in hFSH signaling as identified via

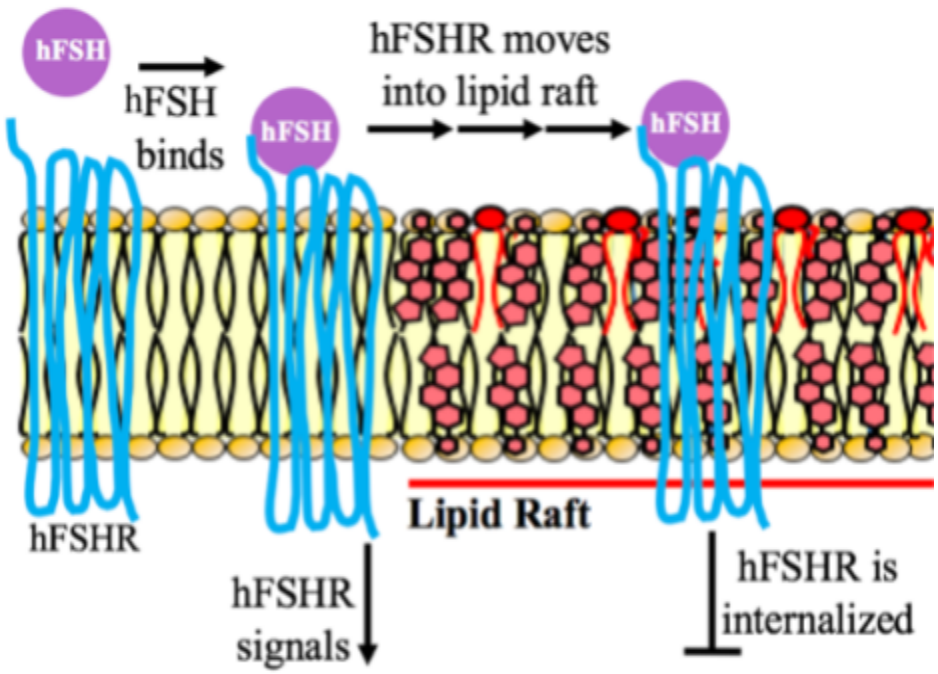

Figure 8: Model of hFSHR signaling based on previous research in our lab. Image taken from Wells & Cohen (2018).

western blot probing for phospho-CREB and phospho-p44. This suggests that hFSH signaling is regulated via residency in lipid rafts. These results further support the aforementioned studies linking receptor-lipid raft interactions to signaling regulation. The author of this study concluded that hFSR binding to hFSHR caused internal signaling from hFSHR, along with relocation of hFSHR to lipid rafts, and that this relocation signaled for internalization of the receptor (Figure 8). Internalization of a receptor halts its signaling, so it was concluded here that residency in lipid rafts causes a net decrease in hFSHR signaling. Thus, when the lipid rafts were eliminated by MBCD treatment, there was no regulation mechanism in place to stop the receptor from signaling continuously (18).

To evaluate the role of caveolin in this regulation mechanism, some hFSHR mutants were created using site-directed mutagenesis of the aromatic residues in the caveolin binding motif. Preliminary testing of these mutants via western blotting probing for phospho-CREB found possibly increased signaling in the mutants as compared to the wild type receptor (18).

Based on the data suggesting hFSHR signaling is regulated by lipid raft residency and the preliminary results suggesting a possible role of caveolin in the regulation, the current study sought to further investigate the role of lipid rafts and caveolin in hFSHR signaling regulation. More complex hFSHR caveolin interaction motif mutants were created with various combinations of changes at the A, B, C, and D sites and the signaling from these mutants was analyzed using western blotting and an EPAC-based FRET assay to investigate the role of the interaction between caveolin and the receptor in signaling. Based on the hypothesis generated by Wells and Cohen (2017), it was hypothesized that this study would find increased signaling from the hFSHR mutants due to the lack of hFSHR-caveolin interactions to allow for signaling

regulation. Further, due to the spatially isolated nature of the B site in the caveolin binding motif, it was hypothesized that mutation of the B site would yield a smaller change in signaling than mutations of the A, C, and D sites due to its orientation away from the other aromatic residues.The EPAC-based FRET assay was also used to analyze hFSHR signaling after MBCD treatment to investigate the role of lipid rafts more generally on signaling. Similar to our other hypotheses, we hypothesized that MBCD treatment would cause increased signaling because there would be no lipid rafts for the receptor to reside in, meaning there could be no signaling regulation through residency in lipid rafts.

Methods

Cell lines:

The human embryonic kidney 293, or HEK293, cell line was used in all experiments because it is easily transfected (19). The HEK293 line was transiently transfected to express mutant hFSHR sequences, as described below, and two lines of HEK293 cells were maintained for the CANDLES assay, one stably transfected with a genetically modified version of the exchange factor directly activated by cAMP (EPAC) protein and one with the wild type hFSHR sequence. Lines were maintained using standard eukaryotic culture methods and antibiotic selection to maintain transfection status.

Mutagenesis:

The New England Biolabs Q5 Site-Directed Mutagenesis Kit and protocol was used to mutate the caveolin interaction motif within the hFSHR gene on a pIRESneo plasmid template

(20). Primers were developed using the NEBaseChanger online primer design tool from New England Biolabs and ordered to introduce the desired mutations (phenylalanine to leucine) in the motif at the A, B, C, and D sites. Mutagenesis was completed per the Kit protocol, consisting of PCR with the mutant-inducing primers, a KLD reaction to form plasmids from the PCR product, and heat shock to transform the plasmids into the New England Biolabs 5-alpha Competent *E. coli* (High Efficiency) (#C2987) chemically competent cells provided with the Kit (20). This site-directed method allowed for complex mutations to be made on the same plasmid by carrying out the mutagenesis protocol on an already-mutated plasmid. For example, an AB mutant could be made by using mutation-inducing primers specific to the B site on a plasmid already mutated at the A site.

The plasmid preparations with mutant hFSHR sequences were stored in a zero degree Celsius freezer until transfected into cells. Mutations were designed specifically to put a new restriction enzyme site at each mutation site, allowing for detection of successful mutagenesis by restriction enzyme digest. Successful mutation of the A site added a BamHI digestion site, while mutation of the B site resulted in a NheI digestion site, of the C site resulted in an EarI digestion site, and of the D site resulted in an AvrII digestion site. Restriction digests with all enzymes were performed, and the products run through a 2% agarose gel electrophoresis, to ensure successful mutation of all desired sites.

Transfections:

HEK293 cells were split lightly into 6-well tissue culture plates and left to grow for about 72 hours. The plasmid preparations were thawed to room temperature prior to transfection. The

Mirus TransIt-293 transfection reagent was used according to its published protocol to encourage uptake of the hFSHR mutant plasmid DNA by the HEK293 cells (21). After treatment with the transfection solution described in the protocol, cells were left to incubate for 24-72 hours before proceeding with isolation of protein for western blot analysis or replating of the cells for a CANDLES assay.

Isolation of protein from transfected cells:

Cells were incubated at 37 degrees Celsius in serum-free media for one hour to induce a state of starvation and eliminate all non-essential metabolic reactions and signaling activation prior to protein isolation. Cells were then treated with hFSH for varied amounts of time. Urinary hFSH was added at a four micromolar concentration, and pituitary hFSH at a two micromolar concentration. Protein samples were obtained from the transiently transfected cells using lysis buffer and douncing to break open the cells, followed by centrifugation to separate all cell debris from the protein fraction. The protein fractions were stabilized with a sample dye containing EDTA and stored in a zero degrees Celsius freezer until run in a SDS-PAGE/western blotting protocol.

Signaling analysis using western blotting:

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins samples isolated from each cell type by size. The gels were made using a stacking gel and running gel to help ensure even run times for each protein within a lane.

After SDS-PAGE, the protein samples were transferred to a Immobilon-P transfer membrane using a semi-dry transfer method and a BioRad Trans-Blot SD Semi-Dry Transfer cell. All membranes were probed first for phospho-p44 using a goat/anti-rabbit secondary antibody to evaluate for activation of hFSHR signaling in the cells, followed by probing for follitropin receptor with mAB 106.105, an hFSHR specific monoclonal antibody and a goat/anti-mouse secondary antibody to evaluate for hFSHR presence. Together, both probing results allowed for a relative comparison of signaling between mutants, with second probing images providing context for the efficiency of each transfection reaction.

Signaling analysis using CANDLES assay:

The cyclic-AMP indirect detection by light emission from sensor cells, CANDLES, assay is an EPAC-based FRET assay originally described in a 2014 paper out of Finland (22). HEK293 cells stably transfected with a gene coding for a genetically-engineered version of the EPAC protein were sent to us from the authors of the paper for use here. These EPAC cells serve as the sensor cells for this assay. The EPAC protein expressed in these cells has a FRET donor and acceptor added on either end of the protein sequence. When EPAC is not bound to cAMP, the FRET donor and acceptor are physically close to each other and FRET occurs, causing the release of 530 nanometer light when the molecule is excited by 430 nanometer light. When EPAC binds cAMP, it undergoes a conformational change that moves the FRET acceptor away from the donor so that FRET no longer occurs. The protein then emits 475 nanometer light, instead (22).

When these EPAC sensor cells are co-cultured with donor cells, the HEK293 cells transfected with the wild type hFSHR gene, the cells form gap-junctions with each other, allowing for transfer of cAMP from the donor cells to the sensor cells. Since cAMP is produced as a result of hFSHR activation, and the ratio of non-FRET to FRET light emission represents the amount of cAMP present, this creates a relationship between the hFSHR signaling in the donor cells and changes in the non-FRET to FRET ratio detected from the sensor cells. Thus, an increase in this ratio correlates to an increase in cAMP levels, which indicates an increase in hFSHR signaling (22).

The EPAC cells are also activated by forskolin in a way that leads to cAMP production, allowing EPAC cells to serve as a positive control by being plated by themselves and treated with forskolin. HEK293-hFSHR cells plated without EPAC cells serve as a negative control, given that these cells should not be florescing at all.

The donor and sensor cells are plated into a 96-well fluorescence-compatible tissue-culture-treated plate and left to incubate for 48 hours. The ratio of HEK293-EPAC cells to HEK293-hFSHR cells was manipulated to find the ideal ratio to be 1:1. The cells

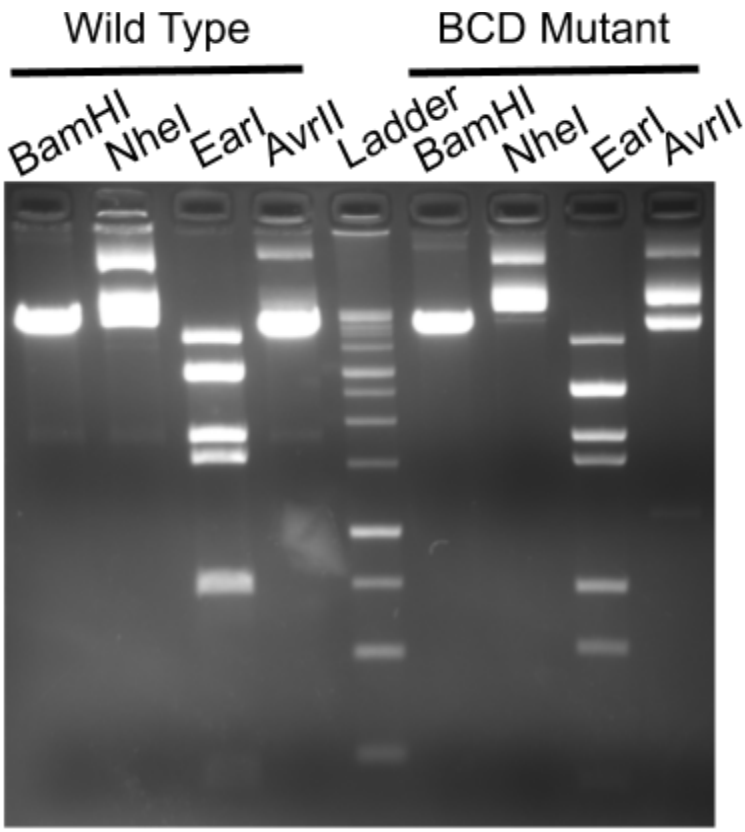

Figure 9: 2% agarose gel electrophoresis of plasmid digests with site-specific enzymes. Note differences between the wild type and mutant plasmids cut with Nhe, EarI, and AvrII, indicating successful BCD mutagenesis.

are first put in serum-free media and incubated at 37 degrees Celsius for one hour to induce a state of starvation and eliminate all non-essential metabolic reactions and signaling activation prior to the assay. If applicable, MBCD treatment was also given during this one hour incubation period by adding MBCD to the serum-free medium at a five micromolar concentration. A "blank" read is taken on a fluorescence spectrophotometer to obtain the level of fluorescence emitted with initial levels of cAMP in the EPAC cells. Cells are then treated with urinary hFSH added at a four micromolar concentration or pituitary hFSH at a two micromolar concentration. Immediately after spiking with hFSH, a kinetic read is taken on the spectrophotometer, tracking changes in the non-FRET/FRET ratio over time while the cAMP levels change due to activation of hFSHR.

Results

Mutagenesis:

Most of the complex mutations of the caveolin binding motif were successfully made using the site-directed mutagenesis technique, except the

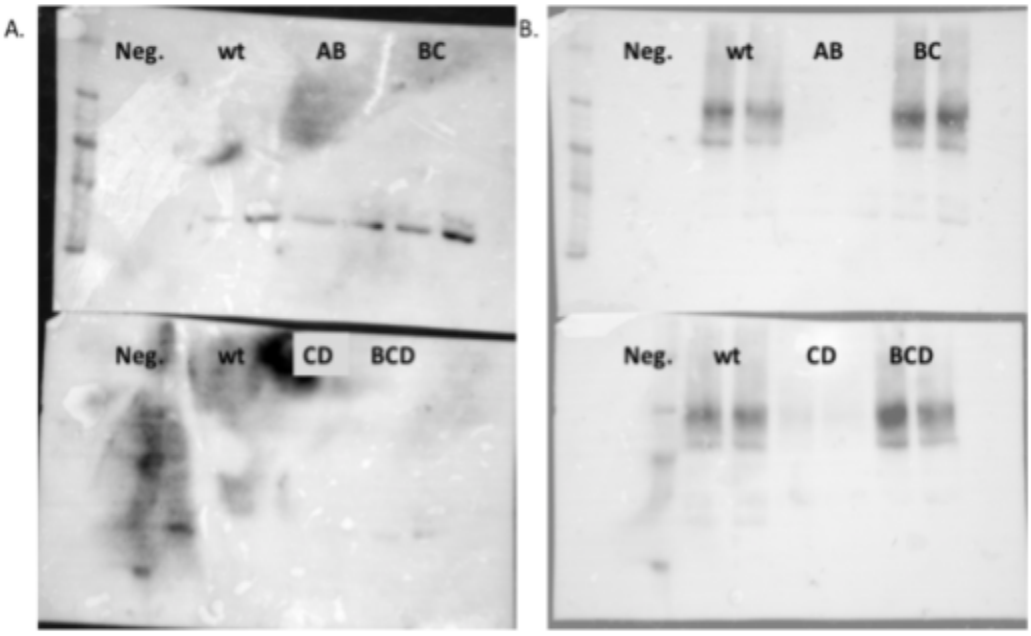

Figure 10: Double mutants show increased basal & hFSH induced signaling by western blot analysis A.) Probed for phospho-p44. B.) Probed for hFSHR.

AC, AD, BD, and ABD mutants. All mutants were verified via restriction digest and agarose gel electrophoresis, indicating successful mutagenesis (Figure 9). Four attempts at making the ABD mutant failed.

Signaling from hFSHR caveolin interaction motif mutants:

Western blots of hFSHR mutants probed for phospho-p44 and the follitropin receptor showed increased basal and induced activation from double mutants, or receptors mutated at two of the four aromatic sites in the caveolin interaction motif, but gave mixed results for the triple mutants (Figures 10 and 11). Compared to the wild type receptor, the phospho-p44 bands on the blots for the double mutants were more intense for both basal and induced signaling when controlled for the amount of receptor in the cells, as shown by the follitropin receptor bands (Figure 10).

Signaling seemed to vary across the triple mutants, as some showed no signaling from the BCD triple mutant, as shown in

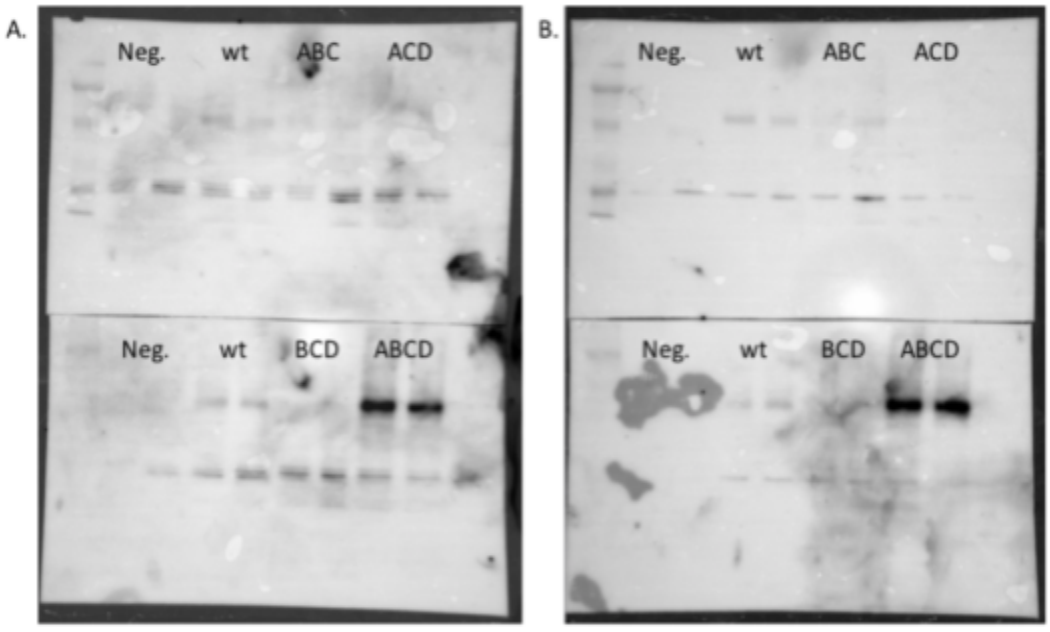

Figure 11: Triple mutants show increased induced signaling in all cases; ACD & BCD mutants show possible increased basal signaling. The Quadruple mutant shows decreased response to hFSH compared to the wt receptor A.) Probed for phospho-p44. B.) Probed for hFSHR.

Figure 10, while others showed increased induced activation from the ABC triple mutant and increased basal activation from the ACD triple mutant, as shown in Figure 11. Bands for the quadruple mutant for both phospho-p44 and the follitropin receptor showed lane streaking and were too thick to interpret (Figure 11).

A CANDLES assay was run using the ABC, ACD, BCD, and ABCD mutants, as well. The results of this assay were inconclusive; the positive controls did not respond as expected, so no conclusions were drawn.

Signaling after MBCD treatment:

Control cells showed near linear increases in non-FRET/FRET ratio with increasing hFSH concentration, as expected (Figure 12). Cells treated with MBCD showed almost no changes in non-FRET/FRET ratio at all doses of hFSH as compared to control cells (Figure 12). The differences in the net change in FRET ratio between the control and MBCD treated cells were statistically significant at the 0.2,

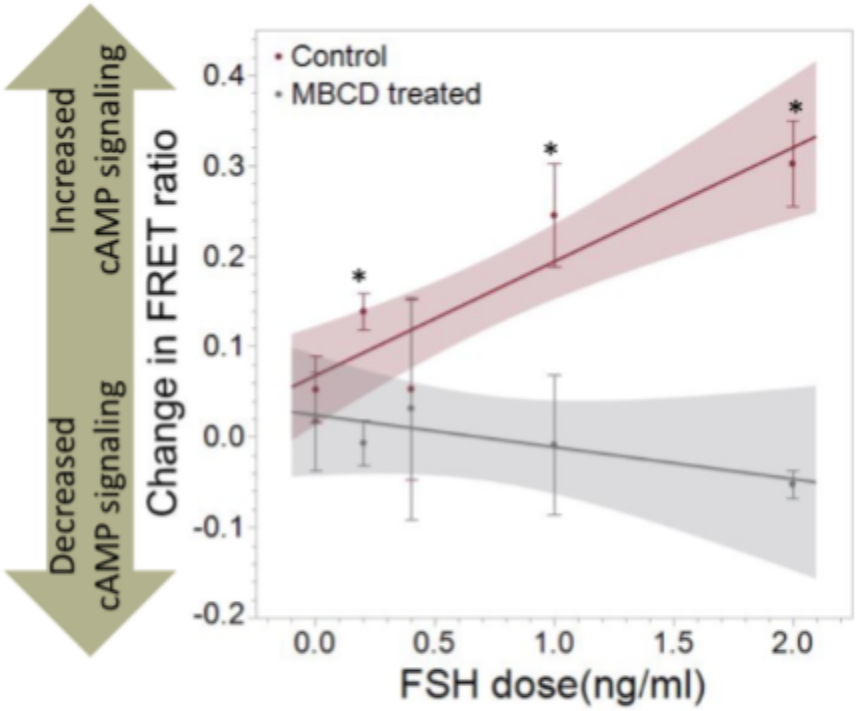

Figure 12: Treatment with MBCD ameliorated hFSH induced cAMP production as measured in a CANDLES assay. A dose dependent increase in the FRET ratio is seen in the control cells but not in the MBCD treated cells. Asterisks denote statistically significant differences in the change in the FRET ratio between the control and MBCD treated cells ($p < 0.05$).

1.0, and 2.0 nanogram per milliliter concentrations of hFSH, with a statistical significance level of $p < 0.05$ (Figure 12).

Discussion

Site-directed mutagenesis was mostly successful to make the mutant hFSHR plasmids, but failed when attempting to insert a mutation at the D site. It is possible that the mutant primers for the D site did not bind to the template DNA strongly enough to allow for successful amplification during the polymerase chain reaction. Since the C and D sites are so close to each other in the sequence, the mutation of the D site after the C site may be more difficult because the mutant primers for the D site are not designed to bind to the mutated C site. Future research should consider using a splicing by overlap extension method to mutate the D site, which may help get around this issue without buying all the possible combinations of primers in an attempt make the polymerase chain reaction more successful.

The increased basal and induced receptor activation seen from the mutant receptors in the western blots supports the hypothesis that mutating the caveolin interaction motif would lead to increased signaling, presumably due to interruption of interactions between hFSHR and caveolin (Figure 10). Based on the data from last year's theses, it would seem that this is occurring because caveolin helps traffic the receptor into lipid rafts, where their signaling is shut off. However, we have no conclusive results regarding the triple mutants or the quadruple mutant (Figures 10 and 11). The mixed results from the triple mutants cannot be reliably interpreted, and the bands representing both the phospho-p44 and follitropin receptor isolated from the quadruple mutant are too concentrated to interpret. The streaking in the lanes for the quadruple mutant,

along with the thicker, blurrier nature of the bands, suggests that the lanes were overrun with protein during the SDS-PAGE protocol and possibly indicate an error during the BCA assay. Due to the inconclusive results for the triple and quadruple mutants, no conclusions can be made about the relative role of the B site versus that of the A, C, and D sites in the caveolin interaction motif.

The CANDLES data regarding MBCD treatment does not support our hypothesis, indicating that decreased cholesterol content in the cell, and presumably decreased lipid raft prevalence in the plasma membrane, actually leads to a drop hFSHR signaling, even with increased hFSH concentration (Figure 12). If cholesterol was playing a role in signaling through lipid rafts, as previously thought, the signaling should have increased because lipid raft residency would have been prevented and the receptor would never have been internalized. The difference in signaling between the control and MBCD treated cells here suggests that cholesterol is integral for hFSHR signaling, just not through lipid rafts.

This data matches some older data from our lab (23). Repeated quantitative radioimmunoassays found that, compared to control cells, cells treated with MBCD showed barely any cAMP production, just like is seen here in the CANDLES assay. However, this data also shows that when the MBCD was washed off of the cells and they were allowed to recover for an hour, cAMP production began to increase in the same pattern seen in the control cells, demonstrating that the MBCD treatment was not killing the cells to result in a lack of cAMP production, but rather was affecting cAMP production in a different way. Lastly, when the cells were treated with cholesterol after MBCD treatment and allowed to recover, cAMP production increased more rapidly, still in the same pattern as seen in the control cells (23). This

radioimmunoassay protocol has not been performed in our lab for about ten years because it exposes the assayer to radioactivity, which has been shown to correlate with higher incidence of cancer. The CANDLES assay is the first quantitative assay we have been able to perform in the lab since the radioimmunoassay, so the success of the method is significant for ongoing research, even if the data does not support our current hypotheses. Further, the data from the CANDLES assay is more compelling because it agrees with the last quantitative data we were able to collect in the lab.

These results could be explained if caveolin and/or cholesterol were involved in hFSHR signaling in other ways. For example, the human beta-2-adrenergic receptor has been found to bind to cholesterol and subsequently take on an alternate structure (24). This alternate structure has been found to be the more functional structure of the receptor, making cholesterol an allosteric regulator for receptor function (25). The role of cholesterol and the role of lipid rafts in this signaling regulation could be further separated through a sphingolipid depletion experimental design. Fumonisin B_1 has a structure very similar to that of sphingolipids, allowing fumonisin B_1 treatment to interfere with sphingolipid synthesis in cells (26). Therefore, treatment with fumonisin B_1 would eliminate lipid rafts in the plasma membrane, but leave the cholesterol content of the cell unchanged. The CANDLES assay could be used to quantify cAMP production after sphingolipid depletion, which would allow for any effects of lipid raft disruption on signaling to be detected without the convolution of other effects of cholesterol depletion on signaling making the data less clear.

Based on the data from the western blots, caveolin does seem to be involved in signaling somehow, but possibly not through lipid rafts, given the MBCD treatment data. Caveolin has

been found to exist stably as a soluble protein with its own functions in such an environment, but since the caveolin interaction motif of hFSHR is found in the fourth transmembrane domain, it would be unlikely that any of the functions of caveolin in a soluble state would be relevant here (27). However, the described functions of caveolin as an integral protein are all related to its presence in caveolae lipid rafts. This data could be alluding to a currently undescribed function of caveolin in the membrane separate from caveolae. The CANDLES assay should be re-attempted on the hFSHR caveolin interaction motif mutants to obtain quantitative data regarding the role of the motif in signaling for comparison to both the western blotting data from the mutants and the CANDLES data from the MBCD treatment.

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