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Human Follicle Stimulating Hormone Receptor Lipid Raft Residency is Hormone and Caveolin Dependent

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Human Follicle Stimulating Hormone Receptor Lipid Raft Residency is Hormone and Caveolin Dependent

By

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INTRODUCTION

All living organisms exist as a product of reproduction; it is one of the most crucial features of life. The inherent desire to reproduce, create offspring, and pass on genetic information regulates much of the behavior of all animals.[1] Without reproduction, life could not continue. Such a large and important topic garners incredible interest. In humans, the interest leads to a desire to achieve a complete understanding of and control over the process.

Control of reproduction strengthens methods of family planning. Family planning gives individuals the ability to decide and regulate when they reproduce. The use of contraception, or birth control, is integral in this process to temporarily prevent reproduction when intended. Contraception is the method of preventing pregnancy using medications or devices.[2] The improvement in the quality of contraceptives and their increased widespread availability over the last half of a century allowed for many cultural and social changes to occur. With appropriate care providers, more women became more informed decision makers regarding their reproductive health. This vast acquired knowledge and increased access to safe and effective contraceptives played a role in empowering women to pursue higher education, compete for high ranking jobs, and decide when they chose to bear children.[3]

The contraceptive industry has exploded since its inception in the 1960s. Each year more women, of child bearing age, seek out a form of contraception. However, many hormonal and non-hormonal products do not work effectively for each user. Consequently, research to create multiple high quality contraceptives has been consistent for decades. Many women cite these products as life changing due to the control they provide over reproduction and the reduction of symptoms of various health conditions. [4] Extensive research has pushed past the standard birth control pill and led to the creation of combination pills, non-hormonal options, and devices, both

temporary and permanent. Current research in the industry is evolving to create more non-hormonal options and explore the possibility of a contraceptive combined with an antiretroviral to protect against sexually transmitted diseases. [4]

While contraceptives still provide those benefits of freedom and control to women today, they are also used to treat the symptoms of reproductive related hormonal imbalances. Many young women are prescribed a combination oral contraceptive pill to manage acne, ovarian cysts, and irregular menstrual cycles. These treatments are exogenous hormones in the form of contraceptives to alleviate painful symptoms, improve confidence, and more. Researchers at Penn State/Hershey Medical Center found combination oral contraceptive pills to be safe and effect for the treatment of acne in females. [5] They conducted a review by analyzing various valid clinical trials for safety and efficacy. The findings were used in conjunction with physiological explanations to draw a conclusion supporting the continued use of contraceptives for treatment of acne. [5]

Control of reproductive health and family planning is a spectrum. Like other physiological processes, there can be too much activity leading to a need to suppress it or not enough creating a need to upregulate it. The latter applies to the reproductive system when conception is intended but not achieved. Medications and treatments within the scope of reproductive health also include those that increase reproductive potential. Infertility is defined as the failure to achieve clinical pregnancy after 12 months. This disorder of the reproductive system effects over 12% of women, aged 15-44, in the United States. [6] The cause of infertility varies from patient to patient, contributing factors can be genetic, epigenetic, or unexplained, and both men and women can be affected. [2] Solving the puzzle of infertility requires extensive study into all components of the female and male reproductive systems. All anatomical and endocrine,

or hormonal, factors must be well understood to achieve the safest treatments and best outcomes. Infertility treatments typically focus on gonadotropin hormone levels as predictors of reproductive capacity and as potential modes of treatment. Procedural treatments such as in vitro fertilization (IVF) and intrauterine insemination (IUI) are both frequently preceded by a regimen of exogenous reproductive hormones, or gonadotropins. The exogenous hormone is used to increase the number of mature follicles in the ovarian reserve and to prepare the body to be more fertile, if hormonal imbalances exist. [7] The gonadotropins, and more recently developed recombinant gonadotropins, are consequently a crucial component to successful fertility treatments and interventions. All components of gonadotropin signaling pathways have the potential to play a role in the correct execution of their functions. Therefore it is important to focus research on all levels in the pathway to fully understand why something may be going wrong.

The physiology of reproduction in men and women is controlled by many hormones of the endocrine system, two of which are gonadotropins called follicle stimulating hormone (FSH) and luteinizing hormone (LH). The two gonadotropins are peptide hormones that

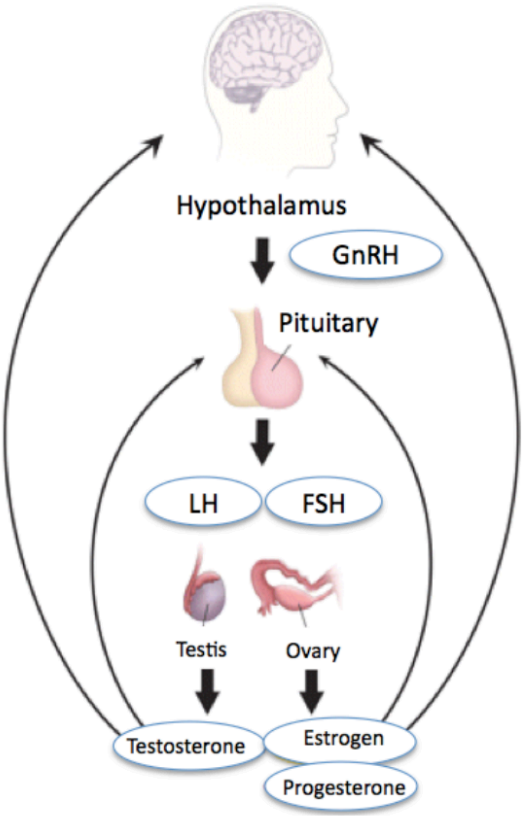

Figure 1. Map of the HPG Axis, showing the origins and target tissues of some of the gonadotropin hormones of the reproductive endocrine system. Koopman, P. 2013.

are part of the hypothalamic – pituitary – gonadal (HPG) axis. [8] When activated, the hypothalamus releases gonadotropin releasing hormone (GnRH) which travels through the hypothalamic portal system to the anterior pituitary. The presence of GnRH in the anterior pituitary stimulates the release of gonadotropins, FSH and LH, into the blood stream to act on target tissues: the Sertoli cells of testes in men and the granulosa cells of ovaries in women. These hormones are at the center of reproductive pathways in women and men and consequently are of high interest in reproductive research. [8]

Human follicle stimulating hormone (hFSH) is a peptide hormone classified as a gonadotropin and is released during the follicular phase of the female menstrual cycle. [9] hFSH mediates the maturation of gametes in both females (oogenesis) and males (spermatogenesis). It is a 35.5 kDa heterodimer, consisting of an alpha and a beta subunit. The alpha subunit is identical to that of LH. The beta subunit is unique to FSH and therefore is required for proper function and signaling. HFSH works by binding to hFSH receptors on the target cell membranes to initiate a signal transduction. [9]

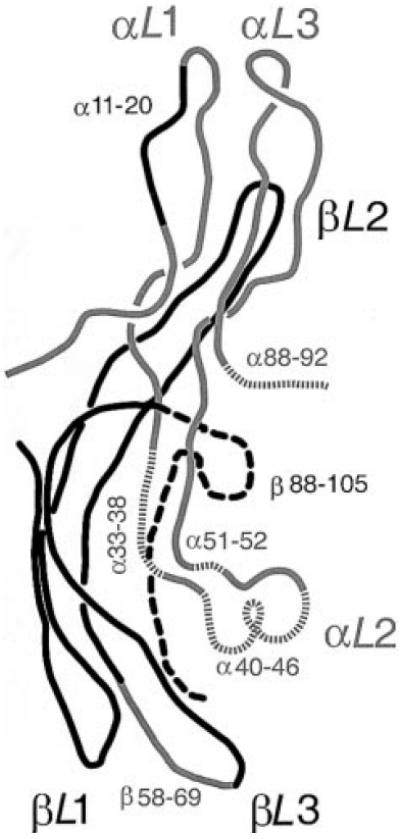

Figure 2. Ribbon structure of hFSH. Alpha subunit colored in grey, beta subunit shown in black. (Dias, et al. 2002)

HFSH cannot generate the signal transduction for these reproductive processes if its receptor (hFSHR) is not present or is not functioning. HFSHR is a g protein-coupled receptor (GPCR). GPCRs are embedded in the cell membrane, with some regions reaching outside of the cell to bind and receive stimuli and others located inside the cell and cell membrane to excite the signal transduction pathway. The receptor is a 76 kDa protein with three main domains. The extracellular domain is responsible for hormone binding. The transmembrane domain is made up of seven transmembrane alpha helices and makes up most of the physical receptor. The C-terminal domain is intracellular and responsible for interacting with the g-protein that will become activated to transmit and execute changes in signaling. HFSHR is a specific receptor, activated only by hFSH, though GPCRs in general are quite abundant. [10]

Cell membranes are incredibly heterogeneous, consisting of many molecules like cholesterol, glycoproteins, and transmembrane proteins like hFSHR embedded within the lipid bilayer. [11] hFSHR has been shown to be located within compartments of the cell membrane known as lipid rafts. [12] Lipid rafts are microdomains of the membrane containing a higher concentration of cholesterol, glycosphingolipids, and a protein called caveolin. Rafts are tightly packed and more ordered than the rest of the lipid bilayer. The high concentration of important membrane proteins and cholesterol allow lipid rafts to become crucial organizing and regulating centers for cell signaling molecules like GPCRs. These unique microdomains are believed to

play a role in cellular signal transduction pathways and membrane trafficking. It is also thought that the rafts serve to protect the signaling molecules from other membrane disruptors or enzymes. [13] Many GPCRs, including hFSHR, are thought to reside within lipid rafts. Previous research in the Cohen Laboratory at Union College has demonstrated that hFSHR is located within lipid rafts.

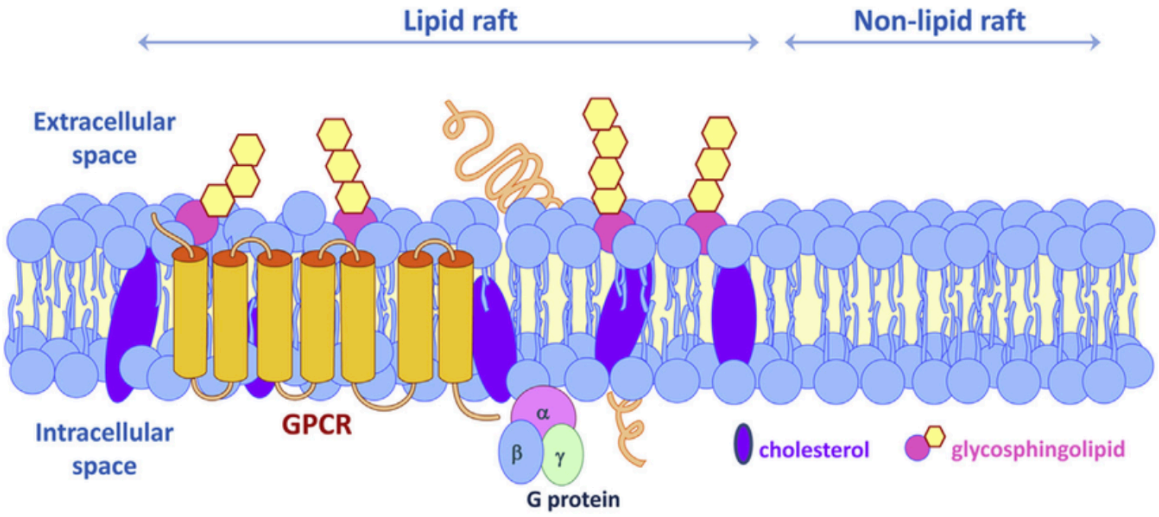

Figure 3. Schematic of the phospholipid bilayer, contrasting a lipid raft and a non-raft area.
Villar, VAM, et al. 2016.

Caveolin is a membrane protein found within lipid rafts. Microdomains of the cell membrane that are rich in caveolin are a subset of lipid rafts called caveolae. These specific regions often take on an indentation, or U-shaped invagination, of the plasma membrane. Caveolin interacts with hFSHR, although the specific location and the physiological relevance of the interaction are still unclear. [14] Researchers speculate that caveolin acts to anchor GPCRs within lipid rafts to improve signaling. Based on homology with other proteins, the most likely

site of interaction with caveolin is a portion of the hFSHR protein found in the fourth transmembrane region. The region contains a specific ten amino acid sequence shared by other proteins that interact with caveolin and it is known as a caveolin interaction motif (CIM) (circled in red, Figure 4). [9]

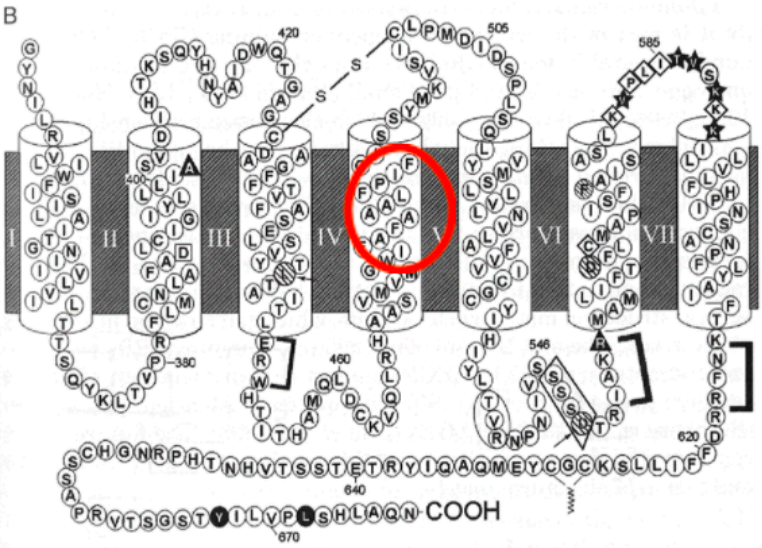

Figure 4. Schematic of hFSHR transmembrane domains, including its sequence. Circled in red is the caveolin binding motif.
Dias, JA, et al. 2002.

We hypothesize that the interaction between hFSHR and caveolin regulates the FSH signaling pathway in some way. Further, we are interested in gathering more evidence pertaining to hFSHR lipid raft residency and investigating alterations in receptor residency. We will attempt to evaluate receptor location, with respect to membrane rafts, under time dependent exposures to hFSH, using discontinuous sucrose gradients. We will also explore the role of caveolin interaction motif (CIM) memetic peptides, both wild type and mutant, and the effects they may have on receptor residency. We are interested in studying receptor residency and the interaction with caveolin because it has the potential to introduce new ways to modulate the receptor. Increased control over the receptor could allow for further investigation into improved contraception methods and infertility treatments that could alter this physiological pathway without requiring exogenous hormone.

METHODS

Human embryonic kidney 293 (HEK293) cells were stably transfected to express human follicle stimulating hormone receptor. The cells were grown and incubated in tissue culture in G418 media. This stable cell line was used in all experiments.

Time Dependent Hormone Treatment

1. Hormone Treatment

Four T75's of HEK293R cells were used, one for each varying timed exposure to hFSH. The G418 media (18 mL) was removed from all T75's and replaced with Serum Free media (5 mL) for 1 hour. Human urinary hFSH was used for all treatments. 4 uL hFSH was added to 1mL Serum Free media. The cells were spiked with hormone using this diluted mix (250 uL). The cells were exposed to hFSH for 0, 5, 15, or 30 minutes. All samples were placed on ice immediately following the expiration of the assigned amount of exposure.

2. Discontinuous Sucrose Gradients

Discontinuous Sucrose Gradient is a differential centrifugation technique used to separate cell parts by density. Sucrose is layered to create a density gradient to allow for buoyant (less dense) lipid raft membranes to be differentiated from other cellular membranes.

At the completion of the respective hormone time courses, all media was removed from the four T75's. All cells were then washed with 1X PBS (5 mL) and harvested from the T75's using 5 mM EDTA/1X PBS (5mL). The four samples (0, 5, 15, 30) of harvested cells were centrifuged at 4400rpm for 10 minutes, to separate them from the harvesting solution. The supernatant liquid was removed and the cells were resuspended in 0.5 M sodium carbonate (pH

11; 0.75 mL). The samples were left on ice for 20 minutes. They were then sonicated three times for 10 seconds to disrupt the cell membranes.

The sonicated samples were mixed with 0.75mL of 90% sucrose in 1X MBS. This mixture then was added to ultracentrifuge tubes. 35% sucrose in 0.5xMBS and 0.25M Na_2CO_3 (~1.5 mL) was layered dropwise on top of the 90% sucrose + sample mixture, using a syringe and hypodermic needle. By the same technique, 5% sucrose in 0.5xMBS and 0.25M Na_2CO_3 (~1.5 mL) was layered on top up to the neck of the tube. Samples were then centrifuged in an SW41 rotor at 34,000rpm (240,000G) for 20 hours.

After 20 hours of ultracentrifugation, the samples were each broken down into fractions from the top of the ultracentrifuge tube to the bottom. 8-12 fractions were created per sample. This method keeps the density gradient intact by the order of fractions, to be used for comparison and analysis.

3. Western Blot Analysis

SDS Polyacrylamide Gel Electrophoresis was used to separate proteins and identify the presence of hFSHR for qualitative analysis. 10% acrylamide gels were cast and loaded with the sample fractions in order of the density gradient. The first sample loaded farthest to the right corresponded with the top of the gradient, or the first fraction taken off. All four gels were run and transferred to Immuno-Blot membranes for western blotting and chemiluminescent imaging.

Western blotting, or immunoblotting, is a technique used to visualize and image proteins separated by SDS-PAGE for identification and qualitative analysis. The technique allows for the identification of hFSHR in each sample fraction. Specifically, it is also useful for qualitative comparison of alterations of hFSHR lipid raft residency by using differences in band darkness as a proxy for comparisons of receptor concentrations between samples.

The gels were transferred to Immuno-Blot membranes using 1X transfer buffer and a transferring instrument, at 15V for 15 minutes. The membranes were then blocked in 5% milk in TBST (10 mL) for an hour at room temperature. Next, the membranes were incubated in an anti-hFSHR monoclonal antibody, 105 (5 uL) in 5% milk in TBST (10 mL), overnight at 4 degrees C. The membranes were then washed for 5 minutes, three times each, in 1X TBST. Then membranes were incubated in the secondary antibody, goat anti-mouse (5 uL) in 5% milk in TBST (10 mL), for 1 hour at room temperature. All four were washed the same way in 1X TBST, again. Next, the membranes were treated with a chemiluminescent developing solution (2 mL/blot) for 5 minutes and developed using ImageLab software, for 10 minutes, to create an image.

Caveolin Interaction Motif Mimetic Peptides

Peptide Pretreatment

The protocol for the Caveolin Interaction Motif mimetic peptides experiment is identical to the protocol described above for the general hormone treatments, except for the addition of the peptides to the cells before the protocol began. Four T75's of HEK293R cells were used. Two were designated to receive wild type peptide and two to receive mutant peptide. The peptides were added to the correct T75's at the first step of the protocol, when the growth media is removed and changed to serum free media (5 mL) for an hour before the hormone treatment. 0.2 mM concentration of each peptide, in serum free media, was used.

RESULTS

Time Dependent Hormone Treatment

Time dependent hormone treatments with urinary hFSH on HEK293R cells showed changes in lipid raft residency of hFSHR across time points. Cells were exposed to hFSH for 0, 5, 15, or 30 minutes. Using western blot analysis, it can be observed that the density of receptor, or darkness of bands, changes across the time points in each fraction. Fractions are divided by the breaking down of sucrose gradients. Fraction 1 corresponds to the least dense components of the plasma membrane. Lipid rafts are assumed to be present across fractions 1, 2, and 3. Therefore, based on this set of blots, it can be suggested that hFSHR lipid raft residency changes with exposure to hormone in a time dependent manner. (Figure 5)

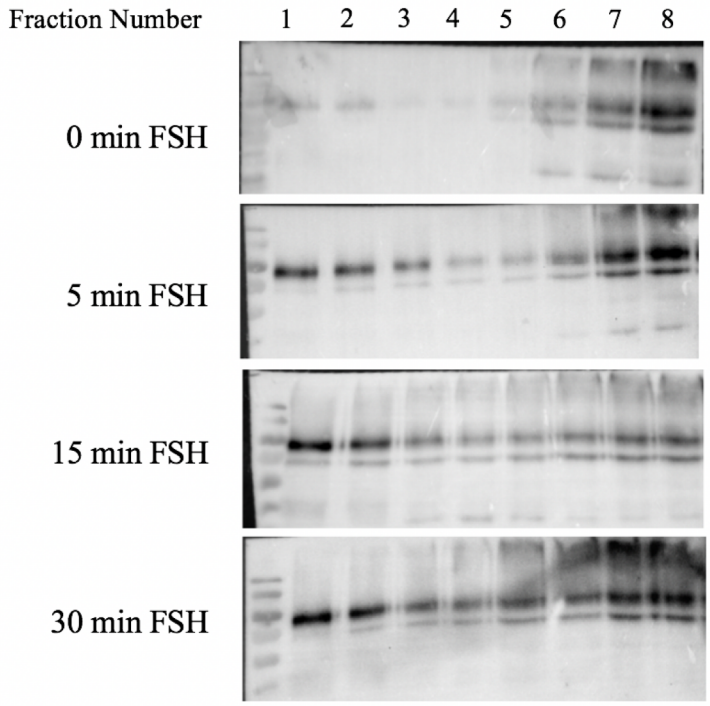

Figure 5. hFSHR presence and residency in sucrose gradient fractions after varying exposure to hFSH. Detected with 105, an anti-hFSHR monoclonal antibody.

Caveolin Interaction Motif Mimetic Peptides

Pretreatment with wild type Caveolin Interaction Motif mimetic peptide showed alterations in hFSHR lipid raft residency in the presence of hFSH. Pretreatment with mutant CIM peptide did not appear to alter hFSHR raft residency. Cells were treated with either wild type or mutant CIM peptide before exposure to hFSH. The samples were broken down into 12 fractions, with lipid raft fractions expected to be within fractions 2, 3, 4, 5, and 6. (Figure 6)

The cells treated with wild type peptide appear to have a higher concentration of hFSHR, darker bands, in lipid raft fractions after exposure to hFSH. Treatment with hormone appears to have affected receptor residency. Cells treated with mutant peptide do not appear to have any change in the concentration of hFSHR in the lipid raft fractions after exposure to hFSH. Hormone treatment does not appear to affect residency in this case. (Figure 6)

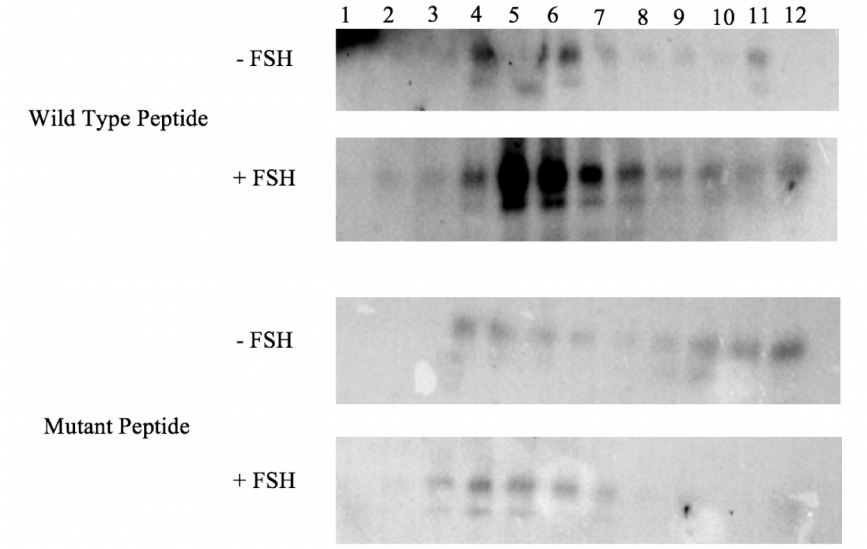

Figure 6. hFSHR presence and residency in sucrose gradient fractions after exposure to CIM peptide. Detected with 105, an anti-hFSHR monoclonal antibody. (Exposure to hFSH = 5 minutes)

DISCUSSION

The time dependent hormone treatment of hFSH on HEK293-hFSHR expressing cells showed alterations in hFSHR lipid raft residency in each exposure time condition (0, 5, 15, 30 minutes). With no exposure to hFSH, 0-minute condition, the hFSHR bands in the raft fractions (lanes 1, 2, 3) are visible but very faint. In the non-raft fractions, especially the heavy fractions, the hFSHR bands are significantly darker, indicating a higher concentration of hFSHR in the heavier (more dense) gradient fractions. This suggests that without exposure to hormone most hFSHRs reside outside of lipid rafts.

The residency pattern of hFSHR appears to change with exposure to hormone. After 5 minutes of exposure to hFSH, the hFSHR bands in lanes 1, 2, and 3 (lipid raft fractions) are much darker than the corresponding bands in 0 minutes hFSH exposure. The bands in the heavy, non-raft, lanes are still similarly dark after 5 minutes of exposure when compared to 0 minutes exposure. This suggests that after 5 minutes of exposure to hFSH, a significant alteration in hFSHR residency occurs. HFSHRs appear to be moving into the raft after 5 minutes of exposure to hormone.

The residency pattern of hFSHR continues to change with longer exposure to hFSH. At 15 minutes of exposure, the bands in lanes 1, 2, and 3 are still dark just like in the condition of 5 minutes of exposure. However, the heavy, non-raft, lanes have much lighter hFSHR bands than in any prior exposure condition. This further suggests that with increased exposure to hormone, more hFSHRs migrate into lipid raft domains.

The final time condition is 30 minutes of exposure to hFSH. At this point the hFSHR bands are almost identical across all lanes, meaning similar concentrations of hFSHR in each gradient fraction. Lanes 1 and 2, and 7 and 8, appear to be the darkest, or highest concentrations.

This either means that hFSHRs are migrating back out of the rafts or receptor recycling is occurring. The latter is more likely. We believe that most receptors are altering their residency within the membrane by migrating into lipid rafts after exposure to hormone. But from there we believe they could be getting internalized inside the cells. The dark bands in corresponding non-raft lanes would be new receptors coming to the surface, as part of the receptor cycling model. The other option suggesting that those bands represent the same receptors that originally resided out of the raft, moved into the raft after exposure to hormone, then migrated back out, is more unlikely.

Based on our results, we believe the residency pattern of hFSHR is dependent on exposure to hormone, hFSH. Our proposed model suggests that without any hormone present most, but not all, hFSHRs reside outside lipid rafts. Once hFSH is present, the receptor appears to be moving into the lipid raft domains. The purpose and mechanism for the alteration of residency is unknown. The changes in receptor residency also do not appear to end one it associates with the raft. We believe the activated receptor goes to the raft to stop signaling and be internalized into the cell. This contradicts previous assumptions that the raft enabled or increased signal transduction pathways. However, these experiments looked only at the structure and function side of receptor residency. Further investigation is needed to link alterations in residency with changes in signaling.

hFSHR is known to interact with the membrane protein caveolin. It has also been demonstrated in the Cohen Laboratory at Union College that mimetic peptides of the caveolin interaction motif (CIM) alter hFSHR downstream signaling. Due to these known interactions we decided to further investigate the structure and function aspect of the interaction between caveolin and hFSHR. The wild type CIM peptide is identical to the 10 amino acid CIM in the

fourth transmembrane region of hFSHR. This is the part of hFSHR that is believed to physically interact with caveolin. We hypothesized that this wild type peptide will have some unknown effect on receptor residency due to its ability to interact with caveolin. Exogenous CIM peptide could interact with Caveolin and therefore reduced the frequency of caveolin interacting with hFSHR, and possibly have some effect on residency. The mutant CIM peptide has been mutated at each phenylalanine to a leucine.

Tat (Blue)-Wild Type Caveolin Interaction Motif (green)

*YGRKKRRQRRR***F**A**F**AAAL**F**PI**F**

*YGRKKRRQRRR***L**A**L**AAAL**L**PI**L**

Tat (Blue)-Mutant Caveolin Interaction Motif (red)

Figure 7. The wild type and mutant CIM mimetic peptide amino acid sequences.

The hormone treatments were conducted on the same cell line, HEK293 FSHR, with the same protocol, only changing to add a pretreatment exposure to wild type (WT) or mutant (Mut) CIM peptide. The results of this experiment show that caveolin does effect receptor raft residency. In the presence of the WT peptide and no exposure to hFSH, some hFSHR bands were evident in the raft fractions and outside of the raft fractions. With the addition of exposure to hFSH, the darkness of the bands and therefore concentration of hFSHR in the raft fractions drastically increases. In the presence of the Mut peptide and no exposure to hFSH, hFSHR bands are very similar across all lanes. The addition of hFSH to the Mut peptide condition does not change the hFSH residency pattern at all. The two Mut blots appear to be the same or extremely similar. Because a large difference occurred between WT (- FSH) and WT (+ FSH) and no difference is evident between Mut (- FSH) and Mut (+ FSH) it can be observed that the interaction between caveolin and hFSHR has effects raft residency. The extent of and mechanism through which it occurs is unknown.

We have observed previously that caveolin alters signaling, through experiments with the CIM peptide and downstream signaling molecules (Cohen Lab, Angelina Stewart). We have also

observed that receptor residency is physically dependent on hormone in a time dependent manner. It would be very interesting to further evaluate how signaling changes as receptor residency changes. We also observed that receptor residency is also caveolin dependent and therefore it would be necessary to further investigate how this is occurring and how it relates to the effects caveolin also has on signaling. Relating the effects of hFSH and caveolin on receptor residency and signaling will give a clear picture to the function and importance of residency to the hFSHR signal transduction pathway. Further understanding of the structural and functional relationship of the receptor and any downstream signaling controls creates more opportunities to modulate the signal transduction pathway through the receptor rather than the hormone. Control through the receptor, without, the use of exogenous hormone allows for the pursuit of non-hormonal interventions for therapeutics like contraceptives.

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