6-2016

Investigation of the Caveolin-Human Follicle Stimulating Hormone Receptor Interaction through Peptide Treatments

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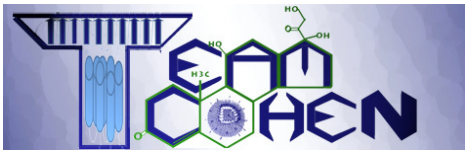

Investigation of the Caveolin-Human Follicle Stimulating Hormone Receptor interaction through peptide treatments

By

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Submitted in partial fulfillment

of the requirements for

Honors in the Department of Biochemistry

UNION COLLEGE

June 2016

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Abstract:

The purpose of my research project has been to determine the nature of the binding relationship between caveolin and hFSHR inside sex cells. FSH plays a role in the maturation of these cells, and interfering with the receptor's interaction with caveolin would prevent cell maturation (down regulating fertility). It is believed the interaction occurs through transmembrane domain IV of the receptor due to its aromatic nature. The treatment of sex cells with synthetic peptides that mimic the hFSHR-caveolin binding sequence should prevent the interaction, shutting down the signaling cascade from hFSHR. This can be tested for by the monitoring of downstream signals given off by hFSHR, including the presence (or absence) of phosphorylated p44, PKA, and CREB. It is hypothesized the wildtype peptide treatment will down regulate all of these signals when compared to the mutant control. Current data points towards this hypothesis holding true, with successful western blots displaying a noted difference in cell signaling between the wildtype and mutant peptide treatments. These results indicate the key interaction between caveolin and hFSHR likely occurs at transmembrane domain IV.

Introduction:

Follicle stimulating hormone (FSH) plays an important role in the maturation of sex cells in both males and females. FSH is part of the hypothalamic-pituitary-gonadal axis in both males and females [1]. Upon proper stimulation, the hypothalamus sends a signal to the anterior pituitary gland to release FSH. Its receptor, Human Follicle Stimulating Hormone Receptor (hFSHR), is only expressed in sex cells. In females, hFSHR is located in granulosa cells, found in the ovaries. A proper interaction between the hormone and its receptor in ovarian cells produces estrogen, allowing proper oocyte development. In males, the receptor is located in sertoli cells in the testis; a proper interaction promotes spermatogenesis [2].

hFSHR is a G-Protein Couple Receptor (GPCR). It requires activation by an agonist to pass an extracellular signal to the intracellular proteins targeted. Upon binding an appropriate agonist, a GPCR's alpha subunit exchanges a GDP for a GTP, becoming active. It then frees up both beta and gamma subunits, allowing those to pass on signals as well. A successful signal transduction cascade requires this activation mechanism to work perfectly, with all members in the right place at the right time.

hFSHR is not readily found on the surface of these cells. It is located in the cytosol, and requires the assistance of caveolin to be brought to the cellular membrane. This is accomplished through a caveolin binding motif in transmembrane domain IV in the receptor. Caveolin, a protein found in the caveolae of the cell membrane, has been proven to transport receptors to the cell surface before. Caveolae are a set of lipid rafts with a structure that allows them to bind and transport many cell receptors. The binding

of hFSHR is believed to be accomplished by an interaction of transmembrane domain IV of hFSHR with the caveolin protein [3].

Any interference with this caveolin-hFSHR interaction would effectively silence the normally observed effects of FSH in sex cell maturation. Inversely, any up regulation of this interaction would allow for increased maturation ability in sex cells that struggle to mature by themselves. It is apparent this interaction can be affected either in the hopes of silencing sex cell maturation (down regulating fertilitiy), or over expressing this interaction (up regulating fertility).

A successful hFSHR signal cascade manifests itself in the appearance of downstream signal transduction, most notably due to the activation of adenylyl cyclase, producing Cyclic AMP that is bound by Protein Kinas A to activate the protein, which in turn activates a multitude of downstream enzymes. Two of the most important downstream signaling enzymes of this cascade are p44 Map Kinase and Cyclic-AMP response element binding protein (CREB), as seen in Figure 1. Both of these are phosporylated (activated) when there is a successful interaction of hFSHR with caveolin, allowing the receptor to come to the cell surface and bind its substrate, FSH. Therefore, monitoring the presence of activated p44 and activated CREB is an extremely effective method of determining if there has been a disruption event in the caveolin-hFSHR binding interaction.

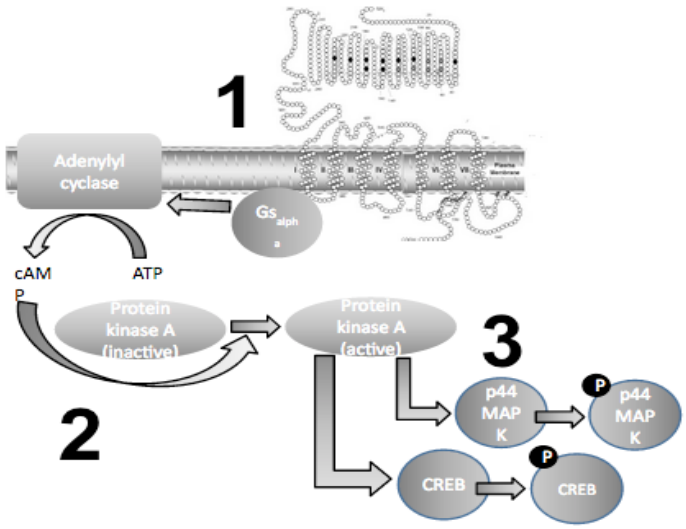

Figure 1. Current model for hFSHR signaling.

It has been shown in previous research projects that this critical binding motif can be blocked by the introduction of

synthetic peptides with the same aromatic caveolin-binding motif as hFSHR. This was noted through monitoring the amount of phosphorylated CREB in cells after treatment with two different synthetic peptide memetics. A decrease in the amount of phosphorylated CREB was observed after treatment with the wildtype binding memetic when compared to the control, a mutant peptide with non-aromatic residues in the binding motif range. There was, however, no testing for the effects of these peptides on the amount of phosphorylated p44. This may in fact be a better indicator of the caveloin-hFSHR interaction interference, as fewer upstream molecules can activate p44 compared to CREB. In other words, CREB can be activated by a multitude of mechanisms, so any drop in its activation could be a result of peptide interference with other activators of CREB.

In order to allow the peptides to enter the cells, they are attached to a truncated version of the Tat protein found in HIV. This protein allows the virus to pass through cell membranes; it is used here to get the peptides through experimental cell membranes. The truncated Tat used also does not change peptide folding due to its incredibly charged nature; it truly is just a vector used to allow peptide entrance [4].

The sequences of these peptides are shown in Figure 2. The important residues are the aromatic amino acids in red. In the wild type peptide, the red residues include the same aromatic residues as those found in the aforementioned transmembrane domain; these are changed to non-aromatic residues in the mutant peptide (as shown by those residues put in bold print).

Figure 2 Custom Peptides used in previous research. Their sequences in italics are from the tat peptide. The remainder of the sequence is from hFSHR amino acids 479-489. Mutated residues are shown in bold.

Tat (Blue) WT Caveolin binding motif (Red)

*YGRKKRRQRRR*FAFAAALFPIF

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

Tat (Blue) Mutant Caveolin binding motif

The wildtype peptide should be able to bind easily to the caveolin in the cell and shutdown the signal cascade. If this occurs, hFSHR would be unable to to bind to its substrate, as it would not be at the surface of the cell. This would result in a decrease in the activation of CREB and of p44. The mutant peptide, on the other hand, won't be able to bind to caveolin in the cell and should not affect the amount of signaling caused by hFSHR.

The primary antibody used in my experiment is Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody #9101, with a secondary antibody Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP conjugate #65-6120. The advantage of using Phospho-p44/42 MAPK antibody 9101 is its specificity for phosphorylated p44 MAP Kinase. It does not cross react with phosphorylated residues of either JNK/SAPK or p38 MAP Kinase, and does not cross-react with non-phosphorylated Erk1/2. This allows for better results in the experiment.

My hypothesis is the cell samples treated with the wildtype peptide should show marked differences in signaling compared to those treated with the mutant peptide.

Methods:

A method developed by Roh et. Al [5] was adapted for use in this experiment.

Cell Culture

HEK 293R cells were used in this experiment due to their stable expression of the desired receptor. Cells were grown in the Union College tissue culture room in sterile conditions and split at 90% confluence to prepare them for the peptide treatments.

Peptide Treatments

Previously prepared stock solution of both wildtype and mutant peptides were used at concentrations of 10-mM. Three 15mL conical tubes had 10 µL of WT, mutant, or no peptide added to 10mL of serum free medium. Then, two milliliters of each mixture was added to the appropriate well in two different 6-well dishes. The layout of these treatments is outlined in Figure 4. Peptide treatments lasted an hour for the cells, allowing them to affect the HEK293R cells in the wells.

Treatment with FSH

4.8 µL of hFSH was mixed into 1.2mL of SFM to create the stock treatment solution while the peptide incubation was occurring. Once the peptide treatment was over, the media in the 6-well dishes was sucked off and replaced with 4mL of fresh SFM to ensure no peptide residue remained. 100µL of the prepared hFSH stock solution was added to the appropriate wells in the dishes as outlined in Figure 5. Incubation took place for periods of 30, 15, 5 and zero minutes to see how signaling changes over time with the

treatments. Once the desired time had elapsed, the media was aspirated off the cells to remove any lingering hFSH.

Cellular Extractions

Immediately following media aspiration, the dishes were placed on ice and each well underwent two washes with 1mL of freezing PBS. In order to lyse open the cells, a solution of lysis buffer was created. One phosSTOP tablet and one cOmplete tablet were added to 10mL of lysis buffer and 500µL of this mixture was added to each well and incubated for 20 minutes. The phoSTOP tablet prevents depshosphorylation of phosphorylated proteins (the data to be examined) and the cOmplete tablet prevented those proteins from being cleaved by proteases (4,5). The well contents were then scraped into twelve microfuge tubes on ice (contents detailed in Table 1). The contents of each tube were transferred into a dounce homegnizer, dounced to ensure all cells had been lysed open, then returned to their microfuge tube. After all cells had been dounced, the tubes were centrifuged at maximum speed for ten minutes with the supernatants collected and placed into different microfuge tubes (Table 2).

BCA Assay

To determine the concentrations of protein in each cell sample, a bicinchoninic acid protein assay was performed. 10 µL of eight standards of pre-determined concentrations were loaded in triplicate on the appropriate 96 well-microplate. The unknown samples were also added in incriments of 10µL in triplicate to the corresponding wells on the plate reader. Once all standards and samples had been loaded,

a working reagent was prepared from the BCA kit at 50:1 ratio of A to B. 200μL of this reagent was pippeted into each well to help with protein determination. A plastic cover was placed on top of the microplate, and it was placed in an incubator at 37C for a half hour. After this incubation period, the plate was loaded into the appropriate assay reader and a standard curve was created. The samples were compared to the curve by the assay to determine their average protein content (from the standard curve). This information was printed out and used to standardize all samples to the lowest concentration sample, diluting the extremely concentrated samples. This allowed for the creation of evenly concentrated samples, as not to incorrectly read a signal as having a stronger reaction to FSH when it was due to the higher level of protein in the sample. Gel samples had a total volume of 200μL, with up to 100μL of sample and the remainder filled in by 2X SDS buffer. These samples were boiled at 70C for 30 minutes once prepped and then frozen for later usage.

Gel Electrophoresis

3.5 mL of 10% SDS-polyacrlyamide resolving gel was added to each gel apparatus, covered in isopropyl alcohol and allowed to sit for an hour to set. Once the hour was up, the isopropyl was poured out, and a 4% SDS-polyacrylamide stacking gel was used to fill the rest of the apparatus with a 10 well comb placed in the gel and allowed to set for 30 minutes. The comb was removed once the gel was set to create the loading wells for the samples. 20μL of a protein molecular weight marker was added in the first well of each gel ran, with the aforementioned prepared gel samples added (20 μL) to the appropriate wells to analyze the amount of signaling the specific well had

undergone. The gels were then run at 60-65 minutes at 100 volts for one hour in prepared 1X electrophoresis buffer.

Western Blotting

In order to visualize the results of the gel electrophoresis step, a western blot was performed. Gels were removed from the glass plates and soaked in transfer buffer for fifteen minutes. In order to transfer the gels, six separate Watmen filter papers were cut for each gel at 3.25X2 inch measurements, with one nitrocellulose membrane for each gel also cut to that size. The membranes were dipped in methanol and soaked in transfer buffer for 10 minutes. Once the gels and membranes had been soaked for the appropriate amounts of time, "sandwiches" were made: 3 of the Watman filter papers dipped briefly in transfer buffer, the membrane, the gel, and 3 more Watman filter papers on top. This set-up was then run on a semi-wet transfer cell apparatus for 15 minutes at 15 volts to pass the proteins from the gel to the membrane. Once the transfer was complete, the membranes were soaked in 5% milk in 1XTBST for 60 minutes for blocking in a sealed bag.

While blocking occurred, solutions of primary antibodies were prepared. The antibodies used were" P-p44 to detect phosphorylated p44 MAPK, P-CREB to detect phosphorylated CREB and P-PKA to detect phosphorylated PKA proteins. The standard concentration was 5µL of antibody to 10mL of 5%BSA in 1XTBST. At the end of the blocking period, the membranes were placed in new bags with the appropriate primary antibody and left to soak in them overnight in the Wold cold room. The next day, solutions of secondary antibody were prepped with the standard concentration being 5µL

of antibody to 10mL of 5% milk. The membranes were then removed from their primary antibody bags, washed 3 times for 5 minutes in 1XTBST before being placed in new bags with the appropriate secondary antibody for another hour long incubation. At the end of this incubation, the membranes were removed from the bags, washed in 1X TBST for 3 sets of 5 minutes each, then soaked in Thermo Scientific SuperSignal® West Pico Chemiluminescent Substrate for 5 minutes to develop an image of the transferred proteins. The membranes were then photographed with the BioRad ImageLab and analyzed for protein expression.

Results:

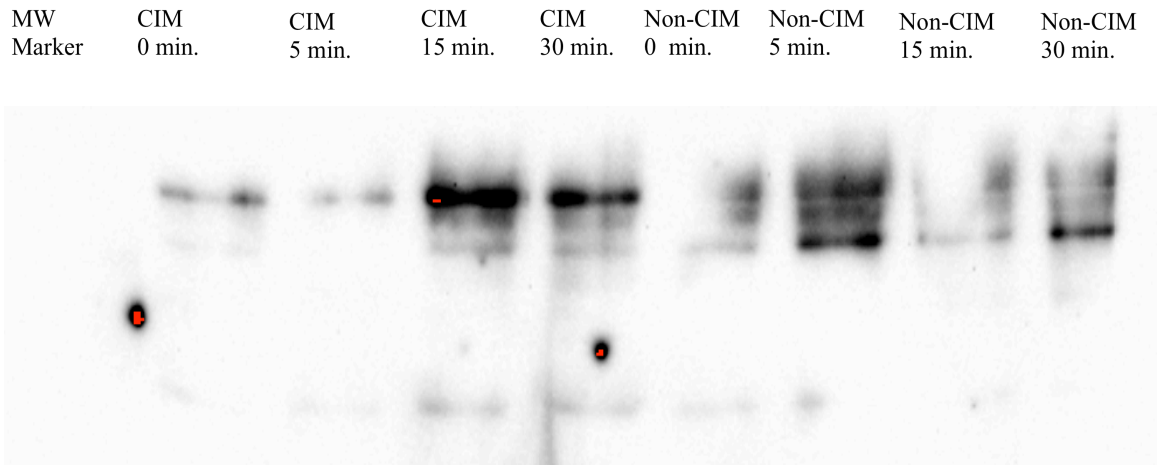

Figure 3. Western Blot of peptide and hormone treatments. Antibody used was for proteins phosphorylated by Protein Kinase A. CIM peptide is the wildtype peptide, non-cim is the mutant peptide.

The western blot shown in Figure 3 shows a clear difference in signaling between cell samples treated with the wildtype peptide, and those treated with the mutant peptide. There is very little downstream phosphorylation of proteins by PKA in the wildtype peptide until the 15 minute mark. This strong signal falls off at the 30 minute mark. The mutant peptide reaches a maximum signal at the 5 minute mark, 10 minutes faster than the wildtype peptide. This signal proceeds to tail off both the 15 and 30 minute mark. The maximum signal for the mutant peptide (5 minute mark) is larger than the maximum signal for the wildtype peptide treatment (15 minute mark). There is a clear difference in the signaling patterns, both in timing and intensity, as anticipated in the hypothesis.

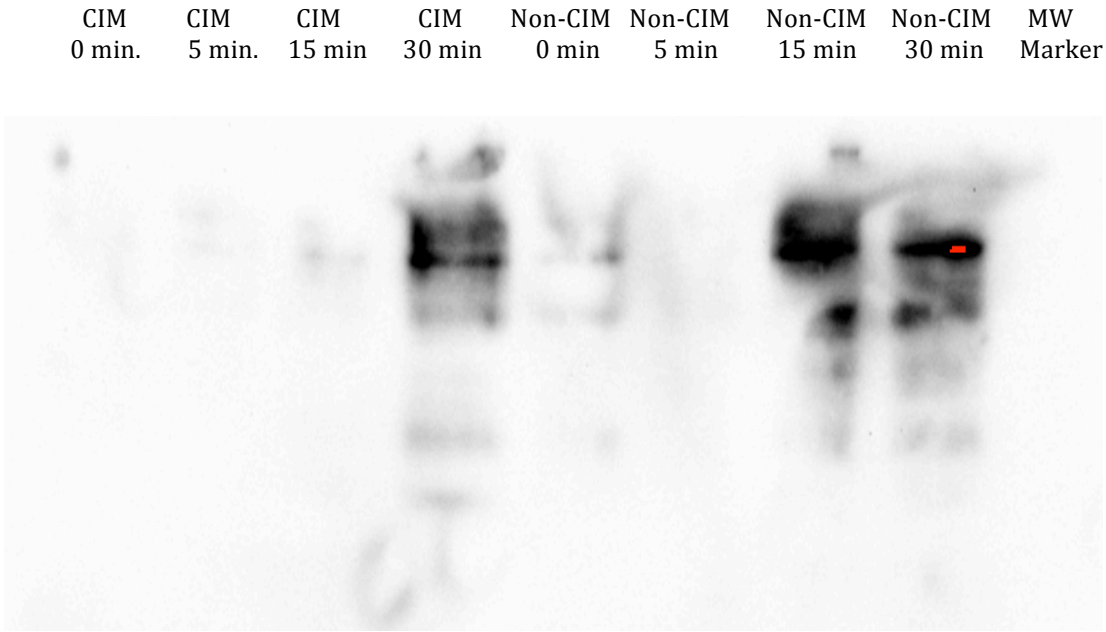

Figure 4. Western Blot for peptide and hormone treatments. Antibody used was anti-phospho-p44

The western blot in figure 4 shows a difference in timing and level of activation of P44 between the mutant and wildtype peptides. The wildtype peptide treatments show almost no signaling for the first 3 time points. It only begins to appear at the 30-minute mark, with the maximum occurring after a half hour of FSH stimulation. The mutant peptide treatments show a maximum occurring at the 15-minute time point, continuing over into the 30 minute time point, There is almost no signal for the mutant peptide for the first two time points. Again, there is clearly a difference between the level and timing of activation of P44 in cell samples treated with either the wildtype or mutant peptides.

Discussion:

The results from this experiment support my hypothesis. Beginning with a detailed observation of Figure 3, there is a clear difference in signaling between the wildtype peptide treated cell samples and those treated with the mutant peptide. Again, the wildtype peptide shares the theorized aromatic amino acid residues believed to exist in the caveolin-hFSHR motif (transmembrane domain IV). The interruption of downstream signaling from PKA shows there has been interference with the hFSHR in these cells. This is likely, although not certainly, due to a binding interaction occurring between caveolin and the wildtype peptides that have entered the cell. This binding prevents the downstream signaling from taking place in the cells. The mutant peptide treatment serves as the control for the monitoring of proteins phosphorylated by PKA, so any difference noted between wildtype and mutant supports the hypothesis. There is a clear difference; therefore the wildtype peptide must have interfered with the signaling, most likely by binding to caveolin and preventing the receptor from reaching the surface.

The results in Figure 4 also support the hypothesis of the experiment. There is a clear difference in the timing and level of activation of P44 in the cells treated with either wildtype or mutant peptide. This is again likely caused by an interference with caveolin-hFSHR localization when cells are treated with the wildtype peptide that is able to bind with the caveolin.

While my results are limited in scope, similar experiments with similar outcomes have been performed before. The possibility that the wildtype peptide is able to interfere with the direct interaction of caveolin with the GPCR is supported in a study done by Kim et Al [6]. They examined the interaction of the mGlu-1 receptor (a GPCR) with

caveolin through the use of both “blocking” and “mutant” peptides. The blocking peptide had the aromatic amino acid residues theorized to mediate the binding between caveolin and the receptor, while the mutant peptide had non-aromatic amino acids at those residues. Upon separate co-immunoprecipitation experiments of both caveolin and mGlu receptor, it was clear they did not associate in cells treated with the blocking peptide. The peptide treatment was preventing the binding of the receptor to caveolin when it contained the aromatic amino acid residues, similar to my experiment. Further experimentation showed this interference with the binding interaction between caveolin and the receptor prevented its localization to lipid rafts. This was accomplished through peptide treatments and double-labeling immunocytochemistry of endogenous mGlu1α receptor and lipid rafts. There were again significant differences in the localization of the receptor in lipid rafts between blocking and non-blocking peptides. All of these data together support a receptor-caveolin interaction being key for the receptor to become localized in lipid rafts. mGlu-1 receptor is a GPCR, like hFSHR, and this similarity shows a related mechanism is likely occurring in my experiment.

Experimental data produced by Bhatanger, et Al., also supports a proposed interaction between caveolin and GPCRs [7]. Instead of using peptide treatments to interfere with the theorized interaction between GPCRs and caveolin, the researchers used siRNA to knockdown the expression of caveolin in a variety of cell lines, most notably C6 glioma cells, that expressed both 5-HT(2A) and Galpha(q)-coupled P2Y purinergic receptor. Prior to the knockdowns, co-immunoprecipitation studies showed the caveolin was associating with the receptors in control cell lines. This of course shows there is an interaction between caveolin and the GPCRs in the cells and is an experiment

that should be performed on hFSHR in the future. Knockouts took place after this first experiment proved the interaction existed. The cells with knocked out caveolin showed decreased cell signaling from both GPCRs when compared to control cell lines with normal levels of caveolin expressed in them. This data was gathered by measuring cell signaling with calcium flux assays in the cells after knockdowns occurred. The results of this experiment further support the conclusion that there is likely an interaction between GPCRs and caveolin that is critical to the function of the receptors. The cells were unable to pass on signals from those receptors after caveolin was eliminated from the cells, showing caveolin to be related to their function. Although this experiment did not investigate where that interaction was taking place on the receptor, its data still supports the results from my peptide treatment experiments.

The evidence in the literature and in my experiment points to supporting a caveolin-GPCR binding interaction to localize to lipid rafts in cell membranes. In the case of hFSHR, this is important for a multitude of reasons mentioned before. The primary field affected by a proper study of the hFSHR-caveolin interaction is fertility [5]. The ability of a drug to interfere with the hFSHR-caveolin interaction motif would revolutionize contraception treatments. Current drugs that serve as birth control are commonly taken in pill form and involve the use of synthetic estrogen and progestin [8]. They aren't quite 100% effective, especially when the drug regimen isn't followed strictly. Hormone imbalances can occur, and improvements can definitely be made in the design of these drugs.

This is exactly where a drug that is able to interfere with the hFSHR-caveolin interaction would be a lifesaver. It wouldn't require synthetic hormone treatments; it

would theoretically simply prevent the maturation of sex cells in both men and women. A birth control treatment for both sexes would revolutionize how we view contraception, with reproductive responsibility not just falling on the shoulders of women if this treatment came to fruition. Given that spermatogenesis and follicular development both require a successful signaling cascade from hFSH, preventing the receptor from reaching the surface would allow the body to produce normal levels of FSH without any sex cell maturation. This type of silver bullet treatment is still far down the road, but there is promise in interfering with hFSHR-caveolin interactions. The data gathered in this experiment and in the literature shows a decrease in GPCR downstream signaling when cell samples are treated with peptides designed to block the aforementioned interaction. However, there are issues with duration of treatment in the experiment. Even the wildtype peptide inhibitor lost its effect between 15 and 30 minutes and normal signaling returned. This could be due to the production of new receptors or new caveolin, or a binding affinity issue with the peptide to the caveolin (i.e. it isn't that strong and eventually dissociates). There needs to be more data gathered on the ability of a drug to block out caveolin-hFSHR binding in sex cells, but this potential drug target would be fantastic. It would eliminate the requirement for synthetic hormone treatments and keep the same efficacy as current drug treatments if not improve upon it.

The idea of creating a drug treatment for birth control that would simply prevent hFSHR from being brought to the surface is sound in logic but still far down the road from being realized. There needs to be a multitude of other studies performed to confirm the localization of hFSHR in lipid rafts is a direct result of its interaction with caveolin. If there were a different mechanism used by the cell to bring the receptor to the lipid rafts at

the surface that is also blocked by the peptide, then the data above are false positives. The first study needs to of course first be replication of the western blots to confirm downstream signaling is affected by the peptide treatments and it was not experimental error. The next step after this would be co-immunoprecipitating caveolin in both peptide treatments and seeing if the peptide is binding to it, or if the receptor is still able to bind to the caveolin. If the wildtype peptide were found to be binding to caveolin, it would confirm that the aromatic amino acid residues at transmembrane domain IV of the hFSHR are almost certainly binding to caveolin to be transported to lipid rafts in the cell surface. This would prove the binding motif is critical for hFSHR function, and open a new door for a potentially longer lasting drug treatment in blocking the receptor from being brought to the surface of the cell.

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