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Caveolin Binding Motif Mutation Yields a Variance in Follicle Stimulating Hormone Receptor Signaling

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Caveolin Binding Motif Mutation Yields a Variance in Follicle Stimulating Hormone Receptor Signaling

By

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Abstract:

Fleischer, Justin Caveolin Binding Motif Mutation Yields a Variance in Follicle Stimulating Hormone Receptor Signaling

Advisor: Brian D. Cohen

The human follicle stimulating hormone receptor (hFSHR) is a glycoprotein hormone receptor belonging to the g protein-coupled receptor family. It is important in both male and female reproductive processes; defects in hFSHR can lead to infertility, delayed puberty, reduced muscle bulk, and osteoporosis. Work in other labs has shown that GPCRs can be localized to microdomains located within the cell membrane called lipid rafts. These regions are highly resistant to detergents because of the high concentration of sphingolipids and cholesterol. Also within these domains, an intracellular protein, caveolin, is present. Our lab has shown that hFSHR also localizes to lipid raft domains. We hypothesize that interaction with caveolin is important in the raft residency of hFSHR through a putative caveolin binding motif (CBM). The interaction between caveolin and hFSHR in these microdomains may regulate intracellular pathways activated by the receptor.

To test our hypothesis, we have constructed CBM mutants and are in the process of creating stable cell lines expressing the mutant receptors to study the effects of mutating the key phenylalanine residues of the CBM. Interestingly, wild type FSHR presents as a doublet on a western blot while the 479L only have a single band. We theorize that one of these bands may be a glycosylation variant however further investigation is required. The mutated receptors qualitatively show normal p44-MAPK signaling in response to FSH, indicating that they are reaching the cell surface and are capable of binding hormone and coupling to signaling partners. We have observed a subtle difference in downstream PKA targets between the mutant and wild type hFSHR, suggesting that there might be a

difference in the ability to activate downstream targets. Studying the interaction between hFSHR and caveolin has the potential to develop new contraceptive antagonists and alternative agonists to regulate fertility.

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Introduction:

The ability of all living organisms to receive and respond to external stimuli is essential for survival. These processes are regulated by cell surface receptors including g protein-coupled receptors (GPCRs). GPCRs account for over 800 types of receptors and are the largest set of drug targets in the pharmaceutical market, thus signifying the importance of their study in greater detail.[1] The human follicle stimulating hormone receptor (hFSHR) is a glycoprotein hormone GPCR important in both male and female reproductive processes.[2] hFSHR is expressed on the granulosa cells of the ovary in females, and the sertoli cells of the testis in males. When activated with human follitropin from the anterior pituitary, hFSHR induces a signal transduction pathway leading to follicular maturation in females and sperm production in males.[2]

Follitropin signaling:

Follitropin is released by the gonadotrope cells of the anterior pituitary in response to gonadotropin releasing hormone (GnRH).[2] GnRH is released from the hypothalamus in pulses based on gonadal steroid signaling.[3] GnRH travels through the hypophyseal portal to the anterior pituitary stimulating the production of lutenizing hormone and follitropin. Slow GnRH pulses stimulate the production and secretion of follitropin in gonadotrope cells.[4] Follitropin is then released into the blood stream. In order for the signal to be received successfully, follitropin must bind successfully to its GPCR, FSHR.[1]

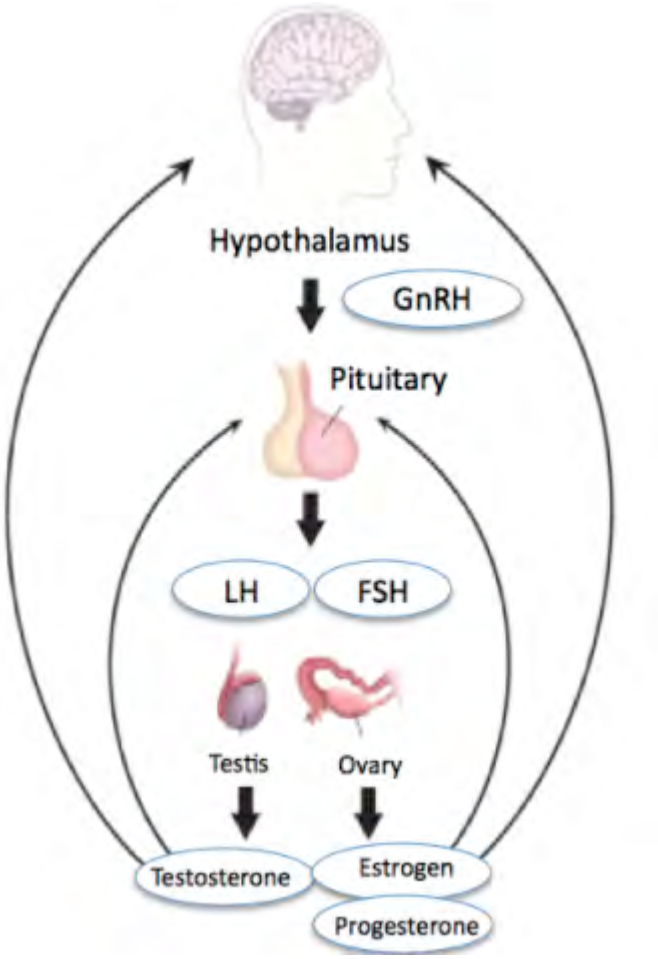


Figure 1. Diagram Illustrating Hypogonadal Axis[5]

FSHR belongs to the glycoprotein family of hormone receptors (GPHRs). Similar to other GPCRs, the protein crosses the membrane seven times as α-helices with a super-secondary structure of α-turn-α in three intracellular loops (IL) and three Extracellular loops (EL).[6]

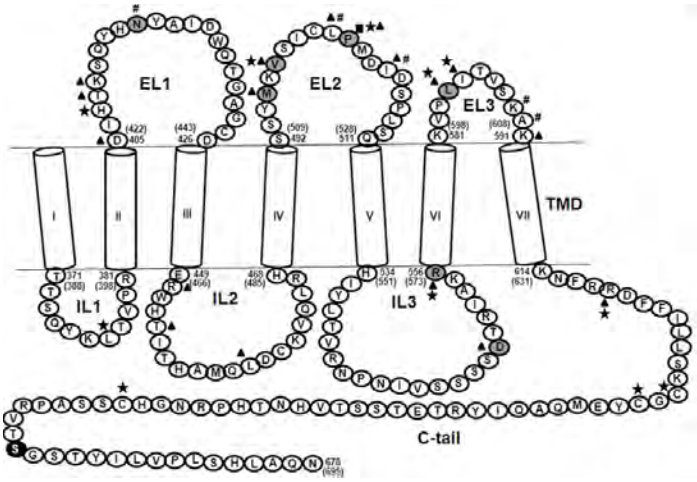


Figure 2. Human Follicle Stimulating Hormone Receptor

The transmembrane domain is highly conserved within the GPHR class. The receptor has three primary domains: Ectodomain, transmembrane domain and endodomain. The ectodomain is composed of two significant subdomains, the hormone binding subdomain and the signal specificity binding domain. Within the hormone-binding domain there are 12 lecuine rich repeats and other hydrophobic residues giving it a cylindrical-horseshoe shape.[5] The hormone-binding domain possesses β-sheets, complementary to follitropin, allowing it to interact electrostatically.

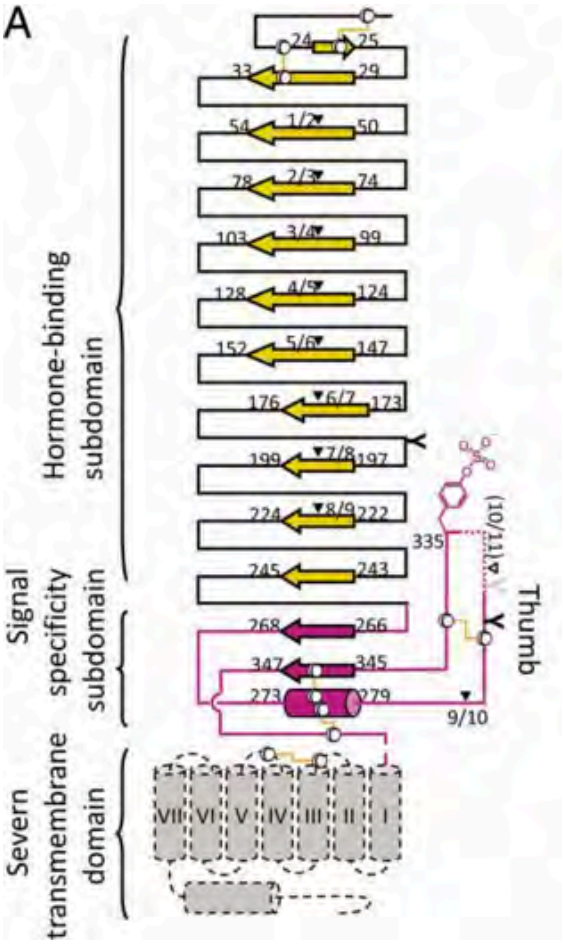


Figure 3. Molecular structure FSHR[6]

The hinge region of the binding domain contains three critical disulfide bonds that communicate a conformational change through the transmembrane domain upon the binding of follitropin. As follitropin binds to the hormone-binding domain, a conformational change in the signal molecule is believed to take place exposing a pocket within the follitropin. This pocket is specific for a sulfated tyrosine that lies on the hinge region. As residues on follitropin stabilize the sulfated tyrosine, a conformational change in the receptor occurs, which is communicated through the transmembrane domain into the endodomain.[5]

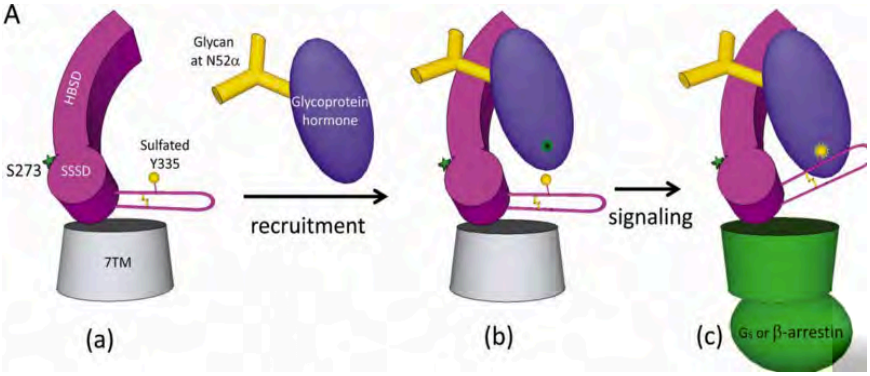


Figure 4. Signal transduction of FSHR[6]

It is believed that this conformational change on the C-terminus (intracellular side) of the GPCR causes a conformational change in the attached $G_s\alpha$-subunit. This structural change causes the subunit to favor the release of GDP and uptake of GTP, which activates the G-protein. $G_s\alpha$ stimulates the activity of adenyl cyclase, which converts ATP cyclic adenosine monophostophate (cAMP).[7] cAMP then activates protein kinase A (PKA), which phosphorylates cAMP response element binding protein (CREB). CREB is a transcription factor that will then act to stimulate steroidogenesis, which is involved in the aforementioned processes of sex cell maturation.

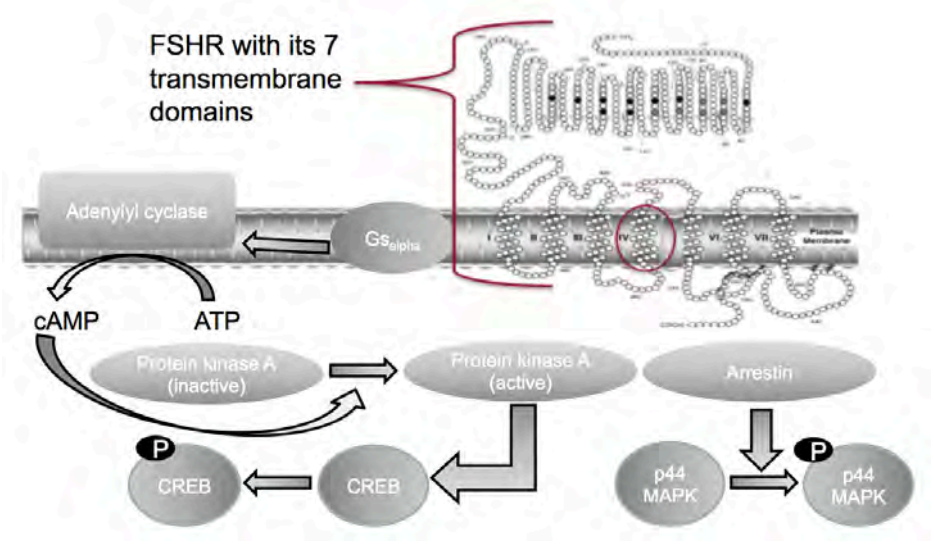


Figure 5. Downstream signaling of FSHR

There are many components within the plasma membrane that are required for appropriate signal transduction, and appropriate activation of secondary messengers.

However, these components are expressed at relatively low levels along the surface. Given this low density, it would be nearly impossible for these components to randomly associate at the correct time to transduce a signal. Yet somehow, this signaling occurs rapidly and efficiently.

Plasma membrane & Lipid Rafts:

One possible mechanism for the efficient transduction of signal is that these components co-localize in a sub-domain of the plasma membrane referred to as lipid rafts. All cells are separated from their surroundings by a plasma membrane. This membrane is semipermeable to certain ions, and small organic molecules, and controls the migration of larger molecules moving in to and out of the cell. Plasma membranes are composed of a lipid bilayer studded with various proteins. The lipid bilayer consists of hydrophobic acyl chains with a polar head group. In an aqueous environment, the hydrophobic head groups are exposed to the surroundings and interior of the cell, while the acyl chains lay within the membrane itself.

Based on the fluid mosaic model, the lipid bilayer functions as a two-dimensional liquid.[8] According to this model, individual proteins and acyl chains are able to move relatively freely within the membrane. However, there is a certain fluidity/permeability balance that must be met for the cell to function. Because some of the acyl chains possess double bonds, there are often openings within the membrane itself.[7] The addition of cholesterol into the plasma membrane allows these gaps to close, and limit the permeability and fluidity of the cell. The intercalation of cholesterol at higher concentrations can also produce micro-domains known as lipid rafts.[9]

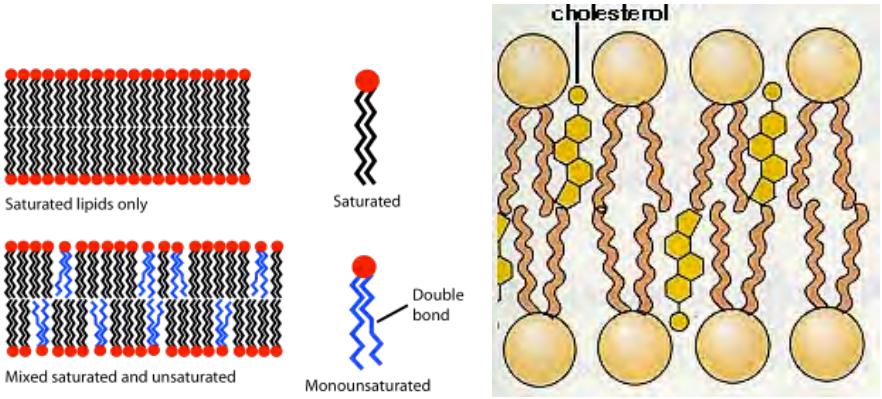


Figure 6. Intercalation of cholesterol in plasma membrane[10]

These sub-domains are characterized by a higher concentration of sphingolipids and high levels of cholesterol, allowing the acyl chains to pack more densely.[8] Lipid rafts are expressed in a wide variety of cells, however, a certain subset of those cells also express the protein, caveolin.

Caveolin:

Caveolin is a 21 kD, structural membrane protein. The presence of caveolin in the membrane causes a 50-100 nm invagination of the lipid raft membrane, referred to as caveolae.[9] It has been demonstrated in literature that these domains enable spatial co-localization of interacting proteins to promote efficiency of signal transduction.[11] Caveolae are primarily found in muscle cells, endothelial cells, and adipocytes.

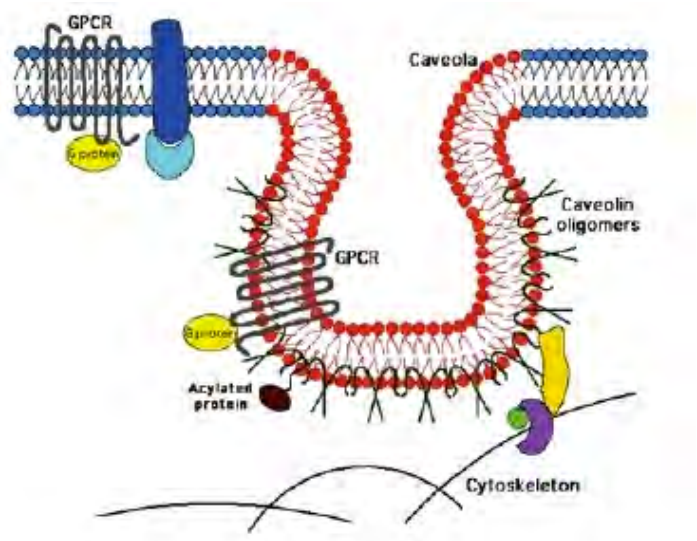


Figure 7. Caveolin promote formation of caveolae[12]

There are three isoforms of caveolin found in mammalian cells, caveolin-1, caveolin-2, & caveolin-3. All three proteins express a similar conformational structure with a cytoplasmic C & N terminus with a hairpin loop transmembrane domain, however, they differ in which tissues they are expressed in.[9] For example, caveolin-3 is exclusively expressed in skeletal and cardiac myocytes. Caveolin-1, the predominant form, tends to dictate caveolae formation through oligomerization.

Caveolin has three major functions when it comes to the formation of lipid rafts and signal transduction. Primarily, it is involved with forming the flask like invaginations within the membrane of the cell. It does this by essentially reducing the physical strain caused by the curvature of the membrane.[13] Caveolin also acts as a scaffolding protein. It has the ability to recruit specific proteins to localize within the caveolae. Finally, caveolin acts as a protein chaperone, and assists with cell membrane trafficking. Within caveolin itself, subdomains exist to carry out these various processes. An example of this is the scaffolding domain of caveolin (CSD).[9] This string of amino acids acts to anchor necessary signaling molecules for efficient signal transduction. The proteins that co-localize with caveolin

express a sequence of amino acids that interact with the CSD referred to as the caveolin-binding motif (CBM).

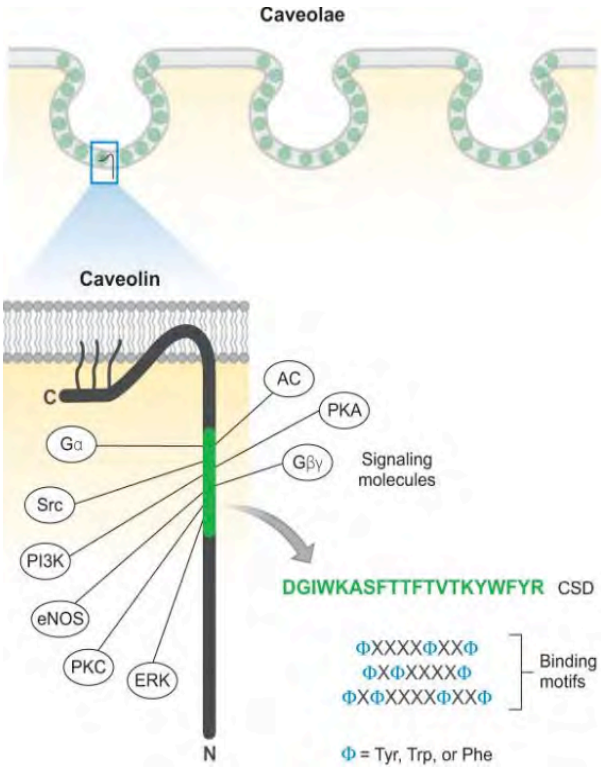


Figure 8. Caveolin scaffolding domain schematic[15]

These short amino acid chains are hydrophobic and rich in aromatic residues. The typical sequences are ϕxϕxxxxϕ or ϕxxxxϕxxϕ or combined sequence ϕxϕxxxxϕxxϕ, where ϕ is a phenylalanine, tyrosine or tryptophan residue, and x can be any amino acid.[14] It has been established that hFSHR expresses the CBM sequence of amino acids between residues between 479 & 489 with each aromatic residue being a phenylalanine.

Caveolin Binding Motif

Φ	X	Φ	X	X	X	X	Φ	X	X	Φ
479		481					486			489

In order to further investigate caveolin-hFSHR interactions, we have set out to establish stable mutant cell lines that express different combinations of mutations in each of the phenylalanine locations. Previous research in the Cohen lab has generated transiently transfected mutants of the caveolin binding motif, but none that readily express the mutated receptor organically. Our research represents the investigation of an F479L polymorphism. By evaluating behavior of the mutated domain we can better understand the relationship between hFSHR and lipid rafts. As the interaction is better comprehended,

research can be focused on the implications of this interaction in infertility and birth control.

Methods:

Cell Culture:

Human Embryonic Kidney cells 293 (HEK293) were grown in culture at 37°C in DMEM with 10% fetal bovine serum and penicillin & streptomycin. Cells were transfected with either wild type hFSHR DNA or mutant hFSHR F479L using SatisFection.

DNA Stable Transfection:

At 75% confluence, HEK293 cells were transfected using SatisFection reagent. Cells were then cultured in G418 antibiotic to select for lines expressing mutated (F479L) receptor or wild type. After several days, colonies of surviving cells were collected and plated in a 96 well dish. Those that continued to proliferate were then transferred to a 12 well and then to a 6 well, and finally transferred to a T25 and a T75 flask as they reached confluence. For more details refer to Satisfection Transfection Reagent manual in Appendix B.

SDS-PAGE Western Blot Hormone Treatment:

Upon T75 confluence, both HEK293 WT and F479L mutant cells were treated with 2000 ng of FSH for either zero, five, 15, or 30 minutes. The cells were then lysed and protein contents evaluated by gel electrophoresis using 7.5% acrylamide gel. The proteins were then transferred to a PVDF membrane using a semi-dry electrophoresis method and blocked with 5% milk in TBST. The membranes were then probed with Anti-hFSHR mAb 106.105 (105) for hFSHR; P-p44/42 MAPK Rabbit Ab for phosphorylated p44; and P-PKA substrates for downstream signaling targets of P-PKA. Membranes were then probed with anti-mouse secondary antibody for 105 and anti-rabbit for P-p44 and P-PKA substrates. Membranes were imaged (BIORAD ChemiDoc MP Imaging System). For more detailed procedures refer to Appendix C.

Immunofluorescence Microscopy:

HEK 293 WT and F479L mutant were grown on glass coverslips and fixed using a 4% paraformaldehyde PBS solution. Cells were probed for hFSHR using mAb 106.105. Goat anti-mouse with an alexa-red fluorophore was used as a secondary antibody. Slides were then evaluated on fluorescent microscope (Olympus BX60).

Discontinuous Sucrose Gradient

2 T75s of each HEK 293 WT and F479L were grown to 80% confluence. One T75 of each was treated with 2000 ng of FSH, incubated for 30 minutes. Cells were collected and resuspended in 0.5 M sodium carbonate (pH 11) and incubated for 20 minutes. Cells were then dounced and sonicated. Lysates were added to 90% sucrose in 1:1 dilution and added to ultracentrifuge tube. A 35% solution followed by a 5% solution were laid on top. Ultracentrifuge (Beckman L-70) was run at 34,000 RPM for 16 hours. Fractions from the gradient were recovered and analyzed using SDS-PAGE and Western Blot. Gel electrophoresis was carried out using 7.5% acrylamide gel. Proteins were then transferred to a PVDF membrane using a semi-wet electrophoresis method and blocked with 5% milk in TBST. The membranes were then probed with Anti-hFSHR mAb 106.105 (105) for hFSHR. Membranes were then probed with anti-mouse secondary antibody for 105. Membranes were imaged (BIORAD ChemiDoc MP Imaging System).

Results:

Establishment of a stable hFSHR-F479L expressing cell line

After HEK293 cells were transfected with F479L SatisFection vector, colonies were harvested and receptor expression were evaluated (See Methods: DNA Stable Transfection for details). Colonies were then labeled A-F and evaluated using SDS-PAGE/Western Blot. Membranes were probed with anti-hFSHR mAb 106.105 (Figure 10).

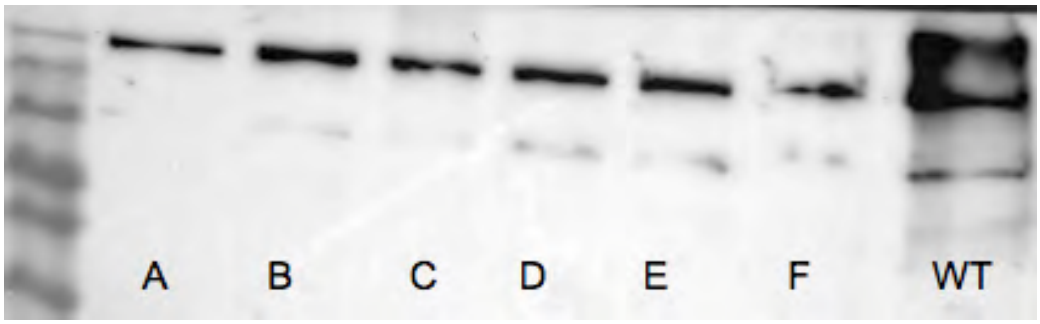


Figure 9. Figure 1. Western blot of protein lysates from HEK 293 cell lines stably transfected with hFSHR-479L mutant (Lanes A-F). Lane WT is control HEK 293 cell line stably expressing wild type receptor. hFSHR probed with anti-hFSHR mAb 106.105. Mutant cell line from lane E was used to carry out subsequent experiments.

There is a single band at 80 kD seen in Lanes A-F of similar intensity. There is also a darker band in the WT lane at 80 kD along with a faint band around 50 kD mark.

Does hFSHR-F479L Localize on the Cell Surface Similar to Wild Type Receptor?

HEK293-hFSHR F479L (Mutant) were compared to HEK293-hFSHR (WT) using immunofluorescence.

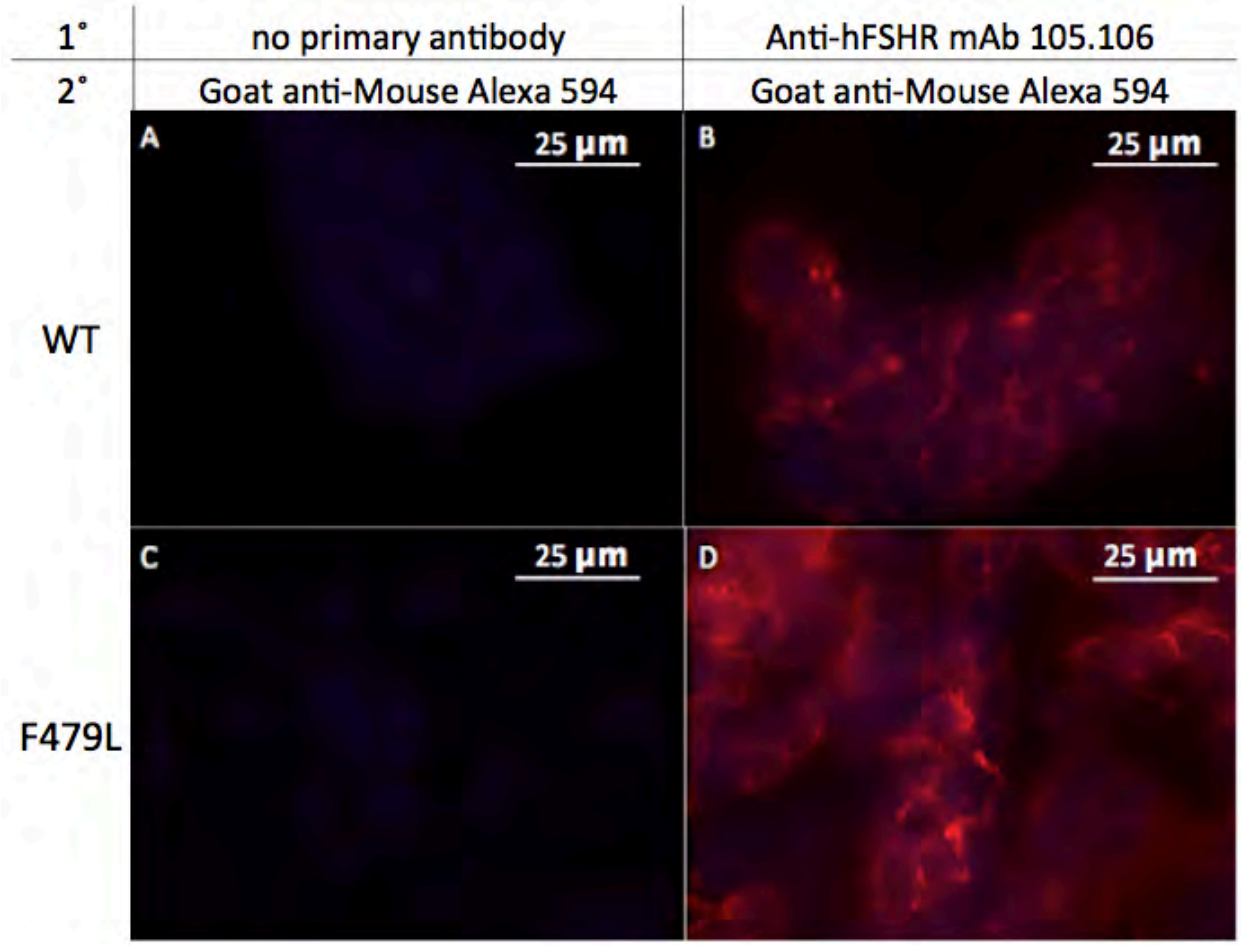


Figure 10. Immunofluorescence of HEK 293 cells stably expressing hFSHR isoforms as indicated. A & B- Wild Type hFSHR, C & D- hFSHR-F479L mutant.

The top row of Figure 10, labeled WT, represents the HEK293-hFSHR wild type cells and the bottom row represents the F479L mutant. A and C represent cells that were not treated with 106.105 antibody and serve as a negative control. In B and D there is increased fluorescence around the perimeter of the cell indicating that both the wild type and mutant receptor are localizing in the cell membrane.

Does the Mutated Receptor Localize in Lipid Rafts?

To investigate the localization of the mutated receptor in lipid rafts, a discontinuous sucrose gradient followed by SDS-PAGE Western blot was carried out (See methods for detailed procedures). The results of the Western Blot can be seen below.

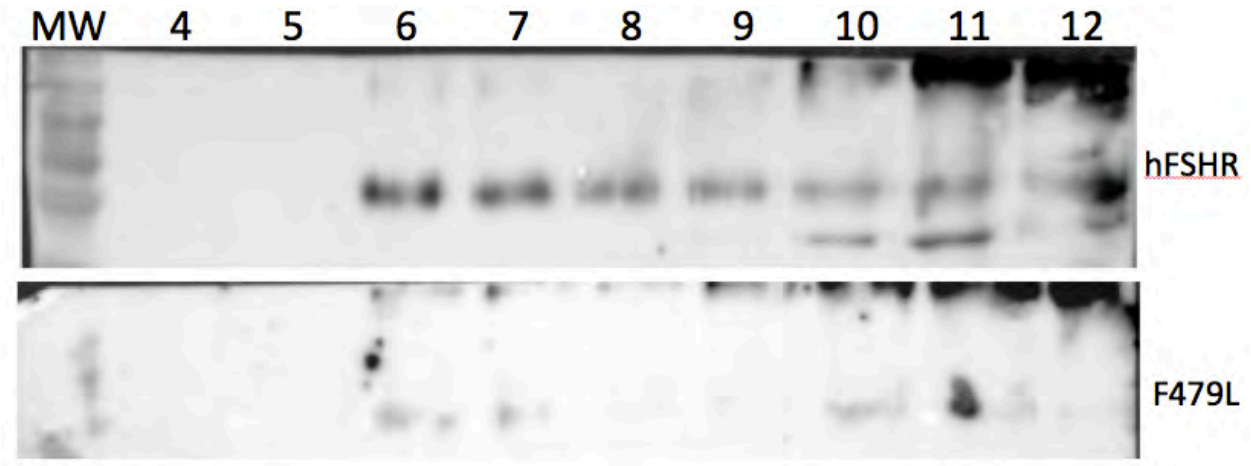


Figure 11. Figure 3. Western blot of protein lysates from HEK 293 cell lines expressing wild type hFSHR or hFSHR-F479L. Cells were treated for 30 min with 20ng/ml hFSH followed by separation on a discontinuous sucrose gradient. Collected fractions were probed for hFSHR using mAb 106.105. Lipid rafts are predicted to be seen in fractions 5, 6, 7.

The red box indicates where the fractions from the sucrose gradient where lipid rafts may be present. It is apparent that there is receptor present within the lipid rafts in both the wild type and F479L mutant.

Does a Mutation in the Caveolin Binding Motif Affect Downstream Signal Transduction?

F479L and wild type were treated with FSH for 0, 5, 15, or 30 minutes. Cells were then lysed and analyzed via Western Blot. Membranes were then probed for phosphorylated PKA substrates and phospho-P44 MAPK. The results of these blots can be seen below.

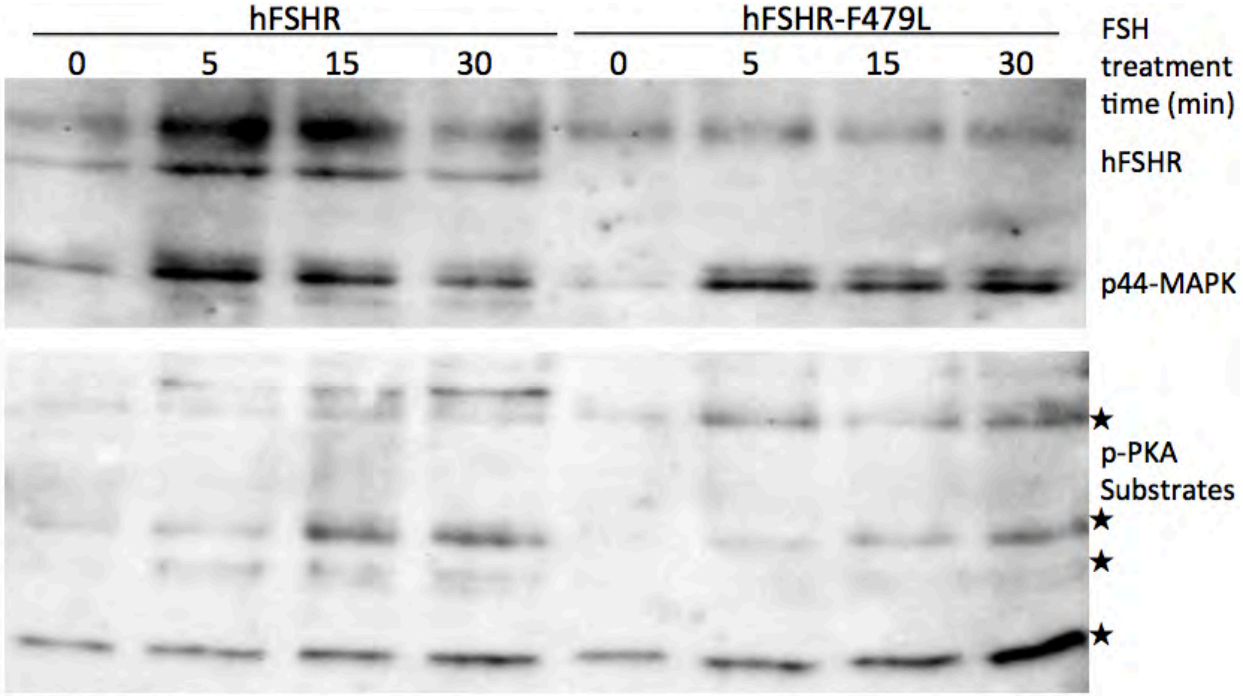


Figure 12. Figure 4. Western blot of protein lysates from HEK 293 cell lines expressing wild type hFSHR or hFSHR-F479L. Probed for hFSHR, p44-MAPK (top), and PKA Substrates (bottom). ★ - indicates bands that are different between the cell lines for PKA substrates

Based on the above blot probed for P44-MAPK, similar density bands can be observed between wild type and mutant. This indicates that P44-MAPK signaling seems to be conserved. On the other hand, there are several differences between wild type and mutant in downstream PKA substrates. These differences are noted by stars on the right side of Figure 12. These differences indicate that there may be slight differences in downstream PKA signaling.

Discussion:

There were two main phases to the experiments carried out to investigate F479L mutant receptor. The first phase, was first to create a cell line that is stably expressing the F479L mutation in the hFSHR. The second phase, was to investigate the raft residency, function, and consequential/downstream signaling of the mutated receptor, and compare it to the wild type receptor.

Previous work in our lab has investigated a transient or temporary transfection of the F479L mutant.[16] Here, we used a stable transfection method to better simulate *in vivo* mechanisms. Also, by establishing a cell line that is stably expressing the F479L receptor, future experimentation can be carried out more efficiently. After HEK293 F479L colonies were established, cells were lysed and evaluated for presence of hFSHR using a Western Blot. The results of this experiment can be seen in figure 9. Here it can be noted that receptors are present in each of the mutant colonies established, as well as the wild type cells. For the purposes of consistency, we used cell line E for future experimentation. Within this figure, it is also important to note within the wild type lane the presence of a second band approximately 20 kD below the receptor. It is thought that this band may be a potential glycosylation variant of the wild type receptor. It is also important to note that this variant is barely visible within the mutant lanes.

Past research has demonstrated that the glycosylation of hFSHR occurs at four different sites within the extracellular domain. Although it has been shown that glycosylation is not directly necessary for hormone binding, certain sites are required for proper conformational folding in order for receptor to properly bind to hormone. [17]

The second phase of our experiment investigated the localization and function of the mutated receptor. First, we used immunofluorescence to establish that the receptor was

residing within the cell membrane. The results of this experiment can be seen in Figure 10A-D. Figure 10A and 10C served as our negative control, and were therefore not treated with primary antibody. This was done to negate any effects of auto-fluorescence or background fluorescence. The presence of red fluorescence around the perimeter of the cells in both the wild type and F479L mutant is indicative of the localization of receptor within cell membrane. It is not quantifiable because this was done using indirect immunofluorescence. However, we can conclude that receptors are present within the cell membrane in both wild type and mutant.

It is believed, that within the cell membrane there lies a region of higher buoyancy designated as lipid rafts. Next, we set out to examine if the mutated receptor localizes within these domains similarly to the wild type. To do this we performed a discontinuous sucrose gradient followed by a Western Blot analysis. The results of this experiment can be seen in Figure 11. In the wild type analysis, receptors can be seen localizing within the lipid raft region of the sucrose gradient indicated by the red box. However, receptors can also be seen in later or denser fractions. There are several reasons this may be the case. As discussed earlier, there are glycosylation variants of the wild type receptor. This glycosylation may occur at different stages. Glycosylation can occur in the endoplasmic reticulum and within the golgi apparatus. Enzymatic glycosylation can also occur while the protein is being transported to the cell surface. These bands may be indicative of "immature" receptor in different stages.

Within the F479L assay, receptor can also be seen present within the lipid raft fractions of the sucrose gradient. However, expression is greatly reduced. Perhaps this is a result of an interruption of the caveolin-binding motif. A disruption of this domain could potentially disturb the co-localization with caveolin and may down regulate the amount of

receptor localizing within lipid rafts. It is also important to note that unlike the wild type, receptor does not seem to be present in denser sucrose fractions.

Our last experiments focused on downstream signaling of receptors to determine if the mutation had an impact of consequential signaling. As seen in figure 5, there are two primary signal transduction pathways that are activated as a result of FSH binding to receptors. The first pathway we examined was the Arrestin/p44 MAPK pathway. This was done by treating the cells with hormone for varying lengths of time, lysing the cells, and analyzing via Western Blot. The results of this can be seen in Figure 12. First, membranes were probed with anti-hFSHR mAb 106.105. Within the wildtype membrane it can be seen that more receptor seems to be recruited with the addition of hormone although this mechanism has yet to be investigated. On the other hand, this did not occur within the mutant. The membranes were also probed with anti-phospho-p44/MAPK. Here it can be seen that signaling seems to be conserved in the mutant relative to the wild type. Although the amount of receptor seen at 5 and 15 minutes in the wild type increased, it is also important to note that the expression of the secondary messenger p44/MAPK seems to be conserved between wild type and mutant. This has also been demonstrated in the work of Stanley Soroka '16. Although Soroka's experiments used a transient transfection technique, it can be seen in Appendix A that p44/MAPK signaling was also conserved with the F479L mutant. Therefore, it can be concluded that the F479L mutation does not affect the arrestin-P44/MAPK pathway.

The other pathway that hFSHR activates is the $G_s\alpha$-PKA pathway seen in Figure 5. Here, membranes were probed with a downstream PKA substrates antibody. Unlike the p44-MAPK pathway, it seems there are slight variations in downstream signaling within this pathway. One potential mechanism for this could be the idea that free caveolin-1

within the cell can inhibit PKA signaling. Expression of activated CREB was noted to drastically decrease with presence of cav-1 in vitro.[17] Therefore, by disrupting the CBM-CSD interaction, more caveolin may be present within the cytoplasm yielding down regulation of PKA substrate activation. On the other hand, although it has been shown that caveolin also interacts with MAPK, there did not seem to be a difference in signaling in our experiments.

For future investigation it would be important to examine certain components of the experiments carried out here. For example, these experiments were done under the assumption that a mutation within the transmembrane domain of the receptor will not affect the hormone-binding region. Therefore, we assumed that hormone would bind equally between the wild type receptor and mutated receptor. If untrue, this would result in altered consequential signaling between receptors. Another assumption made for this study was that the levels of caveolin-1 and caveolin-2 in HEK293 cells are similar to that of Sertoli or granulosa cells. Because caveolin plays such a dynamic role in the localization of hFSHR and its downstream elements, lower or higher levels of caveolin could drastically change this dynamic, and consequentially affect results seen here. Furthermore, caveolin expression and co-localization with receptor should also be investigated to determine if there is decreased co-localization with the mutated receptor. This could be done using immunofluorescence or discontinuous sucrose gradient assay followed by Western Blot analysis. Effects of F481L, F486L, and F489L mutations should also be investigated. Based on previous work, it has been established that the F481 lies in an opposite direction of the other aromatic residues as seen in Figure 13.

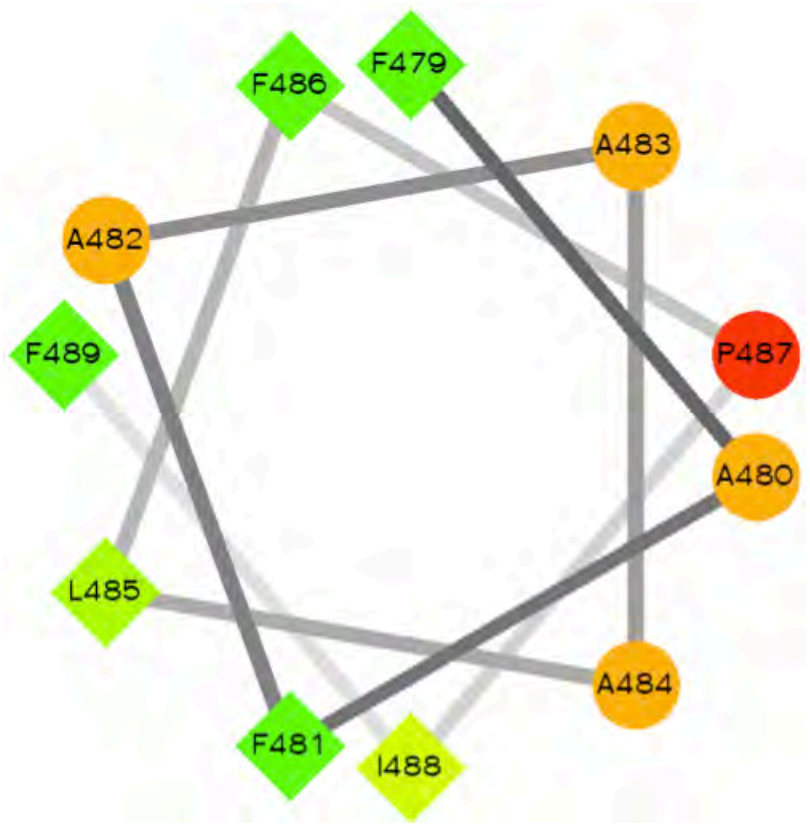


Figure 13. A view of transmembrane domain IV, the caveolin binding motif, of hFSHR. Demonstrates the alpha helix from intracellular to extracellular domain.

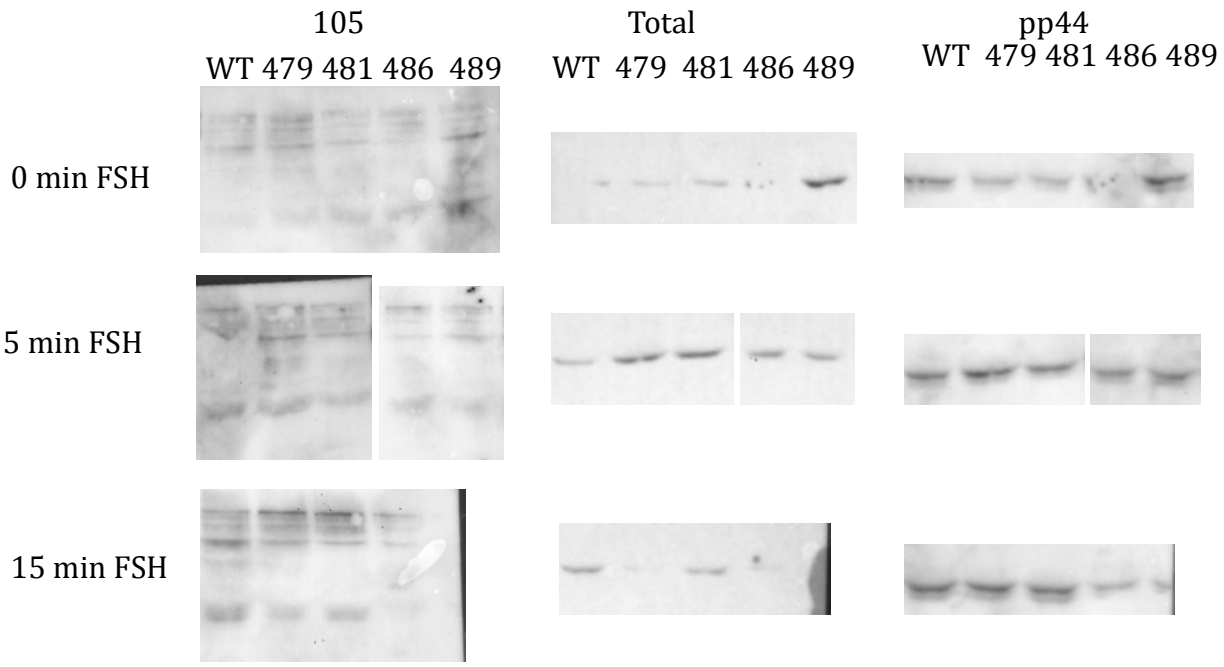
On the other hand, F479, F481, and F486 come before the P487 residue, which interrupts the helix. In this case, the F489 residue may have the least effect on the CBM-CSD interaction. Further experimentation is required to elucidate the dimensional mechanisms of the CBM-CSD interaction. Once these single mutations have been investigated, combinations of these mutations should be examined to determine if any of them have a greater role in the disruption of this interaction.

Disruption of the interaction of caveolin with hFSHR could potentially interrupt reproductive signals of the HPG axis. By interrupting these signals without impacting hormone levels, contraception could be achieved without the negative implications of birth control.

Acknowledgements:

I would like to thank my advisor, Professor Brian D. Cohen, his guidance and assistance was instrumental in my senior thesis work. Professor Cohen orchestrated my research from the start, and guided me in the right direction throughout the year. I would also like to thank my professors in the biology, chemistry, and biochemistry departments for providing me with the academic tools in order to carry out this work. I would also like to acknowledge the Union College Student Research grant for providing me with the necessary funding to complete my thesis. Lastly, I would like to thank Team Cohen for assisting whenever necessary, and for their support.

Appendix A: Soroka '16 Western Blot of transient transfected hFSHR variants



Appendix B: Satisfection Transfection Reagent

SatisFection Transfection Reagent

MATERIALS PROVIDED

Materials provided	Quantity[a]		
	Catalog #204121	Catalog #204122	Catalog #204123
SatisFection Transfection Reagent	0.3 ml	0.75 ml	2 × 0.75 ml

[a] The reagent has been optimized for 625–1250 transfections per 0.75 ml transfection reagent using 24-well tissue culture dishes.

STORAGE CONDITIONS

SatisFection Transfection Reagent: 4°C

ADDITIONAL MATERIALS REQUIRED

Media for preparing transfection mixture (Opti-MEM, DMEM, RPMI or other cell culture media **without serum and antibiotics**)

Media for cell growth (Opti-MEM, DMEM, RPMI, or other cell culture media, including serum, growth factors, and antibiotics if appropriate)

INTRODUCTION

Gene transfection into eukaryotic cells is a fundamental tool for analysis of gene function and for production of recombinant gene products. SatisFection Transfection Reagent gives optimal efficiencies and protein expression levels in common cell lines, while significantly improving efficiencies in many hard-to-transfect and primary cells. Maximum gene expression and protein production is observed 36–72 hours post-transfection.

The SatisFection reagent is a novel cationic polymer suitable for both transient and stable transfections in the presence or absence of serum. This polymer functions by binding and condensing DNA into a polyplex that enters the cell via endocytosis. Properties of the polyplex facilitate both endosomal rupture and protection of the bound DNA from lysosomal degradation. Continued exposure to cellular pH ultimately reduces the polymer into a non-toxic monomer. The monomer is then easily excreted from the cellular compartment, resulting in lowest the levels of cytotoxicity and increased cell viability. As a result, the SatisFection reagent is ideal for experiments that require large, healthy populations of positively transfected cells for downstream applications, e.g., signal transduction pathway analysis.

PREPROTOCOL CONSIDERATIONS

The protocol provided below will allow effective transfections for most cell types, however, optimal transfection conditions may vary. The following parameters should be established for each cell line and plasmid used.

DNA Quality and Concentration

The use of highly purified DNA is critical for successful transfection. Suitable quality DNA is obtained using StrataPrep plasmid miniprep kit, or by cesium chloride purification**.** The optimal concentration for transfection generally falls within the range of 0.4–1.0 µg DNA per well of a 24-well plate, with 0.6 µg as the recommended starting point for optimization.

Cell Density

While we recommend a cell density of 60–80% confluence after 18–24 hours of incubation at the time of transfection for most cell types, the optimal cell density should be determined specifically for each cell type.

Reagent to DNA Ratio

The optimal reagent to DNA ratio should allow the highest transfection efficiencies with the lowest level of toxicity. This is usually achieved within the range of 1–2 µl reagent per µg DNA, with 1.5 µl reagent per µg DNA the recommended starting point for optimization.

Transfection Incubation Time

The optimal time that the transfection reagent is in contact with the cells should be determined by testing a range of incubation times from 36–72 hours.

SATISFECTION TRANSFECTION REAGENT VOLUME GUIDELINES

The following procedure for the generation of transfectants uses a 24-well tissue culture plate. The optimal ratio of SatisFection transfection reagent to DNA must be determined for each plasmid and cell line, however 1.5 µ1 reagent per 1 µg DNA is the recommended starting point for optimization. Reagent volumes for cells not cultured in 24-well tissue culture plates are given in Table I. When preparing the transfection mixture, the volume of the transfection mixture may be scaled up by increasing the components proportionally to accommodate several transfections.

TABLE I

SatisFection Transfection Reagent use for Various Cell Culture Dish Formats [a]

Tissue culture dish format	Diameter of the well (mm)	Recommended number of adherent cells ($\times 10^5$)	DNA solution per well		Transfection reagent per well		Total volume of transfection mixture plus medium (µl)
			Amount of DNA (µg)	Final volume of DNA solution diluted with serum-free medium (µl)	Volume of transfection reagent stock solution (µl) [1.5:1 ratio (µl reagent: µg DNA)]	Final volume of transfection reagent solution diluted with serum-free medium (µl)	
96-well	6.4	0.04–0.1	0.15	7.5	0.225	7.5	15 µl
48-well	10	0.1–0.3	0.3	15	0.45	15	30 µl
24-well	15	0.27–0.67	0.6	30	0.9	30	60 µl
12-well	22	0.6–1.5	1	50	1.5	50	100 µl
6-well	35	1.5–3.7	2	100	3	100	200 µl
35-mm	35	1.5–3.7	2	100	3	100	200 µl
60-mm	60	4.0–11.0	6	300	9	300	600 µl
100-mm	100	12.0–31.0	16	800	24	800	1600 µl

SatisFection Transfection Reagent

Catalog #204121, 204122, and 204123

QUICK-REFERENCE PROTOCOL–ADHERENT CELLS

Seed cells to 2.7–6.7 × 10^4 cells per well of a 24-well plate and incubate 18–24 hours so that cells are at 60–80% confluency at time of transfection

Prepare transfection mixture

- Pipet 30 µl antibiotic-free, serum-free medium (room temperature) into microcentrifuge tube. Pipet reagent (0.9 µl) into medium, do not touch plastic.
- Pipet 30 µl antibiotic-free, serum-free medium (room temperature) into separate microcentrifuge tube. Add DNA (0.6 µg) to medium.
- Add the diluted transfection reagent dropwise to the diluted DNA while gently vortexing or mixing with the pipet tip.
- Incubate transfection mixture 15 minutes at room temperature.

Add the transfection mixture to the cells

- Add transfection mixture dropwise and incubate for 36–72 hours under standard growth conditions.

Perform stable transfection (optional)

- 36–72 hours after the transfection (above), split cells (1:5 suggested).
- After 24 hours, apply selection antibiotics.
- Continue selection, replacing medium every 4–7 days until colonies form.

Appendix C: Protein Extraction and Western Blot

Protein Extraction Protocol

1) Do all steps on ice or at 4^{o}.

2) Rinse cells with 1X PBS (1 ml, ice cold)

3) Add 0.5 ml lysis buffer/well

4) Incubate on ice for 20 min.

5) Scrape into microfuge tube

6) Transfer extract to Dounce homogenizer and homogenize with 10 strokes of Dounce homogenizer (tight pestle)

7) Transfer extract back to microfuge tube

8) Spin in microfuge in cold room for 10 min at max speed

9) Transfer supernatant to new tube. Save pellet.

Igepal-DOC lysis buffer

1% Igepal
0.4% deoxycholate
10mM Tris pH7.
6.6mM EDTA

-add 1X protease inhibitor cocktail before using lysis buffer

SDS polyacrylamide gel electrophoresis and Western Blot

Materials

BCA protein assay (Pierce Biotech, cat. #23225)
Mini-PROTEAN II gel apparatus (Biorad)
Costar gel-loading tips (Krackler Scientific, cat. #MN520R-LRS)
Prestained SDS-Page broadrange molecular weight standard (NEB, cat. #P7708S)
Trans-blot Semi-Dry Transfer cell (Biorad)
Immobilon-P membrane (Millipore, cat. #IPV00010)
heat-sealable bags (Kapak, cat. #TRS-95250)
Kodak Biomax light autoradiagraphy film, 13 x 18 cm (Perkin Elmer Life Sciences, cat. #868-9358)
Radtape (Diversified Biotech, cat. #RAD-10)

Reagents

TEMED (Sigma)
Tween-20 (Sigma)
Price Chopper non-fat dry milk
Isopropanol (Sigma)

Gel Buffers

Acrylamide solution (30% acrylamide/0.8% bis-acrylamide)

-dissolve 30g acrylamide (FW=71.08), 0.8g bis-acrylamide (FW=154.17) in a total volume of 100ml water. Filter through 0.2um filter.

4X Running Gel Buffer (1.5M Tris, pH 8.8)

-dissolve 36.3g Tris Base (FW=121.1) in 150ml water. Adjust to pH 8.8. Add water to total volume of 200ml. Filter through 0.2um filter.

4X Stacking Gel Buffer (0.5M Tris, pH 6.8)

-dissolve 12.1g Tris Base (FW=121.1) in 150ml water. Adjust to pH 6.8. Add water to total volume of 200ml. Filter through 0.2um filter.

10% SDS

-dissolve 10g SDS (FW=288.38) in a total volume of 100ml water.

10% Ammonium Persulfate

-dissolve 1.0g APS (FW=228.2) in a total volume of 10ml water. Store at -20˚C in 50-100ul aliquots.

1M Tris, pH 6.8

-dissolve 12.1g Tris Base (FW=121.1) in 80ml water. Adjust to pH 6.8. Add water to total volume of 100ml. Filter through 0.2um filter.

6X SDS Sample Buffer (0.375M Tris pH 6.8, 12% SDS, 60% glycerol, 0.6M DTT, 0.06% bromophenol blue)

-combine 3.75ml 1M Tris-Cl, pH 6.8, 6ml glycerol, 1.2g SDS (FW=288.38), 0.93g DTT (FW=154.2), 6mg bromophenol blue. Add water to total volume of 10ml. Store at -20˚C in 0.5ml aliquots.

2X SDS Sample Buffer (0.125M Tris pH 6.8, 4% SDS, 20% glycerol, 0.2M DTT, 0.02% bromophenol blue)

-combine 2.5ml 4X Tris pH 6.8 (stacking gel buffer), 2ml glycerol, 4ml 10% SDS (FW=288.38), 0.31g DTT (FW=154.2), 2mg bromophenol blue. Add water to total volume of 10ml. Store at -20˚C in 0.5ml aliquots.

1X SDS Sample Buffer (0.0625M Tris pH 6.8, 2% SDS, 10% glycerol, 0.1M DTT, 0.01% bromophenol blue)

5X Electrophoresis Buffer (0.125M Tris, 0.96M glycine, 0.5% SDS)

-dissolve 30.3g Tris Base (FW=121.1), 144.1g glycine (FW=75.07), and 10g SDS (FW=288.38) in a total volume of 2L water.

1X Electrophoresis Buffer (0.025M Tris, 0.192M glycine, 0.1% SDS)

-add 200ml 5X electrophoresis buffer to 800ml water.

Western Blot Buffers

Transfer Buffer (0.048M Tris, 0.039M glycine, 20% methanol, 0.00375% SDS)

-dissolve 11.64g Tris Base (FW=121.1), 5.86g glycine (FW=75.07) in ~1500ml water. Add 0.750ml 10% SDS (FW=288.38). Add 400ml methanol. Add water to total volume of 2L. No pH adjustment necessary.

10X TBST Wash Buffer (0.1M Tris HCl, 1.5M NaCl, 0.5% Tween-20)

-dissolve 31.52g Tris HCl (FW=157.6), 175.32g NaCl (FW=58.44) in ~1900ml water. Add 10ml Tween-20. Adjust to pH 7.2. Add water to total volume of 2L.

1X TBST Wash Buffer (0.01M Tris HCl, 0.15M NaCl, 0.05% Tween-20)

-add 100ml 10X TBST to 900ml water.

5% milk Blocking Buffer

-Dissolve 5g milk in a total volume of 100ml 1X TBST.

Method

1. Determine cell number or protein concentration to be loaded onto gels and what volume to load per well. Note max volumes for different comb configurations.

2. Usually add sample to Tris or PBS and then add sample buffer (2 ul sample + 18 ul PBS + 3.3 ul 6X sample buffer).

3. Sonicate cells briefly on ice to homogenize. Purified protein samples do not need to be sonicated.

4. Preparing resolving and stacking gels (for BioRad Mini-PROTEAN II): Make sure glass plates are clean. Use Sparkleen or Alconox powder to clean plates. Rinse with distilled water and then

95% EtOH. Wipe dry with KimWipes. Select a comb and spacer (ex. 0.75 mm). Place spacers in between the inner and outer glass plates. Make sure that the spacers are flush with the plates. Insert plates into the clamping assembly and tighten screws. Vacuum grease can be used on the casting stand gaskets to prevent buffer leakage. Snap assembly into the casting stand. Prepare resolving gel. Pour gel and overlay with 1X Electrophoresis Buffer or isopropanol (use 3.5ml resolving gel when using 0.75mm spacers and 7.0ml gel for 1.5mm spacers, this will produce a gel approx. 3.25" x 2"). Look for a division between the overlay and resolving gel, this means the gel is polymerized. Pour off overlay, use thin strips of Whatman paper to remove any excess. Pour stacking gel and insert comb. Remove comb when stacking gel is polymerized. Use a syringe and a 12 gauge needle to wash stacking gel wells. Snap clamp assembly into electrode assembly. Vacuum grease can be used on electrode assembly gaskets to prevent buffer leakage.

5. Boil samples 5 minutes at 100°C, and then spin tubes at 13,000rpm for ~1min. in RT micro centrifuge. Load samples using Costar Gel-Loading Tips.

6. Place electrode assembly with loaded samples into an electrophoresis chamber. Fill the electrode assembly carefully with 1X Electrophoresis Buffer. Fill the electrophoresis chamber with 1X Electrophoresis Buffer. Run gels at 100V for stacking gel, 100-150V for resolving gel (can increase to 200V for resolving gel if running on ice).

7. To transfer mini- gels to Immobilon-P membrane using the BioRad Semi-Dry Transfer Cell: Remove gels from glass plates. Cut stacking gel away and discard. Incubate gels in Transfer Buffer for 20 - 40 min. Cut out 6 pieces of Whatman paper and 1 piece of Immobilon-P membrane for each gel that is being transferred. They should be 3.25" x 2". Incubate Immobilon-P membrane in Methanol for ~10 - 20 seconds. Wet Immobilon-P membrane and Whatman paper in Transfer Buffer for 5 - 10 minutes before assembling the gel sandwich according to the BioRad Reference Guide. Transfer 1 mini-gel for 15 min. at 15 V, max current. Transfer 2 mini-gels for 30 min at 15 V, max current. Make sure that pre-stained MW markers transfer to Immobilon-P membrane.

8. Western Blot:

-Block membrane in 20ml blocking buffer overnight at 4°C or 1h at room temperature on rocker in a seal-a-meal bag.

-Dilute Ab in blocking buffer (1:1000 for serum or 5ug / 10ml for purified Ab). Incubate blots with Ab for 1h at rom temperature or overnight at 4°C on rocker in a seal-a-meal bag.

-Wash blots with 50ml 1X TBST on orbital shaker 3 times, 5 minutes each.

-Dilute HRP conjugated Ab 1:10,000. Incubate blots with secondary Ab for 1h at room temperature or overnight at 4°C on rocker in a seal-a-meal bag.

-Wash blots with 50ml 1X TBST on orbital shaker 3 times, 5 minutes each.

-Add 5ml chemiluminescent substrate to each blot, incubate for 5 minutes. Place blots in between transparency film. Push out air bubbles. Develop Western blot. Do a 1 minute exposure to check signal intensity. Continue with longer or shorter exposures as needed. (Max. exposure length = 10 min. Min exposure length = 60 sec.)

-For high backgrounds, wash in TBST then re-develop.

Resolving Gel

	7.5% gel (15ml)	10% gel (15ml)	15% gel (15ml)
Water	7.5ml	6.25ml	3.75ml
30% acrylamide/ 0.8% bis-acrylamide	3.75ml	5ml	7.5ml
4X Running Buffer	3.75ml	3.75ml	3.75ml
10% SDS	150ul	150ul	150ul
10% APS	150ul	150ul	150ul
TEMED	20ul	20ul	20ul

Running Gel: 7.5, 10, or 15% acrylamide, 0.375M Tris-Cl pH 8.8, 0.1% SDS, 0.33% APS, 0.66% TEMED

Stacking Gel

	4% gel (10ml)
Water	6.1ml
30% acrylamide/ 0.8% bis-acrylamide	1.3ml
4X Stacking Buffer	2.5ml
10% SDS	100ul
10% APS	100ul
TEMED	20ul

Stacking Gel: 4% acrylamide, 0.125M Tris-Cl pH 6.8, 0.1% SDS, 0.05%APS, 0.2% TEMED

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