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Investigating Human Follicle Stimulating Hormone Receptor and its Partners Using the APEX Assay

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By

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Abstract

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Many men and women deal with fertility whether it's needing infertility treatments or contraceptives. The follicle stimulating hormone (FSH) and follicle stimulating hormone receptor (FSHR) play important roles in reproduction and fertility in both males and females. Fertility issues can arise when interactions between FSH and FSHR aren't functioning properly and FSHR signaling is disrupted. FSHR is a G-protein coupled receptor (GPCR) found on the cell surface of granulosa cells in women and Sertoli cells in men. Activated FSHR initiates a cascade of downstream signaling events that result in different biological effects such as ovarian follicular development and estrogen production in women and sperm production and regulation in men. To create better birth control and fertility treatment options, FSHR signaling is a desirable target. Understanding how the receptor functions can offer insights for how it can be manipulated. Therefore, the effector proteins involved in FSHR signaling must be identified and understood. The APEX Assay has been developed to study these interactions for different GPCRs. This assay uses an engineered ascorbate peroxidase attached to the carboxyl terminus of FSHR which tags proteins within a small radius. Using mass spectrometry, the biotinylated proteins can be quantitatively analyzed to give a better understanding of the proteins associated with FSHR. HEK293 cells are currently being utilized to create a stable cell line expressing the FSHR-APEX receptor. The FSHR-APEX receptor can then be used in the APEX Assay to analyze proteins involved in FSHR signaling, allowing for the creation of birth control methods and fertility treatments that target the FSHR signaling pathways.

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Introduction

Fertility is a necessary part of life that allows species to reproduce. However, many men and women suffer from infertility and thus require additional methods to increase chances of pregnancy. Infertility is usually defined as the inability to conceive after one year of unprotected sex (Infertility). Approximately 9% of men and 11% of women of reproductive age experience issues with fertility (How common). The current methods for treatment can include drugs to stimulate ovulation, intrauterine insemination (IUI), and in vitro fertilization (IVF) (Farley, 2007). The cost of fertility treatments, ranging from about $2,000 for medication to about $24,000 for IVF (Advanced Fertility), poses a problem for many individuals seeking treatment for infertility. Thus, there is a need for an effective, low cost option for those who cannot afford IVF or other costly methods. However, there are many possible reasons that cause one to be infertile, bringing forth a challenge when considering new possible treatments. Therefore, understanding the biological pathways and mechanisms that contribute to fertility is important for drug development and other potential treatment options.

One possible mechanism causing infertility in both men and women involves the Follicle Stimulating Hormone (FSH). The human Follicle Stimulating Hormone (hFSH) is a gonadotropin which plays an important role in proper maturation and maintenance of the reproductive systems and is part of the hypothalamic pituitary gonadal axis (Figure 1). The hypothalamus releases gonadotropin releasing hormone (GnRH) which stimulates the anterior pituitary. Under hypothalamic control, the anterior pituitary gland secretes FSH, along with other hormones like luteinizing hormone (LH), which travel to target areas within the body and bind to specific receptors (Rawindraraj, 2019). After secretion, FSH binds to the FSH Receptor (FSHR)

to initiate a response. Upon ligand binding of FSH to FSHR, a biological response occurs in both the ovaries and the testes. In females, the maturation of ovarian follicles and estrogen production occurs and in males, sperm production is initiated and regulated (Lui et al, 2017; Ramaswamy et al, 2014). More specifically, in females, after stimulation by FSH, the ovarian follicle grows and differentiates into preovulatory follicles. Follicles that are FSH responsive are selected to continue maturation. Follicles that either don't respond or have a low level response to FSH undergo atresia. Upon maturation, follicles are continually selected for until the strongest one is released during ovulation. In males, FSH is necessary for Sertoli cell proliferation and to maintain normal spermatogenesis (Ulloa-Aguirre et al, 2008). It is clear that without FSH and its receptor, human fertility wouldn't be possible.

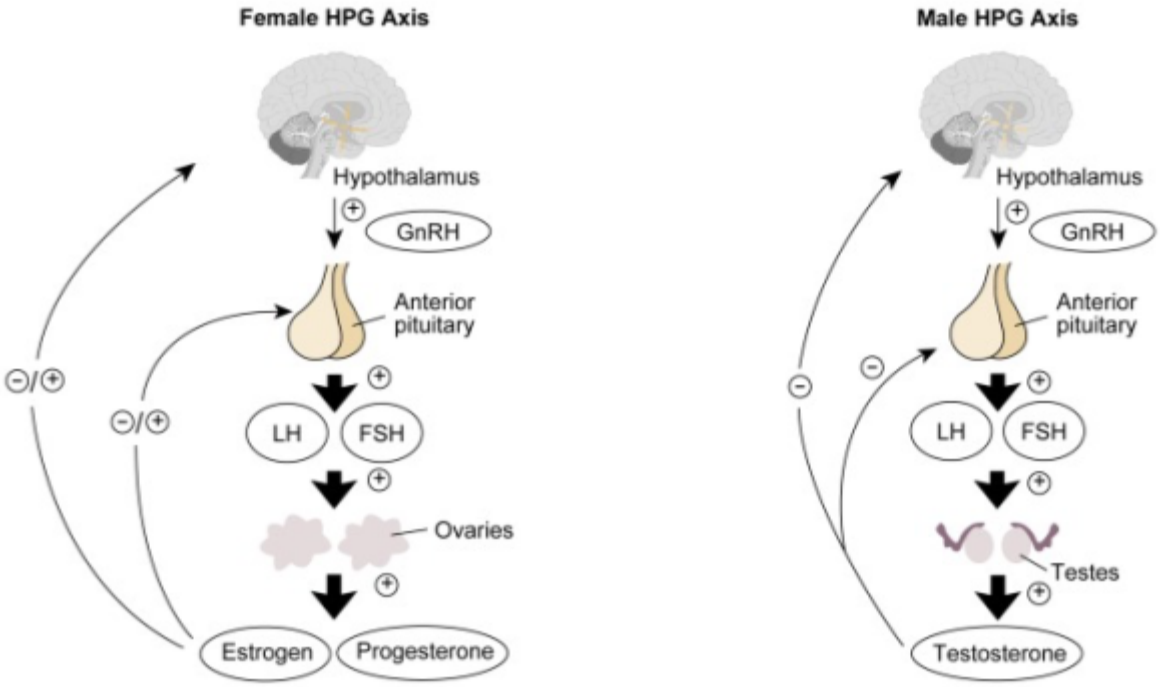


Figure 1. *A diagram of the hypothalamic pituitary gonadal axis (HPG axis) describing the connection between the hypothalamus and the gonads in both men and women.*

On the other side of this story there is the need for better contraceptive methods. Since FSH and its receptor play such a large role in human fertility, it's a desired target for birth control drugs. Current birth control methods for females include but aren't limited to birth control pills and intrauterine devices (IUD). Both are decently effective methods, however, the pill must be taken at the same time everyday to maximize efficiency and the IUD is a foreign object that must be placed inside the body. The average yearly cost is up to $800 for birth control pills and up to $1,300 for an IUD depending on health care coverage (The cost of Birth). Despite being expensive, birth control methods can also interrupt the normal levels of estrogen and progesterone in females. Targeting the FSHR signaling pathway is an attractive target for alternative contraceptive methods because it has the potential to offer effective birth control options while limiting hormonal side effects, thus not only providing an affordable alternative, but also providing an alternative that does not disrupt the normal hormone levels in females. It also gives hope to one day create birth control methods for males since FSH is involved in male reproductivity as well.

The FSHR is part of the G-protein coupled receptor (GPCR) family, and therefore has a variety of signaling pathways and mechanisms that can have different biological effects. GPCRs have an extracellular domain (N-terminus), an intracellular domain (C-terminus), and a transmembrane domain consisting of seven α-helices looping back and forth across the cell membrane (Figure 2). The extracellular domain is where ligand binding and consequently activation of the receptor occurs. The intracellular domain is the part of the receptor that interacts with effector proteins allowing for signal transduction inside the cell.

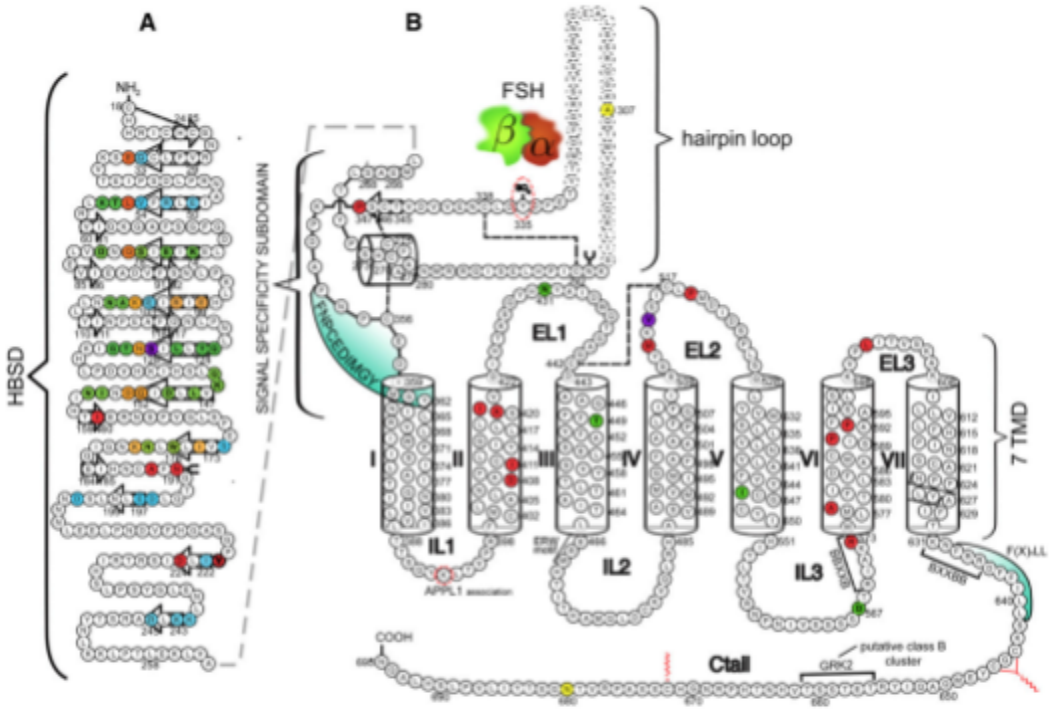


Figure 2. *Follicle Stimulating Hormone Receptor (FSHR) with FSH ligand (Ulloa-Aguirre et al, 2016).*

FSH is a heterodimer consisting of an α-subunit and a β-subunit (Ulloa-Aguirre et al, 2008) which binds to the extracellular domain of the FSHR initiating a cascade of downstream signaling, leading to a variety of biological effects. The network of signaling is complicated with many intertwining pathways. The most researched and understood pathway is through the activation of the Gαs subunit of the heterotrimeric G-protein. Other pathways include signaling via β-arrestin's, Gβ/γ subunit of the heterotrimeric G-protein, and other Gα subunits (Figure 3).

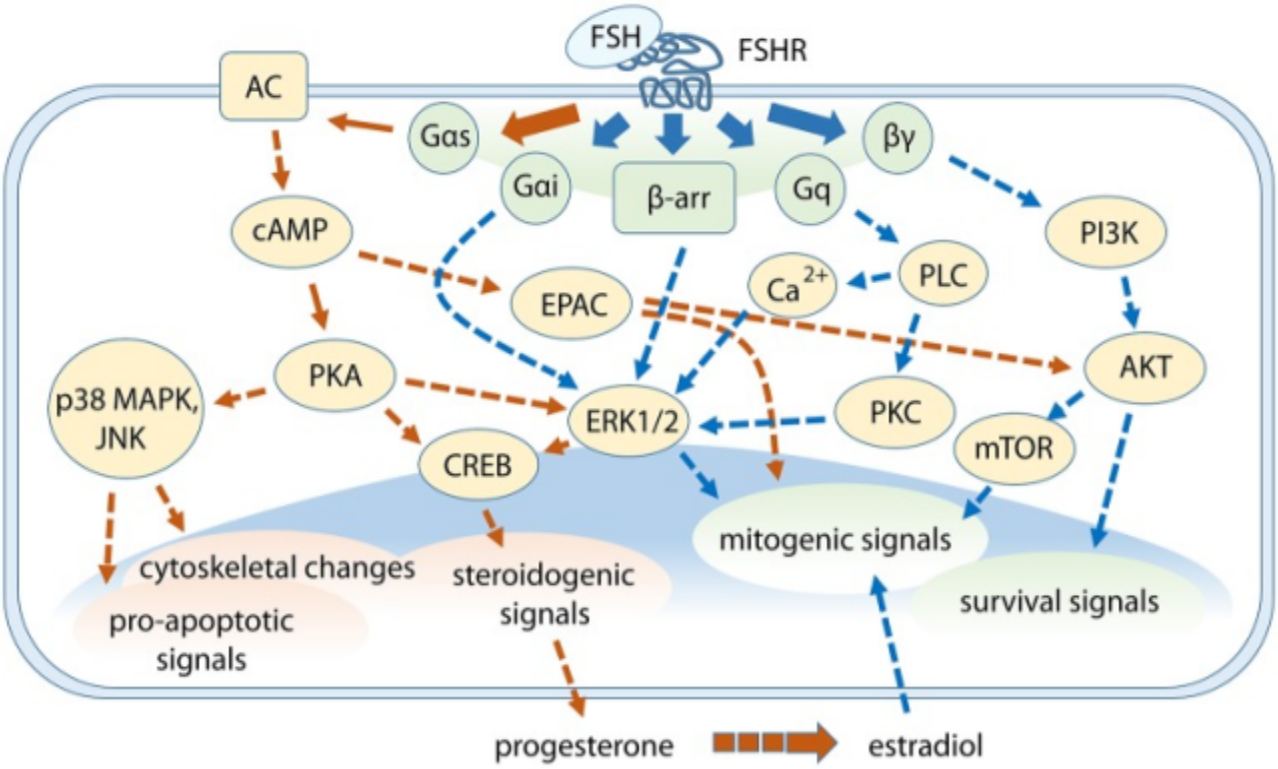


Figure 3. *FSHR binding of FSH results in downstream signaling pathways mediated by G-proteins and β-arrestins (Casarini et al, 2019).*

The most understood pathway for FSHR signaling is through the activation of heterotrimeric G-proteins. Upon ligand binding, FSHR undergoes a conformational change which then activates the G-protein by catalyzing the exchange from GDP to GTP on the Gα subunit (Figure 4). The G-protein then dissociates into its now active Gα and Gβ/γ subunits, both of which subsequently activate other downstream effector proteins. Desensitization occurs when β-arrestin binds to FSHR to halt signal transduction and GTPase hydrolyzes GTP to GDP, rendering the Gα subunit inactive, which then re-associates with the Gβ/γ dimer to form the complete trimeric G-protein complex (Tuteja, 2009).

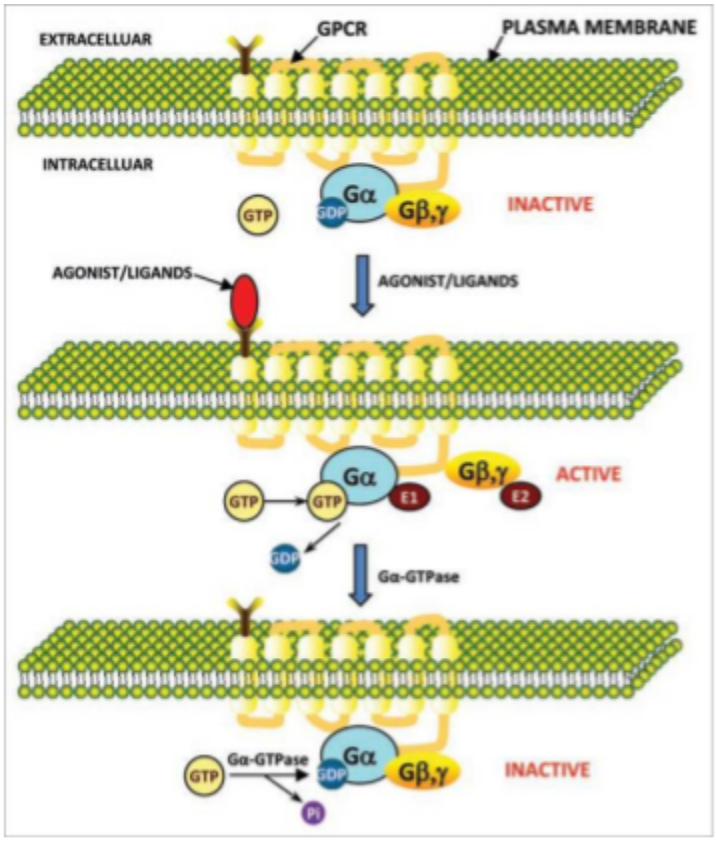


Figure 4. *Activation and inactivation of heterotrimeric G protein through GPCR signaling. Gα subunit is inactive when bound to GDP and is thus associated with Gβ/γ subunit. Gα subunit is active when bound to GTP and dissociates from Gβ/γ subunit where both continue signaling through activation of downstream proteins (Tuteja, 2009).*

The most understood Gα subunit is the Gαs subunit. When activated, it induces adenylyl cyclase which mediates the production of cyclic adenosine monophosphate (cAMP). As the accumulation of cAMP in the cell increases, it binds to and activates two downstream effectors (Gloaguen et al, 2011). These downstream effectors are Protein Kinase A (PKA) and exchange proteins activated by cAMP (EPAC). When PKA is activated, active catalytic subunits are released and able to phosphorylate many targets in the cytosol or nucleus (Gloaguen et al, 2011). Other downstream proteins such as p38 MAPK and ERK1/2 (p44/42 MAP kinase) are activated by PKA (Ulloa-Aguirre et al, 2016) which initiates and maintains transcription factors that can

have different biological effects such as cell growth and proliferation. EPAC on the other hand, promotes Rap1-GDP to GTP and thus activation in granulosa and epithelium ovary cells (Gloaguen et al, 2011). The activation of Akt, p38 MAPK and ERK1/2 are dependent upon successful activation of Rap1 by EPAC. Both effector proteins, PKA and EPAC, are activated by cAMP but have different signaling pathways, yet the downstream effects have some overlap (Figure 5). While there are other pathways involving different Gα subunits, the Gαs is the main target of interest.

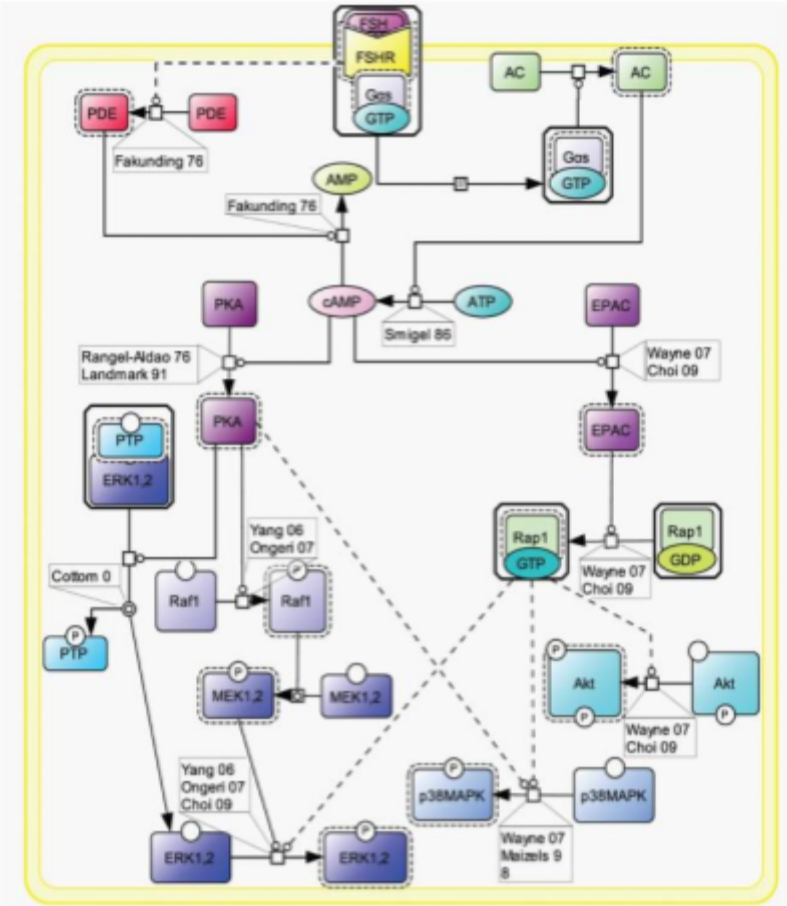


Figure 5. *Signaling pathway of Gαs/cAMP/PKA/EPAC initiated by FSH induced activation of FSHR.*

The β-arrestin pathway is known for its desensitization and recycling of FSHR signaling. However, recent findings suggest that β-arrestins may play a more general role (Figure 6). These β-arrestins are seen as adapters and transducers which lead to the activation of MAPK and ERK (Gloaguen et al, 2011). Desensitization of FSHR signaling occurs in a two step mechanism where GPCR kinases (GRKs) phosphorylate FSHR and β-arrestin binds to the intracellular domain of the receptor, physically blocking and uncoupling the G-protein from the receptor (Jean-Charles et al, 2017). β-arrestins also recruit nucleotide phosphodiesterases (PDEs) and diacylglycerol kinases (DGKs) which work to degrade secondary messengers that are downstream of the activated receptor, including the conversion of cAMP to AMP (Jean-Charles et al, 2017). The decreasing levels of cAMP will thus result in decreased PKA and EPAC activity, resulting in the signaling through the Gαs/cAMP/PKA/EPAC pathway to diminish. With the translocation of β-arrestin to the activated receptor and the recruitment of DGKs, the diacylglycerol (DAG) is phosphorylated and then converted into phosphatidic acid (Jean-Charles et al, 2017). Phosphatidic acid has been found to be involved in many different cellular processes. These processes include cell proliferation, secretion, cytoskeletal organization, cell survival, and vesicular trafficking (Liu et al, 1970). This indicates that even though β-arrestins are involved with FSHR desensitization, they also can initiate separate G-protein-independent signaling mechanisms through the production of phosphatidic acid.

There are other signaling pathways involving FSHR. Some of these include different Gα subunits such as Gαi and Gαq. However, the main pathways that will be focused on are the Gαs/cAMP/PKA/EPAC mechanism and the β-arrestin mechanism. These are the most researched and understood and play the largest role in human fertility.

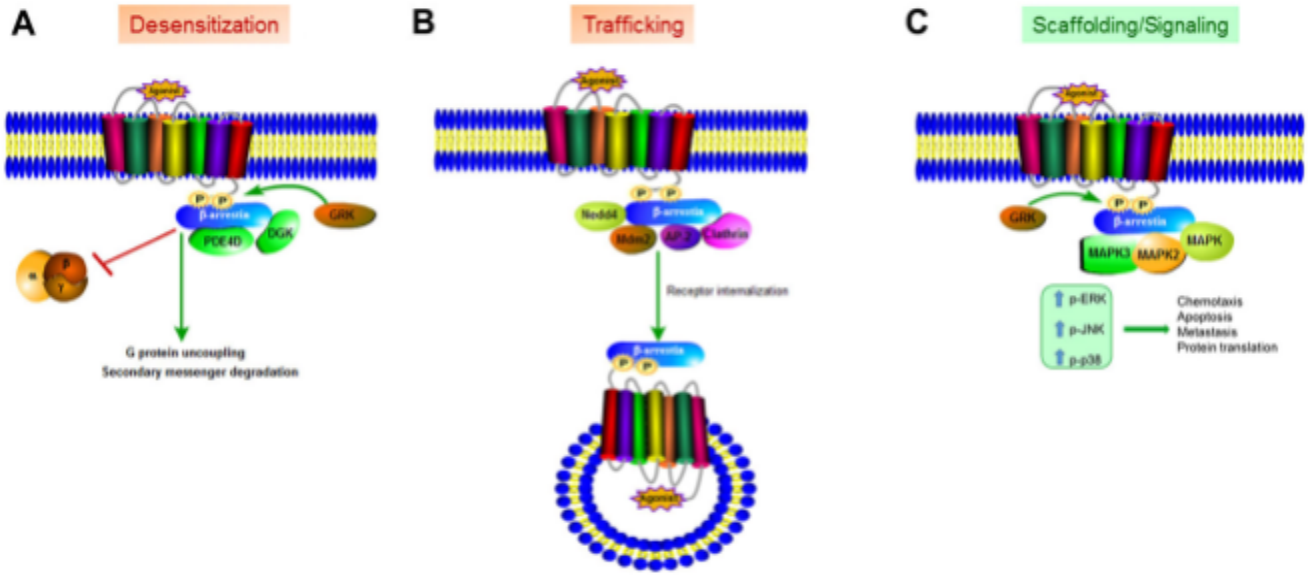


Figure 6. *β-arrestins are involved in desensitization and recycling of FSHR signaling as well as signaling through G-protein-independent pathways (Jean-Charles et al, 2017).*

The objective of this research is to identify proteins involved in FSHR signaling and to gain a better understanding of how FSHR signals. Understanding the signaling pathways that FSH and its receptor are involved in is a critical step. The information can be used for identifying potential drug targets to treat infertility and to provide alternative methods for contraceptives.

The APEX Assay is a relatively new technique that has been used in other recent research to understand the different interactions between GPCR's and effector proteins. APEX is a genetically engineered ascorbate peroxidase. When the substrate biotin-phenol is added along with hydrogen peroxide, APEX creates a biotin-phenoxy radical (Paek et al, 2017) by stripping a hydride from biotin phenol. Since radicals are short-lived and very reactive, the biotin-phenoxy radicals interact with proteins. However, because these radicals are so short-lived, they only interact with proteins within a small radius from the APEX protein, thus tagging these proteins. The biotinylated proteins can be isolated and analyzed using mass spectrometry (Figure 7). By

developing a modified GPCR where the APEX protein is attached to the carboxy terminus, proteins tagged within a small proximity of the APEX protein are ultimately within a small proximity of the GPCR. This enables researchers to better identify the different proteins and understand the protein-protein interactions that occur during GPCR signaling. By creating a modified FSHR-APEX plasmid encoding for FSHR with APEX attached to the carboxy terminus, it will be used to analyze the effector proteins involved with FSHR signaling.

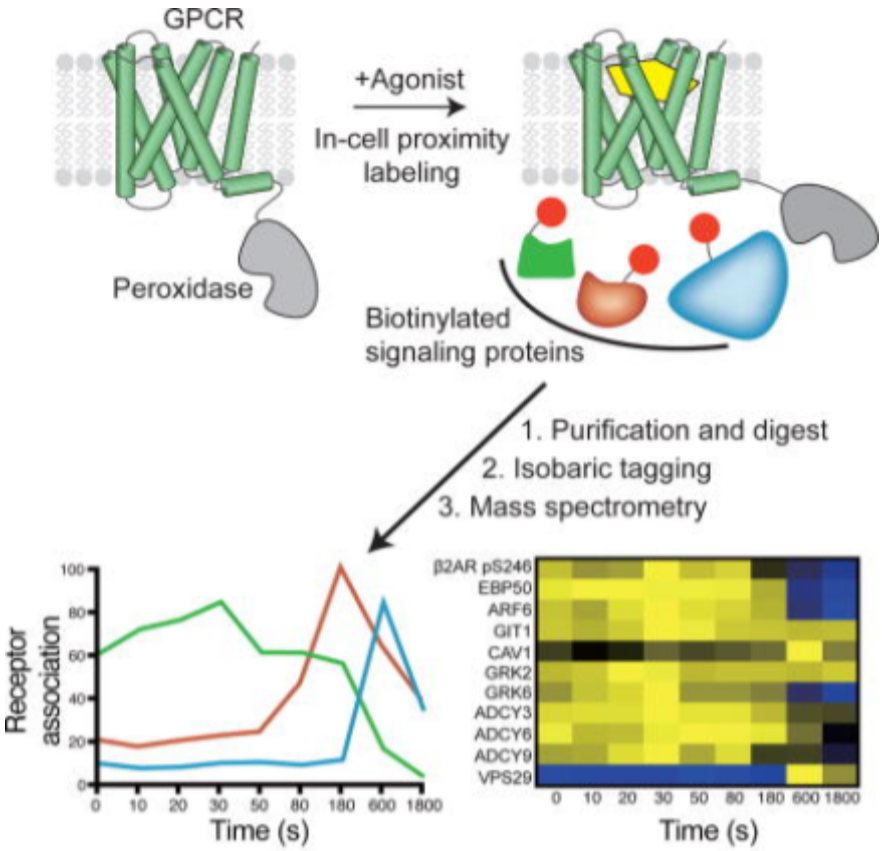
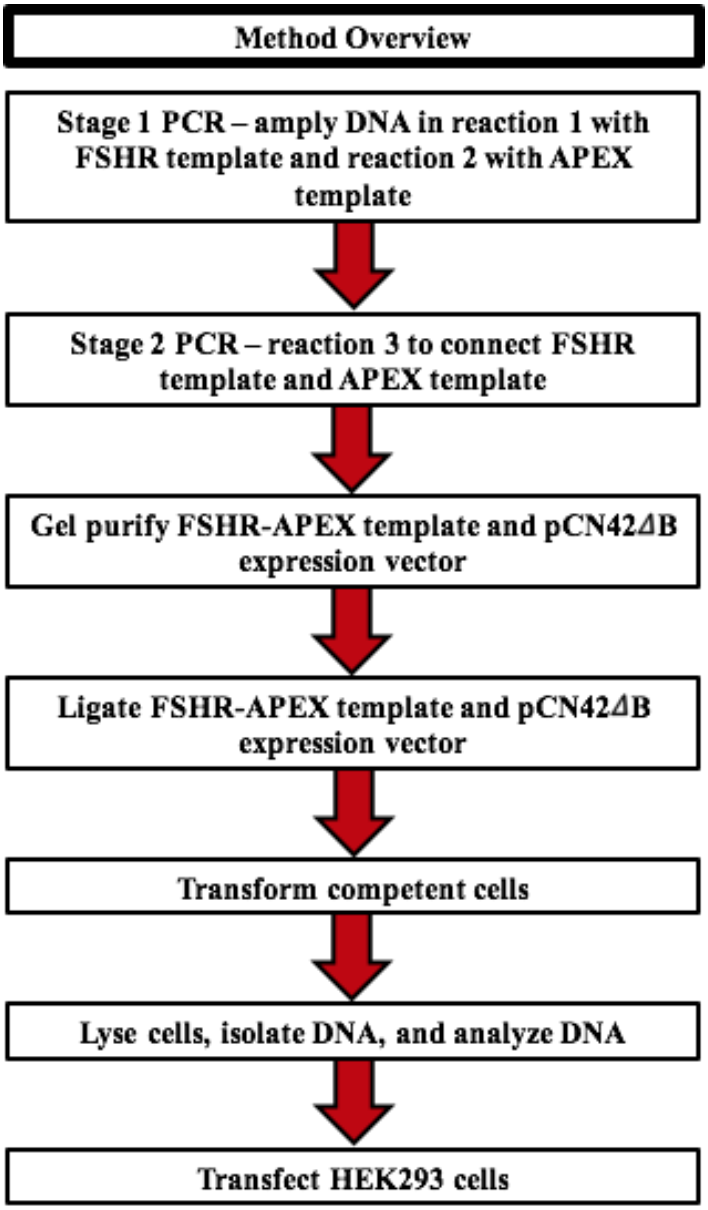


Figure 7. *GPCR-APEX construct produces biotinylated proteins that can be analyzed by mass spectrometry (Paek et al, 2017).*

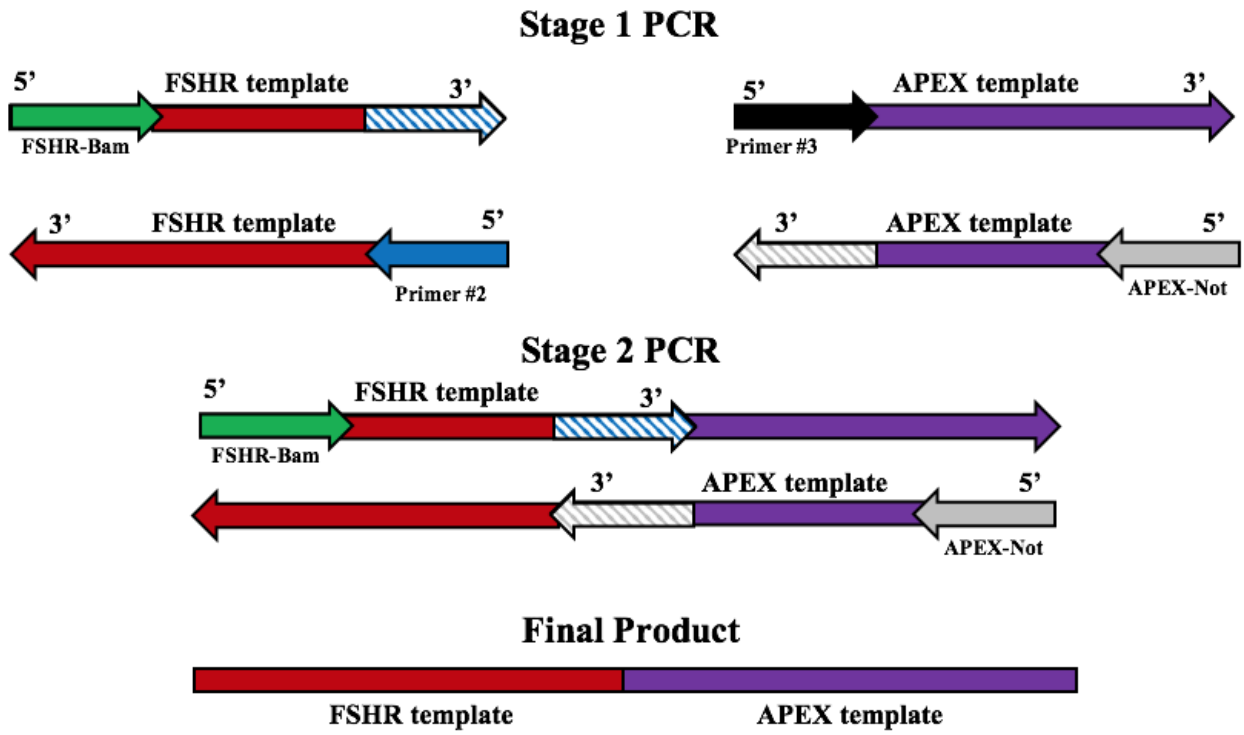
Methods

Overview:



Creation of FSHR-APEX Plasmid:

Stage 1 PCR was performed to amplify both the pCN42ΔB template and the APEX template. The pCN42ΔB plasmid was amplified using the primer FSHR-Bam and the primer #2**.** The APEX plasmid was also amplified using the primer APEX-Not and the primer #3. Stage 2 PCR was performed to connect the FSHR and the APEX DNA products using the FSHR-Bam primer and the APEX-Not primer.



The DNA product was purified with a mini column. Two restriction digests were performed with restriction enzymes Not1 and BamHI to cut both the FSHR-APEX DNA product from the stage 2 PCR and the pCN42ΔB plasmid. Gel electrophoresis was performed and the restriction digest products were gel purified to isolate the FSHR-APEX template and the

pCN42ΔB vector. The FSHR-APEX template and pCN42ΔB vector were ligated together in a 20uL reaction with 10X buffer, water, and ligase and was incubated at room temperature for one hour. Next, competent cells were transformed with the new FSHR-APEX plasmid and shaken in SOC Outgrowth Medium for one hour at 37°C and subsequently plated on LB plates with ampicillin and incubated overnight. Colonies were selected and grown overnight in LB medium supplemented with ampicillin. Cells were lysed and the DNA was isolated using a mini column. Two separate restriction digests were performed: the first using restriction enzyme EcoR5 and the second using restriction enzyme BamHI. A third restriction digest was performed to confirm the results using restriction enzymes BamHI and Not1. Cultures containing the FSHR-APEX plasmid were grown overnight in LB medium supplemented with ampicillin and DNA was isolated and extracted and stored at 4°C. The extracted DNA concentration was determined using the NanoDrop technology.

Stable Transfection of FSHR-APEX Plasmid:

HEK293 cells were grown to approximately 60% confluency in a 60mm dish. The cells were then transfected with the FSHR-APEX plasmid using TransIT 293 and following the transfection protocol from the Team Cohen Master Lab Manual. After approximately a 72 hour incubation period, the media was supplemented with G418. The G418 media was changed every 48 hours until cells that did not take up the plasmid were killed off. Once individual colonies were seen, the media was removed and the cells were washed once with sterile PBS. The colonies were lifted with a trypsinized disc and moved to a 24-well dish. Clones were transferred to a larger dish depending on the rate of growth.

FSH Treatment of Transient FSHR-APEX Plasmid Transfection:

HEK293 cells were grown to approximately 60% confluency in a 6-well dish. The cells were transfected with the FSHR-APEX plasmid using TransIT 293. After a 48-72 hour incubation period, cells were treated with FSH in a time course fashion. Cells were then harvested, lysed, and analyzed by western blotting.

Western Blot Analysis:

A 10% SDS-PAGE gel was made following the protocol in the Team Cohen Master Lab Manual. Cells were harvested and lysed from a 6-well dish and SDS-PAGE gel electrophoresis was performed. The gels were transferred to an Immobilon-P membrane following the Bio-Rad Semi Dry Transfer protocol in the Team Cohen Master Lab Manual. Membranes were incubated in 5% milk in TBST. Primary antibody used was the anti-hFHSR monoclonal antibody 106.105 (kind gift of Dr. James Dias, University of Albany) and the secondary antibody used was goat-anti-mouse HRP.

Fluorescent Microscopy Analysis:

Cells were plated on a poly-lysine coated coverslip in a 6-well dish and grown until confluent. The protocol for fluorescent microscopy was followed from the Team Cohen Master Lab Manual. Coverslips were incubated in 5% BSA in PBS. Primary antibody used was anti-hFHSR monoclonal antibody 106.105 and the secondary antibody used was green goat-anti-mouse fluorophore.

Results

Stage 1 PCR was performed on the pCN42ΔB template and the APEX template. Each template was amplified in separate PCR reactions. Gel electrophoresis was performed to confirm that each template was correct (Figure 8). The blue arrow in Figure 8 indicate the APEX template and the yellow arrow indicates the pCN42ΔB template (FSHR template).

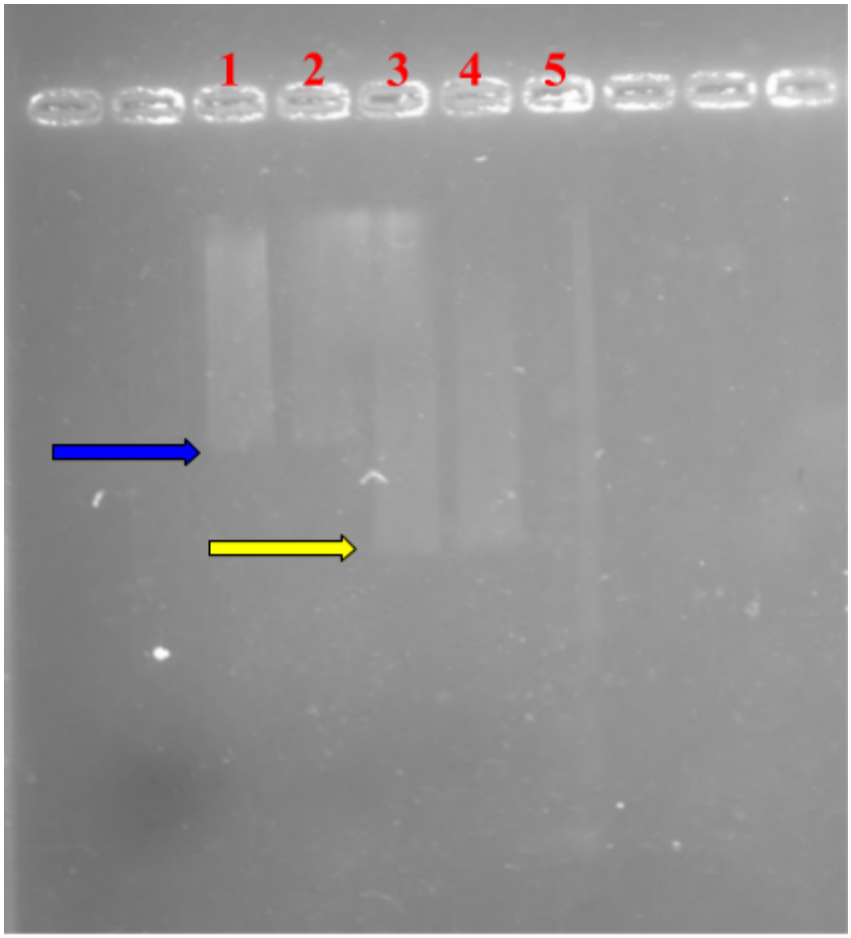


Figure 8. *Stage one PCR products for the APEX template is seen by the blue arrow in lanes 1 and 2 and the PCR products for the pCN42ΔB template is seen by the yellow arrow in lanes 3 and 4.*

Stage 2 PCR was performed to connect the amplified APEX stage 1 product to the pCN42ΔB stage 1 amplified product (containing the hFSHR gene). Gel electrophoresis was again performed to confirm that the two templates were attached (Figure 9).

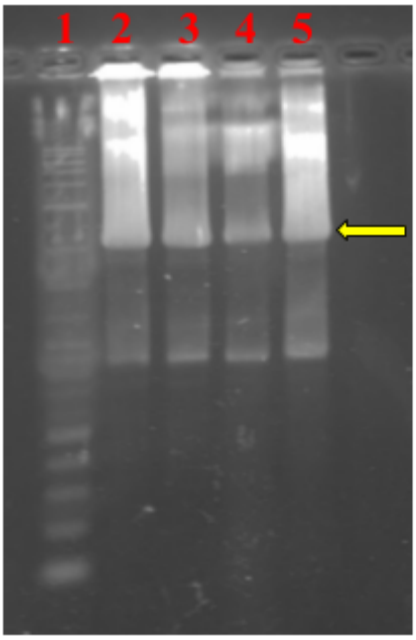


Figure 9. *Stage two PCR products for the connection of the FSHR and the APEX templates seen in lanes 2, 3, 4, and 5 indicated by the yellow arrow. Lane 1 contains the molecular weight marker.*

The FSHR-APEX DNA product from the stage 2 PCR seen by the yellow arrow in Figure 10 and the pCN42ΔB expression vector seen by the blue arrow in Figure 10 were run on an agarose gel and subsequently gel purified. This was done to isolate the FSHR-APEX template and the pCN42ΔB expression vector from the pCN42ΔB template (FSHR template) for ligation.

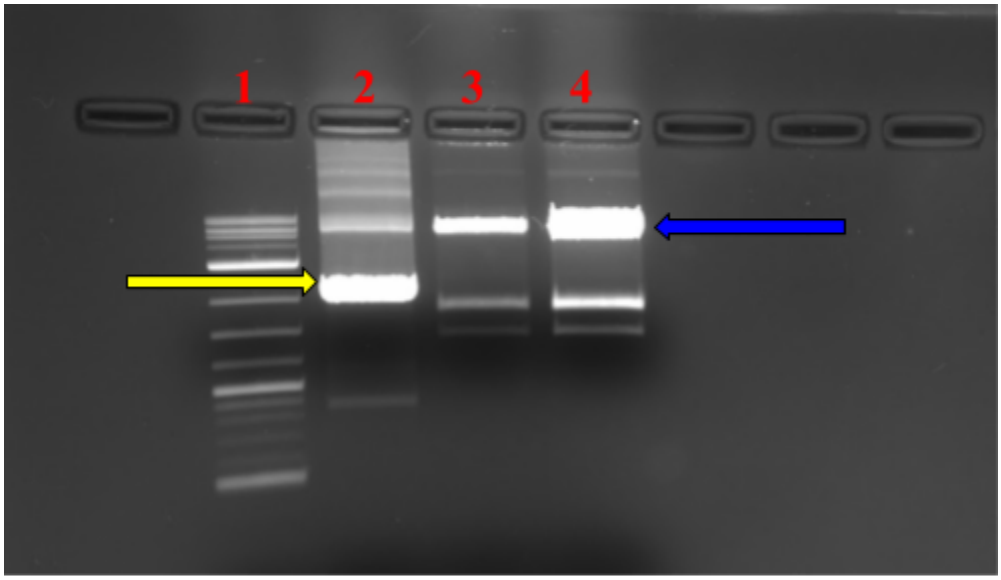


Figure 10. *Lane 1 is the molecular weight marker. Lane 2 holds the FSHR-APEX product after digestion with BamHI and NotI indicated by the yellow arrow. Lanes 3 and 4 contain the pCN42ΔB plasmid after digestion with BamHI and NotI. The vector is indicated by the blue arrow.*

Once the FSHR-APEX DNA product and the pCN42ΔB expression vector were isolated and purified from the gel, they were ligated together to create a new plasmid containing the FSHR-APEX template. Competent cells were transformed, grown, and plated, and 12 colonies were selected. These colonies were grown in an overnight culture and the DNA was isolated and analyzed to determine if the ligation was successful. Restriction digests were performed to look for different products when compared to the wild-type pCN42ΔB template seen in lane 2 of Figure 11 and Figure 12. The first restriction digest used the enzyme EcoR5. Two colonies produced different products when compared to the wild-type FSHR template as indicated by the yellow arrows in Figure 11.

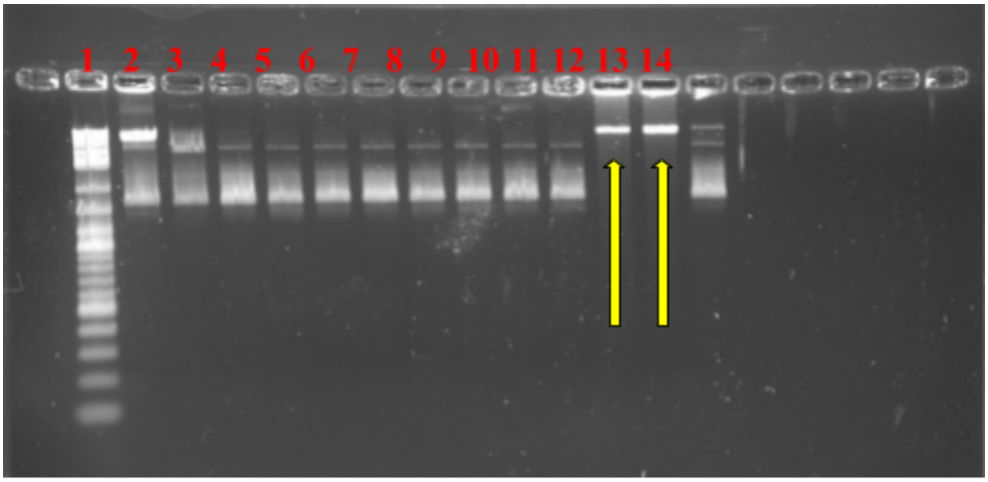


Figure 11. *Restriction digest with EcoR5. Lane 1 is the molecular weight marker. Lanes 12 and 13 contain a different product indicated by the yellow arrow.*

The second restriction digest was performed using the restriction enzyme BamHI. Again, the same two colonies produced different products. This was observed in Figure 12 as seen by the yellow arrows. The plasmids from these two colonies were selected for further analysis.

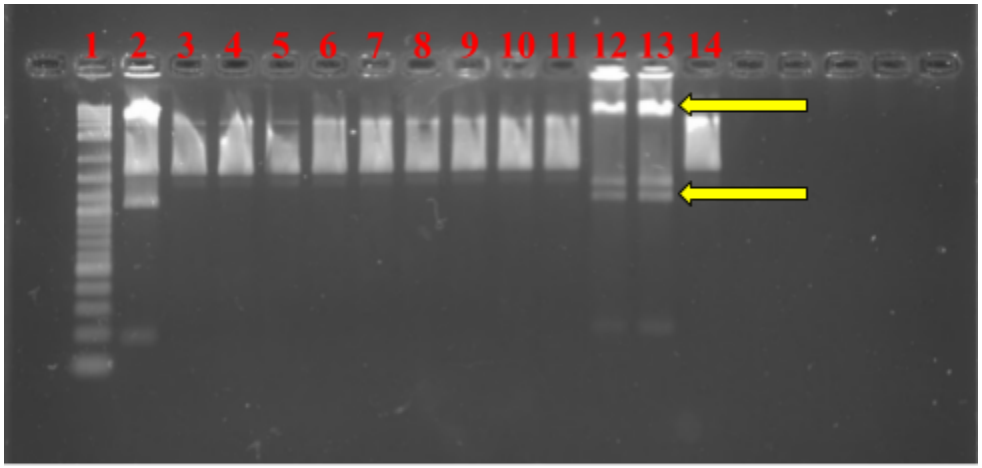


Figure 12. *Restriction digest with BamHI. Lane 1 is the molecular weight marker. Lanes 12 and 13 contain a different product indicated by the yellow arrow.*

A third restriction digest was performed on the selected products using BamHI and Not1 to confirm the results. The expected band sizes are as follows: the pCN42ΔB template is ~ 1400 bp and the FSHR-APEX template is ~ 2300 bp. The blue arrow in Figure 13 indicate the pCN42ΔB template (FSHR template) and the yellow arrow indicates a slightly larger band corresponding to the FSHR-APEX template as desired.

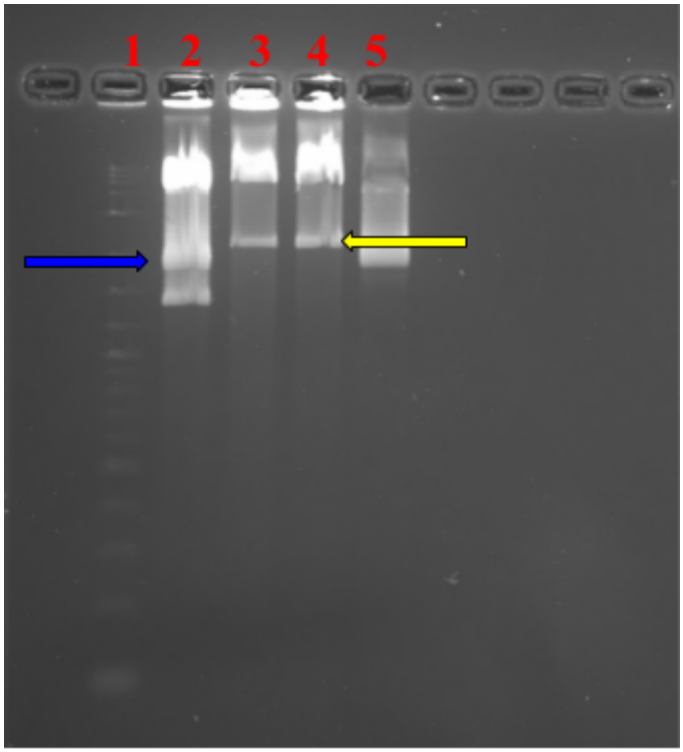


Figure 13. *Restriction digest with BamHI and Not1. Lane 2 contains the pCN42ΔB template indicated by the blue arrow. Lanes 3 and 4 hold the FSHR-APEX template indicated by the yellow arrow.*

Transient transfections of the FSHR-APEX plasmid were performed to use in a fluorescent microscopy experiment to demonstrate receptor presence at the cell surface of

HEK293 cells. Un-transfected HEK293 cells served as the negative control where no fluorescence was seen. The HEK293 cells stably expressing the wild-type FSHR served as the positive control where fluorescence is present. Fluorescent microscopy images of the transient transfections of the FSHR-APEX receptor indicate that the modified receptor was indeed present on the surface of the cell membrane of HEK293 cells (Figure 14).

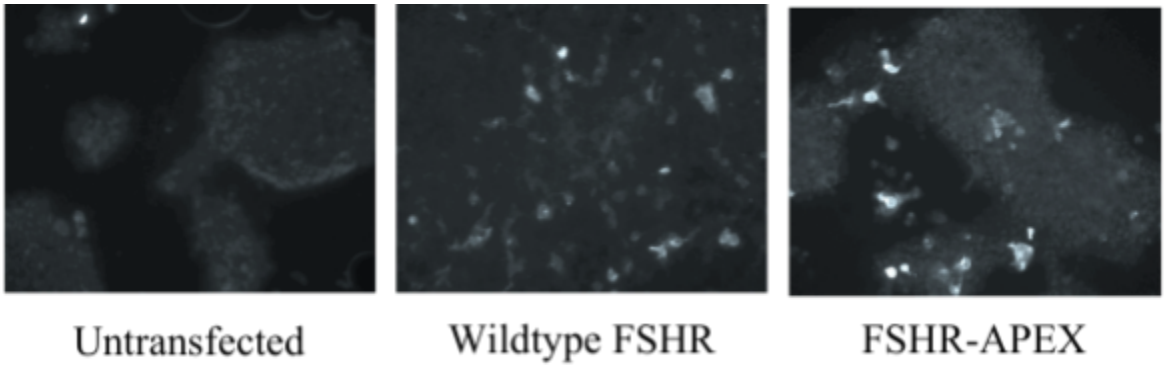


Figure 14. *Fluorescent microscopy images of un-transfected HEK293 cells, HEK293 cells stably expressing the wild-type FSHR, and HEK293 cells transiently expressing the FSHR-APEX receptor.*

An FSH time course experiment was used to analyze receptor signaling (Figure 15). The modified plasmid was transiently transfected into HEK293 cells and FSH was added at 0, 5, and 15 minutes. The cells were harvested and lysed and western blot analysis was used to probe for the downstream signaling product p44/42. Both FSHR-APEX-1 and FSHR-APEX-2, two separate transient transfections, produced p44/42 after FSH activation. However, the un-transfected HEK293 cells also produced a response, indicating that further analysis is needed.

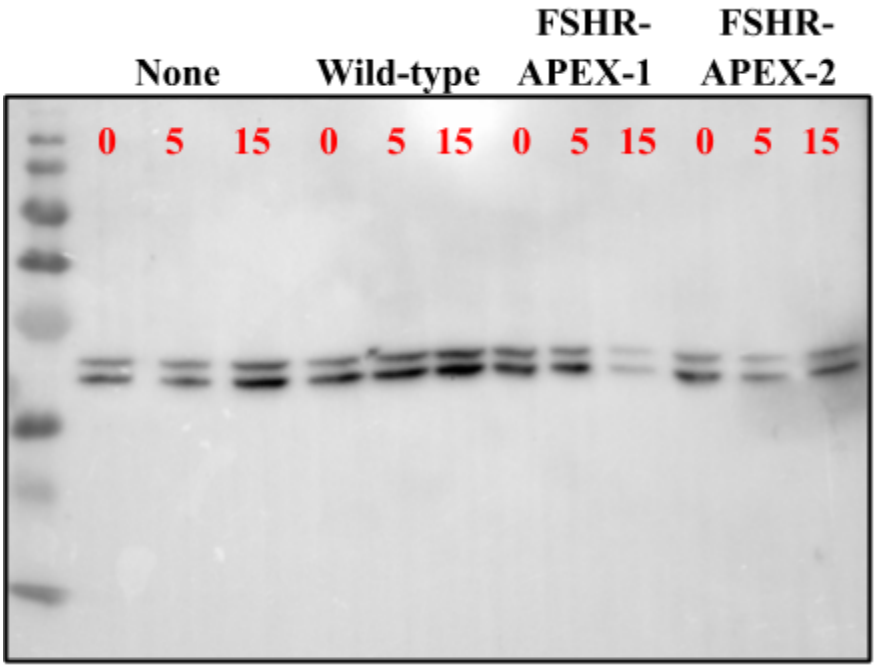


Figure 15. *Western blot from analysis of FSHR-APEX receptor signaling. HEK293 cells were transfected with isoforms of FSHR as indicated and then treated with FSH at 0, 5, and 15 minutes. Lane 1 is a molecular weight marker.*

The FSHR-APEX plasmid was stably transfected into HEK293 cells. Individual colonies were grown until they were confluent in a 6-well plate. The cells were harvested and lysed to determine if the modified receptor was present. Western blot analysis indicated that FSHR-APEX clones 1, 2, and 3 in lanes 2, 3, and 4 are expressing the modified receptor (Figure 16). The molecular weight of the wild-type FSHR is about 70kDa indicated by the green arrow in Figure 16 and the molecular weight of the APEX protein is around 27 kDa. Therefore, the FSHR-APEX receptor would be approximately 97kDa as indicated by the orange arrow in Figure 16.

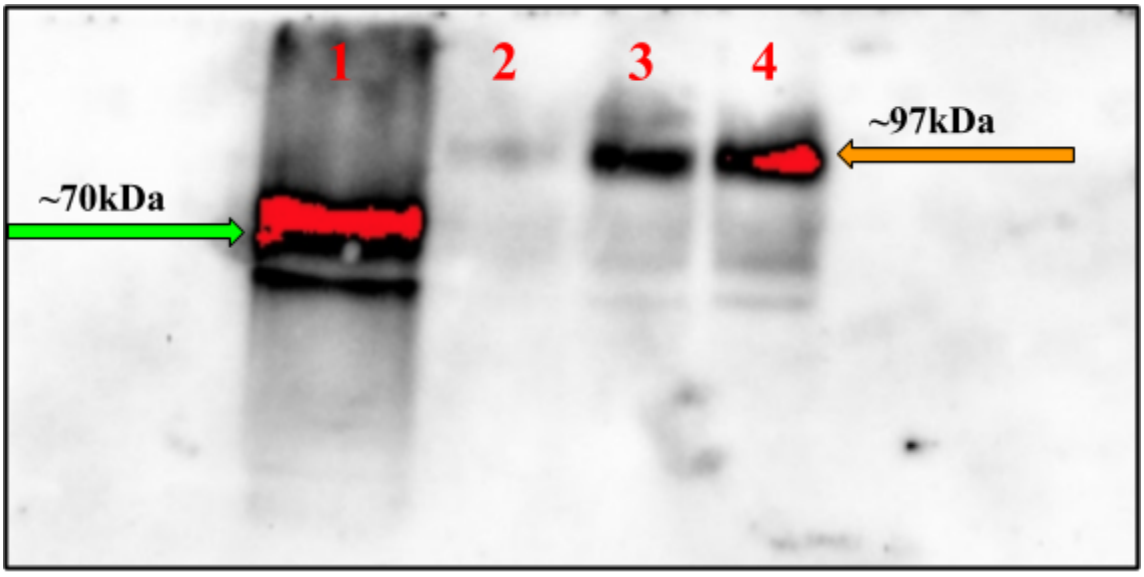


Figure 16. *Western blot analysis of stable transfection of FSHR-APEX modified receptor in HEK293 cells. Lane 1 represents the wild-type FSHR. Lanes 2, 3, and 4 represent three selected clones expressing the modified FSHR-APEX receptor.*

Discussion

Understanding the role of follicle stimulating hormone, follicle stimulating hormone receptor, and the downstream signaling pathways and effector proteins are crucial for explaining how the reproductive system functions and ultimately offering potential targets for fertility drugs and contraceptives. The APEX assay provides a method for studying the downstream pathways of GPCRs and was utilized to develop another option for studying FSHR signaling. To develop this assay, the APEX protein template was attached to the C-terminus of the FSHR template, allowing for effector proteins to be tagged in the cytoplasm.

The fluorescent microscopy results indicate that the modified FSHR-APEX receptor is expressed on the cell surface of HEK293 cells. There is no fluorescence seen in the negative control and there is fluorescence seen in the wild-type FSHR, demonstrating that the fluorescence staining was successful. There is fluorescence seen from the FSHR-APEX receptor, indicating that the modified receptor is indeed expressed on the cell surface. However, the fluorescence is less than the wild-type FSHR. This is most likely due to a lower concentration of the plasmid transfected, resulting in the lower expression of the modified receptor.

The FSH time course treatment was used to determine the modified receptor functionality. The downstream product p44/42 was probed for using western blot analysis. Both FSHR-APEX 1 and 2 produced the p44/42 product at 0, 5, and 15 minutes post FSH treatment. For both FSHR-APEX transient transfections 1 and 2, the highest p44/42 concentration was at 0 minutes indicated by the darker bands in Figure 15, followed by the p44/42 concentration at 5 minutes. The p44/42 concentration for FSHR-APEX-1 decreased between 5 minutes and 15 minutes post FSH exposure, which is confirmed by the faint band seen at 15 minutes. The p44/42

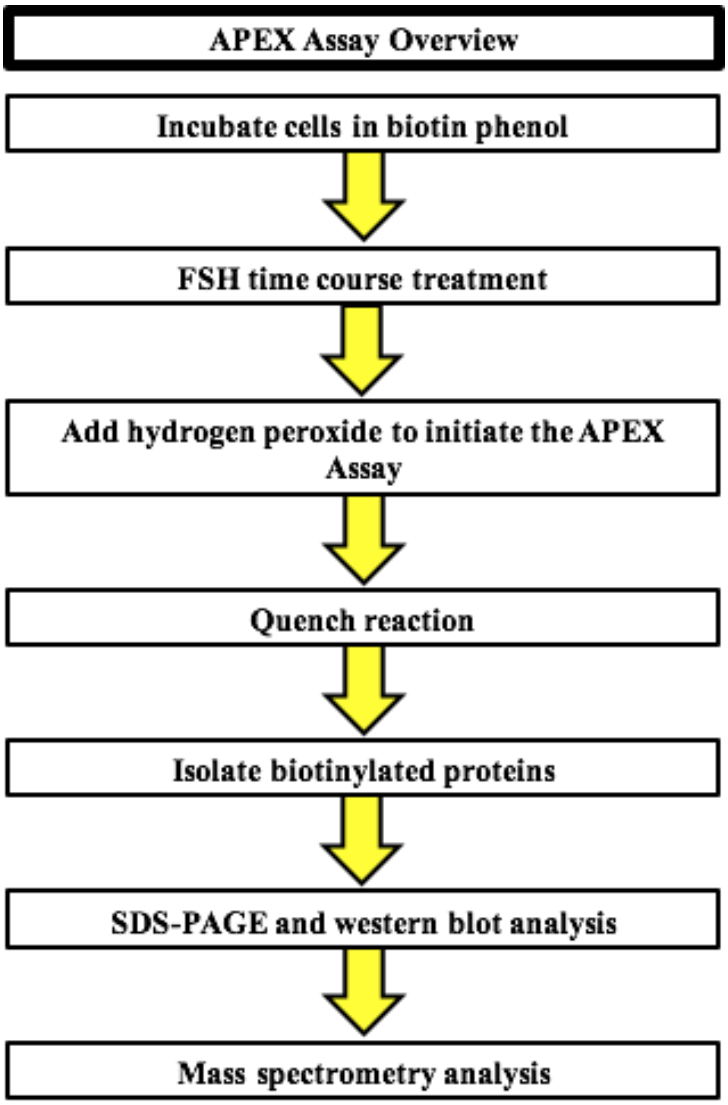
concentration for FSHR-APEX-2 is seen to increase between 5 and 15 minutes after FSH exposure, indicated by the darker band at 15 minutes compared to 5 minutes in Figure 15. The inconsistencies between the two FSHR-APEX transfections could be from the use of a transient transfection. The amount of expression of the modified receptor could differ between the two resulting in different amounts of downstream products produced in the signaling cascade. The wild-type FSHR also shows p44/42 activation at all three time points. There is an increase in p44/42 concentration from 0 to 5 minutes and from 5 to 15 minutes after treatment with FSH. This is seen by the increasing darkness of the bands in Figure 15 for the wild-type receptor. However, at 0 minutes, it would be expected that no p44/42 expression would be seen. Thus, indicating that there were inconsistencies among the experimental design and execution. The un-transfected HEK293 cells also demonstrated a p44/42 response, which was unexpected. These cells do not express any isoform of the FSHR, and were used as the negative control where no response should have occured. Due to this, further analysis is needed to test the FSHR-APEX receptor signaling.

The western blot analysis of the selection of colonies stably expressing the modified FSHR-APEX receptor showed that the receptor was in fact stably expressed in the HEK293 cells, thus creating a new cell line. Three clones were selected for analysis where all three were found to express the modified receptor. The wild-type FSHR is seen at 70 kDa. Since the APEX protein is approximately 27 kDa, then the modified FSHR-APEX receptor is about 97 kDa, which is seen in the western blot. An FSHR specific antibody was also used, further emphasizing that the FSHR-APEX receptor is present. However, since clones 2 and 3 had a much higher expression of the modified receptor as indicated by the darker bands present in the western blot

when compared to clone 1, they were selected for further analysis and continued use in future experiments.

Future Experiments

The future experiments for this research include further analysis of the modified FSHR-APEX receptor functionality. This can be done using techniques like the EPAC assay to test for downstream cyclic AMP production after FSH activation as well as using western blot analysis to determine the p44/42 activation following treatment of FSH. The signaling of the FSHR-APEX receptor can be tested using the stable cell line expressing the modified receptor, which should decrease the amount of inconsistencies found when using a transient transfection. Once the signaling and functionality of the modified receptor is confirmed, the APEX Assay can be utilized to determine FSHR partners during receptor signaling. This assay allows for the determination of proteins in a close proximity to FSHR in a time dependent manner. To use the APEX Assay, cells expressing the FSHR-APEX receptor are incubated in biotin phenol. Next, FSH is added over a specific time course followed by the addition of hydrogen peroxide to initiate the assay. The reaction is then quenched with a quenching solution and the biotinylated proteins are isolated using a streptavidin pulldown. The biotinylated proteins can then be analyzed using gel electrophoresis, western blot analysis, and mass spectrometry to determine potential FSHR partners during signaling. This assay can help to identify potential targets for drug therapies for infertility treatments and contraceptive methods.


APEX Assay Overview
Incubate cells in biotin phenol
FSH time course treatment
Add hydrogen peroxide to initiate the APEX Assay
Quench reaction
Isolate biotinylated proteins
SDS-PAGE and western blot analysis
Mass spectrometry analysis

Conclusions

To conclude, the FSHR-APEX plasmid was created and successfully transfected into HEK293 cells, both transiently and stably. A new HEK293 cell line stably expressing the modified receptor was created for use in future experiments. This was confirmed by western blot analysis. Fluorescent microscopy results indicated that the modified receptor was indeed expressed on the cell surface of HEK293 cells. The functionality of the modified receptor was inconclusive and needs further testing.

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