Use of CRISPR to Develop a Follicle Stimulating Hormone Receptor Knockout in Human Granulosa Cells to Study Lipid Raft Residency

By

Jenna Pradhuman

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ABSTRACT

ADVISOR: Brian Cohen

Human follicle stimulating hormone (hFSH) is a protein hormone responsible for stimulating the gonads and is necessary for regulating reproductive systems in both females and males. The actions of hFSh are carried out by the hFSH receptor (hFSHR), a seven transmembrane receptor that belongs to the G protein-coupled receptor family. Once FSH activates its receptor, the G protein inside the cell that is associated with the receptor is activated and starts a cascade of signaling that results in activation of protein kinase A (PKA) and the p44/42 MAP kinase (MAPK). The activation of these secondary proteins is responsible for follicular stimulation in females and gametogenesis in males and females.

Previous work in the Cohen Lab has shown that hFSHR is located in cholesterol and sphingolipid enriched microdomains referred to as lipid rafts. These microdomains are detergent resistant membranes that "float" in sucrose gradients. Current research is focused on the relevance of hFSHR lipid raft residency in the human granulosa cell line hGrC1; focusing in particular on the activation of signal transduction pathways by hFSHR. To study this in more detail, we are creating a human granulosa cell line where the hFSHR gene has been deleted, known as a knockout mutant. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a revolutionary gene editing technique that can create knockouts by precisely targeting a 20 nucleotide sequence of the genome using the Cas9 protein. This knockout line will enable us to study lipid raft residency and signaling of mutant hFSHR proteins in the native cell background. By studying the regulation of hFSHR signaling we hope to learn more about the receptor's function and identify new ways to modulate the receptor for novel approaches to contraception or treatment of infertility.

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Acknowledgements

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"It was tough, but it was good"- Sydney Spett (Robin Williams)

Introduction

The endocrine system is integrated with hundreds of different hormones and plays a major role in human reproduction and fertility. Human follicle stimulating hormone (hFSH) is a gonadotropin responsible for stimulating gonads and is necessary for regulating reproductive systems in both females and males (Gougeon, 1998). hFSH is a glycoprotein heterodimer released from the anterior pituitary gland and in females it acts on the ovaries (Telikicherla, et al. 2011 and Desai, et al. 2013). The hormone is primarily responsible for signaling the development of follicles and the production of estrogens in females (Figure 1). In males, FSH acts on Sertoli cells in the seminiferous tubules to promote spermatogenesis (Thompson, et al. 2014).

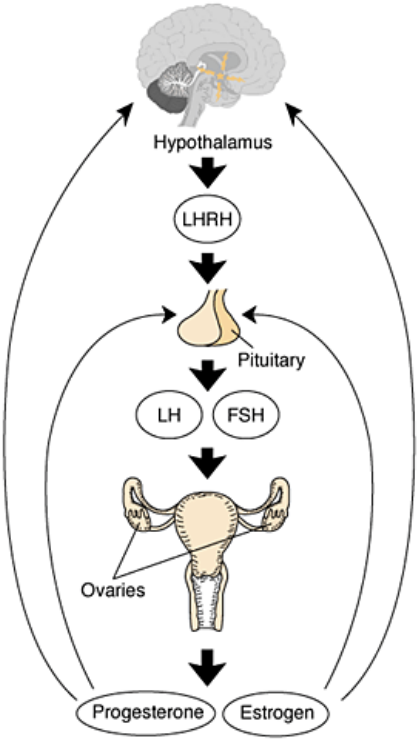

Figure 1: Schematic of FSH Production and Hormonal Affects in the Female Human Body (Emanuele et al. 2003)

Genetic mutations of the hFSH receptor can severely affect gametogenesis as seen in schematic diagram of Figure 1 (Thompson et al., 2014). Loss of function mutations have been seen to cause ovarian dysgenesis (ODG) and primary and secondary amenorrhea in females. In males, these mutations lead to small testes and impaired spermatogenesis. Further studies of hFSH could eventually lead to

therapeutic treatments for female infertility and aspermatogenesis in males (Menon and Menon, 2014).

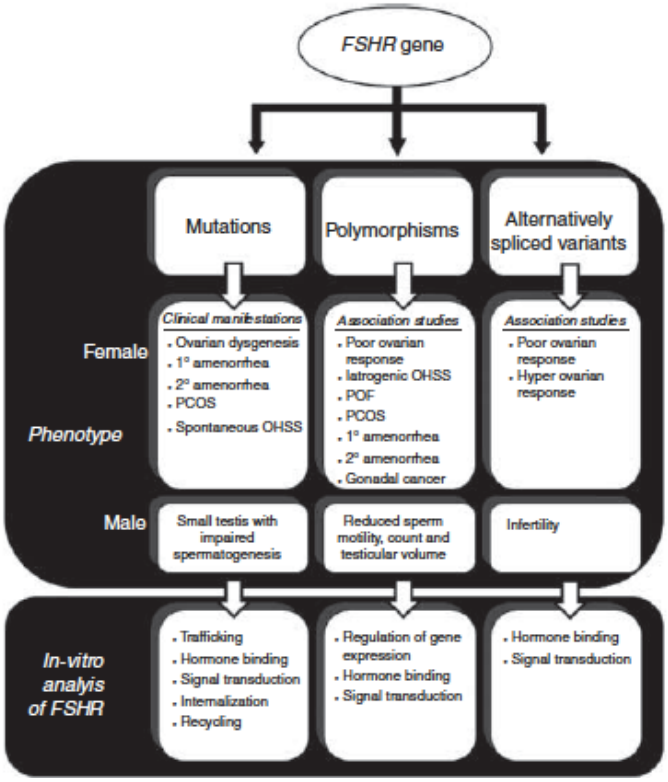

Figure 2: Overview of FSHR gene mutations, polymorphisms, and splice variants (Desai et al., 2013)

The hFSH receptor (hFSHR) contains an N-terminal extracellular domain that binds FSH with high specificity (Menon and Menon, 2014). The receptor also contains seven transmembrane domains and belongs to the g protein-coupled receptor family (GPCR). The receptor is attached to a g protein on the inside of the cell (Menon and Menon, 2014). Once FSH activates its receptor, the G-protein is then activated and starts a complicated cascade of signaling.

The cell membrane, as we know it, is a fluid-mosaic model. Proteins, receptors, channels and other molecules are able to move freely as they wish throughout the membrane. It is made of a lipid bilayer, in which the outside phosphate heads are hydrophilic and the lipid tails are hydrophobic.

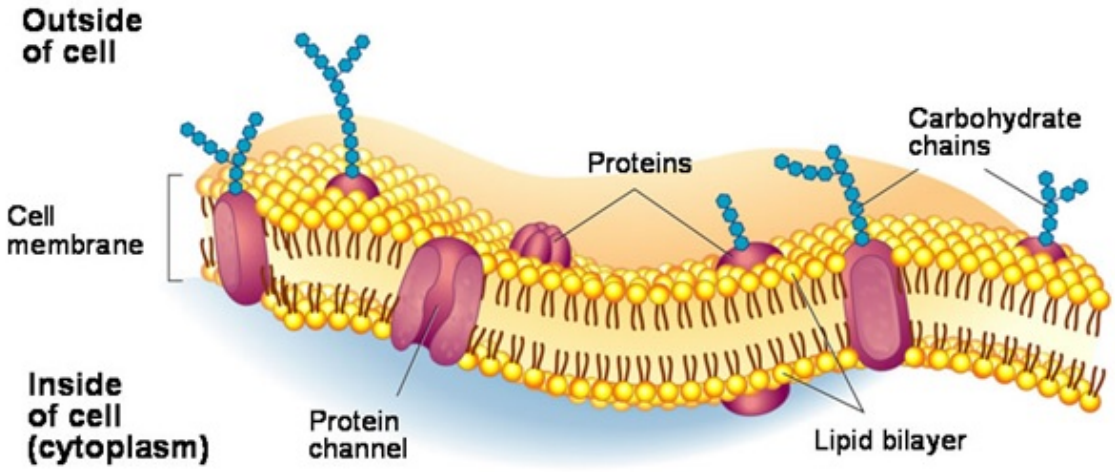

Figure 3: A "Normal" Cell Membrane as a lipid bilayer with

http://biology.tutorpace.com/plasma-membrane-online-tutoring

This idea that the cell membrane is uniform is not correct in reality. There are microdomains of the membrane that are enriched in sphingolipid and cholesterol . These domains are known as lipid rafts (Brown and London, 2000). Sphingolipids have long, largely saturated acyl tails and thus aid in the tight, compactness of these membrane microdomains. Cholesterol gives the rafts the phase behavior between the loosely packed disorder state and the solid-like gel state (Brown and London, 2000). These microdomains can be isolated because they are detergent resistant membranes that float on sucrose gradients. There are also raft markers, such as gangliosides GM1 and GM3 and flotillin- 1 and 2 (Zajchowski and Robbins, 2002). These proteins can be targeted in immunofluorescence to visualize rafts in cell membrane (Brown and London, 2000).

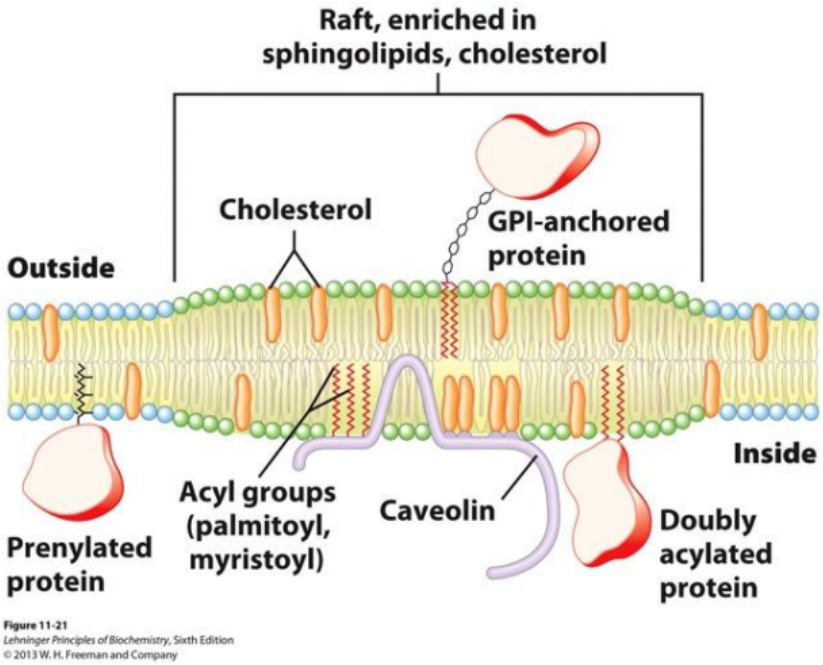

Figure 4: Lipid Raft Organization (Bio380 textbook)

Rafts are important for cell signaling. There are a couple of different theories about the significance of rafts and signaling. First, certain receptors and their secondary signaling proteins can be congregated inside the raft to ensure they can activate the signaling cascade as quickly as possible. Similarly, rafts can segregate proteins inside and outside the microdomain during sorting (Brown and London, 2000). Secondly, rafts might directly affect protein function because of its orderliness.

Previous work in the Cohen lab has shown that hFSHR are commonly located in lipid raft domains. The lab focuses on the importance of rafts and signaling through depletion of cholesterol using reagents such as methyl-beta-cyclodextrin. After FSH binds to its receptor, adenylyl cyclase is activated to turn ATP into cAMP and p44/42 MAP kinase is activated by phosphorylation (Figure 4). The activation of these secondary proteins are responsible for follicle stimulation and gametogenesis and thus are important signaling pathways to study.

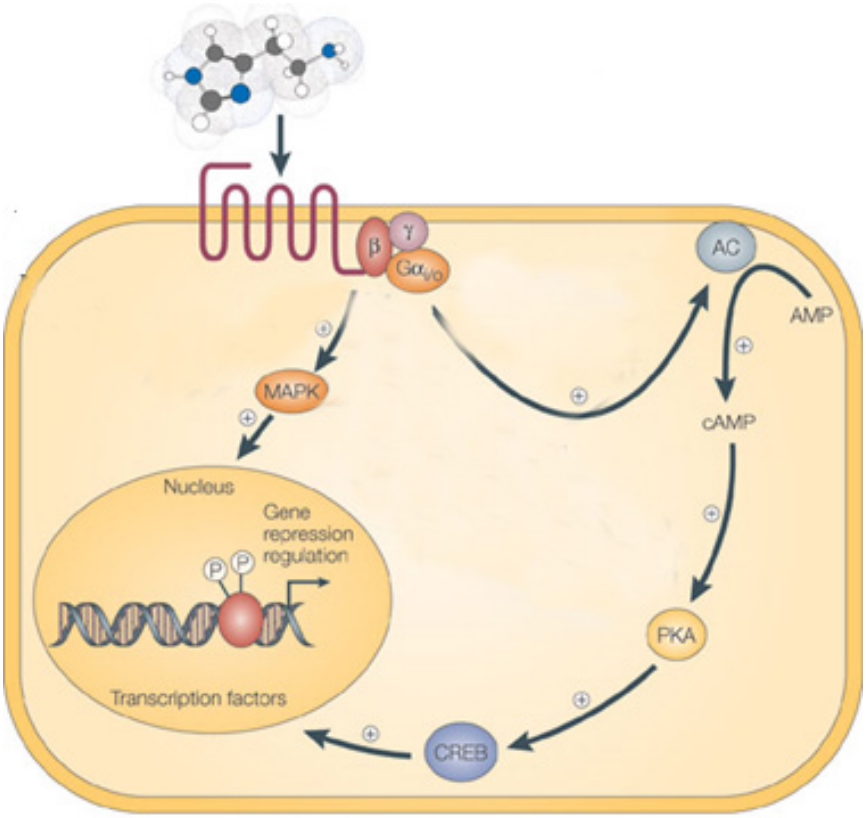

Figure 5: Major Signaling Pathways Involved in Signal Transduction of hFSHR (Leurs, et al., 2005)

Genome editing allows for the investigation of cellular activity with respect to a particular target. In this case our target is the hFSHR. CRISPR (clustered regularly interspaced short palindromic repeats) is a new approach to genome editing. This revolutionary technique, discovered in association with bacterial "immune systems", has allowed for targeted mutation. We can use this method to create knockouts of the FSHR in human granulosa cells, which are immortalized non-luteinized (in which the corpus luteum has not formed) human ovary cells. The introduction of a stable knockout of hFSHR in hGrC1 cells to the Cohen laboratory is vital in order to continue studies of the receptor and its signaling importance to fertility and reproduction.

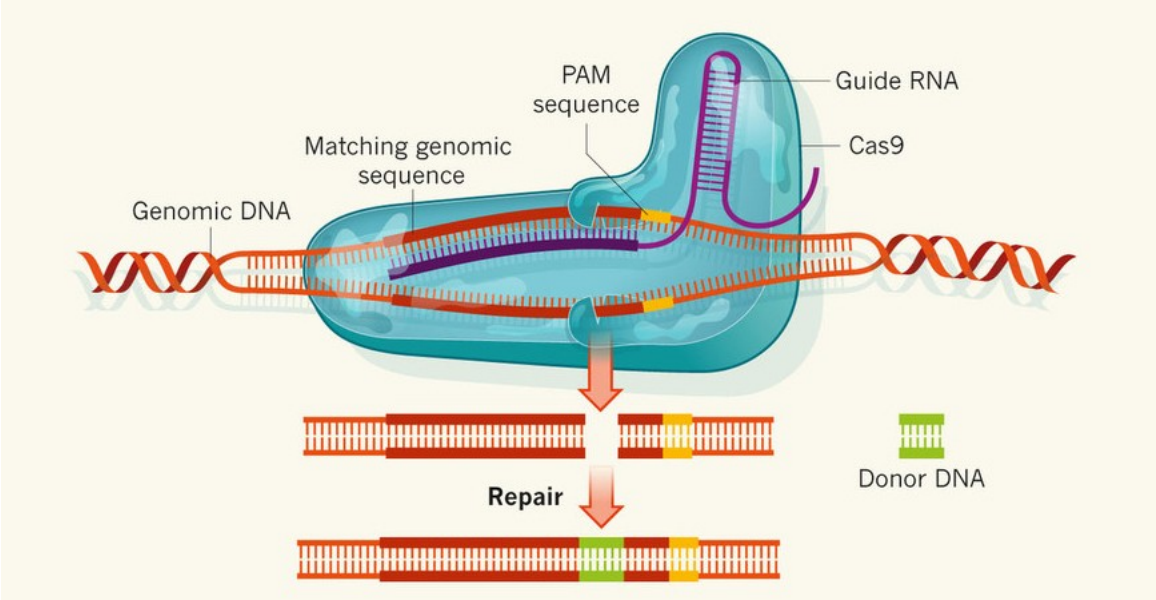

Figure 6: CRISPR and Cas9 Gene Editing Technique Schematic

Methods

Cell Culture

hGrC1 cells and HEK-293-hFSHRs (HEK-293 cells stably expressing hFSHR) were maintained in a confluent monolayer at 37°C and 5% CO_2 in 10% MEM media.

Treatment of hGrC1 cells with lipid raft depletion

Confluent hGrC1 and HEK-293-hFSHR cells were grown in 6 well dishes. Previous Cohen Lab experiments have shown that day 4 old hGrC1 cells have the most hFSH receptor (Rosie, 2016). We set up the experiment using a 150-minute timeline. The following two tables show the arrangement of wells and their respective treatments. We used a sterile Pasteur pipet to remove the medium from all wells and changed it to serum free medium. The volume total for all wells after treatment was 2 mL.

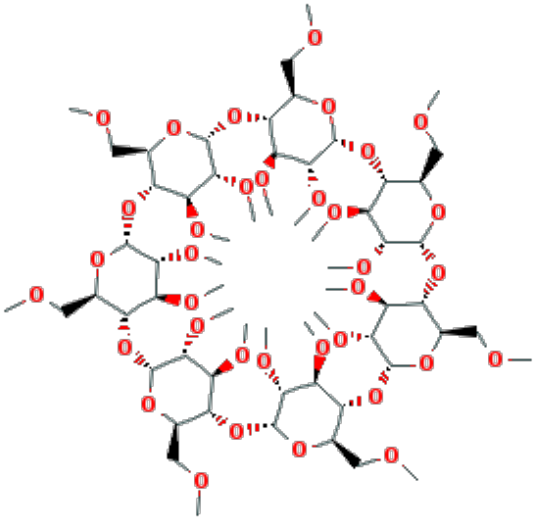

Figure 7: Structure of Methyl-Beta-Cyclodextrin (MBCD), which removes cholesterol from the cell membrane, thus depleting lipid rafts.

https://www.researchgate.net/publication/222573024_Cyclodextrins_and_their_uses_A_review

Table 1: Outline of the overall experiment for one cell line, plate 1

Control, time 0	Control, time 5	Control, time 15
Control, time 30	MβCD, time 0	MβCD, time 5

Table 2: Outline of the overall experiment for one cell line, plate 2

MβCD, time 15	MβCD, time 30	Nystatin, time 0
Nystatin, time 5	Nystatin, time 15	Nystatin, time 30

*Two experiments to test MβCD and FSH concentration were set up differently than above

A 5 mM MβCD solution was prepared for the experimental wells and was added at T-150 minutes for 2 hour pretreatments. Nystatin pre-treatments were given as a spike for 30 minutes before beginning FSH treatments. At T-30 100 µL FSH treatments began and were spiked in the proper wells labeled above. After the FSH treatments were complete, the 6-well dishes were put on ice to stop the reactions. The samples were harvested in order to obtain protein from all samples using lysis buffer and douncing techniques referenced in the Cohen lab manual (Cohen).

Immunoblotting and Chemiluminescence

Western blotting is a technique in which researchers can identify specific proteins from a complex mixture of extracted proteins (Mahmood and Yang, 2012). The mixture is first separated by size through gel electrophoresis. The product is then transferred to a nylon membrane leaving bands at the protein locations. Lastly, the membrane is incubated with probe for a specific molecule through the use of antibodies (Mahmood and Yang, 2012). Chemiluminesence visually locates the proteins that the antibodies tagged and thus contain the target protein. Phosphorylated proteins after hFSH treatment were visualized using this technique to investigate if either cAMP and/or MAPK p44 activation were affected by the drugs. If there was no or significantly less phosphorylation compared to naive hGrC1s then we could determine if the signaling interference was because of lipid raft depletion.

Western blots from 10% acrylamide gels were used to analyze the collected proteins. The samples were diluted according to the BCA assay to ensure equal loading between lanes. The western blots were probed with antibodies specific to the signaling proteins we were interested in investigating. The most common primary antibodies used were 5 µL p-p44 and p-p38 or p-PKA

substrates in 10 mL 5% milk in TBST overnight in the cold room. Then the secondary antibody (goat anti-rabbit HRP)was incubated on the blots for 1 hour at room temperature.

Immunofluorescence

Immunofluorescence on hGrC1 cells was carried out following Professor Danowski's laboratory manual as described in the appendix (Danowski, Cancer Cell Biology, Union College, Fall 2016). Membrane permeabilization through surfactants was skipped because we did not want to puncture holes in the cell membrane since we were studying hFSHR which reside in the membrane. The primary antibody was mAb 106.105 was used at a 1:200 dilution in 1x PBS to stain for hFSH receptor. The secondary antibody used was goat anti-mouse Alexa 594 at a dilution of 1:300 in 1x PBS.

CRISPR

Clustered regularly interspaced short palindromic repeats, CRISPR, was derived from bacterial immune system and now serves as a revolutionary gene-editing technique (Porteus, 2016). It utilizes a protein called Cas9 and a guide RNA component, referred to as, sgRNA. Cas9 has helicase, nuclease, and sgRNA binding activity. CRISPR/Cas9 system is activated specifically through RNA-DNA hybridization activity and not protein-DNA like other methods of genome manipulation. The sgRNA is a 20 nucleotide target sequence allows for the Cas9 to know where to made the DSB and comes right after a PAM sequence NGG (Porteus, 2016).

Double stranded breaks allow for the cells natural DNA repair mechanisms to fix the genome. The RNA polymerase will try to fix the break, but this induces mutations. Eventually

after many mutations, functional protein cannot be made anymore, and this there is a gene knockout (Porteus, 2016).

First we figured out which sgRNA sequence to use, utilizing the online sequence analyzer on MIT Feng Zhang CRISPR online tool website. This allowed us to easily identify which segment of the FSHR sequence to target which was also near a PAM (protospacer adjacent motif) sequence (Figure 8). We grew the Addgene plasmid, pSpCas9(BB)-2A-Puro without the insert overnight in *E. coli* and selected using Ampicillin. Then we followed the mini prep kit to obtain purified CRISPR plasmid. We annealed, kinased using BbsI, and ligated the oligonucleotides into the plasmid following the Cohen protocol handbook. The ligated plasmid was verified using restriction enzyme digests using HinDIII (specific cutter to just the linker) and EcoRI, which should have yielded 3 product bands of weights 670, 3235 and 5270 bp. Once we verified this we could successfully name this plasmid Psp-Cas9-Bb2APuro hFSHR because it contained our linker which would lead Cas9 to create a double stranded DNA break in the FSHR gene of the hGrC1 cells. We then obtained Psp-Cas9-Bb2APuro hFSHR DNA by replicating the *E. coli* overnight culture and the mini prep series. We froze a stock solution in the -80˚C freezer. We then attempted transfected the Psp-Cas9-Bb2APuro hFSHR plasmid into the native hGrC1 cells using Lipofectamine and P3000 reagents. We attempted to select clones by using 2uM puromycin media. After failure, we decided to try other reagents such as, Satisfection, Torpedo, and Transit. Most cells would die after a few days in the puromycin media.

5′-CACCAAGCTTCGAGTCATCCAAAA
TTCGAAGCTCAGTAGGTTTTCAAA-5′

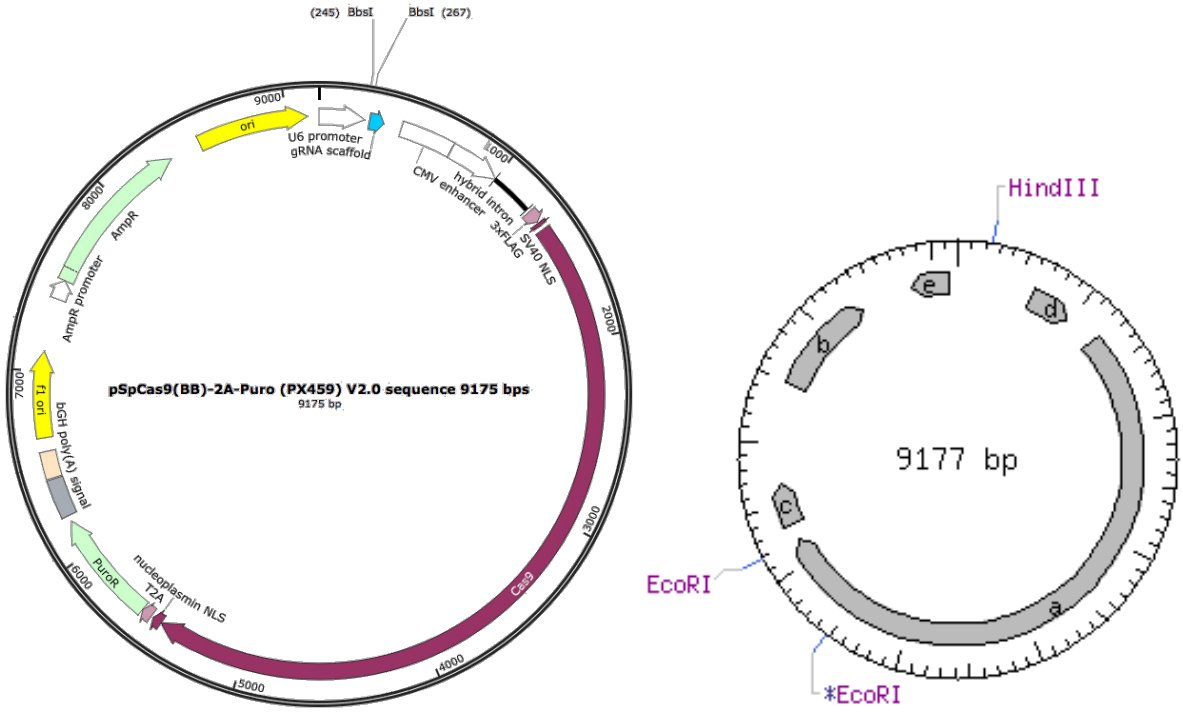

Figure 8: pSpCas9(BB)-2A-Puro Addgene Plasmid and Oligonucleotide Insert and Restriction Enzyme Sites

Results

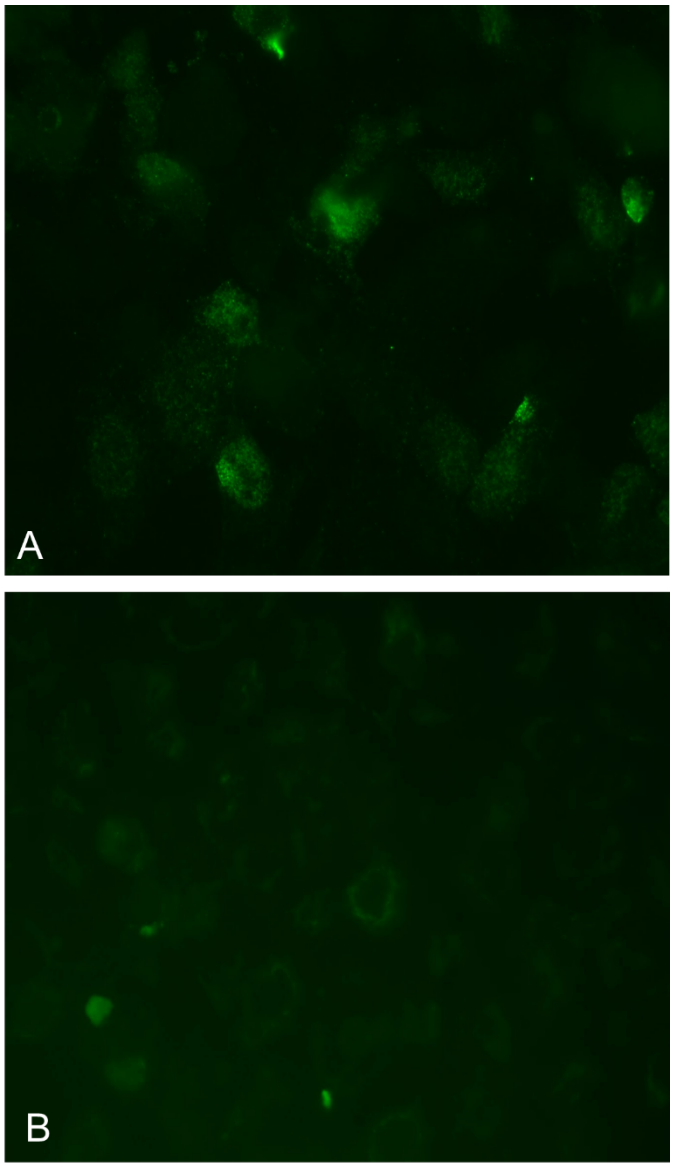

Figure 9: hFSH causes redistribution of hFSHR on hGrC1 cells. hGRC1 cells were grown on coverslips and then treated with hFSH for 0 (A) or 30 (B) minutes. Cells were fixed without permeabilization and stained with anti-hFSHR mAb 106.105 & Goat anti-mouse Alexa 594 antibodies. Cells in A show a broad distribution on the surface, while cells circled in (B) show hFSHR in a more punctate pattern.

Immunofluorescence showed that the receptor is indeed localized to the cell membrane after hormone treatment. This agreed with previous Team Cohen research that concluded that the FSHR resides in lipid rafts of the cell membrane. These results allowed us to continue research upon the assumption that the FSHR resides in these microdomains of the cell membrane.

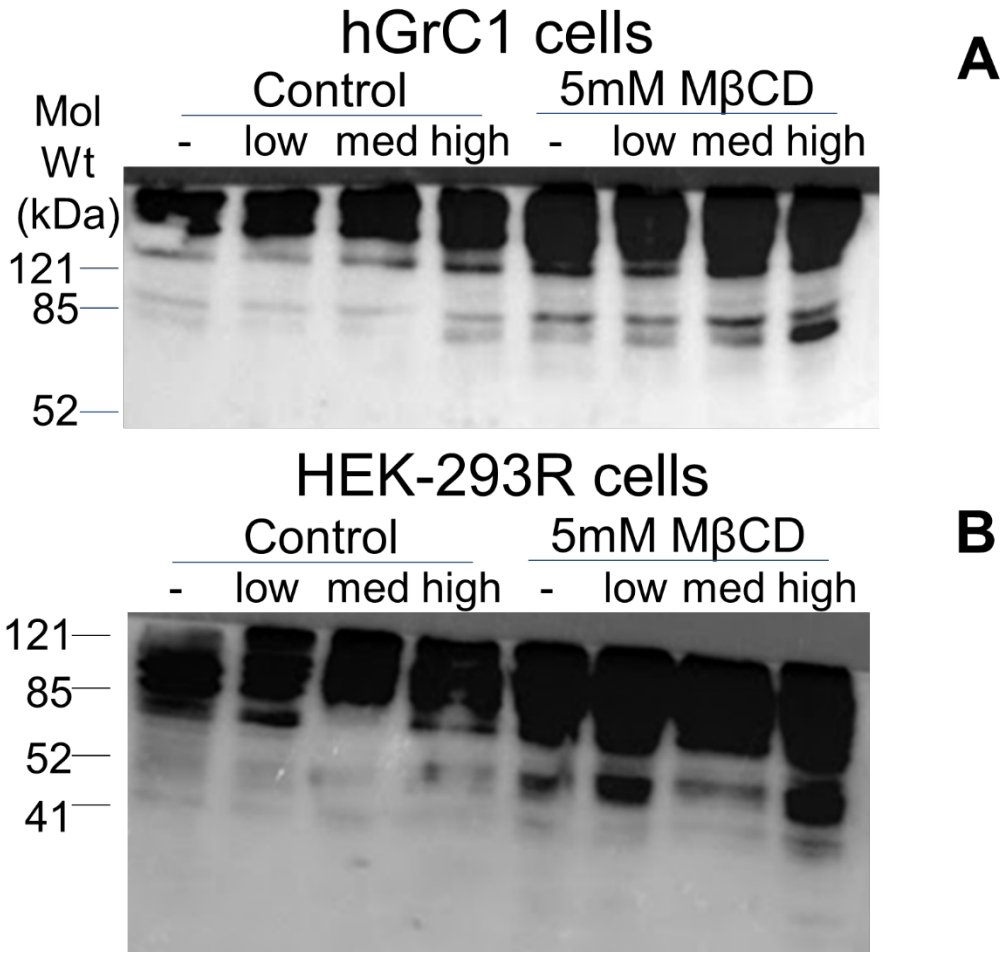

Figure 10: Lipid raft disruption with methyl beta-cyclodextrin (MβCD) alters hFSH induced signaling in human granulosa cell line and in HEK293 cells stably expressing hFSHR cDNA. Cells (hGRC1 in (A), HEK293-hFSHR in (B)) were treated with MβCD as indicated and then treated with varying amounts of hFSH. Cell extracts were probed for phosphorylated substrates of protein kinase A (PKA).

These results showed that intracellular signaling responses are dependent upon the cell type that the receptor resides in. There was a dose response seen in both the hGrC1 and HEK293R cells. Upon lipid raft depletion with 5mM MBCD, there was an increase in amount of phosphorylated substrates by PKA. Therefore, since the phosphorylated substrates of PKA serves as a proxy for cAMP production, we can conclude that there was more intracellular production of cAMP in both cell lines upon lipid raft depletion.

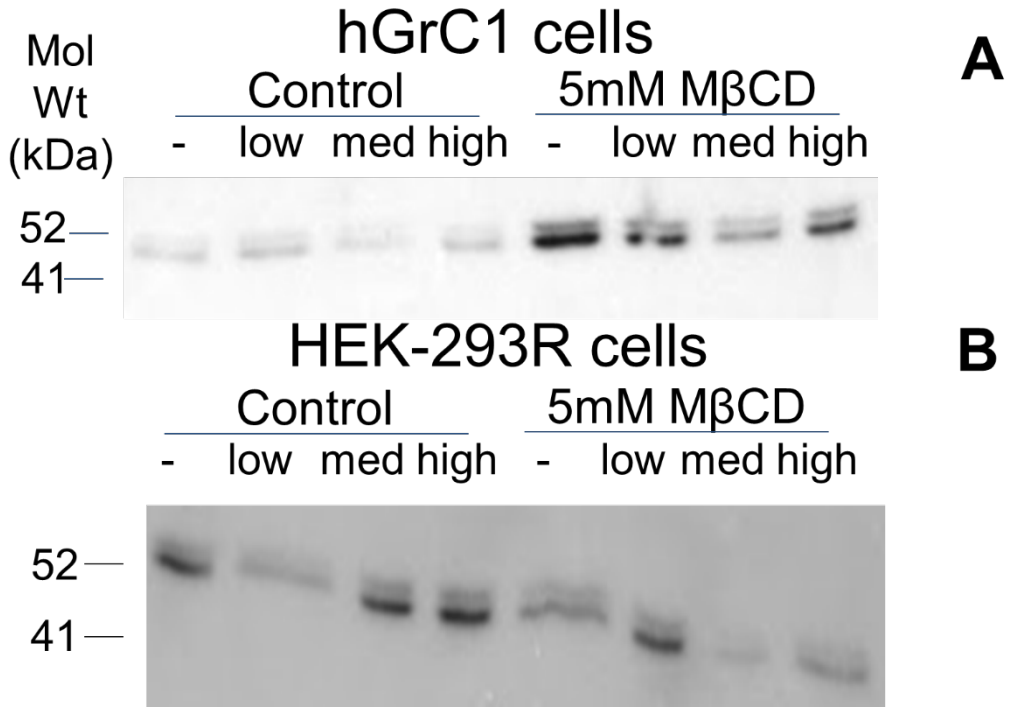

Figure 11: Lipid raft disruption with methyl beta-cyclodextrin (MβCD) alters hFSH induced signaling in human granulosa cell line and in HEK293 cells stably expressing hFSHR cDNA. Cells (hGRC1 in (A), HEK293-hFSHR in (B)) were treated with MβCD as indicated and then treated with varying amounts of hFSH. Cell extracts were probed for phospho-p44 MAP kinase.

Unlike the results we saw for cAMP production upon lipid raft depletion, the two cell lines differed in their p44/42 MAPK response. After lipid raft depletion, the hGrC1 cells showed

an increase in p44/42 MAPK phosphorylation. However, the HEK293R cells showed a relative decrease in activated p44/42 MAPK after lipid raft depletion.

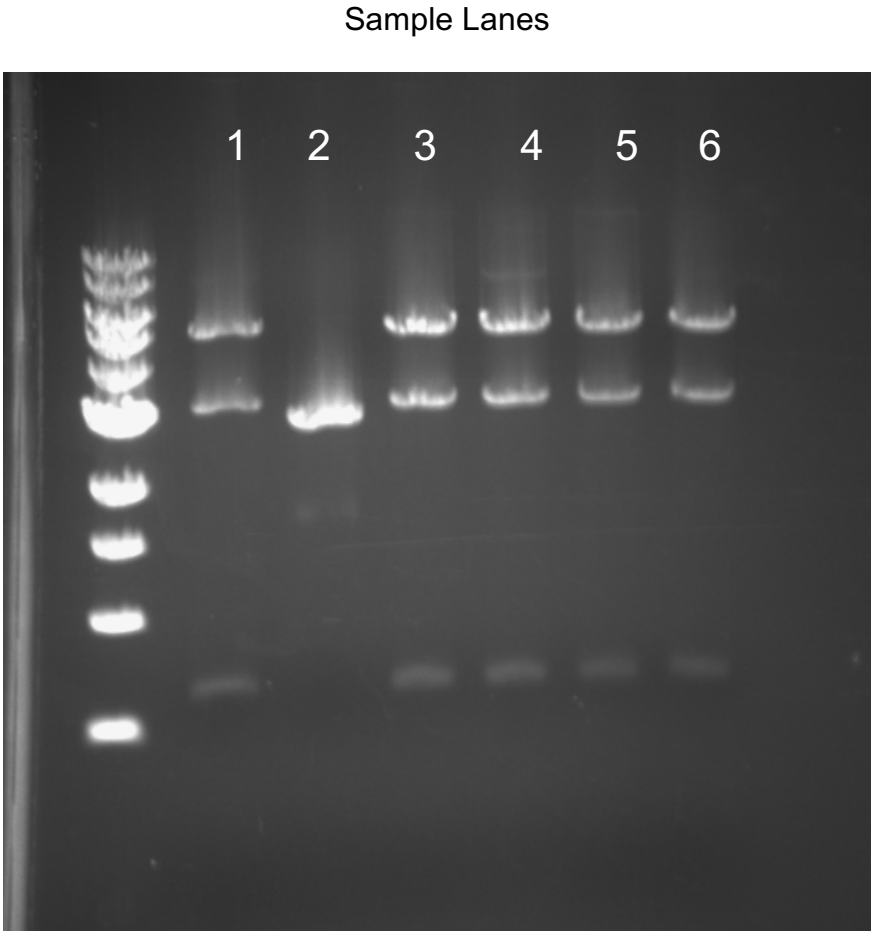

Figure 12: 1% agarose gel of restriction digests run on 6 pSpCas9(BB)-2A-Puro-hFSHR samples cut with HinDIII and EcoRI.

After annealing, kinasing using BbsI and ligation of the pSpCas9(BB)-2A-puro plasmid we wanted to see if indeed we created an all-in-one CRISPR plasmid to create an hFSHR knockout in hGrC1 cells. We used a 1% agarose gel to separate the plasmid segments after the digests. HinDIII only cuts in the sgRNA sequence as seen in Figure 8. If we successfully created

an all-in-one plasmid, there would be 3 plasmid fragments of the following sizes: 670, 3235, 5270 bp. Five of the 6 samples were successful!

Discussion

Follicle stimulating hormone receptor is an important target for future fertility medical care. Before we can design effective treatments, we need to understand more about hFSHR signaling. Our research could explain how to target these receptors by learning more about the importance of lipid rafts for proper hFSHR function. Knowledge of the hormone and its receptor is critical for hFSHR's ability to act as a target for medicines for more effective birth control drugs or for fertility treatment.

Our first major conclusion after immunofluorescence and immunoblotting experiments was that it is really important to study FSHR in a native cell background in hGrC1 cells. The HEK293R cells have shown to differ in their intracellular signaling responses upon lipid raft depletion. This means that the receptor mutants being made in the lab should be studied in a native cell background to yield research that is physiologically relevant.

The CRISPR hFSHR knockout plasmid failed to transfect into the hGrC1 cell line. One potential source of the issue could be that the concentration of puromycin antibiotic was too strong for the cells to stay alive. Previous research suggested 2 uM puromycin, however this was not used with hGrC1 cells, so we cannot rule this out as a potential reason why the cells were dying.

Ovarian follicles undergo a hormonally controlled form of apoptosis, known as atresia. This process can occur at any point in the follicles development (Matsuda, et al. 2012). Deprivation of key survival-promoting factors or stimulation is the main cause of this specific

cell death. Gonadotropins are a major cell survival hormone to the granulosa cells of the follicles. Activation of cAMP-dependent pathways promote survival of these cells in the ovaries. Acting through secondary messenger systems, gonadotropins, coupled with nearby ovarian factors can thwart atresia.

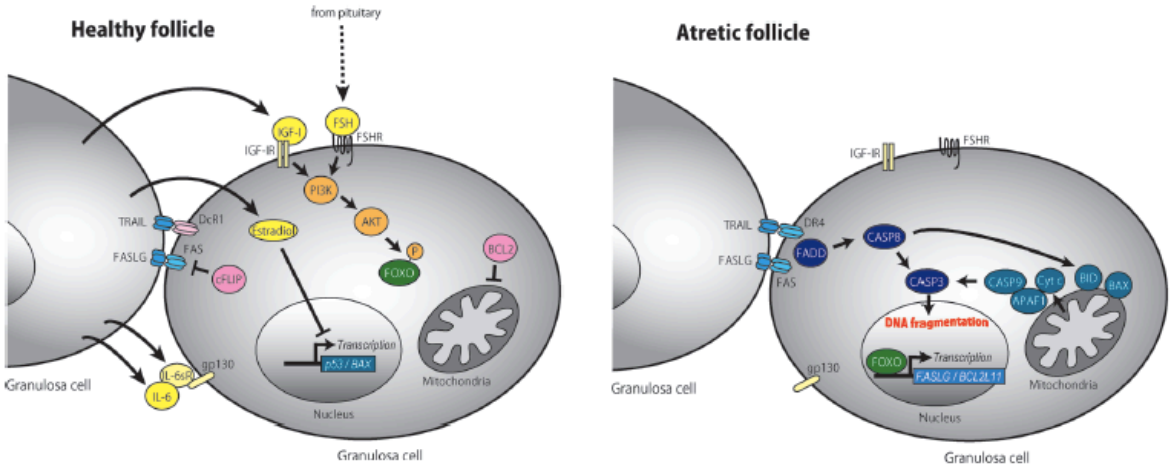

Figure 13: Schematic model of the Intracellular signaling in healthy human follicles compared to that of atretic follicles. FSH that is secreted from the pituitary is essential for granulosa cell survival. (Matsuda, et al. 2012)

In the antral stage of follicle development, follicles become gonadotropin-dependent. Follicle stimulating hormone, is the most important cell survival signal for the cells in this stage. If CRISPR did knockout the hFSHR gene in the hGrC1 cells, then they were not able to respond to FSH. Therefore, because of the absence of this survival signal reaction, the granulosa cells might have undergone atresia. However, the stage of follicle development of the hGrC1 cell line is unknown. Absence of viable cells supports the hypothesis that the hGrC1 cells are in the antral stage.

Future Research

Team Cohen future research on this project might include temporarily silencing the hFSHR gene in the hGrC1 cells. siRNA or shRNA could be utilized in order to investigate if the cells can survive without the FSH receptors. If not, this is an insight as to why CRISPR did not produce any viable cells.

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Appendix

Danowski's Immunofluorescence Manual Excerpt:

Reagents:

-**PBS** (phosphate-buffered saline with Ca++ & Mg++, to wash cells & dilute antibodies)

-**4% paraformaldehyde** (labeled **PF**) in PBS, to fix cells

-**0.1% Triton X-100**, a detergent used to permeabilize the cells. (Without this step, the rather large antibody molecules would be unable to penetrate the plasma membrane.)

-**Primary antibody**, Use at the indicated dilution. Make up 50 ul of diluted antibody for each coverslip. Thus, if you are preparing 2 coverslips, make up ***exactly*** 100 ul of diluted antibody.

-**Secondary antibody**, directed against the primary antibody, and conjugated to a fluorophore. The stock solutions of the second antibodies will be provided to you at a 1:15 dilution. The working dilution is 1:300.

-A **mounting medium** to anchor your coverslips to a glass slide. **WEAR GLOVES** when handling this.

Antibodies **This list might be updated/ changed on the day of the lab.

Anti-cadherin antibody, made in a mouse. Use at 1:100
Anti-paxillin antibody, made in a mouse. Use at 1:100
Anti-alpha-actinin antibody, made in a mouse. Use at 1:100
Anti-vinculin antibody, made in a mouse. Use at 1:200
Anti-non-muscle myosin, made in a rabbit. Use at 1:200

To Make a Moist Chamber:

In order to prevent the evaporation of the diluted antibody, we need to incubate the coverslips in a moist environment. Place 9 or 10 cm filter paper circles into the bottom AND top of a 100 mm plastic petri dish. The larger traditional 'top' of the petri dish will be used as the bottom. Moisten thoroughly with distilled water, and carefully smooth out the filter paper (i.e. get rid of air bubbles) on the bottom of your moist chamber so that the coverslips will lie flat on the filter paper.

Cautions:

WEAR GLOVES when handling Paraformaldehyde (PF) and Alexa-Green PHALLOIDIN
PF should be used IN THE FUME HOODs only!
Phalloidin is a potent poison. Use it with care.

NEVER let your coverslips dry out. There should always be some solution on them, either PBS or diluted antibody.

Procedure

(1) First we must "fix" the cells; we will use a dilute solution of formaldehyde in buffer to kill the cells and cross-link all proteins together to hold them in their proper place. In the fume hood, remove the medium from your dish of cells, using a plastic transfer pipet, and quickly add approximately 0.5 ml of PF. (Why quickly? We don't want the live cells to dessicate before we kill them with PF). Move your dishes to the side of the hood. Incubate 8-10 minutes at RT (room temperature) ****Never squirt anything directly on the cells.** They are fragile! Gently add and remove solutions from the sides of the petri dishes.

(2) When the incubation period is up, remove the PF, place it in the labeled PF waste container, and then **gently** wash the dish 3-4 times with PBS. To do this, fill a transfer pipet up with PBS, and ***carefully and slowly*** squirt the entire contents into the corner of the dish. Then, pour the solution out into a 'dump beaker', and immediately add another pipette-ful of PBS. Repeat this 3-4 times, leaving the PBS on the cells after the last wash for at least 2 minutes. **NEVER** let the cells dry out—always keep liquid on them. **Once you have removed the PF and done one PBS wash, return to your lab benches (remember—there should be PBS in the dishes, on the cells) for the remaining steps.**

(3) DID NOT PERFORM THIS STEP- needed the cell membrane to stay intact

Next step—permeabilize the plasma membrane so that the antibodies can enter the cell. Pour off the PBS and add enough 0.1% Triton X-100 to cover the bottom of the dish (0.5-1.0 ml), and incubate for 3 minutes **EXACTLY** (3') at room temperature (RT).

(4) Wash 3-4 times with PBS. Washing is important! Let cells sit in the last PBS wash for approximately 2'.

(5) **While you are waiting, make the appropriate dilution of primary antibody in PBS. Remember, you need 50 ul of diluted antibody **per coverslip**. (Hint: if you have 2 coverslips, to prepare with the same primary antibody, then make 100 ul of diluted antibody; if you have 3 coverslips, make 150 ul, etc.) Always keep antibodies on ice-they are proteins, and can denature easily.

(6) Using forceps, remove coverslip from dish, carefully blot any excess PBS by tipping the coverslip onto a small piece of filter paper, and letting the edge of the coverslip touch the paper. Place in a moist chamber (see above for instructions on making a moist chamber). Gently add 50 ul of diluted antibody to the coverslip with a micropipet. Carefully carry your chamber to the cell culture incubator and gently place it on a shelf. Incubate for 30-45' at 37 ^{O}C. ****Save the empty petri dishes that the coverslips were in. Rinse them**--you will need them later!

(9) During this incubation, prepare the Alexa green-phalloidin and the second antibody (either goat-anti mouse—Alexa Red, labeled G✔M-Red or Goat-anti-rabbit—Alexa Red, labeled G✔R-Red). You will **combine these 2 reagents**, and incubate them together, but first, you must calculate the appropriate dilution. You want to add exactly 50 ul of solution to each coverslip, and you want the final concentration of Alexa green—phalloidin to be 1:100 and the secondary antibody concentration to be 1:300. **Remember** that your stock solutions of these 2 reagents are already dilute. First, you need to calculate how much further you need to dilute them.

(10) After incubation in the first antibody, remove the antibody by tipping the coverslip onto a clean scrap of filter paper. Return the coverslip to petri dish **pre-filled with** fresh PBS, and wash 3-4 times, as you did earlier, allowing the coverslips to sit in the last wash for ~1-2 min.

(11) Place coverslip back in a moist chamber, and add 50 ul of the 2nd antibody to each coverslip. Incubate for 30 minutes, in the 37 ^{O}C incubator. Label your slides during this incubation. Include the date, your initials, cell type and name of protein being localized. (Write small!!)

(12) After 30 min, remove antibody as before and wash coverslip thoroughly with PBS 3 times **BUT THEN do the last wash in dH2O**.

(13) Place one very small drop of mounting medium (Pro-Long Anti-Fade mounting medium) onto a clean microscope slide. Carefully dry the back of the coverslip, and any excess water. **Invert the coverslip** onto the mounting medium. In other words, the cells are now bathed in (and protected by) the mounting medium. Let harden for ~1 day, and view under the fluorescence microscope.