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Investigating the Use of Aptamers in Binding to the Human Follicle Stimulating Hormone receptor

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By

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Abstract

Ibrahim, Ayon Investigating the Use of Aptamers in Binding to the Human Follicle Stimulating Hormone Receptor (hFSHR). Department of Biological Sciences, June 2013.

Advisor: Brian D. Cohen

Oral contraceptive pills use high doses of hormone to suppress the reproductive arm of the endocrine system in women. However, these drugs can have harmful long term side effects such as increased risk of stroke, heart attack, and breast cancer. Therefore, it has become an important public health goal to look for alternative ways of providing contraception. Aptamers, oligonucleotides that bind to specific molecules due to their unique 3-dimensional structure, are one alternative that has yet to be explored. The goal of our research is to develop an RNA aptamer that will bind selectively to the hFSHR. We first incubated a large pool containing random sequence oligonucleotides with wild type HEK293 cells (which do not have hFSHR) as a counter-selection. Many of the RNA molecules bound to the protein receptors on the cell surface. The unbound molecules were then incubated with HEK293 cells expressing the hFSHR. The oligonucleotides that bound to the hFSHR were eluted, isolated, and then re-amplified. The process of selection was repeated while increasing stringency of binding each time. We were able to successfully perform SELEX on our RNA pool at 4°C and 22°C. We also conducted nanodrop experiments to confirm that our oligonucleotides are indeed binding specifically to the hFSHR. We hope to continue refining our aptamer at higher temperatures such as 30°C and 37°C, determine the exact nature of the oligonucleotide-protein binding complex, and eventually begin *in vivo* trials to determine efficacy.

Introduction

The human follicle-stimulating hormone (FSH), also called follitropin, is a glycoprotein secreted by the basophil cells in the anterior pituitary gland. It is a dimeric protein, consisting of an alpha and a beta subunit, the latter of which interacts with the FSH receptor (FSHR). It is primarily the pulsatile release of gonadotropin releasing hormone (GnRH), from the arctuate nucleus of the medial basal hypothalamus, that causes the production and release of FSH from the gonadotropic cells of the anterior pituitary. In males, follitropin binds to Sertoli cells in the testes, which induces the release of

inhibin, a protein hormone that negatively feed backs on the gonadotropes in the pituitary gland and decreases the synthesis of follitropin, and activin, which also stimulates FSH production, in addition to aromatase production and spermatogenesis [1]. A third hormone, follistatin, works with inhibin by suppressing FSH gene expression. In females, follitropin binds to granulosa cells in ovaries, stimulating the release of inhibin, as it does in the male. It also stimulates the production and release of estrogen/estradiol, a steroid hormone that not only increases GnRH pulses from the hypothalamus but also binds to the anterior pituitary and inhibits FSH production and release. Estrogen is crucial for development of bone, muscle and secondary sex characteristics in the female [2]. Progesterone, a steroid hormone released by theca cells in the ovary, acts upon the hypothalamus to lower GnRH pulses. Progesterone's release is regulated by luteinizing hormone (LH), a dimeric protein similar in structure to FSH that is also released by the basophil cells of the anterior pituitary. In the male, LH also stimulates the release of testosterone which, decreases GnRH pulses and promotes growth of muscle, bone and secondary sex organs. [1][3].

FSH is essential for the proper reproductive development in both sexes. In males, follitropin promotes spermatogenesis and stimulates primary spermatocytes to undergo meiosis. In females, FSH is crucial for developing and sustaining ovarian follicles through the activation of over 100 different genes, all of which leads to multiple physical changes such as increased vascularization of the theca interna layer of cells near the basal lamina and formation of a fluid-filled antrum within the growing follicular cell [1]. FSH effectively prepares the uterus for implantation of a fertilized egg [2]. As levels of inhibin increase, FSH levels decrease and remain low until the end of the luteal phase, the latter stage of menstruation, in which a renewed, steady rise in FSH prepares more follicular cells for the next ovulatory cycle [2][3].

Oral contraception works by interrupting the FSH signaling pathway: the pills contain estrogen and/or progesterone. As mentioned above, high levels of progesterone negatively feeds back on the hypothalamus, causing a decrease in the amount of gonadotropin releasing hormone (GnRH), which in

turn results in a decrease in the release of FSH and LH by the anterior pituitary. Also, high levels of estrogen negatively feeds back on the anterior pituitary gland and inhibit the release of FSH [3]. The presence of oral contraceptives thus prevents proper reproductive maturation by inhibiting the release of FSH and LH, which cause the ovary cells to undergo atresia and therefore suppress the chance of successful ovulation [4].

However, studies have shown that inducing unnaturally elevated levels of estrogen and progesterone by consuming these pills have dangerous side effects. For example, a 2010 paper published in the *Expert Review of Cardiovascular* Therapy journal showed that usage of the combined hormonal oral contraceptive had significant correlations with an increased risk in venous thromboembolism, which is the formation of blood clots in veins, commonly in the leg or pelvic area [4]. A meta-analysis of several studies have shown similar results in that women taking second generation oral contraceptives (containing less than 50 μg of estrogen) have a significantly higher risk of both myocardial infarction and ischemic stroke. Third generation contraceptives are associated with a high risk of vascular arterial complications, especially with women of older age [5]. Additional research supports the conclusion that usage of certain oral contraceptives (such as desogestrel and gestodene) is associated with higher risk of ischemic stroke, deep vein thrombosis and cardiovascular disease [6]. However, it is not just the cardiovascular system that is impacted negatively by the consumption of oral contraceptives. An in-depth study has revealed that both perimenopausal and postmenopausal women who take oral contraceptives on a chronic basis increases their risks of breast, cervical and liver cancer in addition to ischemic stroke. The risk was observed to be greater when the women took the combined estrogen-progesterone pills as compared to estrogen alone. However, the same study showed that there can also be multiple beneficial side effects, such as decreased risk of ovarian and endometrial cancer, pelvic inflammatory disease (PID) and colorectal cancer [7]. Another study showed that high levels of estrogen sustained for over 21 days lead to abnormally high levels of plasma cortisol and tryptophan, as well as high levels of tryptophan metabolites in urine. The

researchers suggest that these elevated tryptophan levels may be related to mental depression and/or rheumatoid arthritis, though the mechanisms remain uncertain. They also postulate that the higher cortisol levels as a result of the oral contraceptive pills led to the liver being exposed to high glucocorticoid concentrations, which led to the finding of impaired glucose tolerance in 75% of the women, while 13% developed diabetes mellitus [8]. Finally, one comprehensive study provides evidence that estrogen/progesterone pills have little to no effect on the risk of developing breast cancer, contradictory to what other studies have shown [9]. Clearly, these pills have an intricate and complex range of effects on the human body, much of which is not completely understood.

Another possible contraceptive option is called the intrauterine device (IUD), which works by inserting a T-shaped object into the uterus, inducing a localized inflammatory reaction in the endometrium, causing a release of the of endometrium's cellular and humoral components. Additionally, some IUD's, such as Cu-IUD's, release ions such as copper into the uterine cavity, which can reach concentrations toxic to sperm and/or developing zygotes [10]. IUD's have higher success rates for preventing pregnancy than do hormonal pills, but have also been known to cause heavier bleeding or more painful menstrual cramps [3][3.5]. There is one possible method of contraception that has not been explored, but may have a high chance of success without harmful side effects, and that is blocking the FSHR directly.

The FSHR belongs to a class of large molecules called G-protein coupled receptors which are embedded in the cell membrane. They have a 7-alpha helical transmembrane spanning domain (TM domain) and a glycosylated extracellular domain (ECD). They are highly prolific in mammal granulosa cells: those from rats are reported to have around 4,600 receptors per cell. It is worthy to note here that this protein, in particular the TM domain, is highly conserved among animal species, especially mammals, with the most variation in the C-terminal tail [1]. Glycosylation of the ECD is also highly conserved. Thus, research done on rat FSHR is highly applicable to human FSHR. Binding of FSH to the ECD of the receptor causes a shift in conformation and activation of the G-protein at the C-

terminus on the intracellular side of the membrane. This action causes adenlyl cyclase to convert ATP to cAMP, beginning a very complex protein signaling pathway illustrated in Figure 1 that eventually leads to gene expression [2]. An important step in the signaling pathway is the activation of protein kinase A (PKA), a necessary action for the directing of granulosa cell differentiation, as signals from cAMP are mainly mediated through PKA. For example, PKA regulates the activity of the transcription factor cAMP response element binding protein (CREB) and protein kinase p38 MAPK, two different signaling pathways that contribute to follicular maturation. As can be seen on Figure 1, there is another signaling pathway that is activated by calcium ion movement (with some unknown protein regulating that activity) rather than by FSH binding. However, even this pathway is eventually influenced by FSHR activity, as one of its proteins, PTP/ERK, is phosphorylated by PKA [2]. The importance of the FSHR in proper reproductive development is further shown by the low occurrences of natural mutations, as such errors would result in lower fertility and inability of the organism to pass down the mutated genes. This is evidenced by the A172V mutation in the ECD domain, something found among a sub-population in Finland, associated with hypergonadotropic ovarian dysgenesis. Many victims presented with infertily, amenorrhea, stunted follicular maturation, and variable secondary sex characteristics [11]. Therefore, it is reasonable to conclude that if a female were to use a form of contraception that blocked FSH binding to its receptor, the follicles would be unable to mature properly and ovulate.

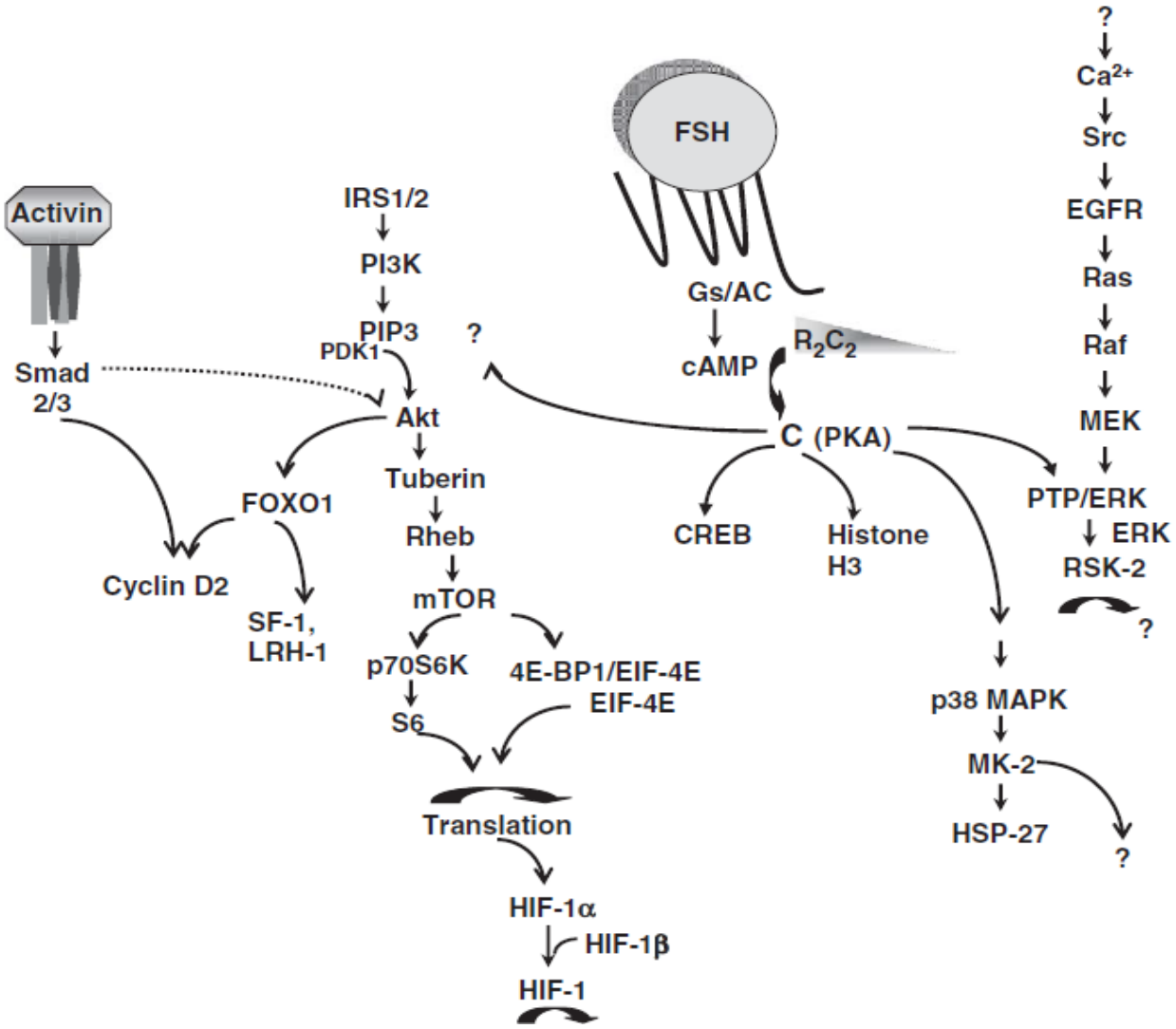


Figure 1. The protein signaling pathways found in human ovary granulosa cells. FSH plays a major role in regulating these pathways. Taken from Hunzicker-Dunn and Miazels (**2006**).

However, it is important to note a major drawback to this strategy. Though blocking the FSHR may prove to be an effective method of preventing pregnancy, it also causes a decrease or even a halt in the production of estrogen, which is important for many others functions in addition to reproductive ones, as mentioned earlier [2]. In order to have normal physiological growth and function while taking this form of birth control, the female would need to take estrogen supplements, which is essentially the same issue with consuming oral contraceptives. However, it is possible that blocking the FSHR can function as a safe form of male birth control. Blocking the FSHR in males will decrease spermatogensis, but will not affect testosterone production and secretion, as that is regulated by LH

binding [2]. This allows for normal, healthy growth in the male while stunting fertility.

Another possible area of interest concerning the blocking of FSHR is animal sterilization. The Michaelson Prize & Grants Foundation offers a $25 million prize for the first, one-shot non-surgical sterilization of cats and dogs, in order to combat the high rates of euthanasia found in pet shelters countrywide [12]. As mentioned earlier, the FSHR is a highly conserved protein, and this fact holds true between humans, cats and dogs [Figure 2]. 88.33% of the amino acids in the extracellular region of the FSHR protein are exactly the same between the three species, and only 3.06% are completely different [Table 1]. Blocking the FSHR with a molecule that was specifically toxic to the reproductive cells it was binding to holds potential as a possible one-shot, chemical sterilization. Thus we propose the usage of aptamers as a novel substitute for traditional oral contraceptives and as a potential sterilization technique in cats and dogs.

Table 1. A quantified comparison of the amino acid similarities and dissimilarities between human, cat and dog FSHR (360 amino acid long extracellular region). “Identity” indicates amino acids that are exactly the same between the three species.

	Number of Amino Acids	Percentage (%)
Identity	318	88.33
Strongly Similar	28	7.78
Weakly Similar	3	0.83
Different	11	3.06

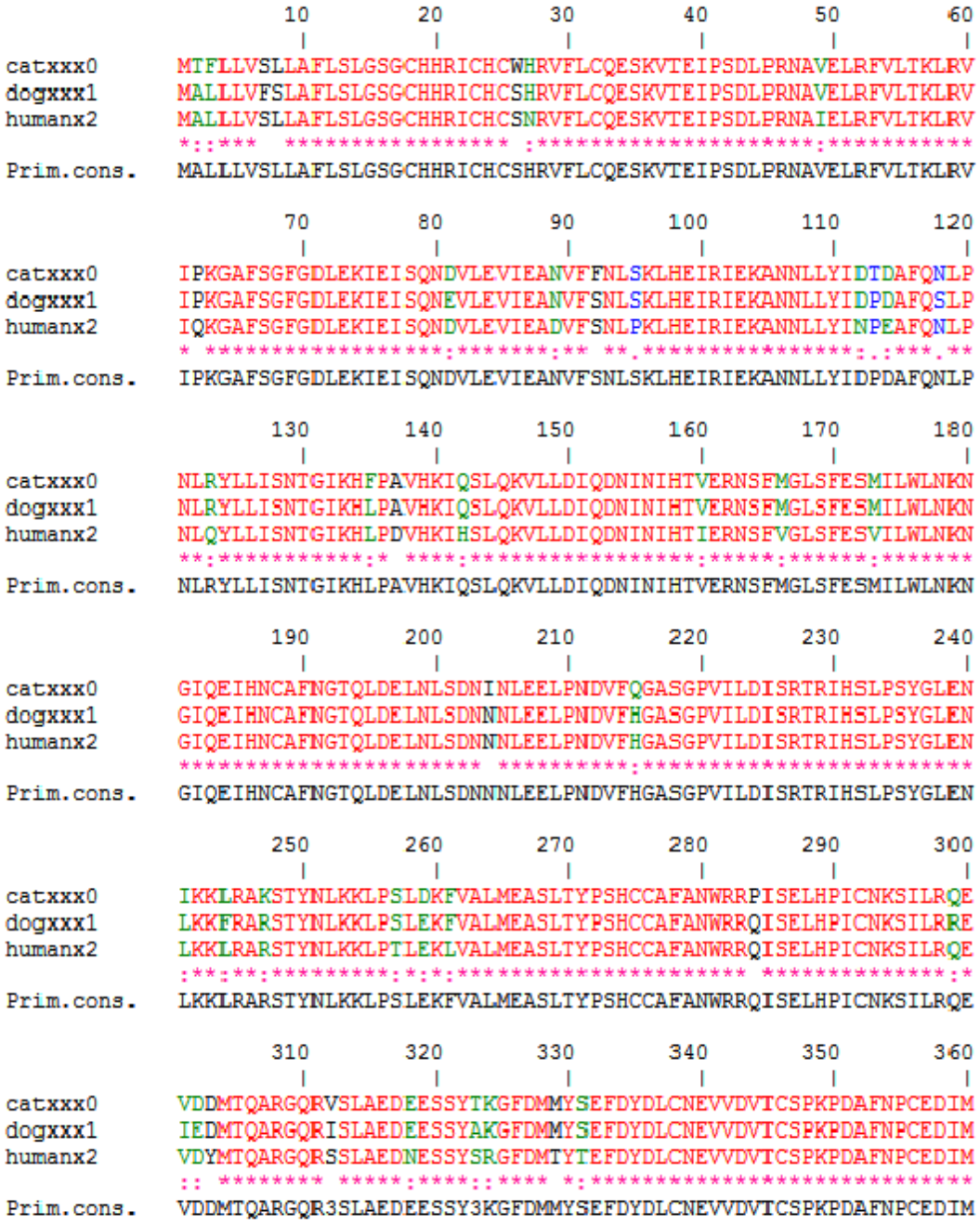
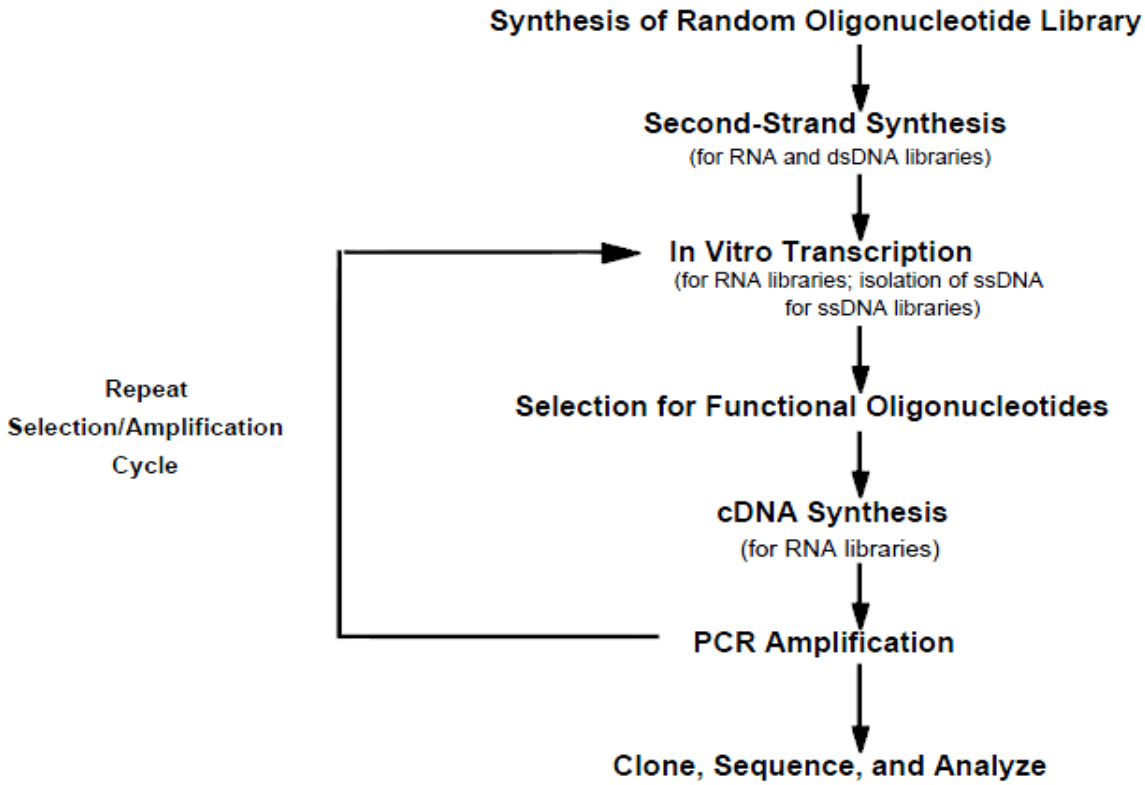


Figure 2. The sequences for the cat, dog, and human FSHR protein (specifically the 360 amino acid long extracellular binding domain). An asterik (*) indicates an exact match between the three species', a colon (:) indicates strong similarity, a period (.) indicates weak similarity and absence of a mark indicates a major amino acid difference.

Nucleic acids (namely, RNA and DNA) have generally been associated with the storage of genetic information. However, their ability to form complex three-dimensional structures have raised interesting possibilities, as this causes the oligonucleotides to exhibit specific intermolecular forces that

may bind to a ligand of interest, such as an enzyme's catalytic domain. They have the potential of becoming very important tools in the science of molecular recognition, a field in which antibodies have long held reign, since before the 1950's [13]. However, the usage of aptamers offers several advantages over the usage of antibodies as molecular sensing probes. Because the aptamer selection process is done *in vitro*, researchers have much greater control over the non-physiological conditions that can be used to strengthen their binding, such as salt concentration, pH, and temperature. Antibodies require an animal host, which in itself presents greater difficulties as the molecule of interest may be toxic to the organism or have little inherent immunogenic response [13][14]. Additionally, they are much more stable than antibodies due to the rigidity of the phosphodiester backbone. High temperatures, harsh pH conditions or drastically high levels of salt would irreversibly denature antibody proteins, while a RNA or DNA aptamer would merely unfold temporarily. In fact, because antibody markers can only be produced *in vivo*, they are restricted to physiological conditions, which means the kinetic parameters of their interactions with target molecules cannot be modified. Finally, due to the protein nature of antibodies, they cannot be stored for a long time, as they will gradually degrade. On the other hand, aptamers have a longer shelf life, as oligonucleotide structures tend to be very stable [11][12].

The *in vitro* method of selection used to make specific aptamers is called the systematic evolution of ligands by exponential enrichment (SELEX), as illustrated in Figure 3 [15]. It begins with the synthesis of a vast library of oligonucleotides (RNA, ssDNA, or dsDNA) that have randomized sequences with fixed ends for primer binding during PCR amplification. After PCR, transcription may be done to get RNA back, if necessary. These oligonucleotides are then incubated with the target molecules. Some of these randomized strands will have a greater affinity for binding than others due to the specific Watson-Crick pairing, non-standard base pairing and novel interactions due to unique three dimensional structures, such as clover shapes and loops and bent "lollipops"[15][16][18]. The more strongly bound oligonucleotides are isolated and amplified by PCR. These eluted molecules are subject to several more rounds of binding under more stringent conditions, such as increased wash time, higher pH, or higher temperature. The oligonucleotides that remain after multiple cycles bind very strongly to



the target ligand. They are then isolated, cloned and analyzed [15].

Figure 3. The SELEX process as shown in a flowchart form. Taken from Nieuwlandt (**2000**).

There exists a more specific form of aptamer selection called cell-SELEX that targets entire cells or ligands bound to cells as opposed to free-floating molecules. The procedure is very similar to normal SELEX, though often with the additional step of counter-selection. As shown in Figure 4, cell-SELEX starts in the expected fashion: a randomized oligonucleotide library is incubated with the target cells, and the bound oligonucleotides are eluted [16]. At this point, the researcher employs the counter-selection technique, in which the eluted molecules are incubated with non-target cells (referred to as negative cells), after which the supernatant containing *unbound* oligonucleotides is collected and the bound aptamer-negative cells are discarded. The unbound oligonucleotides are then amplified by PCR and go through the selection process again, with more stringent measures [16].

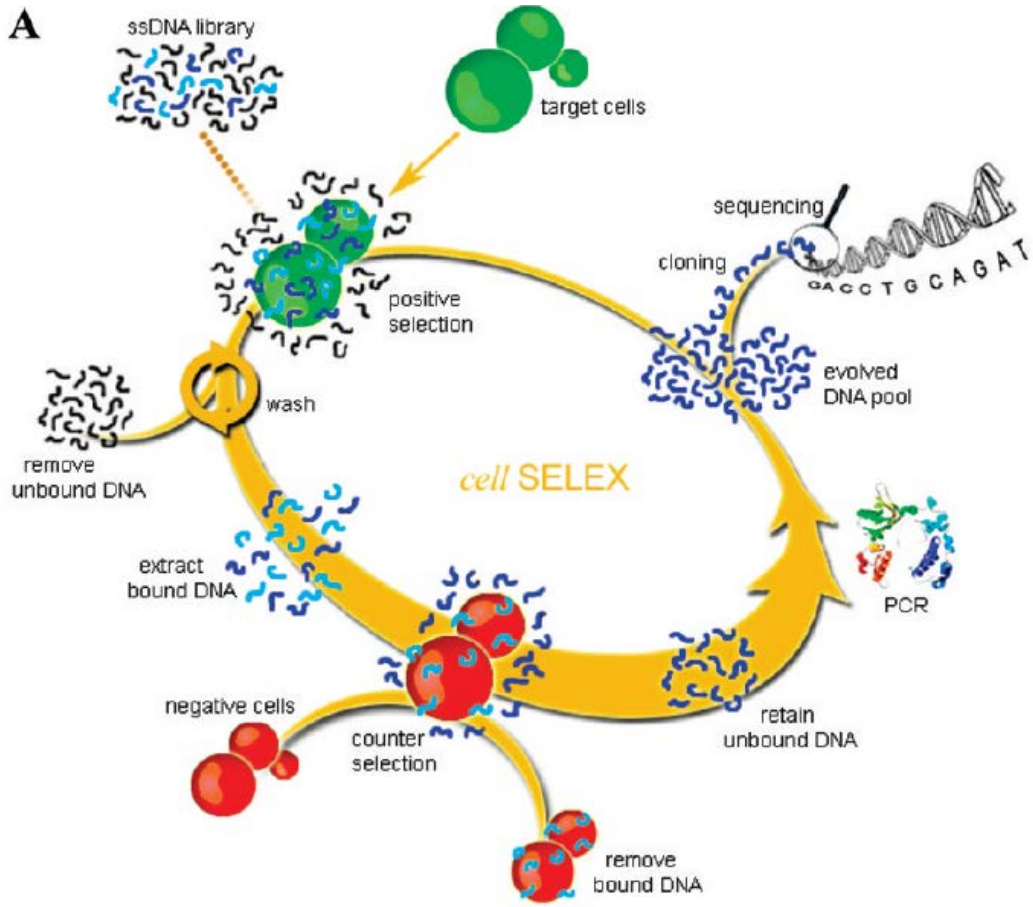


Figure 4. Cell-SELEX, with counter-selection done against negative cells. Taken from Guo *et al*. (**2008)**.

Counter-selection serves to eliminate aptamer candidates that may bind to cells other than the target; thus, aptamers that bind less specifically. This has been shown to be especially useful in the field

of cancer research. Researchers incubated the oligonucleotide library with a cultured precursor T cell acute lymphoblastic leukemia (ALL) cell line, called CCRF-CEM, while using a B-cell line from human Burkitt's lymphoma called Ramos as the counter-selection [16]. This allowed the researchers to select for aptamers that bound much more specifically to CCRF-CEM as opposed to the Ramos cells, which shares several cell surface ligands. In this way, aptamers can be very useful in discovering cancer-specific bio-markers and cell-surface epitopes, which would allow physicians to target and destroy cancer cells specifically, as opposed to current treatments that put somatic cells (that are similar to the cancer cells) at risk [16][17][18]. Aptamers have also shown great potential in early cancer detection. Current methods that attempt to detect specific types of cancer lines have been met with great difficulty due to a low density of cell surface targets for binding. Aptamers conjugated with nanoparticles such as Au-Ag nanorods show much greater efficacy in binding to the cancer cells specifically, as the aptamer-nanorod complexes did not bind to non-target cells [16]. Aptamers can also be conjugated with magnetic and fluorescent nanoparticles (MNP and FNP, respectively), which makes it even easier to detect and diagnose the presence of the target cells. These bioconjugates have the potential to greatly aid drug delivery, as current chemotherapies often result in death of both healthy and cancerous cells [17]. Lastly, aptamers are useful in biological system not only because of their specific binding (which reduces the chance of side effect toxicity), but also because they have relatively short half-lives (due to organic nuclease activity) and are quickly cleared out of the bloodstream by kidneys. Certain modifications such as polyethylene glycol linkage can increase the half-live up to a week [17].

In our research, we expect to develop an aptamer that binds specifically to the hFSHR *in vitro* using a modified version of the standard cell-SELEX procedure. We will perform the counter-selection first, using human embryonic kidney cells (HEK293) provided by James A. Dias from the University of Albany. We will then discard the cells and their bound aptamers, and collect the remaining unbound oligonucleotides and incubate them with hFSHR-transfected HEK293 cells (henceforth referred to as

HEK293r's). These oligonucleotides will only bind the FSHR, as they have already been counter-selected against the other ligands present on the HEK293r cells. Afterward, the bound oligonucleotides will be eluted and amplified and subject to more rounds of selections, with increasing stringency.

Producing aptamers that bind very specifically to the hFSHR will inhibit the natural hormone from binding, which we hypothesize will stunt follicular development, thus acting as a form of oral contraception, most likely for males. . This is a novel project that will open the door to a new, safer and possibly more cost-effective form of contraception that does not introduce extra hormones into the human body. It also holds potential as a sterilization method for cats and dogs, which would reduce euthanasia rates in pet shelters.

Materials and Methods

RNA pool. We purchased our pool of random oligonucleotides from Integrated DNA Technologies, Inc. The total pool contained 100 nmol of ssDNA, which we aliquoted and diluted to several samples that each had a concentration of 100 pm/μL. Each oligonucleotide segment was 77 bases in length, with a randomized 40 base region in the center. The outer 5' and 3' regions were kept constant in order to bind to T7-XbaI and Rev-HinDIII flanking PCR primers, respectively. The sequence for our primers and oligonucleotide were obtained from Bouvet's protocol, and are shown below [17]:

Table 2. The sequences for both primers and oligonucleotide molecules we used.

Nucleic acid strand	Sequence
T7-XbaI flanking primer	3’-TTA CAG CAA CCA CCG GG GAT ATC ACT CAG CAT AAT CCT AGG CGC-5’
Rev-HinDIII flanking primer	5’-CCC GAC ACC CGC GGA TCC ATG GGC ACT ATT TAT ATC AAC-3'
Oligonucleotide	5'-TGGGCACTATTTATATCAAC(N_{40})AATGTCGTTGGTGGCCC-3'

After PCR amplification, we converted our dsDNA pool into an ssRNA pool via transcription.

PCR. PCR reactions contained 10 pm DNA template, 1 μL Rev flanking primer, 1 μL T7 flanking primer, 5 μL Taq buffer, 1 μL Taq polymerase and 1 μL dNTP's. The total volume was 50 μL. Thermal

cycling began with one cycle at 95°C for one minute , 25 repeating cycles at 94°C for one minute, 50°C for one minute, and 72°C for one minute, followed by a cycle at 72°C for ten minutes. After PCR, the presence of product was confirmed by 3% agarose gel (10 μL Ethidium Bromide added) and then subsequently purified by phenol/chloroform extraction and ethanol precipitation.

Cell-SELEX. 135-150 μL of ssRNA was first heated at 95°C for 5 minutes and then immediately put on ice. Afterward, they were incubated with HEK-293 cells with transfected hFSHR (or with untransfected cells if counter-selection was being performed) for 45 minutes at different temperatures (starting at 4°C) depending on stringency level. At room temperature and above, RNAse inhibitor was also employed. Cells were vigorously vortexed and triterated to increase surface area for receptor binding. 1 mL of binding buffer was also added. After selection, centrifugation was done at 13,000 RPM for five minutes and the supernatant was discarded. The cells were then vortexed with 300 μL of wash buffer and heated again at 95°C for five minutes to elute the bound oligonuceotides. Centrifugation was then done once more under the same conditions, and the supernatant was collected and a phenol/chloroform extraction was performed. Afterward, reverse transcription and PCR amplification were done to ascertain the presence of product.

Wash buffer and binding buffer. The wash buffer solution was composed of 450 mg glucose, 10 mL 10x PBS buffer, 5 mM $MgCl_2$ and 90 mL of nanopure water. The binding buffer was created using 10 mL of wash buffer and 10 mg of Bovine Serum Albumin (BSA).

Transcription and reverse transcription. Transcription was done using a HiScribe™ T7 *in vitro* transcription kit, specifically: 76 μL dsDNA template, 10 μL transcription buffer, 1 μL ATP and 1 μL GTP from New England Biolabs (NEB), 1 μL 2'-Fluoro-CTP and 1 μL 2'-Fluoro-UTP from TriLink Biotechnologies, 5 μL 20x high molecular weight (HMW) mix and 5 μL T7 RNA polymerase.

Reverse transcription was done using a Quanta Biosciences, Inc. qScript^{TM} cDNA synthesis kit, specifically: 5 μL of RNA, 10 μL of RNAase-free water, 4 μL mix (reverse transcription buffer, necessary primers) and 1 μL of reverse transcriptase.

Results

For the counter-selection, the random oligonucleotide pool was incubated with wild-type HEK-293 cells. The supernatant RNA was then enriched via reverse transcription and PCR, and a band estimated to be about 123 base pairs was obtained, as shown in lane 2 (Figure 5a), indicating a successful recovery of RNA that did not bind to the wild-type cells. This band presented itself again after the first round of positive selection with HEK-293R cells, which also occurred at 4^{o}C (Figure 5b), representing the fraction of our oligonucleotide pool that binds to the FSHR containing cells at 4^{o}C. This band, however, was absent in the gel after the second round of selection (at 22^{o}C), as shown in Figures 5c and 5d. Only after the addition of an RNAse inhibitor did this band appear again (Figure 5e), which indicates that RNA was now being successfully eluted from FSHR proteins at 22^{o}C.

The nanodrop experiment (Figure 6) done on this current oligonucleotide pool showed that there was a concentration of 55.2 ng/uL of RNA suspended in the solution. The nanodrop experiments (Figures 7 and 8) done on both RNA incubated with both normal type HEK293 cells and FSHR transfected HEK-293 cells yielded concentrations of 154.1 ng/uL and 100.8 ng/uL, respectively.

Selection was carried out at 30^{o}C, but the 135 base pair band was absent again (Figure 5f), even with the addition of the RNAse inhibitor.

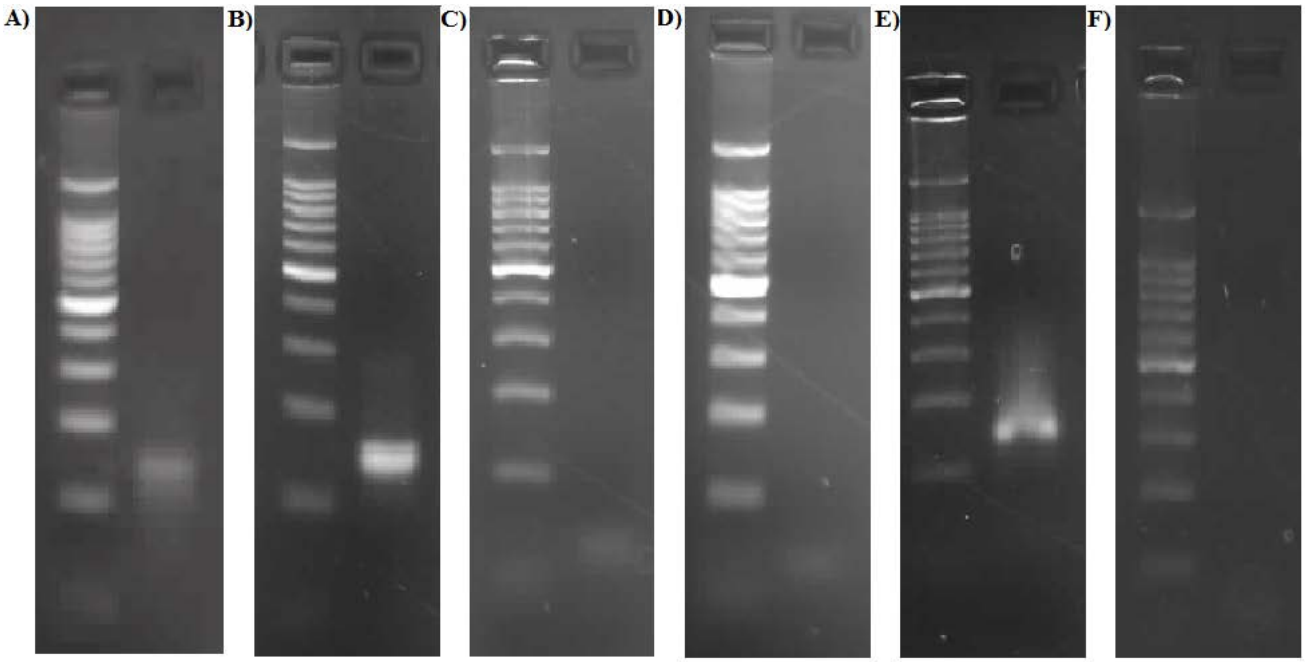


Figure 5. Gels showing results after counter-selection (5a), SELEX at 4°C (5b), SELEX at 22°C (5c-e), and SELEX at 30°C (5f). DNA molecular weight markers are in the first lane, and the product band in the second lane.

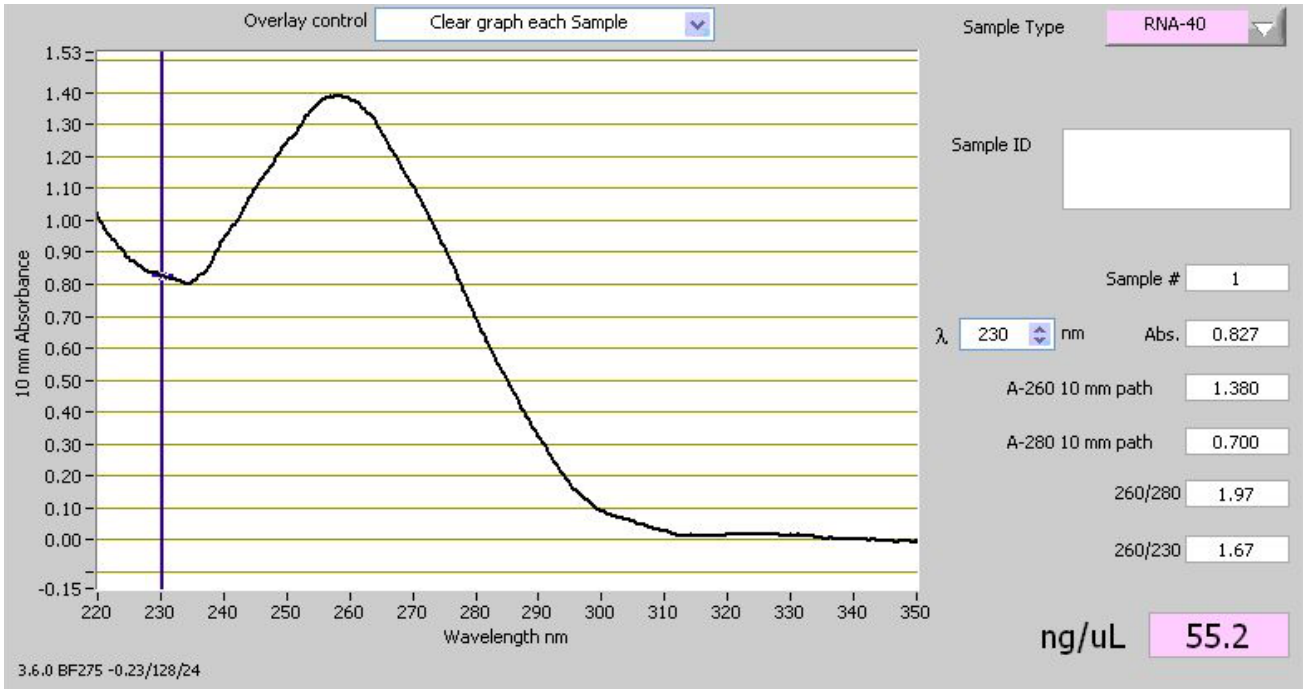


Figure 6. Nanodrop experiment showing the absorbance of RNA at 230 nanometers (nm) for the oligonucleotide pool.

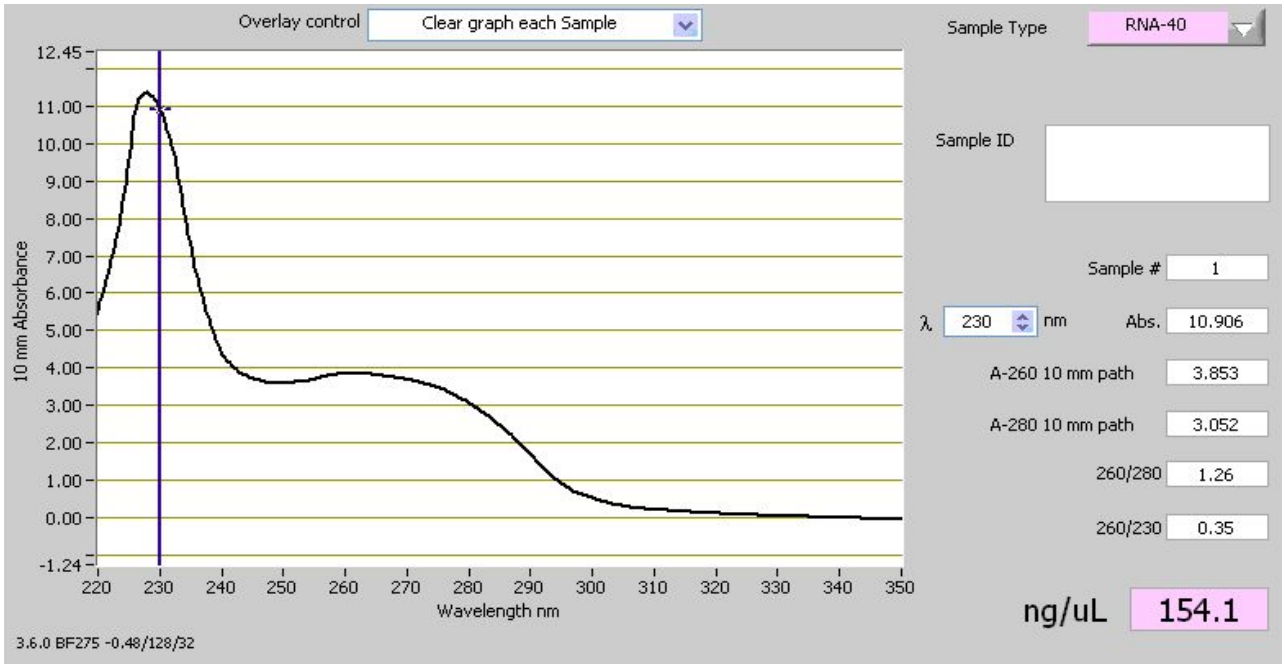


Figure 7. Nanodrop experiment showing the absorbance of RNA at 230 nanometers (nm) for the HEK-293 cells.

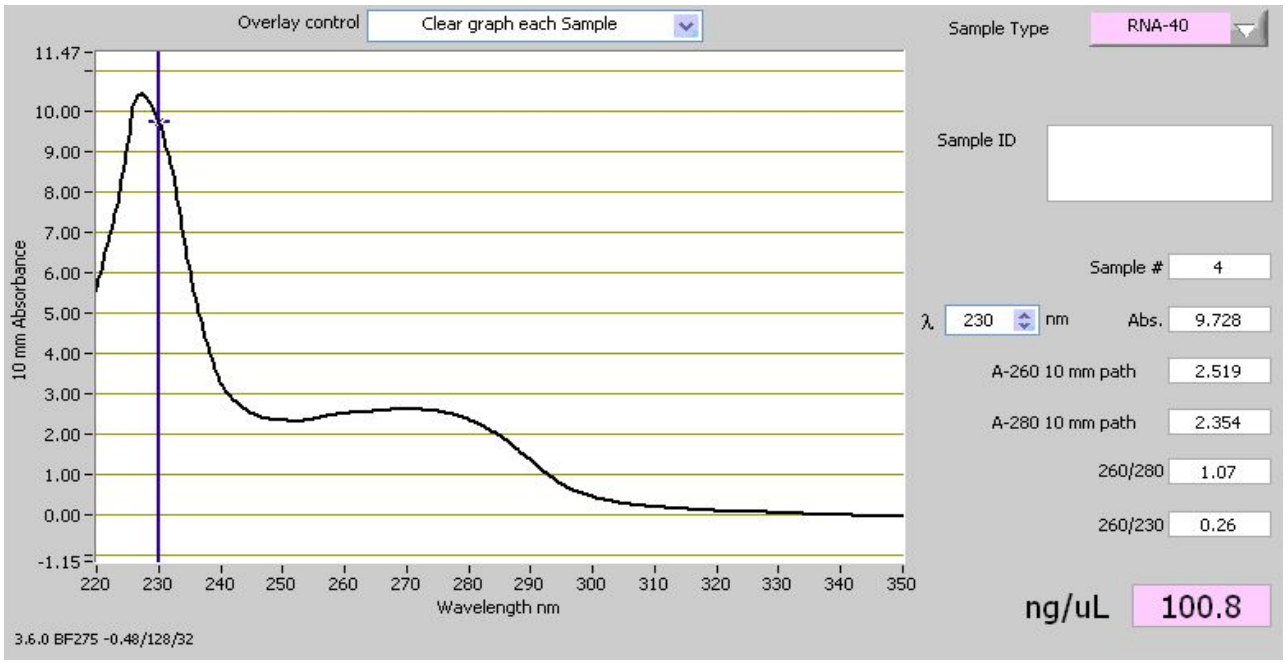


Figure 8. Nanodrop experiment showing the absorbance of RNA at 230 nanometers (nm) for the HEK-293r cells.

Discussion

Using Cell-SELEX, we have been able to select for a group of oligonucleotides that bind to the hFSHR at room temperature. The presence of these oligonucleotides has been determined by both gel electrophoresis of the PCR product after reverse transcription of our RNA and by the nanodrop

experiment. The latter shows a lower concentration of RNA when incubated with the HEK-293r cells, indicating that some portion of the oligonucleotides are binding effectively to the FSH receptor, though more experimentation needs to be done to quantify the level of binding.

Each RNA molecule in our current pool is 123 bases long, though we started from a pool in which the the oligonucleotides were 77 bases long (consisting of two flanking constant regions for primer binding during PCR that are 17 (3' end) and 20 (5' end) bases each and a middle randomized 40 base region). This increase in RNA molecule length is due to the nature of the flanking primers we used, as according to Bouvet's protocol [19]: the T7 flanking primer, binding to the 3' end of our oligonucleotides, is 39 bases long, and the Rev flanking primer, binding to the 5' end, is 44 bases long, as can be seen in Table 2. After PCR amplification, this results in an RNA oligonucleotide pool that is 46 bases longer than the original pool.

Longer constant regions may encourage new, stronger forms of binding, as the molecules have more opportunity for different forms of 3-dimensional binding, such as larger stem loops or three way junctions. However, the fact that primers are longer than the constant regions of the parent oligonucleotides mean that there are several nucleotides on both primers that do not bind predictably to any present sequence (19 and 27 on the T7 and Rev flanking primers, respectively). There is a possibility that parts of these 'extra' sequences may bind to parts of the randomized 40-mer region, masking that sequence's accessibility to binding to the FSHR. This may result in the loss of some potential aptamer candidates.

The actual values for the concentration of RNA for the nanodrop experiments are also important to address. Incubating the oligonucleotides with wild-type HEK293 cells showed a concentration of RNA that was 53.2 ng/uL greater than that of the RNA incubating with HEK293r cells, which shows some degree of specific binding to hFSHR. However, both concentrations are over 100 ng/uL, a seemingly impossible value when we consider that the nanodrop experiment of just our RNA pool showed a concentration of 55.2 ng/uL. We expected that the nanodrop of the RNA incubating with the

wild type HEK293 cells would have showed a concentration equal to or slightly less than that of the nanodrop experiment of the RNA pool (as some of the oligonucleotides may still be binding to the molecules on the surface of the HEK293 cells). As for the RNA incubating with HEK293r cells, we expected a lower concentration than 55.2 ng/uL, if they were binding more specifically to the transfected hFSHR proteins.

We can begin to explain this paradoxical situation by using the simple fact that HEK293 have their own native RNA molecules. The cells would have been somewhat lysed from the beginning of the incubation due to the method of storage. Being frozen in water and stored in a -20^{o}C freezer would cause ice crystals to form and puncture the cells. Next, to thoroughly incubate the cells with the RNA pool in a way that increased potential receptor-oligonucleotide interaction, the cells were triterated and vortexed, causing further strain and breakage. This will lead to the cell's native RNA's to be expelled into the solution that contains our aptamer candidates. The nanodrop machine cannot distinguish between the two different groups of RNA and this likely resulted in the much larger reported concentration of RNA than expected.

In the future, this part of our research can be improved by changing the procedure by which the nanodrop experiment is done: instead of using deionized water as the control, we should use the solution surrounding HEK293 cells. In other words, in addition to incubating the RNA pool with HEK293 and HEK293r cells, a third tube should have only HEK293 without our oligonucleotide molecules present. All other treatments should remain the same: the cells will be triterated and vortexed and left at room temperature for 45 minutes. Theoretically, the native RNA molecules will come out just the same. If this solution were used as the control, the HEK293's RNA should be subtracted out when the nanodrop experiment is done on the other two solutions, and our aptamer candidates should be the only RNA molecules for which the nanodrop experiment reports the concentration.

In addition to the native RNA, ribonucleases (RNAses) are released upon cell lysis. These molecules are used by all organisms to catalyze RNA degradation, a process that is crucial if the parent

RNA is no longer required or if the smaller metabolites are to be used in new molecular pathways [20]. There are many types of RNAses, some of which are not particularly specific. This helps the cell target foreign RNA molecules, such as viral injected RNA, and inactivate them.

This situation presented itself as a problem in our experiment: our aptamer candidates are essentially foreign RNA molecules. At 22^{o}C, we could not recover our RNA molecules after the SELEX process, and it is most likely due to the presence of RNAse molecules that emerged from the lysed HEK293 cells (Figures 5c and 5d). We put this hypothesis to the test by running another SELEX experiment at 22^{o}C, this time adding an RNAse inhibitor, a molecule that binds to RNAse molecules in order to prevent RNA degradation. As can be seen in Figure 5e, this allowed us to recover oligonucleotides after the SELEX process. At 4^{o}C, the SELEX process went to completion, even without the use of the RNAse inhibitor [Figure 5b]. The lower temperature neatly explains this phenomenon: most enzymes, such as ribonucleases, are active at certain temperature ranges, and 4^{o}C is too low for activation of RNAses.

However, even with the presence of the inhibitor, the SELEX did not work at 30^{o}C; The expected 134 base pair band was absent from the subsequent gel (Figure 5f). As all other conditions in this experimental run were identical to that in the 22^{o}C run, the change in temperature must be the main factor that is contributing to the loss of product. Logically, there are three major ways the increased temperature may affect the experiment: it may be reducing the efficacy of the RNAse inhibitor, it may be affecting the structure of the oligonucleotides, or it may be inhibiting the binding between the RNA molecules and the hFSHR protein.

The RNAse inhibitor that we used was a 50 kDa water-soluble protein derived from rat lung, whose recombinant product is produced in *E. coli* (Roche®) [21]. At higher temperatures, proteins will begin to denature and lose their specific tertiary structure, which leads to inhibition or loss of regular function. Our RNAse inhibitor protein is reported to have an active temperature range of 25°C – 55°C, and still retain partial activity at 60°C [21]. Our last SELEX procedure was done at 30°C, which is well

within the active temperature range for our inhibitor, thus eliminating the possibility of enzyme degradation as an explanation for the lack of binding oligonucleotides.

Our RNA molecules form specific three-dimensional shapes via both Watson-Crick and non-standard hydrogen bonding (such as Hoogstein base pairing) between the bases on its own sequence. Heat definitely affects the strength of this binding, as melting curves have been analyzed for both RNA and DNA, in order to the determine the point at which the hydrogen bonds break and the sequence denatures. These studies cast doubt on the idea that increasing the temperature of our SELEX procedure from 22°C to 30°C is causing changes in the structures of our RNA oligonucleotides, as 30°C is not a high enough temperature to truly affect the strength of base pairing. Research done has shown that tRNA, which is similar to our aptamer candidates in that it is a single stranded RNA chain base paired to itself, have melting ranges between 50^{o}C and 75^{o}C, depending on various factors, such as: ion concentrations, GC content, length of the RNA sequence and specific 3-dimensional structure (number, size and length of loops) [22][23]. At some point in the melting curve, there is usually a sudden, sharp increase in denatured RNA. This sharp slope indicates that there is a fast transition between between stable and denatured RNA, and that the heat is not slowly altering the 3-dimensional structure throughout the process. Also, tRNA molecules are generally between 70-95 bases long, and our oligonucleotides are 134 bases long; a longer chain leads to more stability through an increased number of hydrogen bonding[22]. So it may be concluded that our SELEX at 30°C is not altering the actual structure of the oligonucleotides.

Receptor-drug interactions are sensitive to small changes in the environment, such as pH, solubility, and temperature. Another possibility for the lack of binding in our latest SELEX may be that increasing the temperature from 22°C to 30°C is affecting the binding of our oligonucleotides to the hFSHR binding site in some unfavorable way. However, the hFSHR binds to its natural ligand, FSH, at human body temperature, which is 37°C. We have shown experimentally that our oligonucleotides bind at 22°C, so it seems unlikely that the binding interaction would be stunted at 30°C but then at 37°C,

occur readily. One explanation is that our RNA molecules bind less strongly to the active site than the natural ligand. Experimentation must be done to ascertain the binding strengths of our current oligonucleotides to the hFSHR and compare it to that between FSH and the hFSHR.

Additionally, it is possible that our oligonucleotides are not binding to the active site at all; perhaps they are binding allosterically, or as an umbrella agonist/antagonist [24]. That is also a question that remains to be answered: we do not know the exact nature of the oligonucleotide-protein binding. There are many facets to consider: agonism versus antagonism of the hFSHR, the strength of binding (a partial antagonist may end up competing with FSH in binding to the receptor) and the location of interaction. Our RNA molecules may be binding allosterically or as umbrella agonist/antagonist.

After we succeed in using our cell-SELEX procedure to produce a pool of RNA that binds effectively at 37°C, the next course of action will be to quantitatively determine the strength and nature of binding. We will also need to determine the exact sequences of our best aptamers. Our long term future goals are to start *in vivo* trials of our aptamers in models model organisms whose FSH receptor protein is best conserved as compared to humans. Eventually, we can begin a SELEX process specifically for cat or dog FSHR (or other important reproductive proteins). At present, there are still many questions that need to be answered, and much research needs to be done.

Annotated Bibliography

1. Dias, J.A., Cohen, B.D., Lindau-Shepard, B., Nechamen, C.A., Peterson, A.J., Schmidt, A. 2002. Molecular, structural, and cellular biology of follitropin and follitropin receptor. *Vitamins & Hormones*. 64:249-322.

This is a very in-depth study of the human follicle stimulating hormone receptor that gives details about the structure and function of this protein in the human body. I used the information in this paper to help give background information for this receptor protein, in order to highlight its importance in scientific research and why I am interesting in studying it.

2. Hunzicker-Dunn, M., Miazels, E.T. 2006. FSH signaling pathways in immature granulosa cells that regulate target gene expression: branching out from protein kinase A. *Cellular Signaling*. 18(9):1351-9.

This article supplements Dias et. al.'s paper in that it offers additional information about the structure and function of the human follicle stimulating hormone receptor, in particular details pertaining to the

proteins involved in its signaling pathways.

3. Speroff, L., Darney, P. D. 2005. "Oral Contraception". A Clinical Guide for Contraception (4th ed.). Philadelphia: Lippincott Williams & Wilkins.

Speroff and Darney's book provides ample information regarding the mechanism by which medically prescribed oral contraceptives work: specifically, the pathways of the estrogen and progesterone in the pills, and how they cause negative feedback loops that ultimately result in inhibition of FSH release and prevent ovulation. Furthermore, this book provides information about other forms of contraception such as intrauterine devices (IUD's).

4. Blanco-Molina, A., Monreal, M. 2010. Venous thromboembolism in women taking hormonal contraceptives. Expert review of cardiovascular therapy. 8(2): 211–5

This is the first of several papers that I used to elucidate upon medical issues related to taking hormonal contraceptives. Blanco-Molina and Monreal's paper specifically addresses the risk and occurrence of venous thromboembolisms, which are blood clots forming in the veins, in response to the contraceptives.

5. Baillargeon, J., McClish, D.K., Essah, P.A., Nestler, J.E. 2005. Association between the Current Use of Low-Dose Oral Contraceptives and Cardiovascular Arterial Disease: A Meta-Analysis. *Journal of Clinical Endocrinology & Metabolism*. 90(7): 3863–3870.

The results of this paper show that there is an increased risk of cardiovascular disease with chronic usage of oral contraceptives, especially in older groups.

6. Kemmeren, J.M., Tanis, B.C., van den Bosch, M.A.A.J., Bollen, E., Helmerhorst, F.M. van der Graaf, Y., Rosendaal F.R., and Ale Algra. 2002. Risk of Arterial Thrombosis in Relation to Oral Contraceptives (RATIO) Study: Oral Contraceptives and the Risk of Ischemic Stroke. *Stroke (American Heart Association, Inc.)* 33(5): 1202–1208

This extensive study looked at the association between oral contraceptives and risk of arterial thrombosis and ischemic stroke, both of which are usually rarely found in young women. Scientists have found that both diseases seem occur at increased frequency in populations of young women who ingest the contraceptives.

7. Combined Estrogen-Progestogen Contraceptives. 2007. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* (International Agency for Research on Cancer).

This is a very interesting and thorough study of the effect of oral contraceptives on cardiovascular disease and multiple types of cancer. They write that there is “sufficient evidence” to suggest that estrogen-progesterone pills increase the risk of breast, cervical and liver cancer. However, they also found that the same pills helps reduce the the risk of ovarian and endometrial cancer and pelvic inflammatory disease (PID).

8. Rose, D.P., Adams, P.W. 1972. Oral contraceptives and tryptophan metabolism: effects of oestrogen in low dose combined with a progestagen and of a low-dose progestagen (megestrol acetate) given alone. *Journal of Clinical Pathology*. 25(3): 252–8.

Rose and Adams observed that 21 days upon ingestion of the oral contraceptives, serum tryptophan levels were raised to abnormal levels. Additionally, they found elevated levels of tryptophan metabolites in the urine of the subjects. They also observed changes in the way the subjects' somatic cells absorbed glocuse: 75% of the women taking the medicine had impaired glucose tolerance, and 13% developed chemical diabetes mellitus.

9. Collaborative Group on Hormonal Factors in Breast Cancer. 1996. Breast cancer and hormonal contraceptives: further results. *Contraception*. 54(3 Suppl): 1S–106S.

This comprehensive study ultimately found that oral contraceptives have little or no effect on the risk of breast cancer in women, a find that clashes with the data of other studies. Additionally, they found that there is very little difference in the effects of specific types of estrogen and progesterone in the pills.

10. Ortiz, M. E., Croxatto, H. B., & Bardin, C. W. 1996. Mechanisms of action of intrauterine devices. *Obstetrical & gynecological survey*, *51*(12), 42S-51S.

This paper discusses the mechanism by which intrauterine devices (IUD's) work in order to prevent fertilization and pregnancy.

11. Aittomäki, K., Dieguez-Lucena, J., Pakarinen, P., Sistonen, P., Tapanainen, J., Gromoll, J., Kaskikari, R., Sanika, E.M., Lehvaslaiho, H., Engel, A.R., Nieschlag, E., Huhtaniemi, I., de la Chapelle, A. 1995. Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure. *Cell*, *82*(6), 959-968.

This study showed that mutations in the human FSHR protein may produce very serious medical complications, particularly relating to reproduction, indicating the importance of this protein in reproduction.

12. "Michelson Prize." *Found Animals Foundation*. 12 June 2013. <http://michelson.foundanimals.org/michelson-prize>

This is the website for the Michelson Prize & Grants foundation, which offers both grants for research on a safe one-shot, non-surgical sterilization of cats and dogs and a $25 million prize for the first to achieve in finding the the technique.

13. Jayasena, S.D. 1999. Aptamers: An Emerging Class of Molecules That Rival Antibodies in Diagnostics. *Clinical Chemistry*. 45(9):1628-1650.

Jayasena's paper delves into the details of why using an RNA or DNA aptamer may be more advantageous than using an antibody as a binding, targeting or marking molecule; for example, aptamers do not raise an immune response as easily, longer shelf lives and the ability to regenerate after denaturation.

14. McKeague, M., & DeRosa, M. C. (2012). Challenges and opportunities for small molecule aptamer development. *Journal of nucleic acids*, *2012*.

This paper further discusses the usage of RNA and DNA molecules as superior bio-sensors and in binding to specific targets, as compared to the traditional antibody.

15. Nieuwlandt, D. 2000. In vitro selection of functional nucleic acid sequences. *Current Issues in Molecular Biology*. 2(1):9-16.

This paper describes SELEX, the *in vitro* process by which you select the oligonucleotide sequence (out of your random pool) that binds most specifically to your target molecule. This study highlights different selection methods, the process of increasing selection stringency and amplifying the oligonucleotides that could be your target aptamers. This study stresses the idea that as we learn more and more about the three dimensional structure of nucleic acid chains affect their function, we begin to realize the importance of these aptamers as potential research tools (such as for drug design).

16. Guo, K.T., Ziemer, G., Paul, A., Wendel, H.P. 2008. CELL-SELEX: Novel perspectives of aptamer-based therapeutics. *Int J Mol Sci*. 9(4):668-78.

This paper gives a broad yet descriptive overview of the process of aptamer binding to both specific, isolated proteins and whole cells, giving many examples of the clinical significance of this research, such as cancer therapy and gene silencing. Essentially, this paper provides a lot of backbone information that explains the growing importance of aptamers in biomedical research.

17. Fang, X., Tan, W. 2010. Aptamers Generated from Cell-SELEX for Molecular Medicine: A Chemical Biology Approach. *Acc Chem Res.* 43(1): 48-57.

This paper provides further information about producing aptamers that could be used to target entire cells. They used the process of Cell-SELEX (which they outline in a diagram as well) to target cancer cells and they report positive results. However, cancer cells do not have many target molecules on their surface during the early stages for aptamers to bind to, so they tested the efficacy of aptamer-nano-particle complexes, as they bind multivalently to cell surfaces. Their data shows promising results for the ability of aptamers to bind very specifically to cells and one day to be used in chemotherapy.

18. Graham, J.C., Zarbl, H. 2012. Use of Cell-SELEX to Generate DNA Aptamers as Molecular Probes of HPV-Associated Cervical Cancer Cells. *PLoS One*. 7(4): e36103. 1-9.

These scientists also employed Cell-SELEX for targeting a specific strain of cervical cancer cells, showing that aptamers can be used as efficient bio-markers, an important tool in the field of clinical research. As I will be performing SELEX with whole HEK293 cells, the methods and materials provided in this paper will be very helpful as I set up my own experiments.

19. Bouvet, P. 2009. Identification of nucleic acid high-affinity binding sequences of proteins by SELEX. *DNA-Protein Interactions* (pp. 139-150). Humana Press.

We derived our aptamer selection and enrichment process from the protocol Phillip Bouvet published in this book. We used the primers he used, as well as the same sequence for the outer constant regions for our oligonucleotides.

20. D'Alessio, G., Riordan, J.F., eds. 1997. *Ribonucleases: Structures and Functions*, Academic Press.

This book provides a lot of information relating to ribonucleases and their structure and function in cells and as a laboratory tool.

21. Roche® Diagnostics. Protector RNAse Inhibitor. April 2006. PDF file.

22. Beltchev, B., Yaneva, M., Staynov, D. 1976. Thermal Melting Curves of tRNA^{Phe} from Yeast Lacking Different Numbers of Nucleotides from the 3 End. *European Journal of Biochemistry*, *64*(2), 507-510.

This reference gives useful information how temperature affects the tructural integrity of tRNA molecules, which are similar to our potential aptamer candidates in that both are single stranded RNA molecules that fold 3-dimensionally upon themselves.

23. Yakovchuk, P., Protozanova, E., & Frank-Kamenetskii, M. D. (2006). Base-stacking and base-pairing contributions into thermal stability of the DNA double helix. *Nucleic acids research*, *34*(2), 564-574.

This paper talks about about how factors such as G-C content, base-stacking/pairing, and length of sequence affects the thermal stability and melting point of DNA and other nucleotide seuqences.

24. Stevens, E. 2014. *Medicinal Chemistry: The Modern Drug Discovery Process.* Pearson Education, Inc.

This textbook discusses different types of receptor binding, a crucial point to understand as we are not sure how exactly our aptamers are binding to the FSHR protein.