UNIVERSITAT POLITÈCNICA DE VALÈNCIA

ESCOLA TÈCNICA SUPERIOR D´ENGINYERIA AGRONÒMICA I DEL MEDI NATURAL

Studying potential candidate genes for reduced progesterone production in KiSS1/GPR54 mutant mice during pregnancy

TRABAJO FIN DE GRADO EN BIOTECNOLOGÍA

ALUMNO: DAVID MILLÁN ESTEBAN

TUTOR: D. FRANCISCO MARCO JIMÉNEZ
COTUTOR: PROFESSOR WILLIAM H. COLLEDGE

Curso Académico:2014/2015

VALENCIA, MAYO 2015

Título: Studying potential candidate genes for reduced progesterone production in KiSS1/GPR54 mutant mice during pregnancy.

Abstract: Mutant mice for Kiss1/GPR54 gene lack kisspeptin, a neuropeptide required for the activation of the reproductive axis, hence inducing fertility failures. Hormone treatment in these females matures the reproductive axis but pregnancy is not maintained due to abnormally low levels of luteal progesterone. The objective of this study was to evaluate differential expression of candidate genes as PLR, LHR, 20α-HSD, which are involved in the progesterone biosynthetic pathway; and as BCL2 and BAX, which are involved in the cell apoptotic pathway; between the wild-type population and the mutant population. In order to do so, a total of 11 animals, 7 wild-types and 4 mutants were ovariectomized and the expression was analyzed using real time PCR. Our results showed significantly different expression levels of PLR (1,12±0.221 vs. 3.54±0.293, for wild-types vs. mutants, respectively) and LHR (1.16±2.42 vs. 24.15±3.20, for wild-types vs. mutants, respectively). It is concluded that PLR and LHR gene show differential expression between the wild-type population and the mutant one while apoptotic genes BCL2 and BAX are similar between both populations.

Resumen: Los ratones mutantes para el gen KiSS1/GPR54 carecen de kisspeptina, un neuropéptido requerido para la activación del eje reproductivo, lo cual induce fallos de fertilidad. El tratamiento hormonal en estas hembras madura el eje reproductivo pero no logra el mantenimiento del embarazo debido a niveles anormalmente bajos de progesterona lútea. El objetivo de este estudio fue evaluar la expresión diferencial de genes candidatos PLR, LHR y 20α-HSD, que están relacionados con la biosíntesis de progesterona; y de BCL2 y BAX, relacionados con la apoptosis. Para ello, un total de 11 animales, 7 salvajes y 4 mutantes fueron ovariectomizados y la expresión analizada usando PCR a tiempo real. Nuestros resultados mostraron una expresión significativamente diferente de PLR (1,12±0,22 vs. 3,54±0,29, para salvajes vs. mutantes, respectivamente) y de LHR (1,16±2,42 vs. 24,15±3,20, para salvajes vs. mutantes, respectivamente). Se concluye que los genes PLR y LHR muestran expresión diferencial entre salvajes y mutantes mientras que la expresión de los genes relacionados con la apoptosis BCL2 y BAX es similar.

Key words: reproductive axis, mice, kisspeptin, GPR54, progesterone, differential expression.

Palabras clave: eje reproductivo, ratones, kisspeptina, GPR54, progesterona, expresión diferencial.

Student: D. David Millán Esteban
Supervisor: D. Francisco Marco Jiménez
Co-supervisor: Professor William H. Colledge

Valencia, Mayo 2015

Acknowledgments

I would like to thank Professor W. H Colledge for receiving me in his group at the University of Cambridge and for his supervision during the development of this study. I would also like to thank Dr. Shel H. Yeo for her support and help.

I would like to thank Dr. Francisco Marco Jiménez for his advice during the writing of this report.

Finally, I specially thank Dr. Victoria Kyle for her technical help and for her professional and personal support not only during the development of this study but along the year.

INDEX

List of Figures

List of Tables

List of Abbreviations

BAK	BCL2-antagonist killer
BAX	BCL2-associated X protein
BCL2	B-cell Leukemia 2
BLAST	Basic local alignment search tool
cDNA	Complementary DNA
Ct	Cycle threshold value
CYP11A1	Cholesterol Side-Chain Cleavage Enzyme
dNTP	deoxy-nucleoside triphosphate
DTT	Dithiothreitol
E.Coli	*Escherichia coli*
hCG	human Chorionic Gonadotrophin
HPRT	Hypoxanthine-guanine Phosphoribosyl-transferase
LH	Leutinizing Hormone
LHR	Leutinizing Hormone Receptor
IU	International Units
mRNA	messenger RNA
NCBI	National Centre for Biotechnology Information
Oligo-dt	oligomeric deoxy-thymine nucleotides
PLR	Prolactin Receptor
PMSG	Pregnant Mare Serum Gonadotrophin
RQ-PCR	Real Time Quantatiative - Polymerase Chain Reaction
RT-PCR	Revere Transciption - Polymerase Chain Reaction
STAR	Steroidogenic Acute Rgulatory Protein
TAE Buffer	Tris-acetate-EDTA Buffer
Taq	Taq polymerase
µg	Microgram
µg/µl	Microgram per millilitre
µl	Microlitre
20α-HSD	20α-hydroxysteroid dehydrogenase
3ß-HSD	3-beta hydroxysteroid dehydrogenase
3’ UTR	3’ Untranslated region
5’ UTR	5’ Untranslated region

1. Introduction

1.1 The Kisspeptins and GPR54

The kisspeptins are neuropeptides encoded by the KiSS1 gene, which was first identified as a metastasis suppressor gene in melanoma cells (Lee *et al.,* 1996). It was posteriorly discovered that kisspeptins bind to the rhodopsin family/class A G protein-coupled receptor GPR54 in rats and humans (Kauffman *et al.,* 2007).

Kisspeptin-expressing neurons are synthesized in several brain regions, including the anteroventral periventricular nuclei (AVPV) and the arcuate (ARC) (Han *et al.,* 2005; Semaan *et al.,* 2010). However, expression of GPR54 has been found in hypothalamic gonadotropin releasing hormone (GnRH)-releasing neurons in several species, including mice. It has been shown that kisspeptins stimulate GnRH release from these neurons by binding to their receptors (Messager *et al.*, 2005; Smith *et al.*, 2006; Dungan *et al.*, 2007). Consequently, researchers then focused on the role of the kisspeptins in the hypothalamic-pituitary-gonadal (HPG) axis.

Several investigations suggested the possible peripheral roles kisspeptins may have in addition to the central control of the reproductive axis; these include ovulation (Castellano *et al.,* 2006), placentation (Hiden *et al.,* 2007), energy homeostasis and cardiovascular functions (Reynolds *et al.,* 2009*)*. Nevertheless these peripheral roles of kisspeptins are not well understood.

1.2 The hypothalamic-pituitary-gonadal axis

The hypothalamic-pituitary-gonadal (HPG) axis is a neuro-hormonal axis connecting the hypothalamus, pituitary gland and the gonads, hence regulating the reproductive function (Figure 1). Gonadotrophin releasing hormone (GnRH), a neuronal secretory decapeptide, is released by the hypothalamus in a pulsatile manner into the hypophyseal portal circulation. GnRH will travel to the anterior pituitary where it binds to receptors on the gonadotrope cells, promoting synthesis and secretion of gonadotrophic hormones. These hormones are: follicle stimulating hormone (FSH), which stimulates the development of follicles in the ovary by acting on the granulose cells; luteinizing

hormone (LH), which acts on the thecal cells of antral follicles and granulose cells of preovulatory follicles and maintains the corpus luteum (MEDSCAPE, 2014). These gonadotrophic hormones will act on the gonads enhancing secretion of sex steroids (estrogen and progesterone in females and testosterone in males), which will be involved in gametogenesis, sexual maturation, and feedback regulating loops for GnRH, LH and FSH release (Tassigny and Colledge, 2010).

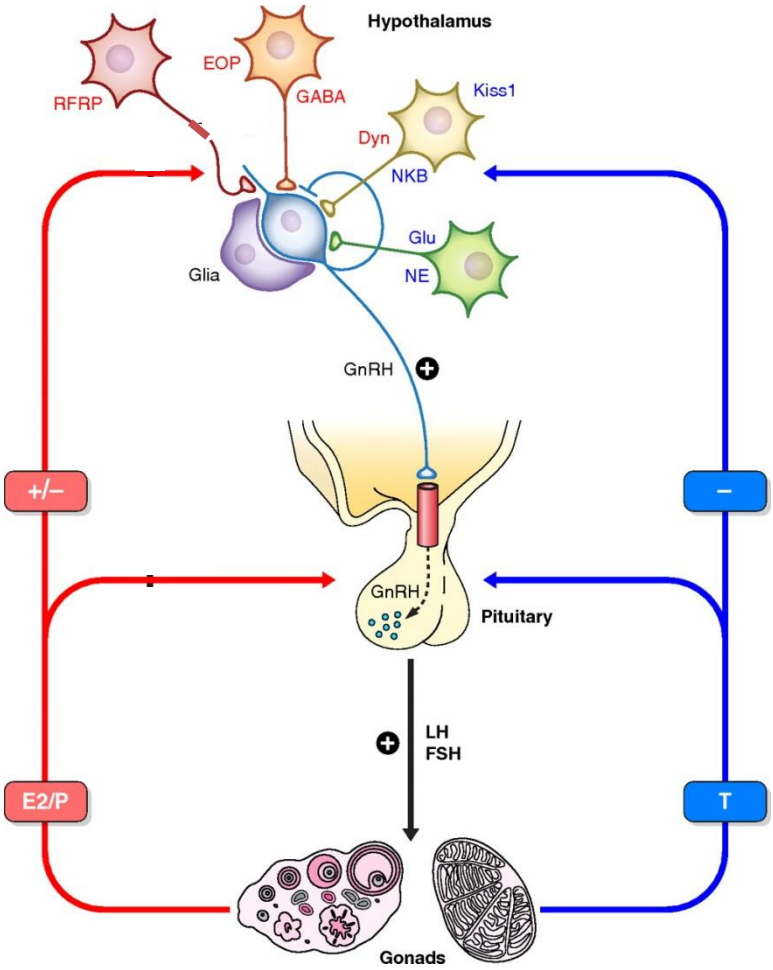

Figure 1. The mammalian hypothalamic pituitary gonadal (HPG) axis. Hypothalamic GnRH neurons, which receive trans-synaptic and glial inputs, release GnRH to the hypophysial portal blood system. In turn, GnRH dictates the pulsatile secretion of gonadotropins, LH and FSH from the pituitary, that stimulate the maturation and regulate the function of the gonads. The gonads will release testosterone, estrogen and progesterone, which are involved in gametogenesis, sexual maturation and feedback loops that regulate the axis activity (Pinilla et al. 2012, modified from original)

Both natural mutations and targeted deletions of the GPR54 gene are associated with failure to undergo puberty, infertility and hypogonadotrophic hypogonadism, proving the important role kisspeptins have in the HPG axis (de Roux *et al.,* 2003*;* Bouligand *et al.,* 2009*;* Kirilov *et al.,* 2013). Conversely, a gain-of-function mutation in GPR54 results in central precocious puberty (Teles *et al.,* 2008) in addition to a missense mutation in the kisspeptin precursor protein (Silveira *et al.,* 2010).

The kisspeptins have a regulatory function upstream the HPG axis since they are able to bind GPR54 receptors on GnRH-releasing neurons, hence stimulating GnRH release from the hypothalamus (Messager *et al.,* 2005). In addition, kisspeptins provide a way to regulate GnRH secretion from the hypothalamus via interaction with sex steroids (Smith, 2013). Although GnRH neurons appear to express the estrogen receptor a (ERa) and estrogen receptor b (ERb) (Shen *et al.,* 1998; Hrabovszky *et al.,* 2000, 2001), it has been shown that ERa, ERb and the androgen receptor are expressed in the periventricular regions of the forebrain where kisspeptin neurons reside, hence mediating the predominant actions of estrogen and androgen in the regulation of GnRH and gonadotropin secretion (Simerly *et al.,* 1990; Herbison 1998; Popa *et al.,* 2008).

1.3 The role of Kisspeptin signalling in pregnancy

Mutations in either KiSS1 or GPR54 result in mutant mice becoming sterile (Colledge, 2009). If female mutant mice receive gonadotrophic hormones their reproductive axes maturate and are able to become pregnant (Colledge and Tassigny, 2010). However, pregnancy is not maintained in the mutant mice past day 6.5 of embryonic gestation. It was shown that this failure of the mutant mice to maintain progesterone levels during pregnancy is caused by inadequate progesterone production by the corpus luteum, which cause has not been explained yet (Herreboudt, 2013).

1.4 Progesterone biosynthesis

Progesterone is a steroid hormone made from cholesterol that is essential for successful reproduction. During the menstrual cycle it is produced by the corpus luteum, hence stimulating the secretory activity of the endometrium. After fertilization and

implantation, the corpus luteum will continue secreting progesterone preventing menstruation and providing the appropriate environment for the developing embryo. By week 6 of gestation, progesterone production will be performed mainly by placenta.
Cholesterol is converted to pregnenolone within the inner mitochondrial membrane by cytochrome P450scc (CYP11A1). Pregnenolone will be subsequently converted to progesterone by type 1 3ß-hydroxysteroid dehydrogenase (HSD), which will diffuse out of the cell (Tuckey, 2014), as it can be seen in Figure 2.

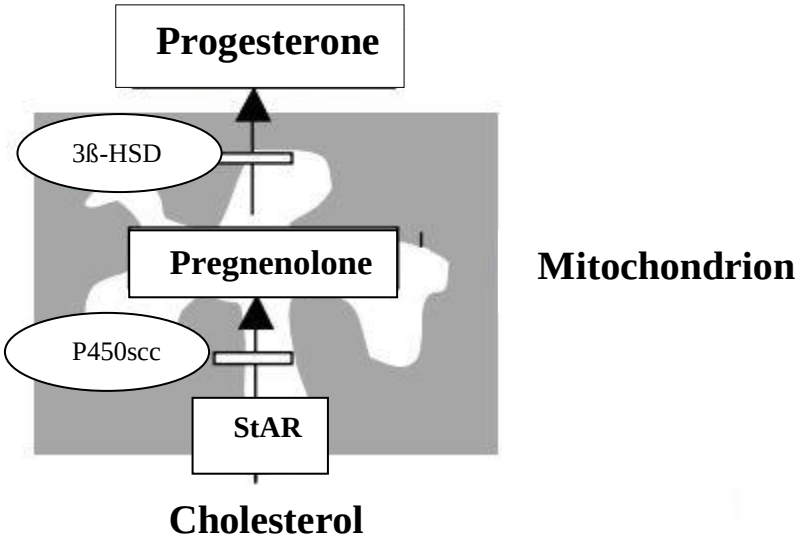

Figure 2. Progesterone biosynthetic pathway. Cholesterol coming from esters, gets into the mitochondria thanks to the activity of the steroidogenic acute regulatory protein (STAR). There, cholesterol is converted to pregnenolone by cytochrome P450scc (CYP11A1), which will be transported out of the mitochondria and converted into progesterone by3ß-hydroxysteroid dehydrogenase (HSD) in order to diffuse out of the cell (Hsia *et al.*, 2006, modified from original).

1.5 Target Genes

There are many biological pathways which would be interesting to study in order to obtain information about what is taking place in the ovaries object of our research. In this case, given the approach of the project we will focus on two: the progesterone biosynthetic pathway and the cell death pathway.

1.5.1 Progesterone biosynthetic pathway

Within the progesterone production there are two different areas. The first one corresponds to the ovarian function, which can be enhanced to produce more or less

progesterone. The second one corresponds to the cellular level and the biochemical transformations in its production.

In the ovarian function, the newly formed corpus luteum is stimulated to secrete progesterone. This stimulation comes from the pituitary prolactin surge, which occurs twice a day (Sapsford *et al.,* 2012) and reflects on the gonads by binding to the prolactin receptor (PLR) hence stimulating the expression of luteinising hormone receptor (LHR). In the pregnant female, the atresia of the corpus luteum must be prevented in order to maintain the production of progesterone so that the pregnany is not threatened. This is achieved thanks to the binding of hCG to LHR on ovarian luteal cells (Latronico and Segaloff, 1999).

In the biochemical aspect, as a steroid molecule progesterone derives from cholesterol. The rate limiting step in this biosynthesis is the transport of cholesterol to the inner mitochondrial membrane which is performed by steroidogenic acute regulatory protein (STAR) (Peters *et al.*, 1998); by the outer mitochondrial membrane protein, peripherial-type benzodiazepine receptor (PBR) (Li and Papadopoulos 1998); and by endozepine which either changes the conformation of PBR so that it can transport cholesterol or facilitates the interaction of Star and PBR (Mishra and Palai, 2015).

Cytochrome P450scc (CYP11A) gene is involved in the cholesterol side-chain cleavage required for the formation of pregnenolone in the progesterone biosynthesis pathway (Simpson *et al.,* 1972). Subsequently, the 3-beta hydroxysteroid dehydrogenase (3ß-HSD) gene produces an enzyme that catalyzes the transformation of pregnenolone into progesterone (Abd-Elaziz *et al.,* 2005).

Further in the progesterone cycle, the 20α-hydroxysteroid dehydrogenase (20αHSD) enzyme converts progesterone into its biologically inactive form, 20α-hydroxyprogesterone (20α-OHP) and in doing so plays a crucial role in the termination of pregnancy and the initiation of parturition (Choi *et al.,* 2008). In rodents, 20α-HSD is expressed in the corpus luteum acting in the process of functional luteolysis during estrous cycle, and its activity is suppressed by prolactin. 20α-HSD is thought to be involved in reducing the cytotoxic effects of progesterone in the developing fetus,

whilst showing an expression increase before parturition when progesterone levels decrease (Naidansuren *et al.,* 2011).

1.5.2 Cell death

Both B-cell leukemia 2 (BCL2) and Bcl2-associated-x-protein (BAX) show a high degree of homology and their role follows a ratio relationship since their products interact with each other causing a different effect depending on which product is present in a higher amount. This is because BAX products are present in the form of homodimers, which induce apoptosis. However, if BCL2 products are also present they will interact with BAX forming heterodimers, hence preventing BAX products performing their apoptotic effect.

That is why BCL2 and BAX are thought to be involved in the regulation of the life span of the human corpus luteum, determining whether they get an extended survival when BCL2 levels are high, usually in the midluteal corpus lutea, or they suffer an accelerated cell death when BAX levels are high as usually happens in the regressing corpus lutea (Sugino *et al.*, 2000; De Falco *et al.*, 2001).

Bcl2-antagonist killer (BAK) gene belongs to the BCL2 family as well and provokes apoptosis by interacting with BAX, hence forming the apoptotic pore in the mitochondrial outer membrane (Dewson 2015).

2. Objective

The objective of this study was to evaluate differential expression of candidate genes as PLR, LHR, 20α-HSD, which are involved in the progesterone biosynthetic pathway; and as BCL2 and BAX, which are involved in the cell apoptotic pathway; between the wild-type population and the mutant population.

3. Materials and Methods

3.1 Animal Procedures

All mice come from a transgenic 129S6/Sv/Ev line, which had undergone targeted loss-of-function mutations in either KiSS1 or GPR54. Following the requirements from the Home Office, the mice were housed in a 12 hours light and 12 hours dark cycle with *ad lib* access to food and water. All procedures carried out were approved by the local ethical review committee. All procedures were stated and approved in Professor W.H. Colledge's project license as well as the personal licences of Dr. V.R. Kyle and Dr. S.H. Yeo.

3.2 Ovary Extraction and Storage

Mice were superovulated with hCG (5 IU) and PMSG injections (5 IU). Due to the impairment in the maturation of the reproductive axis of the KiSS1/GPR54 mutant mice, ovulation does not occur. The role of FSH is fulfilled by PMSG, hence stimulating the growth of ovarian follicle while LH surge role is fulfilled by hCG, hence triggering ovulation.

The mutant mice were injected twice, separated by a two week time period meanwhile the wild-type animals were injected with one round of hCG/PMSG. Injection of the wild-type animals was done at the same time as the second round of injections for the mutant animals. The wild-type animals were injected despite having mature reproductive axes so that similar physiological conditions were achieved in both wild-type and mutant animals.

Once the final hCG injection had been received, the mice were mated with a sterile male. To ensure that copulation had taken place, they were checked for vaginal plugs the following day. On finding a vaginal plug, that day was designated as day 0.5 of embryonic gestation. Wild-type population had 7 individuals (n=7) while mutant population had 4 individuals (n=4). For every animal the ovaries were then extracted at day 7.5 of embryonic gestation and a piece was stored in 500µL of RNALater (Qiagen) in a -80ºC freezer until analysis.

3.3 RNA Extraction

RNA was extracted from the ovaries using the RNeasy Mini Kit (Qiagen-74104) following the manufacturer's protocol with two exceptions. Firstly, in the homogenisation step grinding beads were added. The beads were discarded after the initial homogenisation step. Secondly, the final elution step was repeated twice to maximise the yield of the RNA extraction.

3.4 RNA Quantification

In order to quantify the total RNA content and the purity of each sample, a Nanodrop 3300 spectrophotometer was used.

3.5 cDNA Synthesis

Taking into account the results from the Nanodrop 3300 spectrophotometer, equal amounts of RNA (50ng RNA equivalents) were converted into cDNA using Superscript III Reverse Transcriptase (Invitrogen). The following components were added to each well: random oligonucleotides 10mM (1µL), RNA (1µL), dNTP 10mM (1µL), oligo-dT (1µL) and water (9µL). The solution was heated to 65ºC for 5 minutes using a thermal cycler then incubated on ice for 1 minute. The following components were then added to each well: 5X First-Strand Buffer (4µL), DTT 0.1M (1µL), RNaseOUT 40U/µL (1µL) and Superscript III RT 200U/µL (1µL). As a negative control, one well was kept with no Superscript III RT. In this one, no cDNA is synthesized. The solution was heated to 25ºC for 5 minutes and then at 50ºC for 60 minutes using a thermal cycler then 70ºC for 15 minutes. Then, *E.coli* RNaseH 2U/µL (1µL) was added to each well. Finally, solution was then heated to 37ºC for 20 minutes using a thermal cycler.

3.6 Oligonucleotide Design

All the oligonucleotides used in this study were designed by Dr. V.R. Kyle. Seven target genes were chosen and hypoxanthine-guanine phosphoribosyl-transferase (HPRT) was chosen as an internal housekeeping gene. In the design of the oligonucleotide sequences, standard design rules were followed:

- Melting temperature (Tm=4 x (G+C) + 2 x (A+T) ºC) between 55-65 ºC 18-24 base length.
- G/C content around 50% (including two residues in the five 3' end bases).
- Avoid primer-dimer (homology between both forward and reverse oligonucleotide).
- Avoid intra-oligonucleotide homology (formation of secondary structures).

Oligonucleotide sequence specificity for the gene of interest was assessed using BLAST –Basic Local Alignment Search Tool-database of the NCBI- National Centre for Biotechnology Information. Specificity was 100% for all the genes of interest. The oligonucleotide sequences are outlined in Table 1.

3.7 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

In order to amplify DNA sequences of interest using gene-specific oligonucleotides, the reverse transcription polymerase chain reaction (RT-PCR) method was used. RT-PCR followed by gel electrophoresis was used to make sure that the designed oligonucleotides were binding to the genes of interest. A standard RT-PCR protocol was followed. Each eppendorf tube had 18µL PCR master-mix added to it. The components in the master-mix were: 10X Buffer B (2µL), dNTPs (0.4µL), Forward oligonucleotide (0.8µL), Reverse oligonucleotide (0.8µL), KAPA Taq 5U/µL (0.08µL) and water (13.92µL). Each tube then had 1µL cDNA added to it. Every tube was vortexed and centrifuged before being placed in the thermocylcer and set for a 1.5 hours 40 cycle program as described briefly: 95ºC (3'); 40 cycles of 95ºC (15''), 60ºC (30'') and 72ºC (1'); and 72ºC (2').

Table 1. RT-PCR oligonucleotide sequences

Gene of Interest		5' – 3' Sequence	Sequence Length (bp)	Tm (°C)	GC Content (%)	Accession Number
Prolactin receptor (PLR)	Forward	CGC TGC AAG CCA GAC CAT	156	62.0	61	NM 011169
	Reverse	CAA AGC CAC TGC CCA GAC C		62.6	63	
Luteinising hormone receptor (LHR)	Forward	CAG GAA TTT GCC GAA AGA ACA GAA TT	162	67.5	38	NM 013582
	Reverse	CAG AAG TCA TAA TCG TAA TCC CAG CCA		65.8	44	
20α-hydroxysteroid dehydrogenase (20α-HSD)	Forward	AGT ACA AGC CTG TGT GCA ACC AG	162	62.3	52	NM 134066
	Reverse	CCA AGA GAA CTG GGG TAT CCT CA		62.3	52	
B cell leukemia 2 (BCL2)	Forward	GCG TGG TCT TCT ACT TTG	102	65.86	50	NM009741
	Reverse	AGT CCA GTG TCC AGC CCA TGA TG		66.35	56	
BCL2-associated-X-protein (BAX)	Forward	GTC CCG CCT CAC CTT TCA G	148	65.13	63	NM007527
	Reverse	GAT TCT GGT GTT TCC CCG TTG G		65.43	54	
Hypoxanthine-guanine phosphoribosyl-transferase (HPRT)	Forward	GAT TAG CGA TGA TGA ACC AGG TT	149	60.2	43	NM 013556
	Reverse	CCT CCC ATC TCC TTC ATG ACA		60.8	52	

3.8 Gel Electrophoresis and Gene Sequencing

To ensure that the oligonucleotides were binding to the appropriate sized single cDNA sequence, a gel electrophoresis was carried out. After the amplification step, the samples were run on a 1.5% agarose gel. Twenty microliters of the PCR product and 4μL of loading dye were then added to each of the wells. In addition, 5μL 100bp DNA Ladder (Promega, UK) were added to the first well as a molecular weight marker. The gel was then loaded and run at 135 V for approximately 35 minutes. Using a UV transilluminator, the DNA bands on the gel were visualized.

In order to ensure that the single band products corresponded to the appropriate amplified oligonucleotide sequence, pieces from the gel containing the bands were extracted and processed using a gel extraction kit (QIAQuick, Qiagen UK). Subsequently those were sent off for sequencing (Source BioScience, UK).

3.9 Real Time Quantitative Reverse Transcription Polymerase Chain Reaction

The ABI-PRISM 7500 sequence detection software (Applied Biosystems, UK) system was used to quantify gene expression levels. In order to analyse the samples a neon laser (488nm) was passed through each well of a 96-well reaction plate. Any detected fluorescence was recorded on a computer connected to the ABI PRISM 7500 machine.

Each well of a 96-well quantitative PCR (qPCR) plate had 20μL of a special master-mix (Life Technologies, UK) added to it. The master-mix consisted of Forward oligonucleotide (0.5μL), Reverse oligonucleotide (0.5μL), SYBR Green 2X Mastermix (12.5μL) and Water (6.5μL). Five microliters of the corresponding cDNA were then added to each well. The plate was vortexed and centrifuged previously to being placed in the ABI PRISM 7500 machine and set for the following 1.5 hours, 40 cycle program as described briefly: 95ºC (3’); 40 cycles of 95ºC (15’’), 60ºC (30’’) and 72ºC (1’); and 72ºC (2’).

The dissociation curve was added in order to confirm that the reaction had amplified a single PCR product, shown by the presence of a single melting curve. A total of four plates were run, each plate analysing two target sequences for all mice plus a serial dilution performed in duplicate for each of the analysed oligonucleotides. The serial dilutions were as follows:

- 1, 1/20, 1/40, 1/80, 1/160, and 1/320: These dilutions corresponded to the PLR oligonucleotide sequences.
- 1, 1/200, 1/400, 1/800, 1/1600, and 1/3200: These dilutions corresponded to the LHR oligonucleotide sequences.
- 1, 1/10, 1/100, 1/1000, and 1/10.000: These dilutions corresponded to the 20α-HSD, BAX and BCL2 oligonucleotide sequences.
- 1, 1/2, 1/4, 1/8, 1/16, and 1/32: These dilutions corresponded to the HPRT oligonucleotide sequences.

In order to calculate the amplification efficiency of each oligonucleotide set, cycle threshold (Ct) values were plotted against log10 (template concentration). The slope of the resulting graphs was used to calculate the amplification efficiency with the following equation:

$$\text{Amplification efficiency} = 10^{(-1/\text{slope})}$$

3.10 Statistical Analysis

In order to analyze the data, a relative quantification approach was used. In this, the real-time PCR data is presented relative to another gene, usually a housekeeping gene, which is often referred as an internal control (Livak and Schmittgen, 2001). More specifically, the comparative Ct method, also known as the $2^{-\Delta\Delta Ct}$ method will be applied. This method makes several assumptions, including that the amplification efficiencies of both target and control gene must be similar (Schmittgen and Livak, 2008).

The following explains the procedure of the analysis with the 2-ΔΔCt method. Briefly, Ct values from the duplicates were averaged in order to get a single Ct value for each mouse and each target. Then, the ΔCt value was calculated for each animal within each

oligonucleotide set. In order to do so, the Ct value for HPRT was subtracted from the corresponding Ct value of the target gene. The average ΔCt value for the wild-type and mutant animals was then calculated for each oligonucleotide set.

Subsequently, ΔΔCt values were calculated. For that purpose, the average ΔCt values for HPRT wild-type animals were substracted from the average ΔCt values for each oligonucleotide set for both wild-type and mutant. $2^{-\Delta\Delta Ct}$ was then calculated, but replacing 2 with the actual amplification efficiency of each oligonucleotide.

Differences in mRNA expression among both populations in each oligonucleotide comparison were analysed by one-way ANOVA procedure of Statgraphics Plus 5.1 (STSC Inc., Rockville, MD, USA). Differences of $p \leq 0.05$ were considered significant.

4. Results and Discussion

4.1 Specificity of the designed oligonucleotide sets

The oligonucleotide sequences were designed by Dr. V.R.Kyle as explained in the Material and Methods section. Five genes were chosen as targets and the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) was selected as internal control. The target genes are: luteinizing hormone receptor (LHR), prolactin receptor (PLR), 20a-hydroxysteroid dehydrogenase (20aHSD), b-cell leukemia 2 (BCL2) and bcl2 associated-x-protein (BAX).

To ensure the specificity of the designed oligonucleotides, a RT-PCR was performed for each of the oligonucleotide sets using wild-type cDNA as template. Subsequently these PCR products were run on an electrophoretic agarose gel and the bands were visualized using a UV transilluminator. The results are shown in Figure 3.

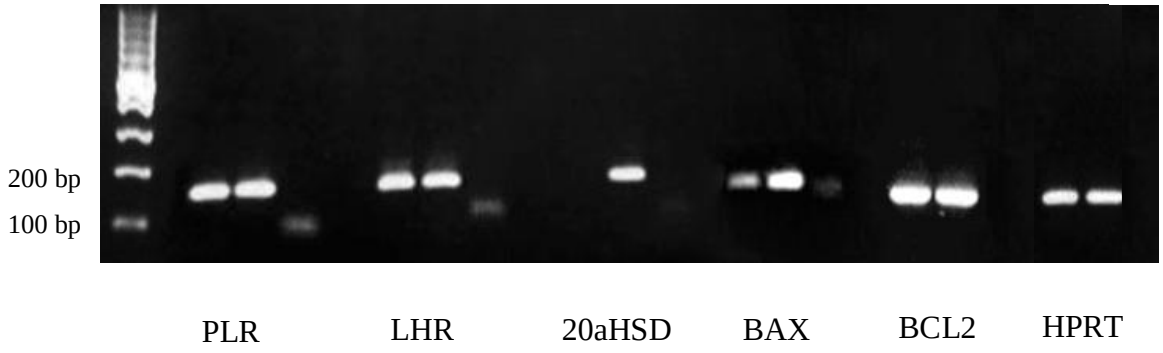

Figure 3. Gel electophoresis identifying target genes. Figure showing single bands corresponding to prolactin receptor (PLR), luteinizing receptor (LHR), 20a-hydroxysteroid dehydrogenase (20a-HSD), Bcl2-associated-x-protein (BAX), cytochrome, B cell leukemia 2 (BCL2) and hypoxanthine-guanine phosphoribosyltransferase (HPRT). For all oligonucleotides wells 1 and 2 are reverse transcriptase positive, while well 3 is reverse transcriptase negative. In some of them a weak band is detected at <100 bp, however since they only appear on the reverse transcriptase negative wells and not in the positive ones it is not believed to be important and possibly will be due to primer-dimer formation.

In addition, the results of the sequencing company were positive for all oligonucleotide sequences. Thus, it can be stated that the oligonucleotide sequences designed for this project are specific and will bind the genes of interest in the subsequent qPCR.

4.2 Amplification efficiencies

qPCR reactions were performed for all oligonucleotide sets for wild-type and mutant samples. In order to analyze the data that will be obtained from this, these oligonucleotides will need to present similar amplification efficiencies so that the proposed $2^{-\Delta\Delta Ct}$ method can be applied. These experiments were developed for every oligonucleotide at different dilutions. The dilutions are:

Table 2. Serial dilutions for each of the target genes.

Oligonucleotide sequence	Serial Dilutions
PLR	1; 1/20; 1/40; 1/80; 1/160 and 1/320
LHR	1; 1/200; 1/400; 1/800; 1/1600 and 1/3200
20α-HSD	1; 1/10; 1/100; 1/1000 and 1/10000
BCL2	1; 1/10; 1/100; 1/1000 and 1/10000
BAX	1; 1/10; 1/100; 1/1000 and 1/10000
HPRT	1; 1/2; 1/4; 1/8; 1/16 and 1/32

As explained in Chapter 2, Ct values were plotted against log2 of the concentration. The resulting slope was used to calculate the amplification efficiency. Results are given in the table below:

Table 3 Amplification efficiencies for oligonucleotide sequences.

Oligonucleotide sequence	Amplification efficiency
PLR	2.09
LHR	2.01
20ALPHA	1.93
BCL2	2.08
BAX	2.11
HPRT	2.05

It is established that the amplification efficiencies are similar to each other, hence allowing for the use of the $2^{-\Delta\Delta Ct}$ method in order to state the fold change. The corresponding value will be used instead of 2 when calculating the fold change.

4.3 Significant differences in target expression

The fold change in expression was calculated for all oligonucleotide sequences for both wild-type and mutant samples. Results are shown in Figure 4. It includes the fold change mean value ± the standard error (n=7 for wild-type, n=4 for mutant) for every gene in order to determine their significant difference. Two genes (PLR and LHR) were found differentially expressed.

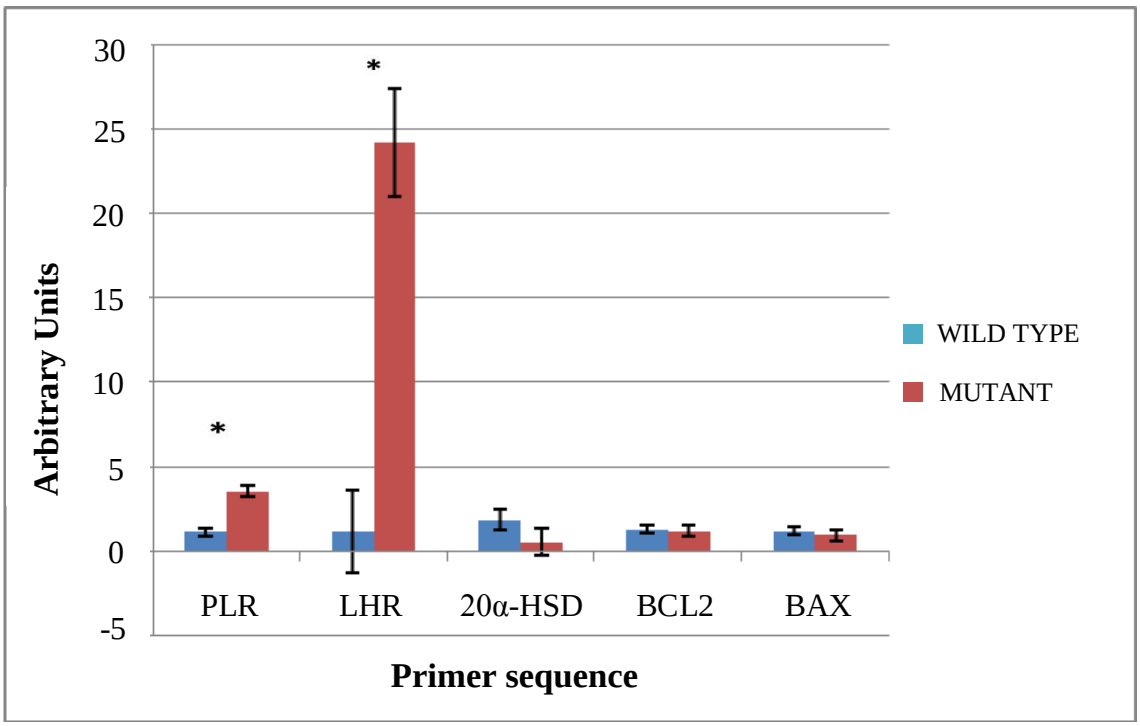

Figure 4. RT-QPCR results for target genes in both populations. These genes are prolactin receptor (PLR), luteinising hormone (LHR), 20α-hydroxysteroid dehydrogenase (20 αHSD), B cell leukemia (BCL2) and Bcl2 associated-x-protein (BAX).

4.4 Discussion of the results

The HPG axis is altered by mutations in either KiSS1 or GPR54 genes resulting in infertile mice (Colledge, 2009). These mice can get their reproductive axes matured when they receive gonadotropic hormones, hence becoming pregnant (Colledge and Tassigny, 2010). However, this pregnancy ends up in abortion by day 6.5 of embryonic gestation due to low levels of progesterone production by the corpus luteum (Herreboudt, 2013).

Previous studies have suggested that a differences in the degree of ovarian apoptosis lead to differences in folliculogenesis and ovulatory potential (Hsu *et al.,* 1996). According to our results, mutant mice show similar levels of apoptotic genes BCL2 and BAX to those in wild-type mice. Hence, this indicates that ovarian cell function is maintained in the mutant ovary and that low levels of progesterone are not a result of an increased cell death rate.

It can be seen in our results that mRNA expression of PLR and LHR exhibit an increase in the mutant population. Previous studies have shown that LHR expression correlates with PLR expression (Manna *et al.,* 2001). There is an established relation between kisspeptin neurons and prolactin secretion. This is based in the fact that prolactin secretion is inhibited by dopamine coming from hypothalamic neurons. It has been shown that the role of kisspeptin signalling is to inhibit dopaminergic neurons from releasing dopamine, hence enhancing the secretion of prolactin (Sapsford *et al.,* 2012; Ribeiro *et al.,* 2015). According to this, mutant mice for Kiss1/GPR54 gene would lack this inhibitory effect of kisspeptin neurons on dopamine release, thus diminishing the levels of prolactin. Perhaps, the increase in the expression of PLR observed in this study could be explained as a physiological response in order to compensate the reduced levels of prolactin. It is widely known that prolactin is necessary in order the corpus luteum to produce progesterone and avoid atresia (Perks *et al.,* 2003). Progesterone, a key hormone for reproduction, stimulates the secretory activity of the endometrium. Once fertilization and implantation have occurred, progesterone will prevent menstruation and will provide an appropriate environment for the development of the embryo.

The pituitary secretes a surge of prolactin twice a day (Sapsford *et al.*, 2012), which will arrive to the gonads and bind to the PLR hence stimulating LHR. These actions will maintain a functional corpus luteum (Latronico and Segaloff, 1999).

On the other hand, 20α-HSD enzyme deactivates progesterone by its conversion into 20α-OHP, its inactive form (Endo *et al.*, 2013). Hence, it plays a role in the termination of pregnancy and reduces the cytotoxic effects of progesterone in the developing fetus (Choi *et al.*, 2008). According to our results, 20α-HSD expression levels do not vary between the wild-type and the mutant population. Hence, the reduced progesterone suggested by Herreboudt (2013) would not be induced by an increase of progesterone deactivation by the activity 20α-HSD.

In summary, we found that the ovary of the mutant mice for KiSS1/GPR54 respond by enhancing the levels of PLR and LHR, maybe due to a decrease of the prolactin secretion, which does not result in normal progesterone levels and provokes premature abortion.

5. Conclusion

It is concluded that prolactin receptor gene and luteinising receptor gene show differential expression between the wild-type population and the mutant one while apoptotic gene B-cell leukemia-2 and Bcl2 associated-x-protein expressions are similar between both populations.

6. References

ABD-ELAZIZ, M.; MORIYA, T.; AKAHIRA, J.; SUZUKI, T. AND SASANO, H. (2005). StAR and Progesterone Producing Enzymes (3ß-hydorxisteroid dehydrogenase and cholesterol side-chain cleavage cytochromes sP450) in Human Epithelial Ovarian Carcinoma: Immunohistochemical and Real-Times PCR Studies. *Cancer Sd*, 96:232-239.

BOULIGAND, J.; GHERVAN, C.; TELLO, J.A.; BRAILLY-TABARD, S.; SALENAVE, S.; CHANSON, P.; LOMBES, M.; MILLAR, R.P.; GUIOCHON-MANTEL, A. AND YOUNG, J. (2009). Isolated Familial Hypogonadotropic Hypogonadism and a GNRH1 Mutation. *The New England journal of medicine*, 360:2742-2748.

CASTELLANO, J.M.; GAYTAN, M.; ROA, J.; VIGO, E.; NAVARRO, V.M.; BELLIDO, C.; DIEGUEZ, C.; AGUILAR, E.; SÁNCHEZ-CRIADO, J.E.; PELLICER, A.; PINILLA, L.; GAYTAN, F. AND TENA-SEMPERE, M. (2006a) Expression of KiSS-1 in Rat Ovary: Putative Local Regulator of Ovulation? *Endocrinology* 147:4852–4862.

CHOI, J-H.; ISHIDA, M.; MATSUWAKI, T.; YAMANOUCHI, K. AND NISHIHARA, M. (2008). Involvement of 20α-Hydroxisteroid Dehydrogenase in the Maintenance of Pregnancy in Mice. *J Reprod Dev*, 54:408-412.

COLLEDGE, W. H. (2009). Transgenic Mouse Models to Study Gpr54/kisspeptin Physiology. *Peptides*, 30:34-41.

COLLEDGE, W.H AND TASSIGNY, X. (2010). The Role of Kisspeptin Signalling in the Regulation of the GnRH-gonadotrophin Ovarian Axis in Mice. *Annales d'Endocrinologie*, 71:198-200.

DE FALCO, M.; DE LUCA, L.; ACANFORA, F.; CAVALLOTTI, I.; COTTONE, G.; LAFORGIA, V.; DE LUCA, B.; BALDI, A. AND DE LUCA, A. (2001). Alteration of the Bcl-2:Bax Ratio in the Placenta as Pregnancy Proceeds. *Histochem Journal*, 33:421-425.

DEWSON, G. (2015). Investigating the Oligomerization of Bak and Bax During Apoptosis by Cysteine Linkage. *Cold Spring Harb Protoc*, 5:pdb-prot086470.

DUNGAN, H.M.; GOTTSCH, M.L.; ZENG, H.; GRAGEROV, A.; BERGMANN, J.E.; VASSILATIS, D.K.; CLIFTON, D.K. AND STEINER, R.A. (2007) The Role of Kisspeptin-GPR54 Signaling in the Tonic Regulation and Surge Release of Gonadotropin-releasing Hormone/luteinizing Hormone. *J Neurosci* 27:12088–12095.

ENDO, S.; ARAI, Y; HARA, A.; KITADE, Y.; BUNAI, Y.; EL-KABBANI, O. AND MATSUNGA, T. (2013). Substrate Specificity and Inhibitor Sensitivity of Rabbit 20α-Hydroxysteroid Dehydrogenase. *Biol Pharm Bull,* 36:1514-1518.

HAN, S.K.; GOTTSCH, M.L.; LEE, K.J.; POPA, S.M.; SMITH, J.T.; JAKAWICH, S.K.; CLIFTON, D.K.; STEINER, R.A. AND HERBISON, A.E. (2005) Activation of Gonadotropin-releasing Hormone Neurons by Kisspeptin as a Neuroendocrine Switch for the Onset of Puberty. *J Neurosci* 25:11349–11356.

HERBISON, A.E. (1998). Multimodal Influence of Estrogen Upon Gonadotropin-releasing Hormone Neurons. *Endocr Rev*. 19:302–330.

HERREBOUDT, A. (2013). The Role of Kisspeptin Signaling in Ovulation and Placentation in the Mouse. Ph.D. Thesis. University of Cambridge.

HIDEN, U.; MARTIN, B.; KNÖFLER, M. AND DESOYE, G. (2007) Kisspeptins and the Placenta: Regulation of Trophoblast Invasion. *Endocrine and Metabolic Disorders* 8:31-39.

HRABOVSZKY, E.; SHUGHRUE. P.; MERCHENTHALER, I.; HAJSZÁN, T.; CARPENTER, C.; LIPOSITS, Z. AND PETERSEN, S. (2000). Detection of Estrogen Receptor-b Messenger Ribonucleic Acid and 125 I-estrogen Binding Sites in Luteinizing Hormone-releasing Neurons of the Rat Brain. *Endocrinology*, 141:3506-3509.

HSIA, S-M; CHIANG, W.; KUO, Y-H. AND WANG, P.S. (2006). Downregulation of Progesterone Biosynthesis in Rat Granulosa Cells by Adlay Bran Extracts. *International Journal of Impotence Research*, 18:264-274.

HSU, S. Y.; LAI, R. J.; FINEGOLD, M. AND HSUEH, A. J. (1996). Targeted overexpression of Bcl-2 in ovaries of transgenic mice leads to decreased follicle apoptosis, enhanced folliculogenesis, and increased germ cell tumorigenesis. *Endocrinology*, 137:4837-4843.

KAUFFMAN, A.S.; PARK, J.H.; MCPHIE-LALMANSINGH, A.A.; GOTTSCH, M.L.; BODO, C.; HOHMANN, J.G.; PAVLOVA, M.N.; ROHDE, A.D.; CLIFTON, D.K. AND STEINER, R.A. (2007c) The Kisspeptin Receptor GPR54 is Required for Sexual Differentiation of the Brain and Behavior. *J Neurosci*, 27:8826–8835.

KIRILOV, M.; CLARKSON, J.; LIU, X.; ROA, J.; CAMPOS, P.; PORTEOUS, R.; SCHUTZ, G. AND HERBISON, A.E. (2013). Dependence of Fertility on Kisspeptin-Gpr54 Signaling at the GnRH Neuron. *Nat. Commun*, 4: 2492.

LATRONICO, A. AND SEGALOFF, D. (1999). Naturally Occurring Mutations of the Luteinizing-Hotmone Receptor: Lessons Learned about Reproductive Physiology and G Protein-Coupled Receptors. *Am J Hum Genet*, 65:949-958.

LEE, J.H.; MIELE, M.E.; HICKS, D.J.; PHILLIPS, K.K.; TRENT, J.M.; WEISSMAN, B.E. AND WELCH, D.R. (1996) KiSS-1, a Novel Human Malignant Melanoma Metastasis-suppressor Gene. *Journal of the National Cancer Institute* 88:1731–1737.

LI, H. AND PAPADOPOULOS, V. (1998). Peripherial-Type Benzodiazepine Receptor Function in Cholesterol Transport. Identification of a Putative Cholesterol Recognition/Interaction Amino Acid Sequence and Consensus Pattern. *Endocrinology*, 139:4991-4997.

LIVAK, K. AND SCHMITTGEN, T. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. *Methods*, 25:402-408.

MANNA, P.R.; EL-HEFNAWY, T.; KERO, J. AND HUHTANIEMI, I.T. (2001). Biphasic Action of Prolactin in the Regulation of Murine Leydig Tumor Cell Functions. *Endocrinology* 142:308-318.

MEDSCAPE, 2014. *Follicle-Stimulating Hormone Abnormalities*. Consulted on April 18th, 2015. http://emedicine.medscape.com/article/118810-overview.

MESSAGER, S.; CHATZIDAKI, E.E.; MA, D.; HENDRICK, A.G.; ZAHN, D.; DIXON, J.; THRESHER, R.R.; MALINGE, I.; LOMET, D. AND CARLTON, M.B. (2005b) Kisspeptin Directly Stimulates Gonadotropin-releasing Hormone Release via G Protein-coupled Receptor 54. *Proc Natl Acad Sci USA* 102:1761–1766.

MISHRA, S. AND PALAI, T. (2015). Steroidogenesis in Luteal Cell: A Critical Pathway For Progesterone Production. *J Invest Biochem*, 3:170-172.

NAIDANSUREN, P.; PARK, C.W.; KIM, S.; NANJIDSUREN, T.; PARK, J.; YUN, S.; SIM, B.; HWANG, S.; KANG, M.; RYU, B.; HWANG, S.; YOON, J.; YAMANOUCHI, K. AND MIN, K. (2011). Molecular Characterization of Bovine Placental and Ovarian 20α-Hydroxysteroid Dehydrogenase. *Reproduction*, 142:723-731.

PERKS, C. M.; NEWCOMB, P. V.; GROHMANN, M.; WRIGHT, R. J.; MASON, H. D. AND HOLLY, J. M. P. (2003). Prolactin Acts as a Potent Survival Factor Against C2-ceramide-induced Apoptosis in Human Granulosa Cells. *Human Reproduction* 18:2672-2677.

PETERS, B.; CLAUSMEYER. S.; OBERMÜLLER, N.; WOYTH, A.; KRÄNZLIN, B.; GRETZ, N. AND PETERS, J. (1998). Specific Regulation of StAR Expression in the Rat Adrenal Zona Glomerulosa: an In Situ Hybridization Study. *J Histochem Cytochem*, 46:1215-1221.

PINILLA, L.; AGUILAR, E.; DIEGUEZ, C.; MILLAR, R.P. AND TENA-SEMPERE, M. (2012) Kisspeptins and Reproduction: Physiological Roles and Regulatory Mechanisms. *Physiol Rev* 92:1235-1316.

POPA, S.M.; CLIFTON, D.K. AND STEINER, R.A. (2008). The Role of Kisspeptins and GPR54 in the Neuroendocrine Regulation of Reproduction. *Annu Rev Physiol* 70:213–238.

REYNOLDS, R.M.; LOGIE, J.J.; ROSEWEIR, A.K.; MCKNIGHT, A.J. AND MILLAR, R.P. (2009) A Role for Kisspeptins in Pregnancy: Facts and Speculations. *Reproduction* 138:1–7.

RIBEIRO, A.; LEITE, C.; KALIL, B.; FRANCI, C.; ANSELMO, J. AND SZAWKA, R. (2015). Kisspeptin Regulates Tuberoinfundibular Dopaminergic Neurons and Prolactin Secretion in an Oeastradiol-Dependent Manner in Male and Female Rats. *Journal of neuroendocrinology*, 27:88-99.

de ROUX, N.; GENIN, E.; CAREL, J.C.; MATSUDA, F.; CHAUSSAIN, J.L. AND MILGROM, E. (2003) Hypogonadotropic Hypogonadism due to Loss of Function of the KiSS1-derived Peptide Receptor GPR54. *Proc Natl Acad Sci USA* 100:10972–10976.

SAPSFORD, T.; KOKAY, I.; ÖSTBERG, L.; BRIDGES, R. AND GRATTAN, D. (2012). Differential Sensitivity of Specific Neuronal Populations of the Rat Hypothalamus to Prolactin Action. *J Comp Neurol*, 520:1062-1077.

SCHMITTGEN, T. AND LIVAK, K. (2008). Analyzing Real-Time PCR Data by the Comparative Ct Method. *Nature Protoc*, 3:1101-1108.

SEMAAN, S. J.; MURRAY, E. K.; POLING, M. C.; DHAMIJA, S.; FORGER, N. G. AND KAUFFMAN, A. S. (2010).BAX-dependent and BAX-independent Regulation of Kiss1 Neuron Development in Mice. *Endocrinology*, 151:5807–5817.

SHEN, E.; MEADE, E.; PÉREZ, M.C.; DEECHER, D.; NEGRO-VILAR, A. AND LÓPEZ, F. (1998). Expression of Functional Estrogen Receptors and Galanin Messenger Ribonucleic Acid in Immortalized Luteinizing Hormone-releasing Hormone Neurons: Estrogenic Control of Galanin Gene Expression. *Endocrinology*, 139:939-948.

SILVEIRA, L.; NOEL, S.; SILVEIRA-NETO, A.; ABREU, A.; BRITO, V.; SANTOS, M.; BIANCO, S.; KUOHUNG, W.; XU, S.; GRYNGARTEN, M.; ESCOBAR, M.; ARNHOLD, I.; MENDONCA, B.; KAISER, U. AND LATRONICO, A. (2010) Mutations of the Kiss1 Gene in Disorders of Puberty. *J Clin Endocrinol Metab* 95:2276-80.

SIMERLY, R.; CHANG, C.; MURAMATSU, M. AND SWANSON, L. (1990). Distribution of Androgen and Estrogen Receptor mRNA-containing Cells in the Rat Brain: An in Situ Hybridization Study. *J. Comp. Neurol*. 294: 76-95.

SIMPSON, E.; JEFCOATE, C.; BROWNIE, A. AND BOYD, G. (1972). The Effect of Ether Anaesthesia Strees on Cholesterol-Side-Chain Cleavage and Cytochrome P450 in Rat-Adrenal Mitochondria. *Eur J Biochem*, 28:442-450.

SMITH, J.T.; POPA, S.M; CLIFTON, D.K.; HOFFMAN, G.E. AND STEINER, R.A. (2006) Kiss1 Neurons in the Forebrain as Central Processors for Generating the Preovulatory Luteinizing Hormone Surge. *J Neurosci*, 26:6687–6694.

SMITH, J. T. (2013). Sex Steroid Regulation of Kisspeptin Circuits. *Adv Exp Med Biol*, 784:275-295.

STOCCO, C.; SJIANE, J. AND GIBORI, G. (2003). Prostaglandin F2a and Prolactin Signaling: PGF2a-Mediated Inhibition of Prolactin Receptor Expression in the Corpus Luteum. *Endocrinology*, 144:3301-3305.

SUGINO, N.; SUZUKI, T.; KASHIDA, S.; KARUBE, A.; TAKIGUCHI, S. AND KATO, H. (2000). Expression of Bcl2 and Bax in the Human Corpus Luteum During the Menstrual Cycle and in Early Pregnancy: Regulation by Human Chorionic Gonadotropin. *J Clin Endocrinol Metab*, 85:4379-4386.

TASSIGNY, X. AND COLLEDGE, W.H. (2010), The Role of Kisspeptin Signaling in Reproduction. *Physiology*, 25:207-17.

TELES, M.G.; BIANCO, S.D.; BRITO, V.N.; TRARBACH, E.B.; KUOHUNG, W.; XU, S.; SEMINARA, S.B.; MENDONCA, B.B.; KAISER, U.B. AND LATRONICO, A.C. (2008). A GPR54-activating Mutation in a Patient with Central Precocious Puberty. *N Engl J Med* 358:709–715.

TELLERIA, C.M.; PARMER, T.G.; ZHOONG, L.; CLARKE, D.L.; ALBARRACIN, C.T.; DUAN, W.R.; LINZER, D.I.H. AND GIBORI, G. (1997). The Different Forms of the Prolactin

receptor in the Rat Corpus Luteum: Developmental Expression and Hormonal Regulation in Pregnancy. *Endocrinology 138,* 4812–4820.

TUCKEY, R. (2006). Progesterone Synthesis by the Human Placenta. *Placenta*, 26:273-281.