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# Regulation of Gonadotropin Expression

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**Dedication**

This dissertation is dedicated to all the individuals who supported me throughout my time as a Master's student. To my parents, my role models of persistence and dedication, who taught me the importance of education, and who supported me throughout my academic journey. Specifically, I want to thank my father who taught me to take on each challenge gracefully with confidence and to realize obstacles are opportunities to learn and build character. Also, to my mother who supported me and shined light on my accomplishments throughout life. Next, I want to thank my sister for teaching me to take advantage of life's opportunities. To my nephew, I want to thank you for giving me the honor of watching you grow, you motivate me to stay tenacious through my strenuous academic journey. To my grandparents, thank you for your enthusiasm and excitement for my academic accomplishments (A mis abuelos, gracias por su entusiasmo por mis logros académicos). Lastly, to Colossians 3:23, no matter what I do, I do it with all my effort, to please God and to be an example for those around me. I look forward to my next steps as an unfaltering woman in Science.

ABSTRACT OF THE THESIS

Regulation of Gonadotropin Expression

by

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Master of Science, Graduate Program in Biomedical Sciences
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Dr. Djurdjica Coss, Chairperson

The hypothalamic-pituitary-gonadal axis (HPG axis) plays an important role in sexual development and reproductive regulation. The hypothalamus releases gonadotropin releasing hormone (GnRH) to the pituitary gland through the hypophyseal portal system. GnRH binds to GnRHR expressed on the gonadotrope cell surface. Gonadotrope cells make up a small population of the pituitary gland, and produce the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), which are composed of an alpha and beta subunit; the alpha subunit is encoded by the *CGA* gene and the beta subunits are encoded by the *FSHb* and *LHb genes*, respectively. There are some transcription factors (TFs) such as, cAMP response element binding protein (CREB) and steroidogenic factor 1 (SF-1), that have an influence on the production of gonadotropins. Additionally, a few of the GATA transcription factors, including GATA-2, GATA-3, and GATA-4, play a role in embryonic development and reproduction. The regulation of the HPG axis, specifically gonadotropin secretion, is an important topic as minor alterations in the endocrine loop can result in reproductive dysfunction. The goal of this study is to use the gonadotrope cell line, LbT2 cells, to determine if the secretion of GnRH has an

effect on the expression of transcription factors like SF-1, CREB-1, GATA-2,GATA-3, and GATA-4, which regulate *FSHb* or *LHb* transcription.

*Keywords*: hypothalamic-pituitary-gonadal axis, gonadotropes, follicle stimulating hormone, luteinizing hormone, transcription factors

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# 1. Introduction

## 1.1 The Hypothalamic-Pituitary-Gonadal Axis

The hypothalamic-pituitary-gonadal axis, also referred to as the HPG axis, is an endocrine loop which consists of the hypothalamus, pituitary, and gonads. The hypothalamus is a region of forebrain located below the thalamus and above the pituitary gland. The pituitary gland is a small endocrine gland located at the base of the brain, which secretes hormones that have an influence on sexual development and sexual maturation. The gonads are reproductive glands which produce gametes and sex steroid hormones. The HPG is driven by the secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus which plays an important role in puberty development and reproduction (Lainez and Coss, 2019).

The gonadotrope cells make up 10% of the pituitary gland, and consist of gonadotropin releasing hormone receptors (GnRHR) expressed on the surface of the cell. GnRHR is a metabotropic receptor, also referred to as a G protein-coupled receptor (GPCR). GnRH, a 10-amino-acid peptide, is a central regulator of the reproductive system (Tsutsumi, et al., 2010; Ferin 2007). GnRH is transported by the hypophyseal portal system to the anterior pituitary where it acts on the receptor GnRHR located on the plasma membrane of gonadotrope cells (Smith et al., 2012). Activation of the GPCR occurs upon the binding of GnRH to the GnRHR which can then activate intracellular signaling molecules, known as second messengers, which can initiate signaling cascades (Syrovatkina et al., 2016). The elevation of second messengers, such as cyclic adenosine

monophosphate (cAMP) activates cAMP-regulated proteins, including protein kinase A (PKA) (Syrovatkina et al., 2016).

The binding of GnRH to GnRHR triggers the synthesis and secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Blair et al., 2015). These hormones act on the gonads and regulate steroidogenesis and sperm production (Stamatiades et al., 2013). Testosterone is a male sex hormone, that can be produced in a woman's ovaries in minimal amounts. However, when testosterone is released in women, aromatization occurs in which the enzyme, aromatase, is used to convert testosterone to estradiol (Stamatiades et al., 2013). Overall, the hypothalamic pituitary gonadal axis plays an important role in regulation of reproductive activity which is maintained by the endocrine loop. A feedback loop is a biological system which helps maintain homeostasis when a product occurs and alters a response in the body. The negative feedback loop relates to the HPG axis because if there is an elevation of testosterone production from the testes, or estrogen from the ovaries, the secretion of GnRH from the hypothalamus will be inhibited.

The LbT2 cell line was created by Dr. Pamela Mellon with targeted tumorigenesis in transgenic mice (Henderson et al., 2008). The LbT2 cell line is important because it recapitulates a mature gonadotrope because they express GnRHR, as well as *FSHb* and *LHb* (Zheng et al., 2011; Ruf-Zamojski, 2018). LbT2 cells are particularly utilized for research to study FSH and LH response to GnRH (Zheng et al., 2011). LbT2 cells have provided significant advancements for researchers to study the gonadotropins, FSH and LH (Thompson & Kaiser, 2014).

Gonadotropes are composed of an α and β-subunit which play a role in their function (Thompson et al., 2014). The α-subunit is common and identical in hormones such as FSH, LH, and thyroid stimulating hormone (TSH) (Thompson et al., 2014). However the β-subunit is specific for each hormone and is responsible for its biological specificity (Thompson et al., 2014). A gonadotrope is an endocrine cell located in the anterior pituitary gland that secretes the gonadotropins, FSH and LH. The release of FSH and LH is regulated by GnRH which is released from the hypothalamus. GnRH is a tropic peptide hormone that is synthesized and released from the GnRH neurons within the hypothalamus. A tropic hormone is a hormone produced and secreted by the anterior pituitary gland, that has multiple endocrine glands as their target.

**1.2 Hypothalamus**

The hypothalamus is located above the midbrain and below the thalamus. The hypothalamus senses changes in external and internal environments. Neurons in the hypothalamus synthesize neurohormones, such as gonadotropin-releasing hormone (GnRH), corticotropin-releasing hormone (CRH), dopamine (DA), growth hormone-releasing hormone (GHRH), somatostatin (SST), and thyrotropin-releasing hormone (TRH), oxytocin (OT), and antidiuretic hormone (ADH) (Schulman, 2018; Papadimitriou, 2009). The hypothalamus contributes to various daily functions by regulating various body systems, through the secretion of these neurohormones. The hypothalamus regulates functions such as pituitary hormone secretion, thermogenesis, appetite control, sexual development, and emotional regulation (Schulman, 2018).

GnRH is secreted by GnRH neurons which regulate fertility and reproduction (Constantin, 2011). There are about 1,000 to 2,000 GnRH neurons located throughout the preoptic area, septum, and anterior hypothalamus (Lainez and Coss, 2019). The neurons are detected to be unipolar or bipolar in structure and function by sending processes to the median eminence (ME) (Lainez and Coss, 2019). The most important upstream neuron and activator of GnRH neurons is kisspeptin (Lainez and Coss, 2019). The neuropeptide, kisspeptin, activates GnRH neurons in hypothalamus, to secrete GnRH. (Constantin 2011; Lainez 2019).

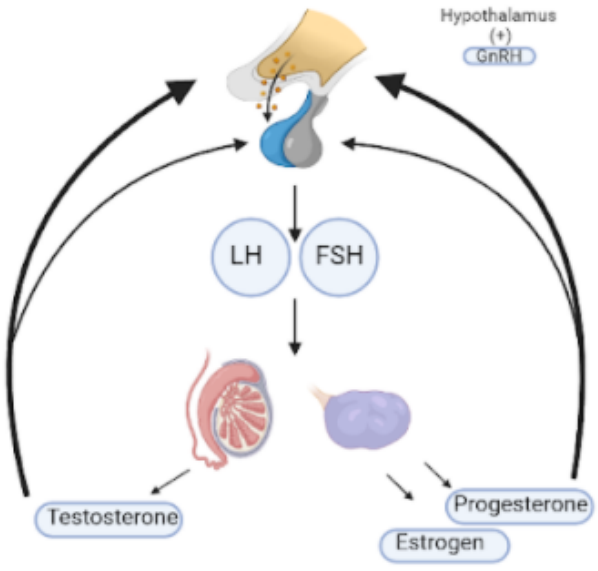



Figure 1. Hypothalamic–pituitary–gonadal axis (HPG axis).

GnRH neurons control GnRH which regulates the synthesis and release of gonadotropins from the pituitary gonadotrope cells (Constantin, 2011). The GnRH

neurons secrete GnRH into the hypophyseal portal capillary blood stream (Constantin, 2011). The hypophyseal portal system is located at the base of the brain and connects the hypothalamus to the pituitary. The hypophyseal portal system contains two capillary beds in the anterior pituitary and median eminence (Clifton & Steiner, 2009). This system allows for rapid exchange of information between the hypothalamus and pituitary gland.

Estrogen, is a steroid hormone produced by the ovaries and plays a role in the development of secondary sexual characteristics however, estrogen is produced until menopause begins (Roby, 2019). Progesterone prepares the endometrium for a possible pregnancy after ovulation (Cable & Grider, 2021). Lastly, testosterone in females is immediately converted to estrogen and aids with growth and maintenance of reproduction tissues, while in males testosterone plays a role in secondary characteristics and sperm production (Isidori et al. 2005).

Receptors for estrogen, progesterone, and testosterone are distributed throughout the brain. Estrogen, progesterone, and testosterone have an effect in regulation of the hypothalamic pituitary gonadal axis and commonly provide negative feedback regulating the HPG axis.

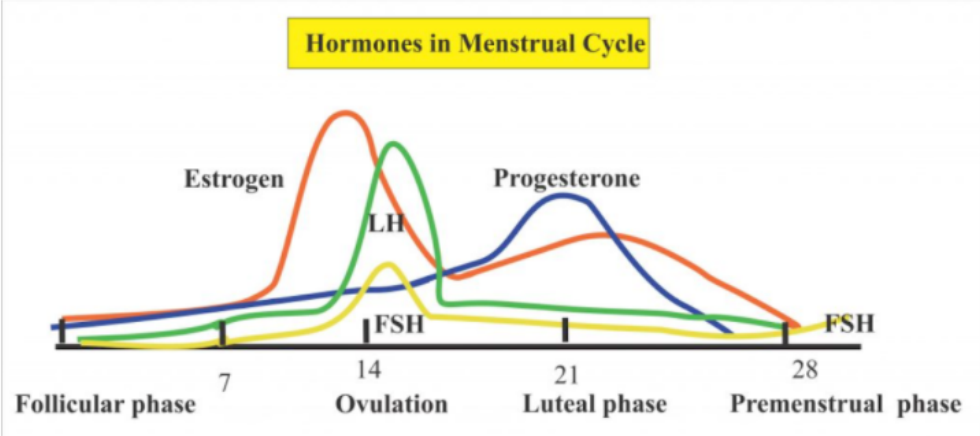



Figure 2. Hormone levels in an average menstrual cycle.

Gonadotropins act via receptors in the gonads. For example, in females, FSH binds to receptors on the granulosa cells of the ovary. The granulosa cells are the only ovarian cells known to have FSH receptors (Goodman, 2009). Granulosa cells respond to FSH by producing estrogen. In males, FSH binds to receptors located on the Sertoli cells of the testes. The Sertoli cells respond to FSH by maintaining spermatogenesis and regulating testicular growth. While LH in females, binds to receptors on the ovarian cells known as granulosa and theca cells. These ovarian cells respond to LH by producing progesterone. In males, LH binds to receptors on the Leydig cells of the testes, which regulate testosterone production (Nielson & Herrera, 2017). HPG is also regulated by protein hormones from the gonads, such as activins and inhibins (Nielson & Herrera, 2017). Activins are crucial for directly stimulating FSH while inhibins inhibit FSH (Nielson & Herrera, 2017).

### 1.3 Pituitary Gland

The pituitary gland, also known as the "master gland", is located inferior to the hypothalamus and has two main parts, the anterior and posterior. The pituitary gland is a major endocrine organ, controlled by the hypothalamus, which plays an important role in metabolism, growth, sexual maturation, reproduction, and blood pressure (Hiller-Strmhofel & Bartke, 1998). Specific cells in the pituitary gland synthesize and secrete hormones depending on the cell type. For example, thyrotropes synthesize and secrete thyrotropin (TSH) while gonadotropes synthesize and secrete LH and FSH (The Pituitary Gland, 2019). The anterior pituitary produces thyroid-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), growth hormone (GH) also known as somatotropin, prolactin (PRL), as well as FSH and LH. The posterior pituitary produces vasopressin, commonly known as antidiuretic hormone (ADH), and oxytocin (Oxt or OT) (Hiller-Strmhofel & Bartke, 1998). The pituitary gland produces hormones which regulate and maintain various systems, such as the thyroid gland, adrenal glands, and gonads (Shulman, 2019).

#### 1.3.1 Follicle-stimulating Hormone and Luteinizing Hormone

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are glycoprotein hormones which have a heterodimeric structure, with an alpha subunit encoded by the *CGA* gene and a beta subunit encoded by *FSHb* and *LHb* genes, respectively (Nwabuobi et al., 2017; Kumar et al., 1997). FSH and LH have the same α-

subunits which is why *FSHβ* and *LHb* regulate specific gonadotropic action (Thompson et al., 2014).

GnRH binds to GnRHR and promotes the secretion of FSH and LH which acts on the ovaries and testes prompting steroidogenesis and gametogenesis (Thompson et al., 2013). In females, FSH stimulates ovarian follicle growth and follicle development. The follicle contains an ovum which is eventually expelled during ovulation. FSH is elevated during the early follicular phase however declines until ovulation. LH in females regulates the menstrual cycle (Hiller-Sturmhofel, 1998). LH plays a role in a women's menstrual cycle because two weeks into a woman's cycle, LH surges prompting the release of an egg during ovulation. If fertilization occurs, LH stimulates the corpus luteum to produce progesterone to support pregnancy by line thickening of the endometrium (Hiller-Sturmhofel, 1998). In males, FSH stimulates spermatogenesis, the production of sperm, and LH stimulates Leydig cells in the testes to produce testosterone (Hiller-Sturmhofel, 1998). These processes in both males and females are essential for reproduction. In the review paper, "GnRH-a key regulator of FSH", Stamatiades et al. (2019) analyze FSH deficiency in females resulting in dysfunction of sexual development. The article also discusses that adult males suffer from azoospermia, the absence of sperm in a male's semen, when there is a lack of testosterone production (Kumar et al., 1997).

According to a study by Thompson et al. (2014) continuous exposure or release of GnRH results in decreased messenger RNA (mRNA) levels and decreased levels of secretion of gonadotropins. An elevated level of GnRH induces an increase in *LHb*

mRNA levels, prompting increased LH production and excretion from the gonadotroph (Kwakowsky et al., 2012). Messenger RNA carries genetic code from DNA in a cell's nucleus to ribosomes which plays a role in protein synthesis. Messenger RNA expression levels are the result of gene transcription. Pulsatile releases of GnRH are necessary for the regulation of FSH and LH production. This displays the complexity and sensitivity of the HPG axis.

### 1.3.2 Additional Pituitary Gland Hormones

Although the main hormones focused on within this thesis are FSH and LH, there are other hormones which are secreted in the pituitary gland. These hormones include thyroid stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), growth hormone (GH), and prolactin (PRL).

Thyroid Stimulating Hormone (TSH) stimulates the release of thyroid hormone from the thyroid gland which controls metabolic processes in all cells (Hiller-Strmhofel & Bartke, 1998). The thyroid hormones regulate metabolism including energy use, thermogenesis, and metabolism of proteins, carbohydrates, and lipids (Du et al., 2019).

The production of ACTH, from the corticotroph cells of the anterior pituitary gland, stimulates the adrenal cortex to release cortisol and aldosterone. Cortisol, a glucocorticoid, maintains homeostasis by carbohydrate, protein, and lipid control and metabolism; however, it also regulates stress response (Papadimitriou, 2009; Aguilera, 1994). Aldosterone regulates water and electrolyte balance (Hiller-Sturmhofel & Bartke, 1998).

GHRH is produced by the hypothalamus and stimulates GH secretion from the anterior pituitary gland which plays an important role in body development (Hiller-Sturmhofel & Bartke, 1998). GH develops organ growth, fat, and regulates endocrine gland development, and reproductive organ development (Hiller-Sturmhofel & Bartke, 1998). Lastly, prolactin plays an important role in female breast development, as well as lactation (Hiller-Sturmhofel & Bartke, 1998). Prolactin is synthesized and secreted from the lactotroph cells of the pituitary gland (Freeman et al., 2000).

### 1.4 GnRH regulation of *LHb* and *FSHb* transcription

GnRH pulses regulate gonadotropin subunit genes, with faster pulses favoring *LHb* transcription while slower pulses favor *FSHb* transcription (Coss, 2017). Early growth response 1 (EGR-1) plays a role in regulation of GnRH to prompt *LHb* transcription (Coss, 2017). Also, activation protein 1 (AP1) transcription factor prompts *FSHb* transcription by GnRH (Coss, 2017).

### 1.5 Transcription Factors

Replication, transcription, and translation are the processes in which genetic information is expressed (Latcman, 1997). Transcription factors (TFs) are proteins which control gene activity by determining if the gene's DNA is transcribed into RNA. RNA polymerase is an enzyme which catalyzes chemical reactions that synthesize RNA by the use of the gene's DNA template (Latchman, 1997). TFs control the efficiency of RNA polymerase functions. TFs are important in organism development, cellular function, and disease repair (Latchman, 1997). TFs bind to a special promoter sequence on the DNA which is upstream of the coding region of genes (Latchman, 1997). The TFs can activate

or repress the transcription of a gene. In other words, TFs regulate genes for expression at the correct time and cell specific manner. In turn, this can direct cell division, proliferation, and cell death (Zheng et al., 2015).

TFs include activators which increase gene transcription or repressors which reduce transcription (Latchman, 1997). There are specific groups of TF binding sites that are either enhancers or silencers which can activate genes (enhancers) or not activate a gene (silencers) in specific body parts (Latchman, 1997). This ensures that the right genes are expressed in the correct cells at the correct time. In some cases, genes need to be expressed in multiple body parts. When a gene needs to be expressed in multiple body parts, the gene will have various enhancers which are binding sites for activators (Latchman, 1997).

### 1.5.1 GATA Transcription Factors

GATA transcription factors are zinc finger DNA binding proteins, located in the nucleus, that regulate transcription (Gao et al., 2015). A zinc finger DNA binding protein acts as a TF by specifically binding to DNA sequences and controlling gene transcription (Isalan, 2013). All GATA family members have conserved DNA-binding proteins that recognize the motif WGATAR, enhancer sites of its target genes on the promoter, by two zinc fingers (Lo et al., 2011). The zinc fingers bind to an individual site which each have their own distinct function (Gao et al., 2015) With this, the C terminal zinc finger binds to the GATA sites, while the N terminal zinc finger promotes GATA and specific DNA binding interactions (Gao et al. 2015).

The GATA family of TFs play a role in embryonic development and the regulation of adult reproductive functions within the hypothalamic-pituitary-gonadal axis (Zheng et al., 2015). According to Lo et al (2011), GATA TF's are found at all levels of the reproductive axis. There are six proteins that make up the GATA family of transcription factors (GATA1-6) which play roles in physiological processes. However, the GATA TF's that will be focused on throughout this dissertation are GATA-2, GATA-3, and GATA-4.

GATA 2 is expressed in the pituitary gland during development as well as in adult gonadotropes and thyrotropes (Charles et al., 2006). GATA 2 is important in gonadotrope and thyrotropic cell differentiation because GATA2 is known to play a role in differentiation toward the gonadotrope cell lineage and away from the thyrotrope lineage. (Charles et al., 2006).

GATA-3 plays an important role in embryogenesis and is found in trophoblastic tissues (Viger et al., 2008). Trophoblasts are the first cells to differentiate from a fertilized egg, and form the outer layer of a blastocyst, which provides nutrition to the embryo, eventually developing into a large section of the placenta (Viger et al., 2008). According to Viger et al (2008), gene mutations in GATA-3 can result in diseases such as dysplasia, the presence of abnormal cells in a tissue. With this, Takaku et al (2016), reports that GATA-3 mutations are observed in 10% of human breast cancers.

GATA-4 TFs are located in the hypothalamus and play a role in early gonadal development and sex determination (Viger et al., 2004). GATA-4 contributes to male gonadal development and testosterone production (LaVoie, 2003). Also, GATA-4 is

highly expressed in ovarian granulosa cells (Bennett et al., 2012). Activation of the promoter is regulated by ovarian hormones, such as inhibin alpha and Anti-Müllerian hormone, which is produced by the granulosa cells (Bennett et al., 2012). Anti-Müllerian hormone plays an important role in the sexual development of fetuses. In the article by Viger et al (2004), dysfunctions of GATA-4 from a mutation of *GATA4* gene results in disruption of testis development. Further, according to the study by Bennett et al (2012) GATA-4 knockout mice have a decrease in fertility which was noted by decreased litters and pups. In accordance, the article by Bennet et al (2012), notes the deletion of GATA-4 prompts subfertility.

According to Lo et al (2011), TFs play an important role in cell differentiation and regulation of gene expression. GATA TFs have been shown to increase glycoprotein alpha subunit gene promoter activity. In the article by Lo et al (2011), they discuss the previous findings of GATA TFs suggesting they can interact with nuclear receptors (NRs) on genes with a vital role in reproductive functions.

GATA-2 is encoded by the *GATA2* gene and stimulates the transcription of *LHb* (Lo et al., 2011). GATA-2 regulates embryonic development and other tissue-forming processes, including hematopoiesis (Crispino & Horwitz, 2017). According to Lo et al (2011), overexpression of GATA-2 influences cell differentiation by guiding the cell toward gonadotrope lineage. In the article by Lo et al (2011) this process was measured by increases in glycoprotein α-subunit and *LHb* mRNA expression.

GATA-3 binds to a GATA motif that is beside the CGA promoter (Banet et al., 2015). According to Banet et al (2015), the mutation of GATA elements resulted in

decreased CGA promoter activity which suggests GATA-3 has a significant role in *CGA* transcription in the placenta. Further, during pregnancy, *GATA-3* gene directs trophoblast differentiation and regulates additional gene expression in trophoblasts or the placenta (Banet et al., 2015). Lastly, according to Ng et al (1994), *GATA-3* mRNA levels in mouse trophoblasts are decreased later in pregnancy which can suggest their importance in placenta development.

GATA-4 is a common TF in regulation of GnRH expression (Zheng et al. 2015). GATA-4 stimulates expression of gonadal genes such as Steroidogenic acute regulator (StAR), aromatase, inhibin, alpha subunit, and Müllerian-inhibiting substance (MIS) (Lo et al., 2011). Also, GATA-4 has been shown to regulate the expression of genes in follicle growth and steroidogenesis (Bennet et al., 2012). The results by Bennet et al (2012) show GATA-4 to be expressed in granulosa cells which are important for folliculogenesis and female fertility. Additionally, it is suggested that GATA-4 regulates cell differentiation because GATA TFs have been found in sertoli cells and leydig cells (Ketola et al., 2000). It is noted that GATA-4 is expressed during periods of androgen synthesis in the testes which suggests an association between GATA-4 and steroidogenesis (Ketola et al., 2000).

According to Zheng et al (2015), GATA-2 and GATA-4 have been shown to stimulate gonadotropin beta subunits. In the article by Zheng at al (2015), the authors discuss additional studies which indicated the removal of GATA-2 and GATA-4 resulting in decreased levels of *LHb* gene expression. In the study by Kumar et al. (2006),

Further, pituitary specific expression has been obtained with sequences for bovine *FSHb* and ovine *FSHb* genes (Kumar et al., 2006).

GATA-3 and GATA-4 manage the HPG, specifically, GATA-3 is found in pituitary and placental cells regulating alpha-glycoprotein subunit gene expression. Additionally, GATA-4 is expressed in the hypothalamus, pituitary, gonads, and adrenal glands. GATA-4 regulates GnRH expression as well as has a role in gonadal steroidogenesis.

### 1.5.2 SF-1 Transcription Factors

Steroidogenic factor (SF-1), also known as Ad4BP (adrenal 4 binding protein), is a member of the orphan nuclear receptor family, encoded by the *NR5A1* gene (Chen et al., 2020). Nuclear receptors (NRs) are a family of TFs that regulate physiological processes like metabolism, sexual development, inflammation, and circadian rhythm (Weikum et al., 2018). Nuclear receptors bind to DNA sequences known as hormone response elements, which regulate gene expression usually in the presence of their respective ligands (Meinsohn et al., 2019). However, SF-1 has no identified ligand, prompting its name "orphaned receptor" (Zheng et al., 2015). The nuclear receptors are ligand-regulated TFs that can be activated by steroid hormones, including estrogen and progesterone (Mangedsorf et al. 1995).

SF-1 is expressed in the gonadotropes of the anterior pituitary where it plays a role in regulation of gonadotropin synthesis (Woodson et al., 1997). Additionally, gonadotrope-specific element (GSE) is found in the promoter of the alpha *GSU* gene which has SF-1 binding sites (Fowkes et al., 2003). SF-1 regulates the transcription of

genes involved in steroidogenesis, reproduction, and sex differentiation (Chen et al., 2020). Specifically, SF-1 is found in steroidogenic tissues such as the leydig cells, theca, and granulosa cells (Woodson et al., 1997). This relates to gonadotropin transcription regulation because the gonadotropins have a beta subunit which determines specificity which allows for the expression of *FSHb* gene or *LHb* gene. This prompts a transcription factor, such as SF-1, to bind to either gene and promote transcription for FSH and LH proteins. With the production of FSH and LH, granulosa cells respond to FSH by producing estrogen while both granulosa and theca cells respond to LH by producing progesterone. Also, Leydig cells respond to LH with testosterone production. However the regulation of activity is unclear (Chen et al., 2020).

### 1.5.3 CREB-1 and SF-1 Transcription Factors and Gene Expression

The transcription factor, cAMP response element binding protein (CREB) is located in the nucleus, encoded by the *CREB1* gene, and binds to the DNA sequence known as cAMP response element (CRE) which regulates gene expression (Wang et al., 2018). The protein is phosphorylated by protein kinase which prompts transcription (Wang et al., 2018).

CREB plays a key role in *FSHb* and *LHb* expression regulation (Kwakowsky et al. 2012). The presence of cAMP response element in the promoter region induces transcription (Wang et al., 2018). Cyclic AMP response element (CRE) is the response element for CREB, which has conserved nucleotide sequences of 5'-TGACGTCA-3' (Thompson et al., 2013). According to Thompson et al (2013), CREB is present in the *FSHb* promoter and is bound by CBP. In the study by Thompson et al (2013) the LbT2

cell line, the FSHb gene promoter has an appreciated GnRH responsive element which has CRE sites. In other words, GnRH stimulates CREB phosphorylation which increases the recruitment of CBP resulting in an increase of *FSHb* (Thompson et al., 2013).

SF-1 is located in the pituitary gland, hypothalamic neurons, and in steroidogenic cells (Zheng et al., 2015). In the anterior pituitary gland, the expression of SF-1 is restricted to the gonadotrope subpopulation as well as contributes to cell specific gene expression (Zhang et al., 2015). SF-1 binds DNA to the sequence 5'-AGGTCA and three specific 5' adjacent nucleotides (Ferraz de Souza et al., 2011). As stated, SF-1 is an important TF for *LHb* gene expression (Kwakowsky et al. 2012). In the review paper by Zhang et al (2015) they discuss that animals without the SF-1 TF had a decreased expression of LH and FSH, resulting in dysfunctional sexual development. Further, Lo et al (2011) and Kumar et al (2006) found that *LHb gene* promoter activity was increased with the presence of SF-1 in combination with EGR-1 which is encoded by the *EGR-1* gene. In other words, *EGR-1* binds to the promoter and interacts with SF-1 to regulate *LHb* gene expression which demonstrates that *LHb* is mediated by the EGR-1 TF (Thompson et al., 2013).

Overall, normal reproductive function requires alpha and beta subunits (Kumar et al., 2006). Kumar et al (2006), states the different regulation of *LHb* and *FSHb* within the pituitary gonadotroph may be due to the differences in sequence specific binding requirements for distinct transcription factor combinations. Further, *LHb* and *FSHb* are regulated by various factors such as pulsatile GnRH and gonadal steroids (Kumar et al., 2006). However, the response of *LHb* and *FSHb* responds to each regulation factor

differently (Kumar et al., 2006). According to Kumar et al. (2006), alpha subunit gene expression is not affected by gonadal peptides, however, the alpha subunit is stimulated "independently" of GnRH pulses (Kumar et al., 2006). The gonadal peptides include inhibins, activivs, and anti-Mullerian hormones which are produced in the ovaries and testes by the granulosa and Sertoli cells (Lahlou et al., 2009). The *FSHb* gene is regulated by gonadal steroids (Kumar et al., 2006). For example, estrogen has been shown to mediate its repressive effects with modulating GnRH secretions (Kumar et al., 2006). Lastly, according to Lo et al (2011), GATA-2 and GATA-4 stimulate the *LHb* promoter activity when working with SF-1.

The goal of my research is to determine transfection efficiency of LbT2 cells using two protocols, known as reverse and forward, which have different cell seeding times. In this experiment, there is an attempt to improve transfection efficiency because LbT2 cells are known to have a transfection efficiency of 10-20%. Additionally, since CREB, SF-1, and GATAs may regulate *LHb* or *FSHb* transcription and GnRH also regulates their transcription, the goal is to determine if CREB, SF-1, or GATAs are influenced by GnRH.

## 2. Materials and Methods:

### 2.1 Cell Culture

LbT2 cells were cultured in DMEM, 1x (Dulbecco's Modified Eagle's Medium), supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin cocktail and grown in an incubator at 37 °C. Prior to experiments cells were split into new 10cm cell culture dishes to distribute cells from the original confluent plate. Media is

aspirated, then cells are rinsed with 6ml Phosphate Buffered Saline (PBS), which is then quickly aspirated. 1ml of trypsin is then added to lift the cells from being plated.

**2.2 Nuclear Extract**

Cells were serum-starved overnight and treated with GnRH at 37 C prior to nuclear extraction. Depending on the incubation time, GnRh is added with a final concentration of 10nM. Cells were washed with 1X PBS, with trypsin added to lift the cells, then scraped and pelleted. The cell pellet was resuspended in a hypotonic buffer in order to obtain a cytoplasmic and nuclear fraction. The remaining fraction will then be spinned down.

Once cells finish centrifugation, aspirate PBS from Eppendorf tube and add 500ul of hypotonic buffer to the pellet with resuspension. Incubate Eppendorf tubes on ice for fifteen minutes. To help break the membrane, use a syringe and resuspend slowly within the Eppendorf tube. The remaining product will  be spun down. Then, a hypertonic buffer is added and spun down.

**2.3 Bradford Protein Assay**

Create stock BSA (1ug/uL) (0.1% BSA) in PBS. To create Bradford Standards, the assay is diluted 5 fold and 200uL of Bradford with 800uL of PBS. Add protein concentrations to samples, and use a spectrophotometer to measure light absorption of the samples. After samples are measured, collect values and make a standard curve. With this standard curve, determine the amount of protein needed for Western Blot samples.

**2.4 Transfection**

Aspirate media in 10cm cell culture dish and rinse with 6ml PBS, quickly aspirate. Add 1ml of trypsin to lift cells off the plate. The microscope should be used periodically to observe the efficiency of the cells lifting. To resuspend the cells, 6ml media is added to the plate. A 5ml serological pipette is used to take up cell suspension and repeatedly take up and eject the suspension.

Remove 6ml of cell suspension and transfer to a conical tube labeled 'cell suspension'. From the conical tube, remove 10ul of cell suspension and eject into the hemocytometer. Put the hemocytometer under the microscope and count the cells in the quadrants for an average cell count. Calculate the amount of cells/ml in reverse and forward wells. Create a transfection mix by pipetting DMEM and fugene into a small conical tube. Wait an incubation time of 15 minutes at room temperature. After incubation, pipette 6ul of expression vector into a small conical tube, and incubate for ten minutes.

To seed and transfect for forward wells, use the calculation to use the needed amount of solution to a reverse transfection and forward transfection conical tube. Resuspend multiple times. Transfer 1ml from the conical tube labeled reverse transfection and add to each well in the reverse column. Once cell suspension is in each reverse well, add 50ul of created transfection mix into each well. The reverse transfection wells will have a cell count within 48 hours.

To seed for forward transfection, use the calculation to use the needed amount of solution to a reverse transfection and forward transfection conical tube. Resuspend multiple times. Transfer 1ml from the conical tube labeled reverse transfection and add to

each well in the reverse column. The expression vector will be added 24 hours after the wells have been filled.

After 24 hours, forward transfection will begin. Create a transfection mix by pipetting DMEM and fugene into a small conical tube. Wait an incubation time of 15 minutes at room temperature. After incubation, pipette expression vector into a small conical tube, and incubate for ten minutes. Once the transfection mix is created, add 50ul of created transfection mix into each forward well. The forward transfection wells will have a cell count within 24 hours.

On day three and four of the experiment, cells will be counted. On day three, reverse transfected cells, with a 48 hours incubation, will be counted. On day four, forward transfection cells, with a 24 hour incubation, will be counted.

**Transfections**

Schedule Example:
(Monday) Day 1: Split cells
Reverse Transfection (Seed and Transfect) Wait 48 hours
Forward Transfection (Seed) 24H
(Tuesday) Day 2:
Transfect
(Wednesday) Day 3:
Count Reverse Cells
(Thursday) Day 4:
Count Forward Cells 48H

Figure 3: Transfection scheduling of reverse and forward transfection.

### 2.5 Western Blot

Protein for Western blot was isolated by nuclear extract.

#### 2.5.1 Run Gel

To run the gel, a 10% separating gel is made with Acrylamide, Tris pH 8.8, SDS 10%, and water. Then Ampersulfate and TEMED are added to begin the solidifying reaction. This gel is then poured into an SDS apparatus with an incubation until the solution becomes solidified. After, stacking gel is created with Acrylamide, Tris pH 6.8, SDS 10%, and water. Then Ampersulfate and TEMED are added to begin the solidifying reaction. The stacking gel is poured on top of the separating gel and a 10 well plate comb is placed into the solution. The stacking has an incubation until solidification.

Sample proteins are maintained at -80C incubation and must be thawed at room temperature. Once sample concoctions are created, vortex, and heat samples at 95C for 15 minutes. During incubation, create a 1L transfer buffer.

Set up gel apparatus, and pour a 1x running buffer into the center chamber of the apparatus. Remove comb and add protein ladder to desired wells. After the protein ladder is inserted, add the final volume of each protein sample into each well. Then, run the gel at 80V for 20 minutes, after 20 minutes increase voltage to 100V and run until samples reach the bottom of the gel.

### 2.5.2 Transfer

While the sandwich is assembled, maintain the set up of sponge, filter paper, nitrocellulose membrane, gel, filter paper, and sponge. Run transfer for 1hr at 100V while on ice. After the run is completed, remove the nitrocellulose membrane from the sandwich and put it into a lab box.

### 2.5.3 Primary and Secondary Antibody

Put cellulose membrane into a lab box with 1g milk and 9ml wash buffer at 4C on a rocker. After incubation, add primary and secondary antibodies.

### 2.6 Development

Remove wash buffer solution from the lab box, remove nitrocellulose membrane and lay it within the development cassette. Create ECL solution 1:1 with reagent 1 and reagent 2, pour onto the membrane for one minute. Transfer cassette to dark room and begin development. Expose film paper in cassette and develop. After development, chemidoc is used to quantify results.

## 3. Results

### 3.1 Transfections

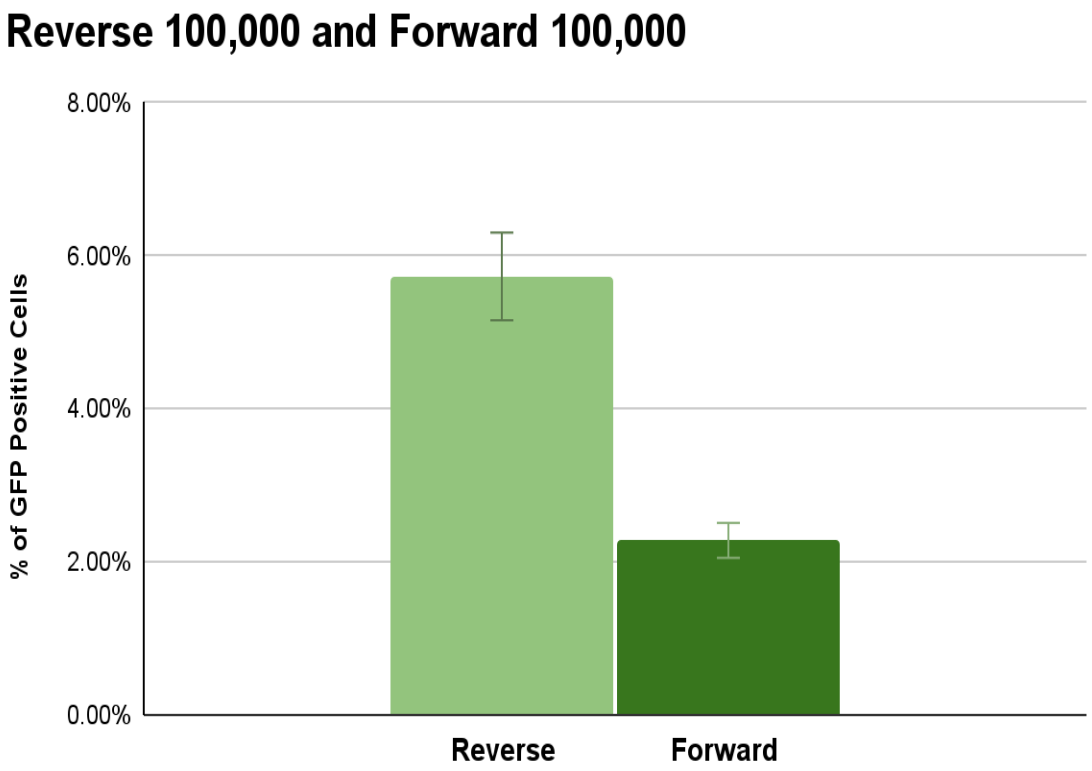


Figure 1. Averages of three transfection trials for Reverse and Forward protocol in wells with 100,000 cells/wells.

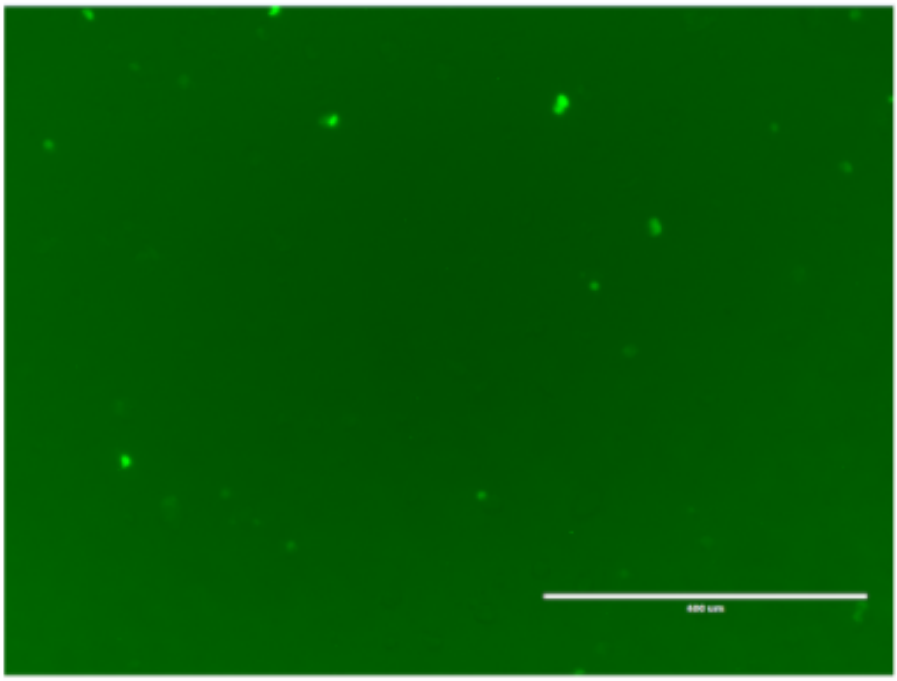


Figure 2. Reverse protocol with 100,000 cells/well.

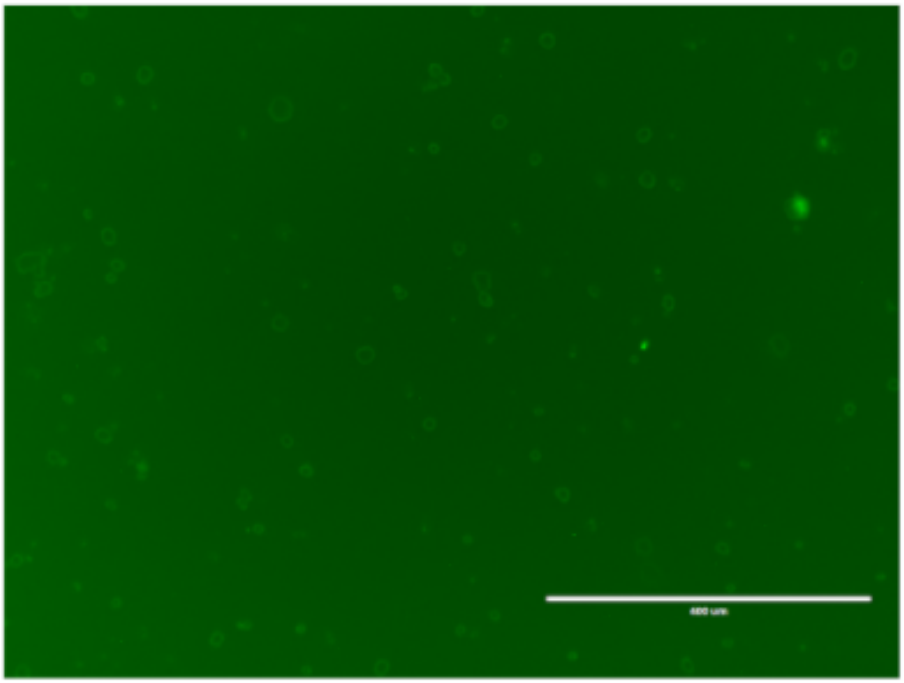


Figure 3. Forward protocol with 100,000 cells/well.

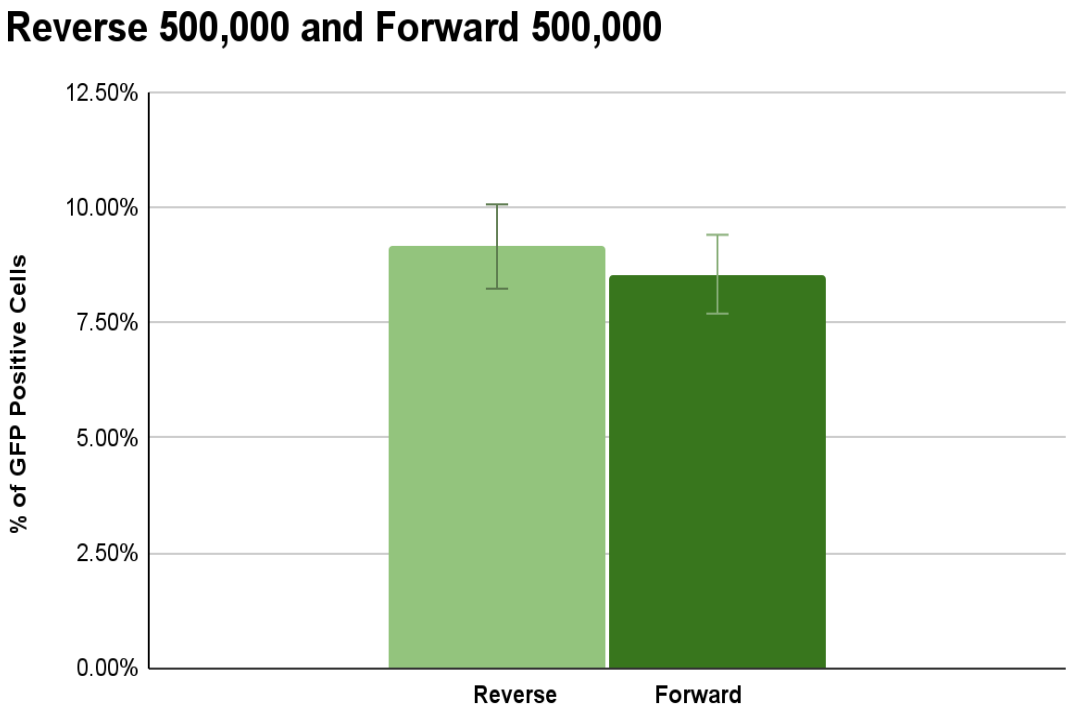



Figure 4. Averages of three transfection trials for Reverse and Forward protocol in wells with 500,000 cells/well.

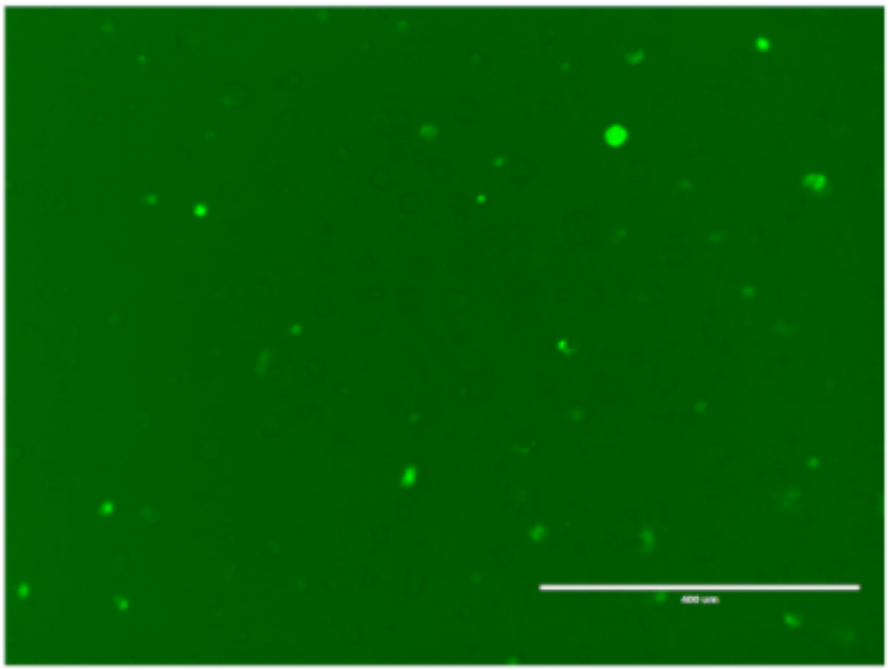


Figure 5. Reverse protocol with 500,000 cells/well.

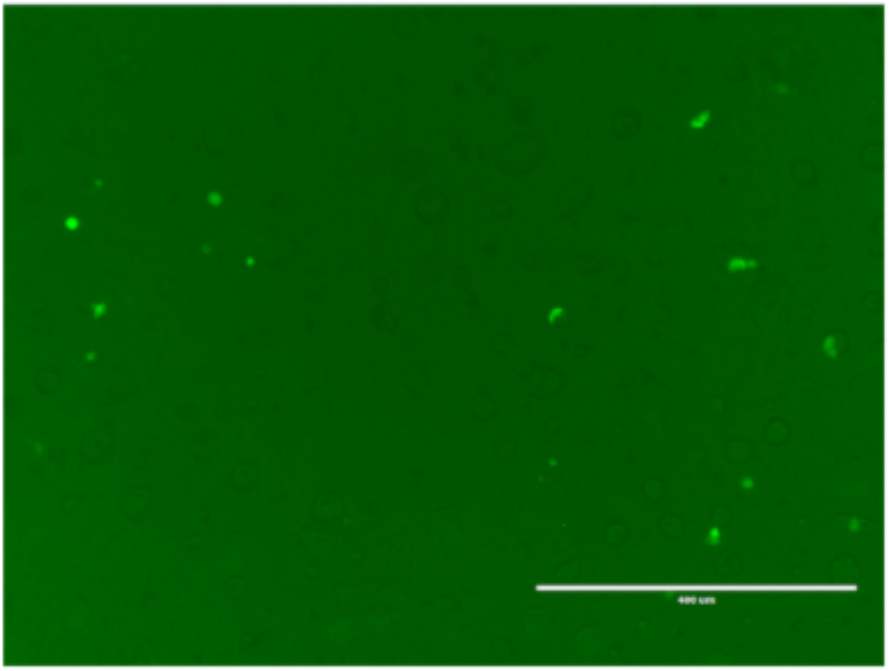


Figure 6. Forward protocol with 500,000 cells/well.

| | Average of Averages | Reverse vs Forward |
|---|---|---|
| | **Reverse 100,000** | **Forward 100,000** |
| **Transfection #4** | 5.08% | 2.50% |
| **Transfection #5** | 6.63% | 1.74% |
| **Transfection #6** | 5.48% | 2.61% |
| Average | **5.73%** | 2.28% |
| Standard Deviation | 0.008018204288 | 0.004760465663 |
| SEM | 0.004629312404 | 0.002748456132 |
| | **Reverse 500,000** | **Forward 500,000** |
| **Transfection #4** | 13.59% | 9.14% |
| **Transfection #5** | 8.53% | 11.06% |
| **Transfection #6** | 5.32% | 5.44% |
| Average | **9.15%** | 8.55% |
| Standard Deviation | 0.04172926591 | 0.02855507194 |
| SEM | 0.02409240291 | 0.01648627847 |
| | | |
| | | |
| | Reverse 100,000 | Forward 100,000 |
| Reverse Forward | 5.73% | 2.28% |
| | | |
| | | |
| | | |
| | Reverse 500,000 | Forward 500,000 |
| Reverse Forward | 9.15% | 8.55% |

Figure 7. Average of averages of three trials for reverse 100,000 and forward 100,000 as well as reverse 500,000 and forward 500,000.

### 3.2 Western Blot

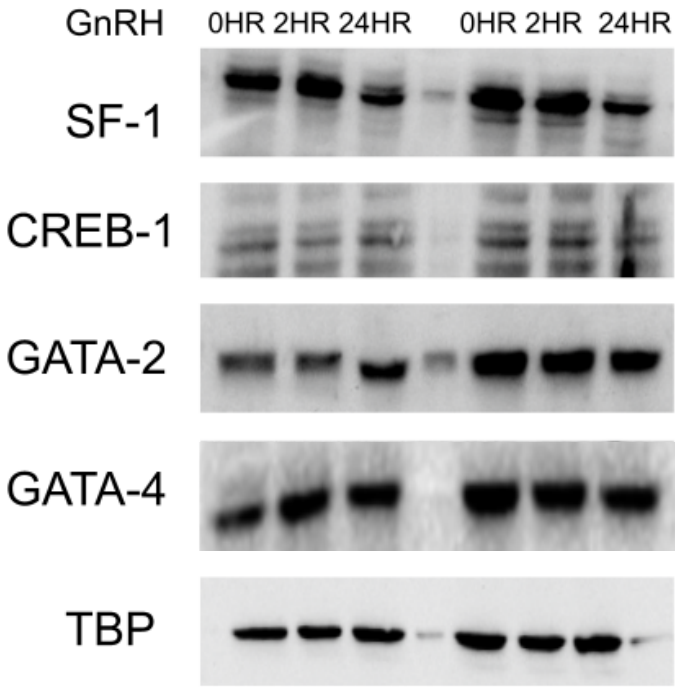
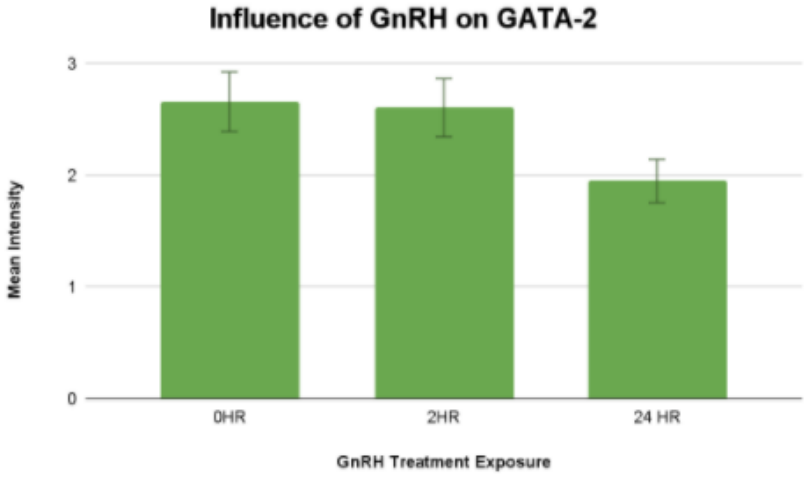
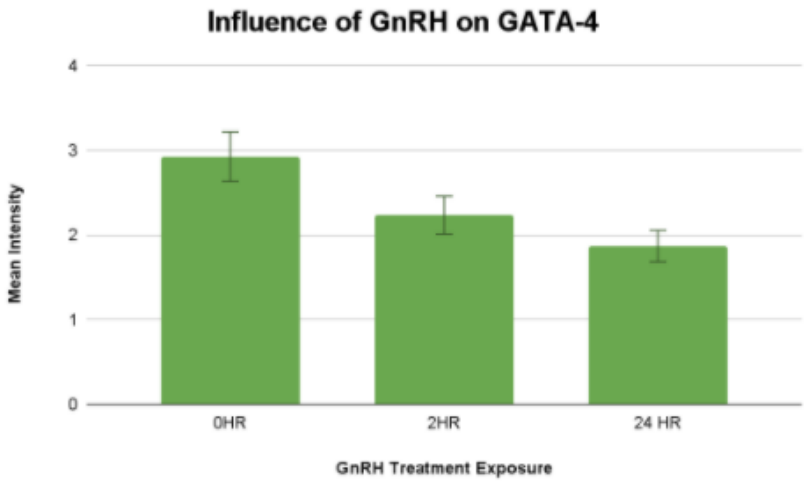



Figure 8. Western blot analysis of transcription factors present in LbT2 cell nuclear extracts with a 0 hour GnRH treatment, 2 hour GnRH treatment, and 24 hour GnRH treatment.

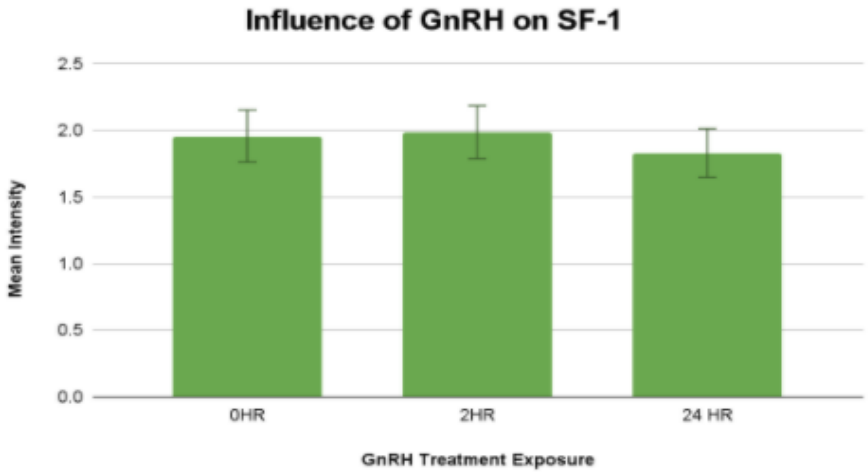
A


Influence of GnRH on GATA-2
Mean Intensity
3
2
1
0
0HR
2HR
24 HR
GnRH Treatment Exposure

B


Influence of GnRH on GATA-4
Mean Intensity
4
3
2
1
0
0HR
2HR
24 HR
GnRH Treatment Exposure

C




D

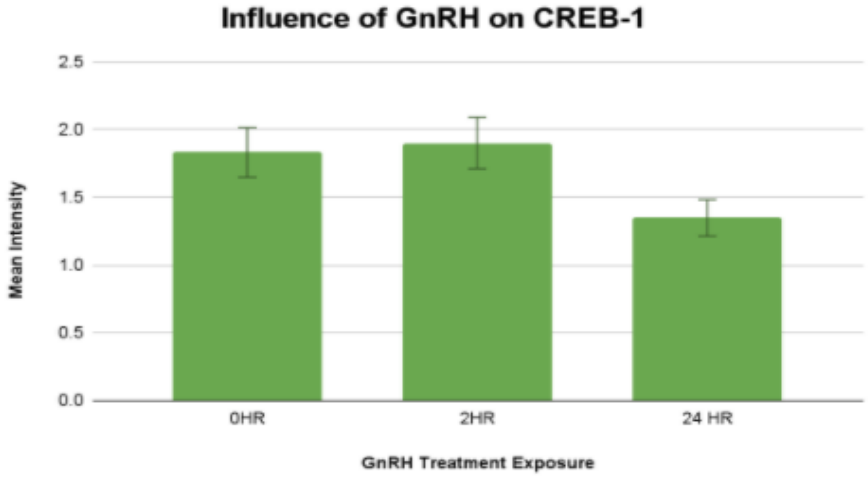



Figure 9. (A) Influence on GATA-2 transcription factor levels at 0 hour GnRH treatment, 2 hour GnRH treatment, and 24 hour GnRH treatment. (B) Influence on GATA-4 transcription factor levels at 0 hour GnRH treatment, 2 hour GnRH treatment, and 24 hour GnRH treatment. (C) Influence on SF-1 transcription factor levels at 0 hour GnRH treatment, 2 hour GnRH treatment, 24 hour GnRH treatment. (D) Influence on CREB-1 transcription factor levels at 0 hour GnRH treatment, 2 hour GnRH treatment, 24 hour GnRH treatment.

| | SF-1 mean intensity | TBP mean intensity | SF-1/TBP (normalized) | Average |
|---|---|---|---|---|
| 0Tx | **4,152.71** | **1,667.20** | 2.490831506 | **1.958173788** |
| 0Tx | **4,905.93** | **2,060.69** | 2.380719758 | |
| 0Tx 11/12 | **6,316.75** | **3,047.08** | 2.07 | |
| 0Tx 11/28 | **7,982.17** | **8,988.00** | 0.89 | |
| 2Tx | **3,996.72** | **1,616.32** | 2.47273053 | **1.986846932** |
| 2Tx | **4,724.19** | **1,754.29** | 2.692930759 | |
| 2Tx 11/12 | **7,592.58** | **5,062.15** | 1.499872103 | |
| 2Tx 11/28 | **7,587.00** | **5,918.77** | 1.281854336 | |
| 24Tx | **4,521.67** | **1,853.23** | 2.439888626 | **1.83093736** |
| 24Tx | **5,080.32** | **1,842.08** | 2.757927891 | |
| 24Tx 11/12 | **7,825.00** | **6,199.92** | 1.262112433 | |
| 24Tx 11/28 | **6,449.42** | **7,466.15** | 0.8638204891 | |

| | CREB-1 mean intensity | TBP mean intensity | CREB-1/TBP (normalized) | Average |
|---|---|---|---|---|
| 0Tx | **2,184.88** | **1,712.23** | 1.276045143 | **1.829917647** |
| 0Tx | **2,620.48** | **2,142.63** | 1.223019344 | |
| 0Tx 11/12 | **11,076.82** | **3,047.08** | 3.635227617 | |

| | | | | |
|---|---|---|---|---|
| 0Tx 11/28 | **10,654.18** | **8,988.00** | 1.185378484 | |
| 2Tx | **2,316.05** | **1,682.57** | 1.376492896 | **1.899267732** |
| 2Tx | **3,022.88** | **1,820.23** | 1.660715033 | |
| 2Tx 11/12 | **11,913.27** | **5,062.15** | 2.353399973 | |
| 2Tx 11/28 | **13,059.55** | **5,918.77** | 2.206463024 | |
| 24Tx | **2,450.48** | **1,928.29** | 1.270805551 | **1.347077423** |
| 24Tx | **2,672.83** | **1,939.54** | 1.378073871 | |
| 24Tx 11/12 | **11,628.64** | **6,199.92** | 1.875609781 | |
| 24Tx 11/28 | **6,449.42** | **7,466.15** | 0.8638204891 | |

| | GATA2 mean instensisty | TBP mean intensity | GATA2 /TBP (normalized) | Average |
|---|---|---|---|---|
| 0Tx | **2,468.46** | **1,712.23** | 1.44166333 | **2.659429829** |
| 0Tx | **3,245.83** | **2,142.63** | 1.514881587 | |
| 0Tx 11/12 | **14,645.71** | **3,047.08** | 4.806478045 | |
| 0Tx 11/28 | **25,837.77** | **8,988.00** | 2.874696354 | |
| 2Tx | **2,251.14** | **1,682.57** | 1.337918152 | **2.606473965** |
| 2Tx | **2,958.23** | **1,820.23** | 1.625196208 | |
| 2Tx 11/12 | **19,726.73** | **5,062.15** | 3.896904315 | |
| 2Tx 11/28 | **21,105.60** | **5,918.77** | 3.565877185 | |
| 24Tx | **2,460.23** | **1,928.29** | 1.275863091 | **1.94940289** |
| 24Tx | **2,183.77** | **1,939.54** | 1.125920689 | |
| 24Tx 11/12 | **19,139.98** | **6,199.92** | 3.087131716 | |
| 24Tx 11/28 | **17,237.08** | **7,466.15** | 2.308696064 | |

| | GATA4 mean instensisty | TBP mean intensity | GATA4/TBP (normalized) | **Average** |
|---|---|---|---|---|
| 0Tx | **1,358.07** | **1,712.23** | 0.7931573448 | **2.927609669** |
| 0Tx | **1,481.93** | **2,142.63** | 0.6916426641 | |
| 0Tx 11/12 | **15,970.20** | **3,047.08** | 5.241154196 | |
| 0Tx 11/28 | **29,502.01** | **5,918.77** | 4.984484473 | |
| 2Tx | **1,296.73** | **1,682.57** | 0.7706854584 | **2.235503144** |
| 2Tx | **1,374.40** | **1,820.23** | 0.7550700071 | |
| 2Tx 11/12 | **21,640.29** | **5,062.15** | 4.274918146 | |
| 2Tx 11/28 | **23,453.72** | **7,466.15** | 3.141338966 | |
| 24Tx | **1,473.47** | **1,928.29** | 0.7641329583 | **1.869916496** |
| 24Tx | **1,321.33** | **1,939.54** | 0.6812601888 | |
| 24Tx 11/12 | **20,424.40** | **6,199.92** | 3.294298937 | |
| 24Tx 11/28 | **20,457.07** | **7,466.15** | 2.739973899 | |

Figure 10. Average mean intensity of each transcription factor with an average of the normalized value.

LbT2 cells are known to have a low transfection efficiency of about 10-20%. With this, we decided to see if the transfection efficiency can be increased by comparing two different protocols (reverse and forward) as well as cell density. In figure (1) it is shown that reverse protocol has a higher transfection efficiency with lower cell density compared to the results of forward transfection efficiency with lower cell density. Further, in figure (4) it is noted that reverse or forward protocol does not provide significant difference in transfection efficiency with higher cell density. Higher cell density provides higher transfection efficiency compared to lower cell densities. This result can suggest that higher density of cells can increase transfection efficiency. The clumping of cells can influence fugenes' ability to transfect the expression vector into the cell. Transfections were tested for statistical significance, using TTEST. Overall, my transfection results suggest that cell density has a significant effect on transfection efficiency while difference in reverse or forward protocol does not.

In the western blot experiment, the goal is to observe if GnRH treatment has an influence on SF-1, CREB-1, or GATAs. The nuclear extract samples were treated with GnRH for 2 hours, 24 hours, and a 0 hour GnRH treatment to compare. By using ordinary one-way ANOVA, my results propose that there is no significant difference in my results. This suggests that GnRH does not have an influence on CREB-1, SF-1, or GATAs.

**4. Discussion**

In the transfection experiments, comparisons are made between reverse and forward protocols. Reverse and forward protocols differ in whether the cells are plated the day before transfection (forward) or seeded at the same time of transfection (reverse). As LbT2 cells have a transfection efficiency of 10-20%, the goal of the experiment is to increase transfection efficiency and to determine if efficiency is increased when cells are lifted. This allows for a whole individual cell to be transfected rather than attempting a transfection when cells are plated, which can result in cell crowding or clumping making it more difficult for the enveloped expression vector to enter the cell.

In the western blots, the goal is to determine if GnRH has an influence on GATAs, SF-1, or CREB-1 in LbT2 cells. Transcription factors are proteins that play an important role in gene expression. The presence of transcription factors can play roles in gonadotropin regulation and expression. In this thesis, we specifically look at the influence of GnRH on SF-1, CREB-1, GATAs. This is because in previous studies, it has been suggested that GnRH in combination with EGR-1 plays a role in *LHb* transcription. While GnRH in combination with AP-1 regulates *FSHb* transcription. For this reason, we wanted to observe if GnRH has an influence on SF-1, CREB-1, or GATAs.

As briefly mentioned, GnRH regulates the expression of beta subunits with immediate early genes such as EGR-1 and AP-1 (Coss, 2017). With this, EGR-1 prompts the expression of *LHb* while cFOS, another transcription factor, regulates the expression of *FSHb* (Coss, 2017). This process results in heterodimerization with cJUN, a protein encoded by the *JUN* gene, which forms AP-1 (Coss, 2017). The genes arrange a complex on their target gene promoters and gonadotrope specific factors (SF-1), respectively

(Coss, 2017). According to Coss (2017), the complex has low beta subunit expression on account of the repressors present such as Nab for *LHb*, and JDP2, or ICER for *FSHb* transcription (Coss, 2017). Once GnRH induces the immediate early genes, EGR-1 and cFOS, they replace the repressors Nab and JDP2 which then prompts the expression of the beta subunit (Coss, 2017). According to Coss (2017), EGR-1 and cFOS have short half-lives as well as rapid degradation.

The study of using LbT2 cells is a way to study reproductive function due to its recapitulation of a mature gonadotrope by the expression of GnRHR, *FSHb* and *LHb*. As the production of FSH and LH acts on the ovaries and testes, malfunctions can cause reproductive dysfunction. For example, FSH and LH have a common alpha subunit which is encoded by *CGA* while the beta subunit is specific and encoded by *FSHb* and *LHb*.

In the article by Ferraz-de-Souza et al. (2011) note SF-1 variation and mutations are found in individuals with disorders of sexual development such as hypospadias, anorchia, male factor infertility, or primary ovarian insufficiency in women. Additionally, Ferraz-de-Souza et al. (2011) note the overexpression of SF-1 is reported in some individuals with endometriosis. In the article by Kopalli et al (2017) CREB-1 is highly expressed in the testes during spermatogenesis which suggests CREB-1 plays an important role in fertility.

Overall, the secretion of GnRH from the hypothalamus travels to the pituitary gland by the hypophyseal portal system and binds to GnRHR of the gonadotrope cell. The pituitary gland then secretes FSH and LH which is composed of a heterodimeric,

alpha and beta subunit. In order for FSH and LH to be secreted, the *FSHb* and *LHb* gene must be expressed. If *FSHb* or *LHb* expression does not occur, there will also be malfunction in the production and secretion of FSH and LH which can cause sexual development problems.

**5. Conclusion**

As stated previously, LbT2 cells are known to have a transfection efficiency of about 10-20%. With transfections, normally, cells are given time to attach to the cell plate prior to transfection. However, to attempt to increase transfection efficiency by these protocols, we wanted to observe transfection efficiency while cells are lifted. When cells are lifted, they are individual whole cells in a single layer rather than seeded. Our results suggest that reverse or forward protocol does not have a significant effect on transfection efficiency. However, it is suggested that cell density plays a role in transfection efficiency. In future studies, transfection efficiency can be increased by solely conducting transfections with higher cell densities such as 500,000 cells/ml regardless of the reverse or forward protocol being used.

Aforementioned, GnRH pulses regulate gonadotropin subunit genes such as *CGA*, *FSHb*, and *LHb*. It is suggested that GnRH and Egr-1 play a role in the regulation of *LHb* transcription. While GnRH and AP-1 induce *FSHb* transcription. Additionally, it is important to note that GnRH is released from the hypothalamus in a pulsatile manner allowing for *FSHb* and *LHb* transcription, which then plays a role in gonadotropin secretion.

In the western experiments, GnRH was added in a tonic fashion for 2 hour GnRH treatment and 24 hour GnRH treatment. Based on my results, there is no statistical significance in the influence of GnRH on CREB-1, SF-1, or GATAs which are the TFs utilized in this thesis. Due to adding GnRh in a tonic manner to LbT2 cells for GnRH treatment time points, rather than its necessary pulsatile manner for the expression of *FSHb* and *LHb*, the western blot experiment may suggest the importance of pulsatile GnRH release to the gonadotropes. As my results suggest that adding GnRH to the cells in a tonic manner had no statistical significance on the effects of SF-1, CREB-1, or GATAs.

Unlike past findings, my results suggest that GnRH does not have an influence on the regulation of SF-1, CREB-1, or GATAs. Although the results of this experiment do not suggest that GnRH has an influence on GATAs, SF-1, or CREB-1, future studies can continue to be conducted in order to further study the effects of GnRH on SF-1, CREB-1, or GATAs.

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