UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of the Homeodomain Protein Six6 in Pituitary Gonadotrope Gene Expression

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requirements for the degree Master of Science

in

Biology

by

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The Thesis of Chiara Maruggi is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2013

Dedication

I would like to dedicate this thesis to my loved ones:

To my Mom and Dad for their eternal love and support.

To my brother Marco for always being there for me.

To Tina and Francisca for constantly encouraging and believing in me.

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ABSTRACT OF THE THESIS

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Essential to reproduction and fertility is proper functioning of the axis that is formed from the interaction of the hypothalamus, pituitary and gonads. *In vivo* studies have shown the crucial role played by the sine oculis-related homeobox gene-6 (Six6) in the proper development of the pituitary as well as fertility in general. In this research study, we investigated the role played by Six6 in the regulation of the gonadotropin-releasing hormone receptor (GnRH-R) and its subsequent effect on reproductive function in an *in vitro* setting. Experiments involving embryonic mice point to the crucial role played by Six6 in the regulation of GnRH-R expression as evidenced by the sharp

difference in GnRH-R mRNA levels in wild type mice as compared to Six6-null mice. Further *in vitro* studies of Six6 have revealed its role in the repression of GnRH-R through binding at an ATTA site located in the -517 bp to -741 bp region of the GnRH-R promoter. We hypothesized that the repression by Six6 could be aided by the recruitment of co-repressor proteins of the Groucho/TLE family proteins and transient transfections using Grg1 and Grg4 were conducted to test this hypothesis. Finally, aiming to identify the exact sites at which Six6 binds on GnRH-R to exert its repressive function, electrophoretic mobility shift assays were performed using sequence elements of GnRH-R from within the -517 bp to -741 bp promoter region.

I

Introduction

The Hypothalamic-Pituitary-Gonadal Axis

Reproduction has been a topic of much interest in today's scientific community as approximately 1 of 10 couples in the United States face infertility issues [1]. Aside from the effects on societal structure, infertility has become such a hot topic due to many nations' concerns for the eventual effects on the economic structure of future communities [2]. Thus, with the goal of trying to tackle this pressing issue, much research has focused on the causes and eventual treatments for infertility. Of particular relevance to this field are the coordinated actions of the hypothalamus, pituitary and gonads via the hypothalamic-pituitary-gonadal axis. Malfunction of the hypothalamic-pituitary gonadal axis has been linked with many fertility and developmental problems including hypogonadotropic hypogonadism [3].

Standing at the top of this axis is the hypothalamus, a neuroendocrine brain region that secretes gonadotropin-releasing hormone (GnRH) in a pulsatile fashion. GnRH is a tropic peptide hormone that is released by hypothalamic neurons into the hypophyseal portal system to the adenohypophysis of the pituitary gland. In the anterior pituitary, GnRH binds onto its receptor protein to stimulate the secretion of the glycopeptides luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In turn, FSH and LH act on receptors on the gonads to affect the production of sex steroid hormones (mainly estrogen in females and testosterone in males). In particular, LH is responsible for steroidogenesis, while FSH controls gamete production (in both males and females).

The circadian and pulsatile secretion of GnRH appears to be necessary for the proper secretion of LH and FSH as a constant presence of GnRH in the anterior pituitary

has been shown to inhibit the release of LH and FSH by down-regulating the GnRH receptors or depleting gonadotropin stores in the pituitary [4, 5]. Aside from receiving regulatory neuronal inputs from various parts of the brain, GnRH secretion is also influenced by the negative feedback provided by sex steroids at the pituitary and hypothalamic levels, as well as environmental cues [6].

GnRH and the GnRH receptor

GnRH neurons originate in the olfactory placode around embryonic day e11.5 and then migrate, along with the olfactory neurons, through the cribriform plate. The GnRH neurons then continue their migration to the rostral hypothalamus where they extend their axons into the hypothalamic area known as the medial eminence [7]. Improper migration of these neurons has been associated with a pathophysiology known as Kallmann's syndrome. Aside from presenting with gonadotropin insufficiency, known as hypogonadotropic hypogonadism, patients suffering from this disease also have an inability to sense smell, known as anosmia. This is due to the olfactory axons and GnRH neurons remaining above the cribriform plate in the meninges [8].

Although previous research has not identified mutations of the GnRH hormone, it has been shown that about 20% of patients presenting with idiopathic hypogonadotropic hypogonadism may have a mutation of the GnRH receptor. Patients with mutations in the GnRH receptor can have a wide variety of pathophysiological states including incomplete pubertal development and resistance to GnRH [7].

Because of its crucial role in reproductive health and development, much research has focused on better understanding the regulatory mechanisms by which GnRH and the GnRH receptor (GnRH-R) are controlled. GnRH has been shown to play a central role in the regulation of transcription of gonadotropins by binding to its seven-transmembrane, G-protein coupled receptor located primarily on the surface of pituitary gonadotrope cells [9]. About 60 kDa in size, the GnRH receptor is activated when GnRH first binds to the G_q receptor and leads to the production of the second messengers 1,4,5-trisphosphate and diacylglycerol (DAG) and the eventual activation of the Protein Kinase C pathway [10]. Located in a 1.2 kilobase 5' flanking sequence, various *cis*-regulatory elements have also been shown to be necessary for tissue-specific expression of GnRH-R [11]. These include the steroidogenic factor-1 (SF-1) binding region, an activating protein-1 (AP-1) binding site (located at SURG-2), and a GnRH-R activating sequence (GRAS) [12]. Stimulation of GnRH-R transcription has been shown to be induced by binding of Activin, via Smads, to the GRAS element on GnRH-R [13, 14]. The different transcriptional regulators in the 5'-flanking region of the GnRH receptor are displayed in Figure 1 [15].

Six6 and Groucho-related family proteins

Six6, a member of the SIX/Sine oculis family of homeobox genes, stimulates progenitor cell development in the growing pituitary, eye, and brain. Looking at the developing forebrain, Six6 expression has been shown in those regions that give rise to the neurohypophysis and the hypothalamus [16]. Murine expression studies conducted during pituitary development have also determined the crucial role that Six6 plays in the

formation of Rathke's pouch [16]. Images from the Allen Brain atlas depicting the presence of Six6 in the developing mouse anterior pituitary are shown in Figure 2.

In mice, an absence of Six6 has been found to cause a hypo-pituitary phenotype and a striking decrease in fertility [17]. Further research on humans has shown that patients with deletions in the chromosomal region in which Six6 maps (chromosome 14q22-23) exhibit bilateral anophthalmia and pituitary anomalies [18].

Previous studies conducted in the laboratory have identified Six6 as one of the most up-regulated genes in the fully differentiated GnRH neuron (represented by the GT1-7 cell model) as compared to a developmentally earlier GnRH neuron (represented by the GN11 cell model) [17]. Both GT1-7 and GN11 cells are immortalized hypothalamic neuronal cell lines that were created by targeted oncogenesis in transgenic mice [19, 20]. Further, *in vivo* experimentation in mice has shown that Six6 expression correlates with an increase in GnRH expression and that loss of Six6 results in a significant decrease in the total GnRH neurons found in all areas of the brain [17].

While several members of the Six family of proteins have been shown to bind to a TCAGGT sequence motif, Six6 has been shown to bind to the homeodomain sequence ATTA—a binding site that is conserved across various species [21]. Deletion of ATTA binding sites on the GnRH promoter have been shown to significantly reduce the inductive effect of Six6 on GnRH—thus indicating the importance of these sites in regulating the action of Six6 [17]. *In vivo* experiments implicate that the infertility observed in Six6 knock-out mice is primarily due to LH deficiency resulting from a disruption of signaling between the hypothalamus and the anterior pituitary [17].

Further, previous tissue culture experiments have suggested that the observed repressive effect of Six6 on transcriptional activity vastly depends on its interaction with the Groucho family of co-repressor proteins (Grg proteins) [17, 22]. Groucho-related proteins are non-DNA binding co-repressors that interact with various DNA-binding transcription factors to repress transcription [23]. There are two main groups of Grg proteins: "long" Grgs compromising of Grg1, Grg2, Grg3, and Grg4 and "short" Grgs which are made up of Grg1-S, Grg3b, Grg5, and Grg6 [24, 25]. While "long" Grgs have been found to enhance the repression by certain homeodomain proteins necessary for embryonic development, "short" Grgs appear to exert a dominant-negative effect on this homedomain-dependent repression [24]. Previous work has established that the interaction between Grg co-repressor proteins and Six proteins is mediated by an engrailed homology 1 (eh1)-like motif in the Six protein [22]. This aligns with the finding that Grg proteins interact with transcription factors containing the eh1 motif in order to physically interact with DNA-binding domains [25].

***In vivo* studies of Six6 knock-out mice**

To better understand the function of the neuroendocrine system underlying fertility and development, experiments were conducted to analyze the role of the homeodomain transcription factors Six3 and Six6 in an *in vivo* setting. Findings showed that when compared to wild-type mice, Six6 knock-out mice (Six6KO) presented with a striking decrease in fertility. Moreover, while at embryonic day e13.5 the Six6KO mice contained normal levels of GnRH neurons, adult Six6KO mice exhibited a large decrease in the levels of GnRH mRNA as well as total GnRH neuron number. Looking at another

member of the sine oculis family of homeodomain proteins, Six3, *in vivo* studies showed that GnRH neurons were also affected in Six3Flox/GnRH-Cre mice. All together, the experiments confirmed that these homeodomain proteins are not only necessary for proper migration of GnRH neurons, but also for the correct functioning of the pituitary gonadotrope and the presence of the suprachiasmatic nucleus (SCN)—which is absent in Six6KO mice. Moving forward, *in vivo* studies using mice models will aim to further study the actions of these homeoproteins in the regulation of development, migration, gene expression and circadian rhythm.

LβT2 Cell Model

To study the effect of Six6 on the GnRH receptor, the LβT2 cell line was used. The LβT2 cell line was generated by Dr. Mellon's laboratory from a pituitary tumor induced by targeted oncogenesis in transgenic mice with a rat LHβ region linked to the SV40 T-antigen oncogene [26]. These cells express αGSU, LHβ, FSHβ and the GnRH-R and have been shown to respond to GnRH. [27, 28]. Representing a fully differentiated gonadotrope cell, this model has been used in various studies to study the molecular regulation underlying gonadotrope cell function. In this thesis, the LβT2 cell model was used to analyze the transcriptional regulation and binding of the Six6 protein in gonadotropes.

Overall, the main goal of this project is to analyze the repressive effect of Six6 on GnRH-R and try to localize the site of binding of the Six6 protein on the receptor and the possible recruitment of Grg proteins.

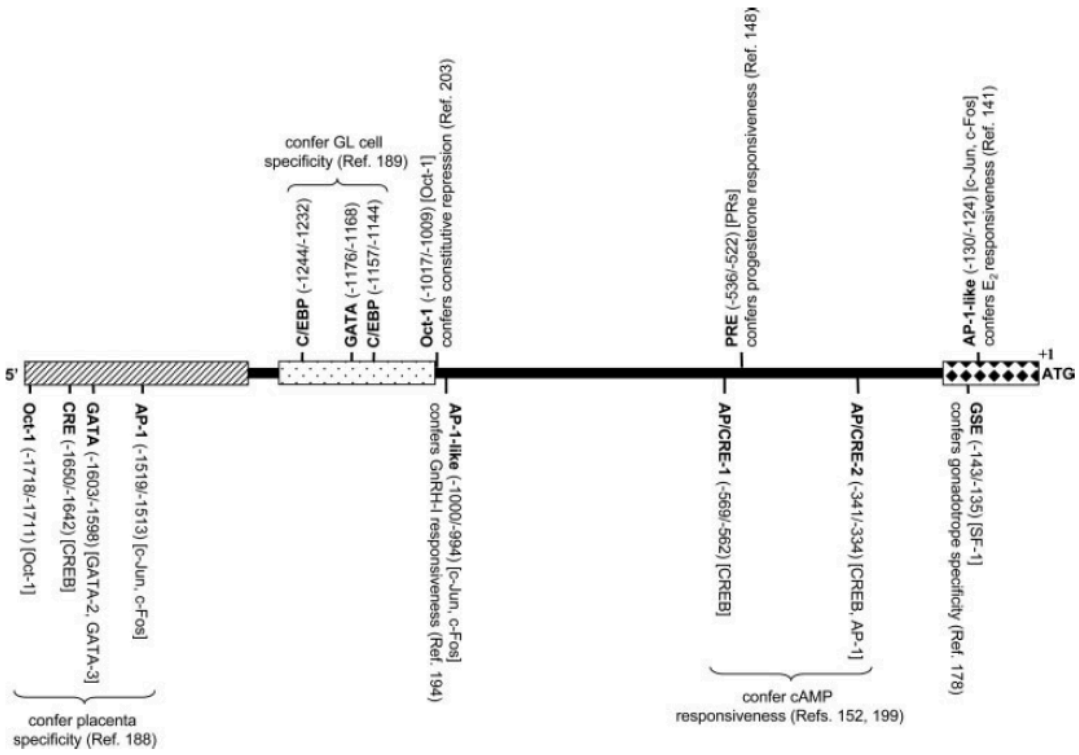

Figure 1: Transcriptional regulators of the GnRH-R. Figure displays different transcriptional regulators found on the GnRH-R promoter 5' flanking region [15].

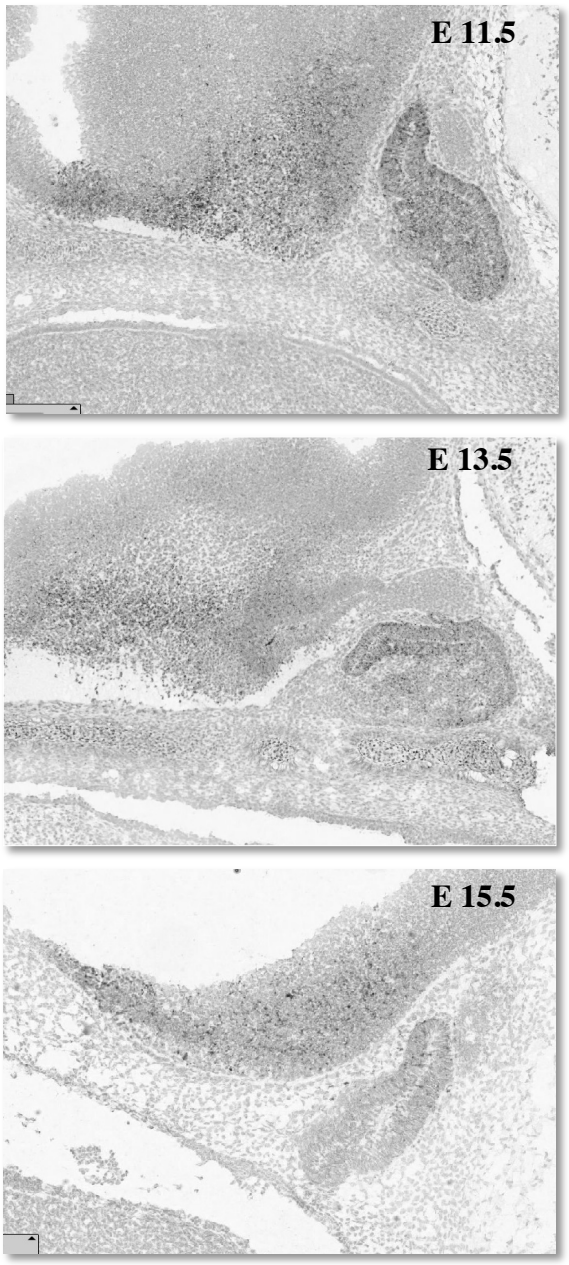

Figure 2: Six6 in situ in mouse anterior pituitary from Allen Brain Atlas. Medial sagittal cross-sections of mouse brains. Figure displays presence of Six6 in pituitary in mice at different developmental ages—embryonic ages: 11.5 days, 13.5 days, and 15.5 days.

II

Materials and Methods

Cell culture

LβT2 cells [27] generated by Dr. Mellon as well as COS cells [29] were cultured in Dulbecco's modified Eagle's medium with 4.5 g/L glucose, L-glutamine & sodium pyruvate (DMEM; Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA) and 1% penicillin-streptomycin (Life Technologies, Inc./Invitrogen, Grand Island, NY) at 37°C under 5% CO_2/ 95% O_2 atmosphere.

Plasmids used for transient transfections

In transient transfections used for luciferase assays with Six6 and various reporters, the plasmids used were: the luciferase reporter GnRH-R promoter (-1100 bp), the luciferase reporter LHβ promoter (-1800 bp), the luciferase reporter FSHβ promoter, the pGL3 luciferase reporter with SV40 promoter, the Six6 expression vector with p3X Flag-CMV-7.1 (Flag-CMV) backbone and promoter, and empty vectors. The mouse Six6 Flag-CMV was designed by Rachel Larder in our lab and has been previously described [17]. The GnRH-R (-1100 bp) reporter plasmid has also been previously described [30]. The -1800 LHβ luciferase and FSHβ luciferase reporters were also previously described [31, 32]. For experiments in LβT2 cells, the following plasmids were co-transfected into cells: 100 ng of Tk-βgal; 200 ng of either empty pGL3 vector, GnRH-R luciferase reporter plasmid, LHβ luciferase reporter plasmid, or FSHβ reporter plasmid; and either 100 ng, 200 ng, or 300 ng of either Six6 or empty Flag-CMV.

In transient transfections used for luciferase assays with Six6, the Six6 eh1-mutant, and Six6 with a deletion of the eh1 domain, the plasmids used were: the luciferase reporter GnRH-R (-1100 bp), the pGL3 luciferase reporter with SV40 promoter, the Six6 expression vector with Flag-CMV backbone and promoter, the Six6 expression vector with pSG5 backbone, the Six6-mutant expression vector in pSG5 backbone, the Six6 expression vector with Flag-CMV backbone and a deletion in the eh1 domain (Six6 eh1-deletion), and empty vectors. The Six6 expression vector in pSG5 backbone was made by Rae Larder and has been previously described [17]. The mouse Six6-mutant and Six6 eh1-deletion plasmids were also created by Rachel Larder. The Six6 mutant contains a *cis*-mutation in the eh1 domain that was made using site-directed mutagenesis using the QuickChange Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol (Fig. 4). The Six6 eh1-deletion plasmid was generated from the wild-type Six6 plasmid by PCR and corresponds to the eh1 domain of Six6 (Fig. 4). For experiments in LβT2 cells, the following plasmids were co-transfected into cells: 100 ng of Tk-βgal; 200 ng of empty pGL3 vector or 200 ng of GnRH-R luciferase reporter plasmid; and 200 ng of either Six6, Six6-mutant, Six6 eh1-deletion, or empty Flag-CMV or empty pSG5.

For the GnRH-R truncation transient transfections in LβT2 various truncations of the -1.1 kb GnRH-R reporter plasmid were used. These include -840 bp, -738 bp, and -517 bp truncated GnRH-R promoter constructs. The -517 bp reporter plasmid was obtained from Dr. Huimin Xie while the other truncations were received from Dr. Emily Whitman and were created using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). For experiments, the following plasmid concentrations were

transfected into cells: 100 ng of Tk-βgal; 200 ng of empty pGL3, GnRH-R (-1100 bp), GnRH-R (-840 bp), GnRH-R (-738 bp), or GnRH-R (-517 bp); and 200 ng of Six6 expression vector in Flag-CMV backbone or empty Flag-CMV.

For the co-transfection experiments using GnRH-R and the groucho-related protein-1, Grg1, the following plasmid concentrations were used for transfection of the LβT2 cells: the luciferase reporter GnRH-R (-1100 bp), pGL3 luciferase reporter with SV40 promoter, Six6 expression vector with pSG5 backbone, Grg1 expression vector in pSG5 backbone, and empty pSG5 vector. The Six6 expression vector in pSG5 was generated by Rachel Larder and previously described [17]. For experiments in LβT2 cells, the following plasmids were co-transfected into cells: 100 ng of Tk-βgal; 200 ng of empty pGL3 vector or GnRH-R (-1100 bp) luciferase reporter plasmid; 200 ng of Six6, Grg1, or empty pSG5; and either 200 ng of Grg1 (co-transfected into cells with the Six6 expression vector) or 200 ng of empty pSG5 (co-transfected into cells only containing Six6, Grg1, or empty pSG5).

For the co-transfection experiments using GnRH-R and the groucho-related protein-1, Grg4, the following plasmid concentrations were used for transfection of the LβT2 cells: the luciferase reporter GnRH-R (-1100 bp), pGL3 luciferase reporter with SV40 promoter, Six6 expression vector with Flag-CMV backbone, Grg4 expression vector in pKW2T backbone with a CMV promoter and Flag tagged, and empty Flag-CMV vector. The Grg4 expression obtained from Antonio Simeone. For experiments in LβT2 cells, different conditions were used. In the first condition, the following plasmids were co-transfected into cells: 100 ng of Tk-βgal; 200 ng of empty pGL3 vector or GnRH-R (-1100 bp) luciferase reporter; and 200 ng of Six6 or Grg4 or empty Flag-CMV.

In the second condition, the following plasmid concentrations were used: 100 ng Tk-βgal; 200 ng of either pGL3 or GnRH-R (-1100 bp) luciferase reporter; 200 ng of either Six6 or empty Flag-CMV; and 400 ng of either Grg4 or empty Flag-CMV. In the third condition used for the experiment, the following plasmids were co-transfected into cells: 100 ng of Tk-βgal; 100 ng of either pGL3 or GnRH-R (-1100 bp) luciferase reporter; 100 ng of either Six6 expression vector or empty Flag-CMV; and 400 ng of either Grg4 or empty Flag-CMV.

Luciferase reporter assays

For transient transfections with LβT2, 350,000 cells were seeded into each well of a 12-well tissue culture plate and incubated at 37°C for 18 to 24 hours before transfection. The transient transfections were done using FuGENE 6 transfection reagent (Roche Applied Science) and following the manufacturer's protocol.

Approximately 48 hours after transfection, the cells were washed with PBS and lysed with 60 μl of 0.1 M phosphate buffer (pH 7.8) containing 0.2% Triton X-100. After lysis, a Veritas Microplate luminometer (Turner Biosystems, Sunnyvale, CA) was used to first quantify the luciferase activity in the cells. This was done by injecting 100 μl of luciferase assay buffer (100 mM Tris-HCl (pH 7.8), 15 mM $MgSO_4$, 10 mM ATP and 65 μM luciferin) into wells containing 20 μl of the cell lysate. Following this, the β-galactosidase activity was measured by using the Tropix Galacto-light-β-galactosidase assay (Applied Biosystems, Foster City, CA) and following the protocol provided by the manufacturer. After the data collection, the measurements of relative luminescence unit

(RLU) for luciferase for each well were normalized to β-galactosidase to account for transfection efficiency.

All experiments were performed in triplicates and most were conducted a minimum of three times (exception noted in figures involving Grg4 co-transfection with second and third conditions).

RNA Isolation

LβT2 cells were plated at a 1:4 concentration in 6-well plates using antibiotic-free DMEM supplemented with 10% fetal bovine serum. After 24-hour incubation, the cells were transfected using DharmaFECT 1 - and the siRNA (Thermo Fisher Scientific). For control, Scramble and CylcoB siRNA were used along with Six6 siRNA. All of the wells were transfected with 10μL of 5 μM siRNA following the manufacturer's protocol. Different amounts of transfection reagent were used for the wells transfected with Six6 siRNA. Of the wells transfected with either DharmaFECT 1 the following concentrations of transfection reagent were used: 1μl/well, 5μl/well, 10μl/well. All the wells transfected with Scramble or CyclophilinB siRNA were transfected using 5μl/well of DharmaFECT 1.

Approximately 24 hours after transfection, the media was replaced with 10% FBS and 1% penicillin-streptomycin in DMEM. After 48 hours of incubation post-transfection, the cells were harvested for RNA. The media was first aspirated and the wells rinsed with cold PBS. Using the RNeasy Mini Kit (Qiagen), the RNA from each well was isolated following the manufacturer's protocol. DNA was removed from the

RNA by using a DNA-free kit (Ambion). Reverse transcription was then carried out on the RNA samples using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) using random hexamer in a total volume of 20 μl.

Harvesting nuclear extracts and protein quantification

LβT2 and COS cells that were grown and transfected on 10 cm dishes were rinsed with 5 ml of PBS (Mediatech) and lysed with cell lysis buffer [20 mM Tris-HCl (pH 7.4), 10mM NaCl, 1mM $MgCl_2$, 1 mM PMSF, 10 mM NaF, 10 μg/ml protease inhibitors (Sigma)]. The lysates were then transferred to 1.5 ml tubes and placed on ice to swell for 15 minutes. A 25 5/8 gauge needle was used to pass the lysate four times before the cells were centrifuged at 4000 rpm for 4 minutes at 4°C. The supernatant was aspirated and the pelleted cell nuclei were resuspended using 35 μl of nuclei lysis buffer [20 mM Hepes (pH 7.9), 20% glycerol, 420 mM KCl, 2 mM $MgCl_2$, 1 mM PMSF, 10 mM NaF, 10 μg/ml protease inhibitor (Sigma), 0.1 mM EDTA, and 0.1 mM EGTA]. The nuclei were placed on ice to swell for 30 minutes prior to being centrifuged at 14,000 rpm for 10 minutes at 4°C. The resulting supernatant containing the nuclear lysate was then divided into 10 μl aliquots which were flash frozen using a dry ice-ethanol bath and stored at -80°C for later use.

The protein concentration of each sample was determined by using 1 μl of cell lysate and the Bradford assay reagent (Bio-Rad Laboratories, Hercules, CA) and following the protocol for the Bio-Rad Protein Assay provided by the manufacturer.

Electrophoretic mobility shift assays

The oligonucleotide probes used for the electrophoretic mobility shift assays were obtained from Integrated DNA Technologies, Inc. (San Diego, CA) and their sequences are listed in Table 1. The oligonucleotides (1 pmol/μl) were first annealed and end-labeled with T4 Polynucleotide Kinase (New England Biolabs) and [γ^{32}P]ATP (5000 Ci/mmol; MP Biomedicals, Solon, OH). To purify the probes, Micro Bio-Spin 6 Chromatography Columns (Bio-Rad) were used. Binding reactions used 2 fmol of ^{32}P-labeled oligonucleotide in 10x GSB, 0.1 M PMSF, 5% BSA, 0.1 M dithiothreitol, and 2 μg/μl poly dIdC, as well as 2 μg of nuclear protein. The nuclear protein extracts used were from either COS cells or LβT2 cells transfected with either Flag-CMV or Six6-CMV Flag. For supershift assays, 2.5 μg of anti-FLAG M2 monoclonal antibody (Sigma-Aldrich), or normal mouse IgG control (Santa Cruz Biotechnology) were added to the reactions and allowed to incubate for 15 minutes on ice prior to addition of the labeled probe. Competition assays were also conducted by incubating the reactions with 500-fold excess of unlabeled oligonucleotide for 10 minutes prior to addition of the labeled probe. Once the probe was added, the samples were incubated at room temperature for approximately 10 minutes before being loaded on a 5% non-denaturing polyacrylamide gel in 0.25X Tris-borate EDTA buffer. The gels were electrophoresed at 250V for 1.5 to 2 hours at 250V, dried under vacuum for 2 hours, and then exposed to a film with L-Plus intensifying screens (Fisher Biotech) while being stored at -80°C.

Statistical analyses

Statistical analysis was carried out using the JMP Version 9.0 software (SAS Institute, Inc., Cary, NC). Raw data was analyzed using the Student's 2-tailed T-test as or 1-way ANOVA followed by Tukey honestly significant different (HSD), as indicated in the legends of the figures. For differences to be deemed significant, the measured p-value was determined to be less than or equal to 0.05.

Table 1: Oligonucleotide probes generated for EMSA

Probe Name	Oligonucleotide Sequences for EMSA (sense strands)
-1635 bp GnRH Forward	5' AAAATTGTGACAATTATAAAGCCCA 3'
-1635 bp GnRH Reverse	5' TTTTAACACTGTTAATATTTCGGGT 3'
-742 bp GnRH-R Forward	5'AATAGGATTATAAATTATAA 3'
-742 bp GnRH-R Reverse	5' TTATCCTAATATTTAATATT 3'
-726 bp GnRH-R Forward	5' ATAAATTAGCATATTATATC 3'
-726 bp GnRH-R Reverse	5' TATTTAATCGTATAATATAG 3'
-640 bp GnRH-R Forward	5' TTGGTATGATAATAACAAGT 3'
-640 bp GnRH-R Reverse	5' AACCATACTATTATTGTTCA 3'
-615 bp GnRH-R Forward	5' ATTTACCCAATTATAAAATC 3'
-615 bp GnRH-R Reverse	5' TAAATGGGTTAATATTTTAG 3'

III

Results

Overexpression of Six6 decreases transcriptional activity of the GnRH-R, LHβ, and FSHβ promoters in the LβT2 cell line

Having determined the crucial role of Six6 in the regulation of fertility and development, we sought to investigate the role of Six6 in the transcriptional control of the expression of various promoter genes: GnRH-R, LHβ, FSHβ, and αGSU. To determine the effect of Six6 overexpression on the promoter activity of the different reporter genes, Six6 was co-expressed with the various gene promoters fused upstream of a luciferase reporter gene by transient transfection in LβT2 cells. Varying concentrations of Six6 were used in the transient transfections. Results indicate that when Six6 is overexpressed at various concentrations (100 ng, 200 ng, and 300 ng), there is a notable repression of the GnRH-R, LHβ, and FSHβ promoters. However, Six6 overexpression does not appear to affect αGSU activity (Fig. 3). Moreover, the experiments indicate that the repressive effect of Six6 on GnRH-R, LHβ, and FSHβ is greatest when the cells are transfected with 200 ng of Six6 (for GnRH-R there is a slight increase in repression when 300 ng is used versus 200 ng, but the difference in the repression between the two is not significant).

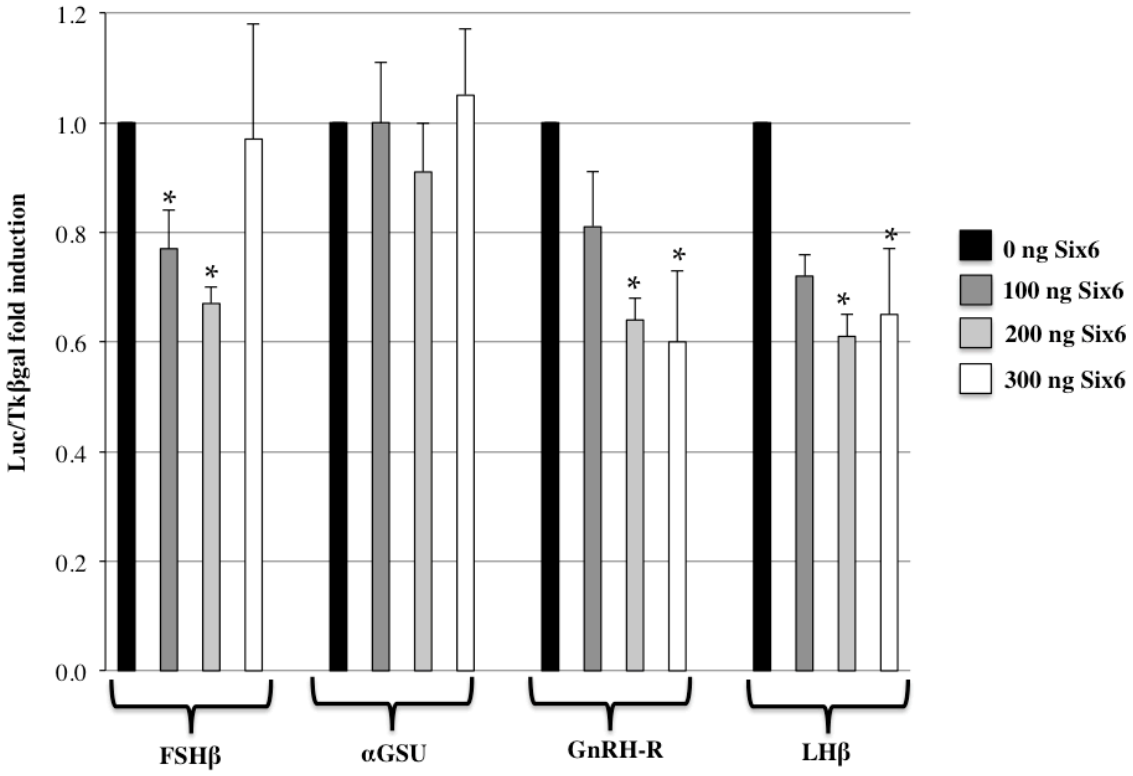

Figure 3: Six6 represses LHβ, FSHβ, and GnRH-R but not αGSU. Over-expression of Six6 in LβT2 cells represses the FSHβ, LHβ, and GnRH-R promoters but does not appear to repress the αGSU promoter. The Six6-CMV Flag expression vector or CMV-Flag were co-transfected along with the GnRH-R-luciferase reporter gene (GnRH-R) or pGL3 reporter into LβT2 cells. `Experiments were done in triplicates and the results displayed are an average of an N=3 study. The asterisk * indicates a p-value <0.05 using a 2-tail T-test. The error bars represent the standard deviation.

(Experiments conducted in collaboration with Dr. Susan L. Mayo)

Mutation and deletion of the eh1 domain of Six6 relieves repression of the transcriptional activity of GnRH-R, LHβ, and FSHβ in the LβT2 cell line

Previous research has shown the important role that the eh1 domain of the Six6 protein plays in its repressive function due to its interaction with TLE co-repressor proteins [22]. Thus, we studied the role of this domain in an *in vitro* setting via transient transfections of LβT2 cells with two Six6 mutants: one containing a *cis*-mutation in the eh1 domain (Six6-mutant) and one with a deleted eh1 domain (Six6 eh1-deletion) (Fig. 4). Results indicate that when Six6 is overexpressed in the cells there is a repression of the promoters for the GnRH-R, LHβ, and FSHβ genes. This repression is relieved when the eh1 domain of the Six6 protein is mutated (Fig. 5). Further, deletion of the eh1 domain from the Six6 protein also appears to relieve the repression of Six6 on these promoters (Fig. 6).

1 | GACAGCTCTTCCAGGCGGGAAGGCTCCCCCCGACTCTGTAACCCCCAGCCGCAC

CCCGCTCGGCCCGCGGGCATCTGCTGCGTGTCCCACCCGGGGCGCCCGCGCAGCCT

CGATGTTCCAGCTGCCCATTTTGAA**TTT**CAGCCCCCAGCAAGTAGCCGGGGTATGT

GAGACCCTGGAGGA | 180

Figure 4: Six6 sequence with eh1 domain. This figures shows part of the Six6 nucleotide sequence including the eh1 domain contained in the 116 to 177 region. In the Six6 eh1-mutant, the eh1 domain contains a *cis*-mutation that changes an F to an A (highlighted and bolded). In the Six6 mutant containing an eh1-deletion (Six6 eh1-deletion), this domain was deleted in its entirety (boxed).

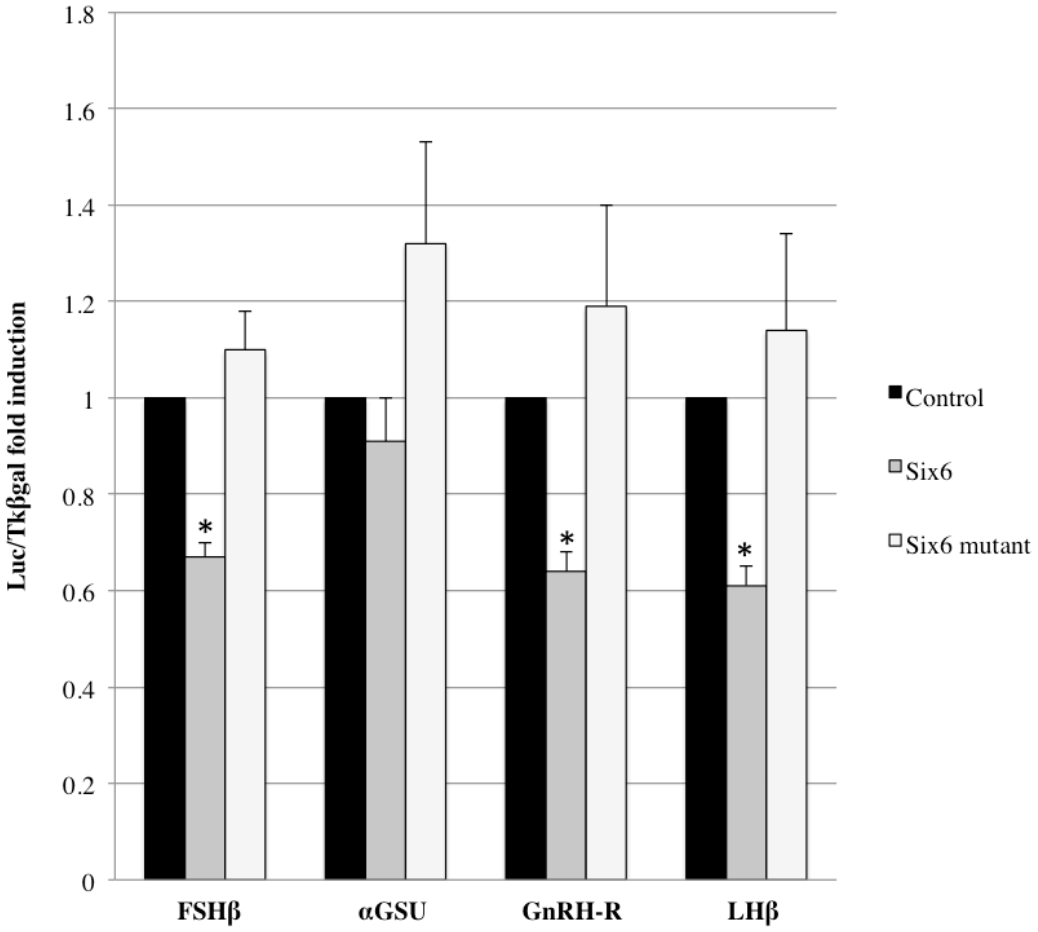

Figure 5: The Six6 eh1-mutant does not repress LHβ, FSHβ, and GnRH-R.
200 ng of Six6 expression vector or Six6-eh1 mutant expression vector were co-transfected along with the different reporter genes and pGL3 reporter (for control) into LβT2 cells. Over-expression of Six6 in LβT2 cells represses the FSHβ, LHβ, and GnRH-R promoters. The repression by Six6 of the different reporter genes is statistically significant for all (2-tail T-test *p-value<0.05). This repression is lost upon mutation of the eh1 domain of Six6. Experiments were done in triplicates and the results shown indicate an average of an N=3 study. Luc/Tkβgal data values are normalized to the values obtained for cells co-transfected with the respective reporter gene and CMV. The error bars represent the standard deviation.

(Experiments conducted in collaboration with Dr. Susan L. Mayo)

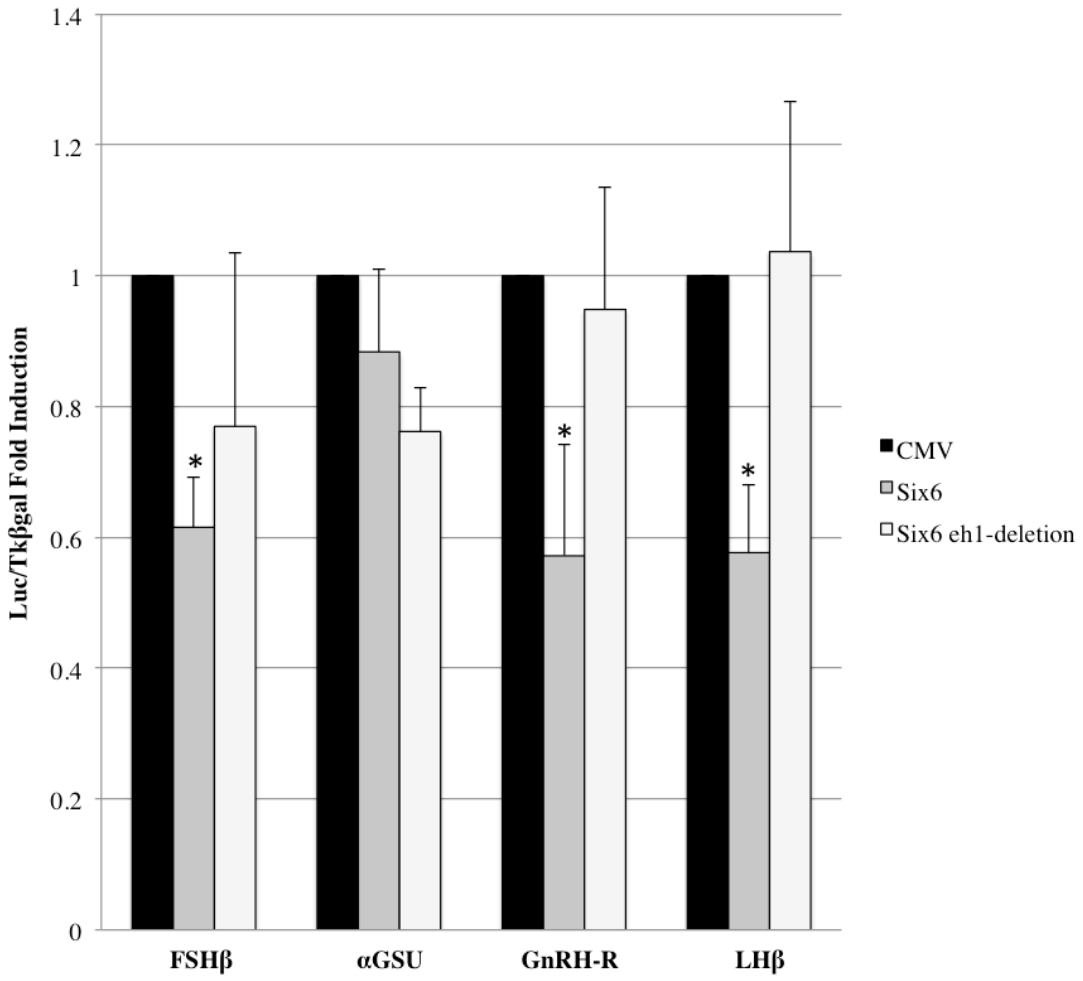

Figure 6: The Six6 eh1-deletion does not repress LHβ, FSHβ, and GnRH-R. 200 ng of Six6 expression vector or Six6 eh1-deletion expression vector were co-transfected along with the various reporter genes and pGL3 reporter (for control) into LβT2 cells. Over-expression of Six6 in LβT2 cells represses the FSHβ, LHβ, and GnRH-R promoters. This repression is lost upon deletion of the eh1 domain of Six6. The relief of repression by Six6 on GnRH-R was statistically significant (2-tail T-test p= 0.004). Results shown for FSHβ, αGSU, and LHβ are an average of an N=3 study while results for the GnRH-R reporter gene are averages of an N=4 study. Luc/Tkβgal data values are normalized to the values obtained for cells co-transfected with the respective reporter gene and CMV. The error bars represent the standard deviation.

(Experiments using FSHβ, αGSU, and LHβ and N=3 of GnRH-R conducted in collaboration with Dr. Susan L. Mayo)

Six6 repression on the GnRH-R reporter gene is relieved at the -517 bp truncation of the promoter region of GnRH-R

Observing the role of Six6 in the transcriptional control of the expression of the GnRH-R reporter gene, we aimed at finding the site on the promoter to which Six6 binds. Using various truncations of the GnRH-R reporter gene we conducted transient transfections in LβT2 cells using the Six6 expression vector and either the 1.1 kb GnRH-R reporter gene or truncations at: -840 bp, -738 bp, and -517 bp of the promoter. Results indicate that when Six6 is overexpressed, it leads to a repression in the 1.1 kb GnRH-R reporter gene as well as in the GnRH-R -840 bp that is statistically significant (2-tail T-test). However, though there appears to be a repression by Six6 in the transcriptional activity of the GnRH-R -738 bp truncation, more trials are needed to reach statistical significance for this results. Looking at the results, it is observed that the repression by Six6 on the transcriptional activity of GnRH-R appears to be relieved in the -517 bp truncation (Fig. 7). The relief of Six6 repression observed in the -517 bp truncation, when compared to the repression by Six6 on the 1.1 kb GnRH-R promoter is statistically significant with a p-value of 0.019 following a 2-tail T-test. The results indicate an average of an N=7 study conducted in triplicates.

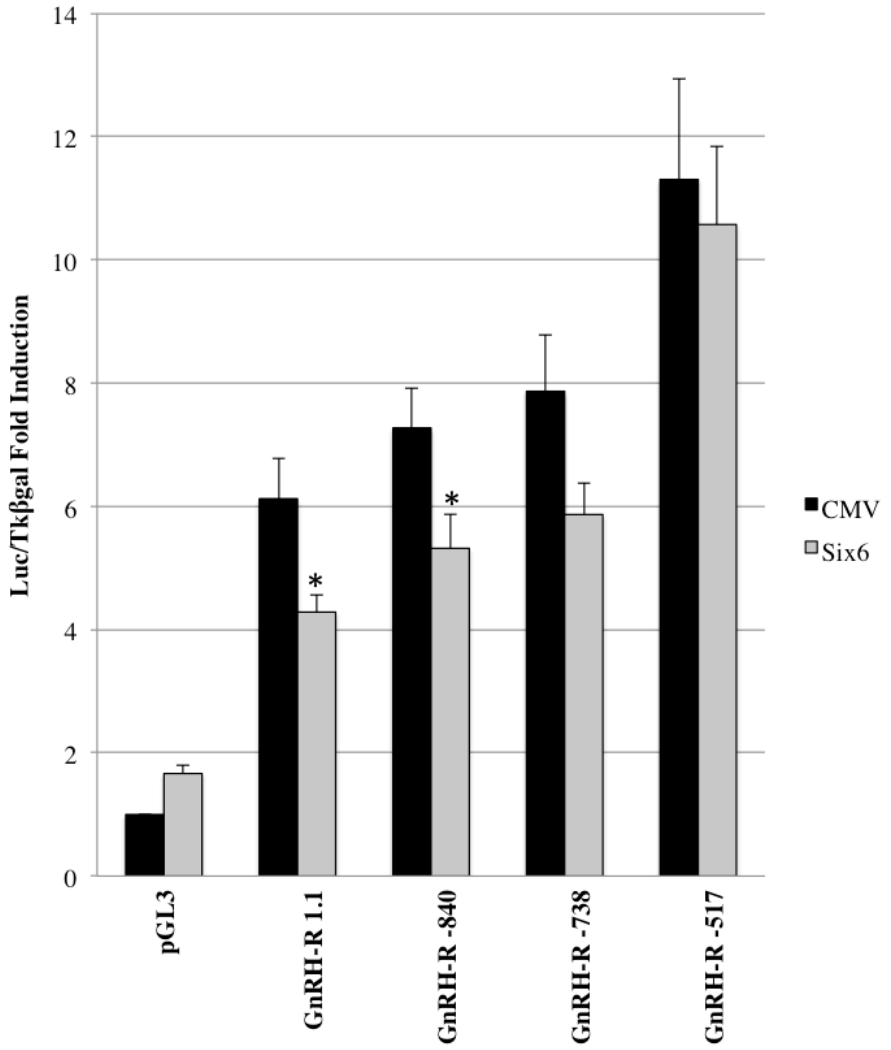

Figure 7: Relief of repression by Six6 at the -517 bp truncation of GnRH-R. 200 ng of Six6 or CMV-Flag expression vector was co-transfected along with the different truncations of the GnRH-R-luciferase reporter genes (GnRH-R) or pGL3 reporter into LβT2 cells. The Six6 expression vector was co-transfected along with the different truncations of the GnRH-R-luciferase reporter genes (GnRH-R) or pGL3 reporter into LβT2 cells. 200 ng of Six6 or CMV-Flag expression vector were used to transfect the cells. Experiments were done in triplicates and the results shown indicate an average of an N=7 study. Luc/Tkβgal data values are normalized to the values obtained for cells co-transfected with pGL3 and CMV. The repression by Six6 on the 1.1 kbp and -840bp truncation of the GnRH-R promoter is statistically significant (2-tail T-test *p-value< 0.05). The error bars represent the SEM.

Recruitment of TLE/Groucho-family related proteins in the repression of the transcriptional activity of the GnRH-R reporter gene by Six6—Grg1

With previous research showing that Grg proteins interact with transcription factors containing the eh1 motifs [22], we sought to determine the possible recruitment of these co-repressors by Six6 in its repression of the GnRH-R reporter gene. Long Grgs have been shown to enhance the repression of homeodomain proteins [24]. Thus, focusing on this subgroup of Grg proteins, we first decided to study the possible recruitment of Grg1 and Grg4 in the co-repression of GnRH-R with Six6.

Investigating the recruitment of Grg1 in the repression of GnRH-R by Six6, transient transfections in LβT2 cells were conducted using the 1.1 kb GnRH-R reporter gene along with Six6 and Grg1 expression vectors. Results indicate that when the cells are transfected with the Six6 plasmid (200 ng) along with the Grg1 expression plasmid (200 ng), there is no observable added repression than when the cells are simply transfected with Six6 (Fig. 8). Further, when the cells are transfected solely with Grg1, there is no observable repression in the transcriptional activity of the GnRH-R reporter gene. This indicates that Grg1 does not co-repress the GnRH-R promoter with the endogenous Six6 present in LβT2 cells. Thus, overall, the results suggest that Grg1 does not affect repression of GnRH-R by the Six6 protein.

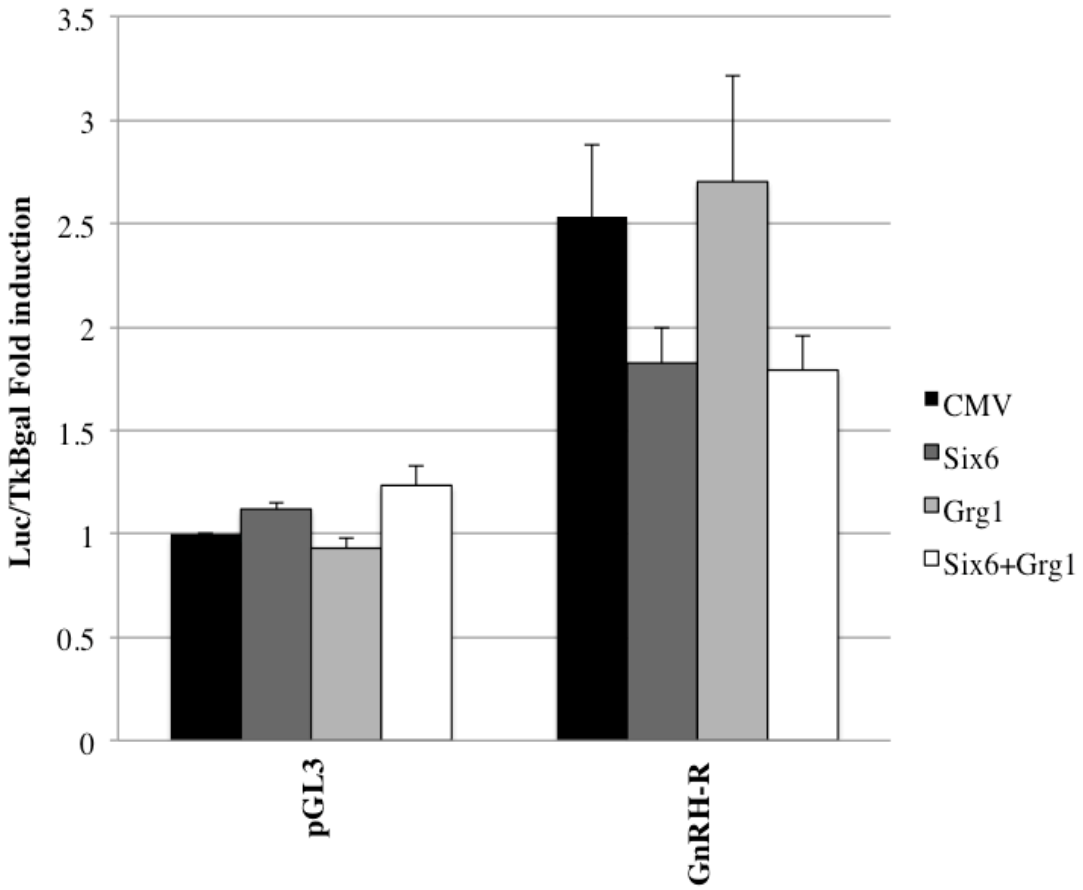

Figure 8: Grg1 does not enhance repression of GnRH-R by Six6. The Six6 expression vector was co-transfected along with the GnRH-R-luciferase reporter gene (GnRH-R) or pGL3 reporter into LβT2 cells. 200 ng of Six6 and 200 ng of Grg1 expression vectors were used to transfect the cells. Experiments were done in triplicates and the results shown indicate an average of an N=3 study. Luc/Tkβgal data values are normalized to the values obtained for cells co-transfected with pGL3 and CMV. The error bars represent the standard deviation. No statistical significance was observed by analysis with the 1-way ANOVA.

Recruitment of TLE/Groucho-family related proteins in the repression of the transcriptional activity of the GnRH-R reporter gene by Six6—Grg4

Investigating another long Grg protein, experiments were carried out using Grg4, a co-repressor protein that had been previously shown to strongly interact with mouse Six3 and Six6 in an *in vitro* setting [22]. Again, transient transfections in LβT2 cells were carried out using expression vectors for the Six6 and the Grg proteins (Grg4).

In the first condition for this experiment, cells were co-transfected with 200 ng of Six6 and 200 ng of Grg4 or simply with 200 ng of Six6 or Grg4 and empty vector. Results from this experiments indicate that cells co-transfected with Six6 and Grg4 could display a greater repression in the transcriptional activity of GnRH-R than cells simply transfected with Six6 (Fig. 9). Though this difference in repression was observable, it did not showcase statistical significance by the 1-way ANOVA test.

In the second condition, transient transfections in LβT2 cells were conducted using 200 ng of Six6 or CMV and 400 ng of Grg4. Similar to the first condition, cells transfected with Six6 did display what appears to be a decrease in the GnRH-R transcriptional activity when compared to cells transfected with CMV-Flag empty vector (Fig. 10). Co-transfection with Six6 and Grg4 led to what appears to be a greater repression of the transcriptional activity of GnRH-R than is observed in cells simply transfected with Six6. However, because the error bar is too big, these results have not shown statistical significance, no definite conclusions can be drawn regarding the recruitment of Grg4 by Six6 in the repression of the transcriptional activity of the GnRH receptor. More trials need to be done in the future.

In the third condition, 100 ng of Six6 or pGL3 was co-transfected with 400 ng of Grg4 in LβT2 cells. The results obtained from this experiment indicate that when cells are transfected with 100ng of Six6, this concentration is too low to lead to a statistically significant repression of the GnRH-R transcriptional activity (Fig. 10). Because of this, no conclusions can be drawn regarding the co-repression of Six6 and Grg4 since the low amount of Six6 makes it unable to significantly repress GnRH-R.

Thus, further trials are needed, using condition #2, to draw conclusions regarding the possible recruitment of Grg4 by Six6 in repressing the GnRH receptor.

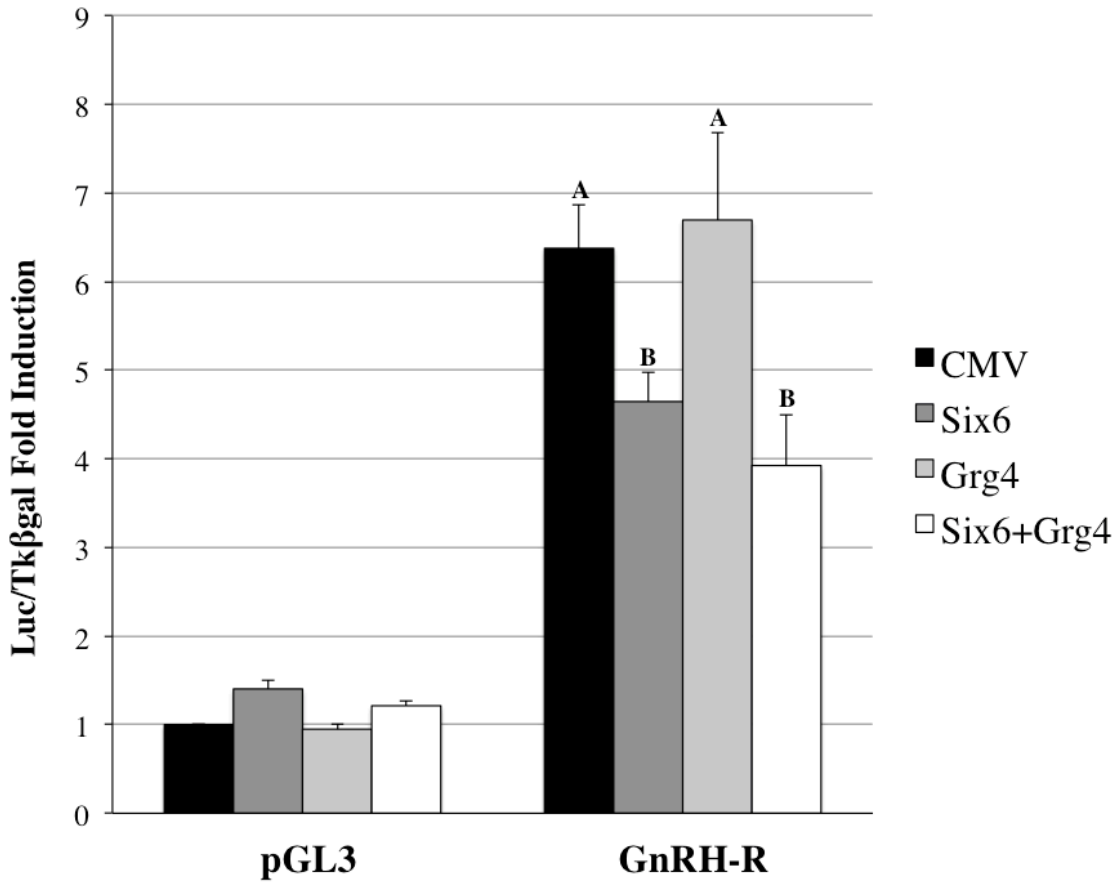

Figure 9: Grg4 possibly enhances repression of GnRH-R by Six6 (Condition #1). The Six6 expression vector was co-transfected along with the GnRH-R-luciferase reporter gene (GnRH-R) or pGL3 reporter into LβT2 cells. 200 ng of Six6 and 200 ng of Grg4 expression vectors were used to transfect the cells. Experiments were done in triplicates and the results shown indicate an average of an N=3 study. Luc/Tkβgal data values are normalized to the values obtained for cells co-transfected with pGL3 and CMV-Flag. Groups labeled with different letters are significantly different from each other (p-value< 0.05). The error bars represent the standard deviation.

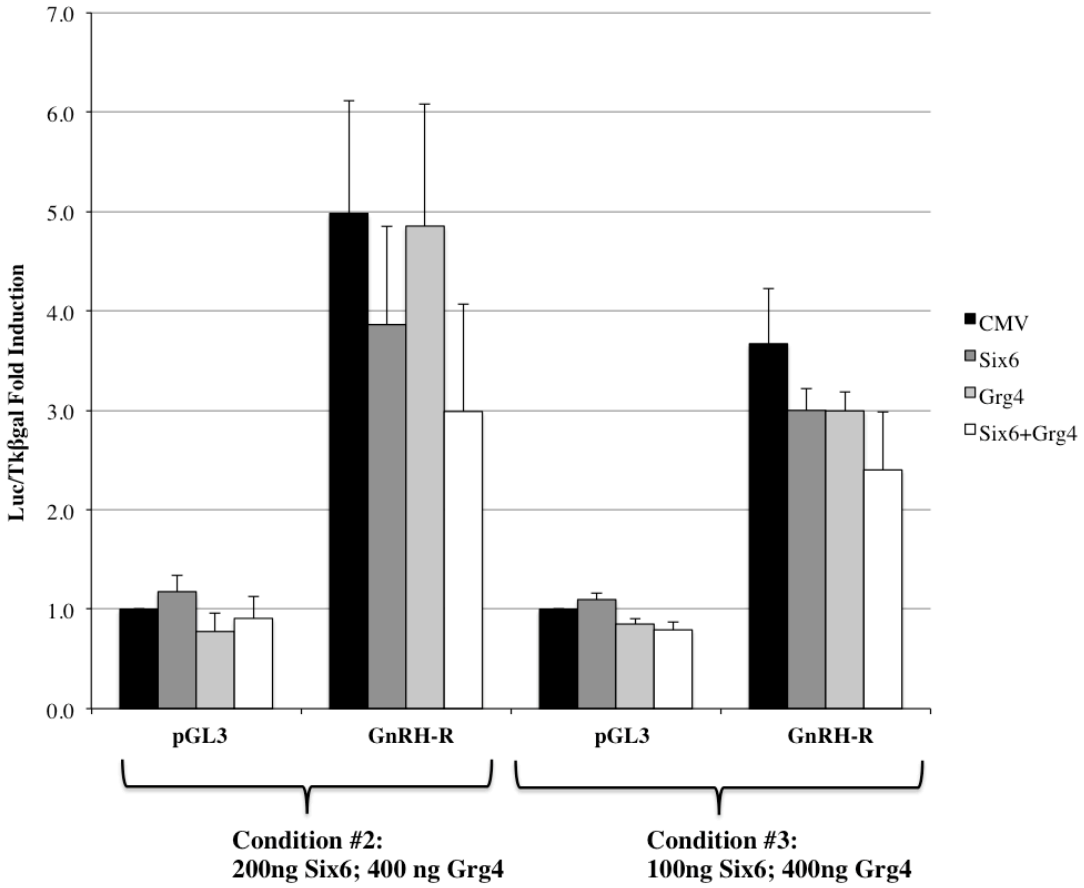

Figure 10: Grg4 possibly enhances repression of GnRH-R by Six6 (Conditions #2, 3). Over-expression of Six6 in LβT2 cells represses the GnRH-R promoter (Conditions #2 and #3). Co-transfection experiments with Six6 and Grg4 were carried out with two conditions—condition #2 and #3—that differ in the concentration of DNA used. In condition #2, 200 ng of Six6 or pGL3 were co-transfected with 400 ng of Grg4 expression vector. In condition #3, 100 ng of Six6 or pGL3 were co-transfected with 400 ng of Grg4. Both conditions represent an N=3 study where experiments were conducted in triplicates and the Luc/Tkβgal values normalized to pGL3/CMV. Data were analyzed using 1-way ANOVA but no statistical significance was found. The error bars represent the standard deviation.

Electrophoretic Mobility Shift Assay

Previous work has demonstrated that the Six proteins Six3 and Six6 bind to the classical homeodomain core sequence ATTA [22, 33]. Thus, characterizing the 1.1 kb GnRH-R promoter sequence, several ATTA sites were identified. Having confirmed the relief of repression by Six6 on the transcriptional activity of the GnRH-R promoter when it is truncated between the -740 bp and the -517 bp promoter region sites, we analyzed the mouse GnRH-R promoter sequence and identified nine ATTA sites located in this promoter region (Fig. 11).

To determine whether Six6 showed affinity for these ATTA binding sites, we conducted electrophoretic mobility shift assays using radioactively labeled probes containing the various ATTA sites. The DNA probes used for the experiment consisted of radiolabeled oligonucleotide containing the ATTA sites identified at -735, -728, -721, -713, -630, and -605 in the mouse GnRH-R promoter. Four radioactively labeled probes were used for the EMSAs: one containing the ATTA sites at -735 bp and -728 bp (labeled "-740" probe), one containing the sites at -721 bp and -713 bp (labeled "-726"), one containing the site at -630 bp (labeled "-627"), and one containing the site at -605 bp (labeled "-600") (Fig. 11, Table 1). In the experiment, nuclear protein extracts from COS cells transfected with either CMV-Flag reporter plasmid or pCMV-Six6-Flag were incubated with the individual probes.

A positive control was also used in the experiment, this consisted of a radiolabeled oligonucleotide containing an ATTA site at -1635 bp of the rat GnRH regulatory region [17]. Results show that a complex corresponding to Flag-tagged Six6 is observed in the -1635 radiolabeled probe after incubation with extracts from COS cells

transfected with pCMV-Six6-Flag but not with extracts from cells transfected with the empty CMV-Flag vector (Fig. 12, lanes 1 and 2). This complex is super-shifted or completely removed when a Flag antibody is added, however, it is not affected when a non-specific antibody (mouse IgG) is used [17]. Moreover, addition of a 500-fold excess of unlabeled, WT probe significantly decreased the formation of the complex (Fig. 12, lane 3).

Looking at the -740, -627, and -600 radiolabeled probes, no complex appears to form upon incubation with COS cells transfected with either pCMV-Six6-Flag or empty CMV-Flag vector (Fig. 12, lanes 4, 5, 10, 11, 13, and 14). This perhaps indicates the lack of affinity of Six6 for the ATTA sites found at in this mouse GnRH-R promoter region.

Looking at the -726 radiolabeled probe we do see a complex forming but this does not correspond to Six6 as it appears upon incubation with COS cells transfected with either pCMV-Six6-Flag or empty CMV-Flag vector (Fig. 12, lanes 7 and 8). Moreover, the complex does not match the size of Six6 as seen in the positive control used (Fig. 12, lane 2).

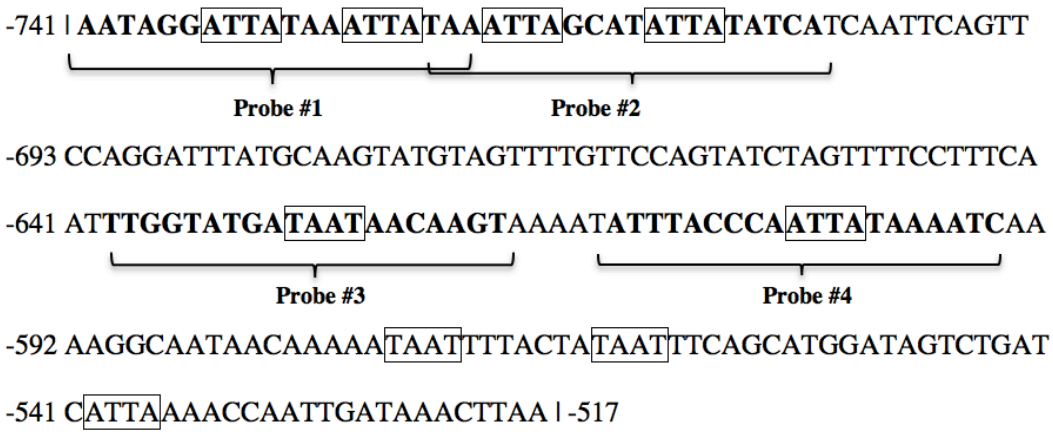

Figure 11: ATTA sites between -517 bp and -741 bp on GnRH-R promoter. GnRH-R reporter gene sequence displaying ATTA sites contained in the -517 bp to -741 bp promoter region. Figure displays the sequences of the oligonucleotide probes used for the electrophoretic mobility shift assay experiments. Probe #1 is also labeled as the "-740 bp" probe, probe #2 is labeled as the "-726 bp" probe, probe #3 is labeled as the "-627 bp" probe, and probe #4 is labeled as the "-600 bp" probe.

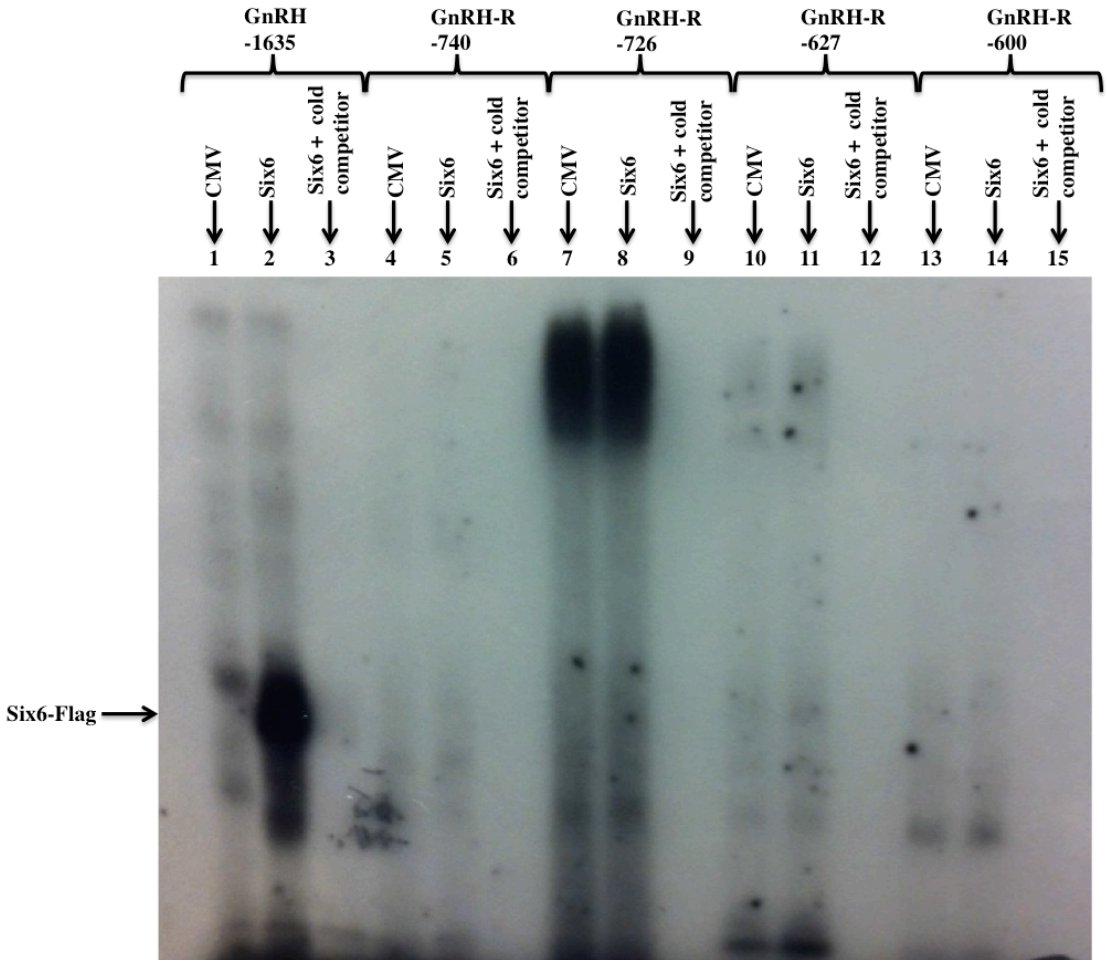

Figure 12: Six6 does not bind to several of the ATTA sites in the -517 bp to -741 bp GnRH-R promoter region. An EMSA was performed using nuclear extracts from COS cells that were transfected with either CMV-Flag (lanes 1, 4, 7, 10, and 13) or pCMV-Six6-Flag (all other lanes). The DNA probes consisted of radiolabeled oligonucleotides containing the ATTA sites identified at -735, -728, -721, -713, -630, and -605 in the mouse GnRH-R promoter. A probe containing the ATTA site at -1635 bp of the rat GnRH promoter was used as a positive control (lanes 1, 2, and 3). A 500-fold excess of unlabeled WT probe was included in the reaction mixtures (as indicated) in lanes 3, 6, 9, 12, and 15. With the exception of the positive control, no Six6-Flag complex formation is observed in any of the probes (lane 2, 5, 8, 11, and 14).

IV

Discussion

As the topic of infertility continues to remain at the forefront of much of today's research, a lot of focus has been placed on the hypothalamic-pituitary-gonadal axis and its functioning. A key player in this axis and the proper progression of development and fertility is GnRH and its receptor, GnRH-R. Both the hormone and its receptor have intricate mechanisms and thus elucidating these holds the key to much of the progress in this field.

In this specific project, we aimed to further our understanding of the GnRH receptor by studying the important regulatory role of the Six6 transcription factor on this promoter as well as other promoters such as LHβ, FSHβ, and αGSU.

Past data has shown the crucial role played by Six6 in the proper development of the eye and pituitary [34]. Moreover, *in vivo* studies have shown Six6 to be an important hypothalamic regulator of fertility in mice as mice lacking the Six6 protein display a striking decrease in fertility [17].

Studying the effect of Six6 on various promoter genes in the pituitary, the LβT2 cell line was used. Overexpression of Six6 in these cells led to a significant decrease in the transcription of the LHβ, FSHβ, and GnRH-R promoter genes. However, transcription of the αGSU promoter gene did not appear to be repressed by Six6 when transient transfection experiments were conducted.

Trying to understand the role of the eh1 domain of Six6 in its repressive function on the various promoters, two Six6 mutants were constructed by Rachel Larder—one containing a *cis*-mutation in the eh1 domain region (Six6-mutant) and one with a deleted eh1 domain (Six6 eh1-deletion). Transient transfection with these plasmids, along with the Six6 expression vector, showed the importance of this domain in the repressive action

of Six6 on the various promoters. In fact, transfections with either the Six6-mutant or the Six6 eh1-deletion led to a relief of the repression on LHβ, FSHβ, and GnRH-R that was observed in cells transfected with the wild-type Six6.

Focusing our attention on the GnRH-R promoter, we then aimed to identify the region to which Six6 binds and represses the transcriptional activity of the promoter gene. Using various truncations of the GnRH-R promoter, transient transfections revealed that while Six6 represses GnRH-R transcriptional activity in the full-length, -840 bp, and -738 bp truncations, the repression by Six6 is relieved between the -738 bp and -517 bp truncations. This indicates that the site at which Six6 binds and represses the GnRH-R activity could be contained in the region between -738 bp and -517 bp of the promoter. This N=7 study did display statistical significance in the repression by Six6 observed in the full-length GnRH-R promoter as compared to that seen in cells transfected with the -517 bp truncation. However, the large standard deviation has led to this project being on-going as transient transfections with the various truncations are being carried out in other LβT2 cells. A shorter truncation of the GnRH-R promoter will also be used in other transfection—this truncation would be downstream of the -517 bp truncation and it would eliminate an additional ATTA site.

With past data showing the binding of Six6 to ATTA sites, we analyzed the region between -738 and -517 bp on the GnRH-R promoter for that sequence [22, 33]. Within this region, nine ATTA sites were identified. Using electrophoretic mobility shift assays, radiolabeled oligonucleotide probes containing the various ATTA sites were then incubated with COS cells transfected with either CMV-Flag or pCMV-Six6-Flag. The data gathered does not suggest that Six6 binds to the ATTA sites used to construct the

various oligonucleotides. In fact, no Six6 complex was observed in any of the probes besides the -1635 probe used as a positive control. Because this is an N=1 study, this could be due to experimental error or it could indicate that Six6 does not show affinity for any of these ATTA sites. Rather, Six6 could be binding to one, or all, of the three ATTA sites that are also present in the region between -738 bp and -517 bp (but were not used in prior work). Future experiments using electrophoretic mobility shift assays, will aim at localizing Six6 binding on the GnRH-R promoter and will thus include the current probes as well as oligonucleotides including the other three ATTA sites identified in the -738 bp to -517 bp region of the promoter.

The TLE/groucho-related family of proteins has been shown to act as co-repressors of a variety of transcription factors containing an eh-1 motif [22, 24]. Looking at Grg1, transient transfections with the Six6 plasmid and Grg1 in LβT2 did not appear to lead to an increase in the repression of Six6 on the GnRH-R transcriptional activity. Moreover, transfection of cells with only Grg1 did not appear to repress the GnRH-R transcriptional activity. Together, these findings point at the conclusion that Grg1 does not act as a co-repressor of Six6 in the regulation of GnRH-R transcription.

Focusing on another "long" Grg, transient transfections were carried out in LβT2 cells using the Six6 plasmid and Grg4. For this experiment, three different conditions were used—each using a different ratio of Six6 plasmid to Grg4 plasmid (condition #1- 1:1; condition #2- 1:2; condition #3- 1:4). In conditions #1 and #2, it appears that transfection with the Six6 plasmid reduces GnRH-R transcriptional activity. However, due to the low amount of Six6 used for transfection in condition #3, no significant repression on the transcriptional activity of GnRH-R could be observed. Moreover, from

the data collected in Condition #2, it appears that this repression on GnRH-R could be enhanced by co-transfection of Grg4 with Six6—a conclusion that cannot yet be drawn due to the lack of statistical significance in the present results. Thus, overall, what the transient transfections using Six6 and Grg4 indicate is that Six6 could in fact be interacting with Grg4 to repress the transcriptional activity of the GnRH receptor. However, more trials need to be conducted to confirm these hypothesized results.

In conclusion, this thesis project has identified Six6 as an important regulator of transcriptional activity of a variety of promoters that are crucial to the proper functioning of the hypothalamic-pituitary-gonadal axis and, as a result, fertility and development. The action of Six6 on the LHβ, FSHβ, and GnRH-R promoters, appears to require the eh-1 domain of Six6—without which repression is lost. For the GnRH receptor, this repression by Six6 is hypothesized to be enhanced by the recruitment of Grg4. Conducting a variety of studies, we have narrowed the region of Six6 binding on the GnRH-R promoter to -738 bp to -517 bp. This region contains several ATTA sites that are currently being studied via electrophoretic mobility shift assays to try to identify the exact site used by Six6 to repress the promoter. Overall, continuing this project, we will further study the action of Six6 on GnRH-R as well as other promoters so as to further elucidate the intricate mechanisms used by Six6 to affect fertility and development. These findings could then have possible applications in a medical setting to try to better understand and treat various cases of hypogonadotropic hypogonadism and infertility.

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