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Anteroventral Periventricular *Kiss1* Gene Expression is Regulated by Estradiol through Estrogen Response Elements

A thesis submitted in partial satisfaction of the requirements
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in

Biology

by

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2020

The Thesis of Teresa Chou is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2020

DEDICATIONS

I would like to dedicate my thesis to:

My family, for their endless love and encouragement.
Ryan, for supporting me at every step of my journey
and
Berthie, for lifelong friendship.

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ABBREVIATIONS

ARC	Arcuate Nucleus
AVPV	Anteroventral Periventricular Region
DMEM	Dulbecco's Modified Eagles Media
DPBS	Dulbecco's Phosphate Buffered Saline
E2	17β-estradiol
E2 + ERα	E2-bound ERα
ER	Estrogen Receptor
ERE	Estrogen Response Element
EtOH	Ethanol
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
HPG axis	Hypothalamic Pituitary Gonadal Axis
LH	Luteinizing Hormone
mKiss-Luc	*Kiss1* promoter-driven luciferase reporter plasmid
Tk-βgal	Thymidine kinase promoter-driven βgal expression plasmid
TSS	Transcriptional Start Site

ACKNOWLEDGMENTS

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ABSTRACT OF THE THESIS

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Master of Science in Biology

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Professor Pamela L. Mellon, Chair
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In females, estrogen signaling is necessary for coordinating ovulation and the menstrual cycle. *KISS1*, a gene expressed by kisspeptin neurons and critical for reproduction, is positively and negatively regulated by 17β-estradiol (E2), an endogenous estrogen. E2 regulates gene transcription through estrogen receptor α (ERα), but the exact mechanism of *KISS1* gene regulation by E2 and ERα remains unclear. I identified key regions for ERα binding and regulation on the mouse *Kiss1* promoter by creating 5'

deletions of a *Kiss1*-reporter plasmid in kisspeptin neuronal cell cultures. Within these regions, I created *cis*-mutations of putative EREs, revealing one imperfect ERE sequence on the *Kiss1* promoter that contributes to ERα regulation in anteroventral periventricular (AVPV) kisspeptin cells. These findings provide further support that E2 bound to ERα acts through an ERE-dependent mechanism for positive feedback in AVPV kisspeptin neurons, providing insight to the molecular mechanism that drives ovulation in females.

INTRODUCTION

The hypothalamic-pituitary-gonadal (HPG) axis is the neuroendocrine pathway responsible for regulating reproduction. Reproductive neuroendocrinology describes the hormone signaling that regulates reproductive development and coordinates reproductive events, allowing species to survive by passing down their genetic information. In females, hormone signaling underlies critical reproductive events, such as the menstrual cycle and ovulation (1). Hormonal imbalances in the HPG axis contribute to reproductive disorders such as infertility, hypogonadism, and irregular menstruation (2). A deeper understanding of the HPG axis and mechanisms of neuroendocrine signaling will provide the knowledge to discover better treatment targets and therapeutics.

The HPG axis, depicted in Figure 1, consists of three regions that use hormones to communicate: the hypothalamus, a brain region important for sleep, hunger, temperature control, and sexual reproduction; the pituitary gland, which releases critical hormones throughout the body; and the gonads (ovaries or testes), which are the site of sperm and egg maturation (3). Within the hypothalamus, kisspeptin neurons produce and secrete the neuropeptide kisspeptin, stimulating gonadotropin-releasing-hormone (GnRH) neurons. In turn, activated GnRH neurons secrete GnRH onto the gonadotrope cells of the anterior pituitary gland, stimulating the pulsatile release of gonadotropins (3). The gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), travel through the bloodstream to the ovary or testis, stimulating the production and secretion of sex steroid hormones. Estrogens, androgens, and progestins are sex steroid hormones, and their widespread travel through the bloodstream and lipid solubility allow them to alter gene transcription at many tissue targets (1). Importantly, 17β-estradiol (E2),

a natural estrogen produced by the ovaries, feedbacks onto the hypothalamus and pituitary, regulating the expression and release of upstream HPG axis hormones to coordinate female reproductive events, such as ovulation and the menstrual cycle (1).

Kisspeptin neurons are critical regulators of the HPG axis because of their ability to mediate the E2 feedback that is essential for reproduction; that is, maintaining basal GnRH secretion as well as initiating ovulation in females (4). Kisspeptin neurons are found within two regions of the hypothalamus—the arcuate nucleus (ARC) and the anteroventral periventricular region (AVPV) (5). The ARC and AVPV kisspeptin neurons differ in function, as the *Kiss1* gene, which encodes for the neuropeptide kisspeptin, in these two neuronal populations is differentially regulated by E2. Studies using qPCR to quantify the *Kiss1* mRNA in female mice demonstrated that E2 inhibits *Kiss1* expression in ARC kisspeptin neurons but stimulates *Kiss1* expression in AVPV kisspeptin neurons (6). This dichotomy is physiologically relevant: E2 inhibition in ARC kisspeptin neurons is necessary to regulate basal GnRH pulsatile release, and thus reproduction in both males and females (7). On the other hand, E2 stimulation in AVPV kisspeptin neurons is required for producing the LH surge necessary for ovulation in females (8). Notably, AVPV kisspeptin neurons exhibit sexual dimorphism, as male mice, who do not ovulate, have fewer AVPV kisspeptin neurons and express less AVPV *Kiss1* mRNA than females (8). The specific mechanism by which ARC and AVPV kisspeptin neurons mediates E2 feedback requires further study.

As a steroid hormone, E2 exerts its transcriptional effects by binding to estrogen receptor (ER). ER is a nuclear transcription factor of the steroid receptor family and is thought to act through two mechanisms: the classical and nonclassical mechanisms. In

the classical mechanism, the ligand-bound ER (E2-ER) readily dimerizes and translocates to the nucleus, directly binding to sequence motifs called estrogen response elements (EREs) (9). In the nonclassical mechanism, E2-ER does not directly bind DNA, but is instead recruited by other DNA-bound proteins in a process called "tethering" (9,10). Via these mechanisms, E2 and ER can alter gene expression at target tissues, although which specific mechanisms are involved in regulating the HPG axis needs further study. While both subtypes of ER, α and β, are expressed throughout the body, only ERα is necessary to produce the LH surge and generate basal GnRH secretion that are critical to reproduction (11). In the HPG axis, the kisspeptin neurons are a key target of E2 as they express ERα, unlike the GnRH neurons which they directly innervate (11).

In vivo studies have suggested that ARC and AVPV kisspeptin neurons mediate E2-ERα feedback through nonclassical and classical nuclear receptor action, respectively, but these mechanisms have not been confirmed *in vitro*. In mutant mice that either lack ERα completely or express ERα without the DNA-binding domain, E2 is no longer able to stimulate AVPV kisspeptin neuron activation, as indicated by *c-Fos* expression (12,13). This supports the hypothesis that ERα directly binding to ERE sequences on DNA is necessary for E2-ERα induction in the AVPV kisspeptin neurons. However, in the mutant mice that express ERα without the DNA-binding domain, E2 can still repress ARC kisspeptin neuron activation, suggesting that E2-ERα repression in the ARC kiss neurons may not require direct ERE binding alone, but may also involve a nonclassical tethering mechanism (12,14). However, these mutant mouse lines—either lacking ERα completely or expressing ERα without the DNA binding domain—may not be the best model, as functional ERα may be important for kisspeptin neuron development (15).

Thus, the mouse lines may exhibit reproductive defects due to incorrect development, masking the true functionality of the kisspeptin neurons. Instead, an *in vitro* approach can be used to characterize the specific regulatory actions of E2-ERα on the *Kiss1* promoter.

To elucidate the mechanism in which E2-ERα regulates *Kiss1* in ARC and AVPV kisspeptin neurons, functional EREs on the *Kiss1* promoter must be identified *in vitro*. Luciferase reporter gene plasmids present a useful approach to investigate these E2-ERα regulatory sites on the *Kiss1* promoter, as DNA plasmids are easily manipulated, and transcriptional activation is easily quantified through luciferase light production. Previous studies have used reporter plasmids to locate EREs on various gene promoters, but this has not been done for *Kiss1* (16). While the established consensus sequence of an ERE is 5'-GGTCAnnnTGACC-3' (where n is any nucleotide), functionally imperfect EREs that differ by one to three bases have been discovered in multiple genes (16). Based on the known consensus sequence, previous work in our lab has identified 3 putative imperfect EREs on the mouse *Kiss1* promoter (17). However, it has not been confirmed whether ERα can actually regulate *Kiss1* transcription through these EREs.

This research aims to identify functional EREs on the mouse *Kiss1* promoter that mediate E2-ERα feedback in AVPV kisspeptin neurons. To study this, I used the immortalized kisspeptin cell lines KTaV-3, which was derived from the AVPV of an adult female mouse (18). The cells were transfected with a plasmid containing the *Kiss1* promoter fused directly upstream of a luciferase reporter gene (mKiss-Luc), allowing transcriptional activation of the *Kiss1* promoter to be quantified using a luciferase assay (19). To confirm that the immortalized cells recapitulate the physiological response to E2, I established that E2-ERα can induce transcription of the mKiss-Luc reporter in

KTaV-3 cells. The *Kiss1* promoter on the mKiss-Luc plasmid was then manipulated *in vitro* via deletions and point mutations. Systematic deletions from the 5' end of the *Kiss1* promoter identified promoter regions important for E2-ERα regulation. Within these key regions, I performed point mutations of the putative EREs to disrupt ERα binding. Ultimately, this will elucidate whether the putative EREs on the *Kiss1* promoter are sufficient for E2-ERα regulation in AVPV kisspeptin neurons.

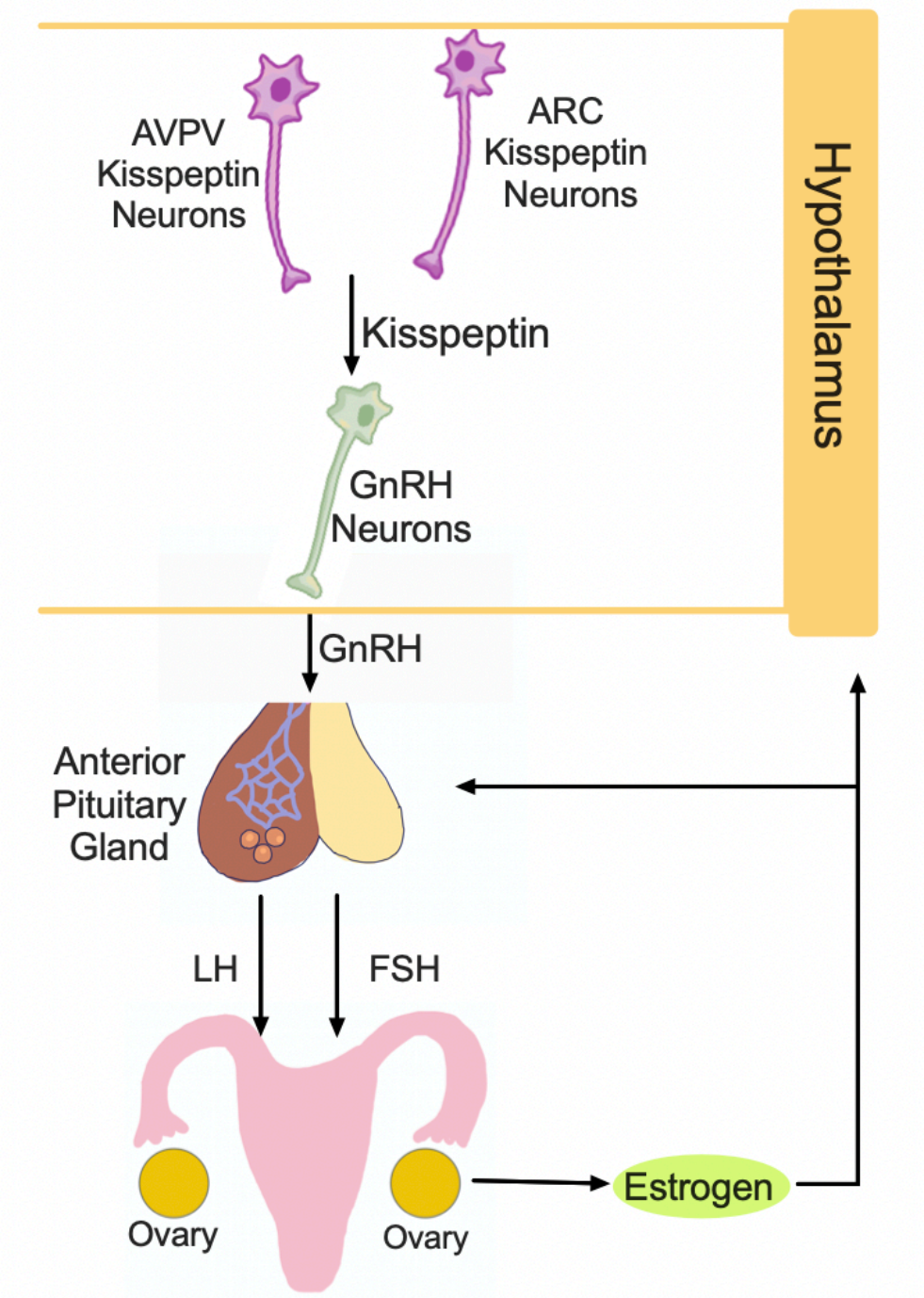


Figure 1. Schematic of the HPG axis. Kisspeptin neurons in the anteroventral periventricular region (AVPV) and arcuate nucleus (ARC) of the hypothalamus release the neuropeptide, kisspeptin onto gonadotropin-releasing hormone (GnRH) neurons. GnRH neurons release GnRH, stimulating the release of LH and FSH from the anterior pituitary gland. LH and FSH stimulate the ovaries to produce estrogen, which can feedback onto the hypothalamus and pituitary gland to regulate the release of upstream hormones.

MATERIALS AND METHODS

Cell Culture

The immortalized KTaV-3 cell line, kindly provided by Dr. Patrick Chappell (Oregon State University), was derived from the AVPV region of an adult female mouse. The cells were maintained in complete media, consisting of DMEM supplemented with 10% fetal bovine serum (Omega) and 1% penicillin-streptomycin, and incubated at 37 degrees Celsius with 5% CO_2. Cells were passaged at 80-90% confluency by washing with DPBS and resuspending in trypsin. Cells were then diluted and seeded in 10 mL complete media, which also deactivates the trypsin.

Plasmids

Transfection experiments were performed using a plasmid containing 4058 base pairs upstream and 455 base pairs downstream (-4058/+455) of the mouse *Kiss1* transcriptional start site (TSS), which drives the expression of a luciferase reporter gene on a pGL4.10 backbone (Fig. 2A). This plasmid (mKiss-Luc) was kindly provided by Dr. Steven Kliewer (UT Southwestern). To account for transfection efficiency, all cells were co-transfected with a reporter plasmid containing β-galactosidase driven by the Herpes virus thymidine kinase promoter (TK-βgal). An ERα expression vector in pcDNA3.1 backbone was used to overexpress ERα in the cells.

Deletions and *cis*-mutations of the mKiss-Luc plasmid were made according to the Q5 Site-Directed Mutagenesis kit using primers designed with the NEBase Changer tool (New England BioLabs). The primers used to create broad deletions of the *Kiss1* promoter resulted in five plasmid constructs, named after the base pairs upstream and downstream of the *Kiss1* TSS: -3877/+455, -3668/+455, -2558/+455, and -1711/+455,

which truncate the *Kiss1* promoter from the 5' end; and ∆-2706_-580/+455, which deletes the putative intergenic region of the *Kiss1* promoter (Table 1). The primers used to create *cis*-mutations of the putative EREs resulted in three plasmids, named after the ERE mutated: µERE a, µERE b, and µERE c (Table 2). The TRANSFAC database was used to predict that the mutated ERE sequences would prevent binding of ERα and other known transcription factors (20).

Plasmids were transformed and amplified in DH5α competent cells and isolated using Qiagen Maxiprep kits. Plasmid sequences were confirmed by Eton Biosciences sequencing.

Table 1. Site-directed mutagenesis primers used to create mKiss-Luc plasmid constructs. Forward and reverse primers were designed for site-directed mutagenesis to create deletions of the mKiss-Luc plasmid. Constructs are named after the base pairs upstream and downstream of the *Kiss1* TSS.

mKiss-Luc Plasmid Construct	**SDM Primer Sequence 5'-3'**
-1711/+455	F: GGC TGG CCT CGA ACT CAG R: GGT ACC GGC CAG TTA GGC
-2558/+455	F: GGC ATG TGT GTT GCT TGT GG R: GGT ACC GGC CAG TTA GGC
-3307/+455	F: TGA TCA GGG AGC CAG ATA GAG R: GGT ACC GGC CAG TTA GGC
-3668/+455	F: CTC CAG CCT GTG TCG TCA TC R: GGT ACC GGC CAG TTA GGC
-3877/+455	F: GCT TGT CAA ACC CCT GTG TC R: GGT ACC GGC CAG TTA GGC
Δ-2706_-580/+455	F: GCT CCC AAG AGA ATA GCT G R: TTA TGT GGT CTG TCA TGG

Table 2. Site-directed mutagenesis primers used to create *cis*-mutations of putative EREs on mKiss-Luc plasmid. Plasmids were designed using NEBase Changer. Cis-mutations to each ERE are denoted in red. Mutated sequences were checked via TRANSFAC database to confirm no unintended binding targets.

ERE	**Sequence** **5’ -3’**	**Mutation (μ)** **5’ -3’**	**SDM Primer Sequence** **5’ -3’**
ERE a	GAGGCCAATCTGACCTG	ATAAAAAATCTAAAAGA	F: CTAAAAGACTTTCTTTCATGTGGGCACTG R: ATTTTTACACCAGAGGTGGCTCCTG
ERE b	TGGGTCAGACTGTGCCT	ATAAAAAGACTAAAAGA	F: CTAAAAGATGCTCCTCCTCCTCCAGC R: TCTTTTATGAGGCTCCTCAGTGCCCA
ERE c	TCCCTCATCGTGACCTC	ATAAAAAAGCTAAAAGA	F: CTAAAAGGTTTAGAGAAACCAGGAGGCG R: CTTTTTTATGGAAGGGGAGGGGAGGTT

Transient Transfections

Cells were transfected using PolyJet reagent following the manufacturer's protocol. 500 ng of the full length mKiss-Luc plasmid construct was transfected per well. Although ERα is endogenously expressed in both the KTaV-3 and KTaR-1 cell lines, the cells were co-transfected with a pcDNA3.1 vector containing the gene coding ERα. Cells transfected with the mKiss-Luc plasmids and/or ERα were matched with cells transfected with the empty backbones; pGL4.10 and pcDNA3.1, respectively. To account for transfection efficiency, all cells were co-transfected with TK-βgal. Within each biological replicate, transfections were performed in triplicates.

Hormone Treatments

Solid 17β-estradiol (E2) was dissolved in 100% ethanol and diluted to 1 mM and 1 μM stock concentrations. Immediately before treating the cells, hormone treatments were made through a 1:1000 dilution of E2 stock in either serum-free or charcoal-stripped DMEM, and the same volume of EtOH was used as a vehicle treatment. Media was aspirated from cells and replaced with 1 mL of treatment: either 1 nM E2, 1 μM E2, or vehicle.

Luciferase Assays

Following hormone treatment, KTaV-3 cells were harvested for a luciferase assay. Media was aspirated off wells and washed with 1X DPBS. Cells were lysed and transferred to two separate 96-well plates for the luciferase reaction and the internal control TK-βgal reaction. Luciferase reaction buffer, which contains TC water, $MgSO_4$, TRIS, luciferin, and ATP was injected into each well of the luciferase reaction plate in the GLOMAX Luminometer, which measured light absorbance of each well directly after

injection. TROPIX TK-βgal reagents were added to the TK-βgal 96-well plate and incubated for 30-60 min. The TROPIX Accelerator reagent was injected into the incubated TK-βgal reaction plate, and absorbance of each well was measured. For all experiments, luciferase expression is normalized to the TK-βgal expression.

Sequencing the mKiss-Luc plasmid product

KTaV-3 cells were plated at 30,000 cells per well in complete DMEM and transfected for 24 hours. KTaV-3 cells were harvested and total RNA extracted using TRIzol (Invitrogen). RNA was isolated using RNA Clean and Concentrator Kit (ZYMO). RNA was converted to cDNA through reverse transcription in the iScript cDNA Synthesis Kit (Bio-Rad). Polymerase chain reaction was performed on the cDNA, using a forward primer located in the putative noncoding exon (5'-AGAGTAAGCCCAGGAGCCAGTGGC-3') and a reverse primer located in the luciferase reporter gene (5'-AAAGCCACCATGGAAGATGCCA-3'), using an annealing temperature gradient. Polymerase chain reaction products were separated on a 2% agarose gel via gel electrophoresis, and distinct bands were excised and extracted using the QIAquick Gel Extraction Kit (Qiagen). Extracted DNA was sequenced by Eton Biosciences sequencing.

Promoter Truncation Assays

KTaV-3 cells were plated at 30,000 cells per well in complete DMEM. One day after plating, cells were transiently transfected with the following: either mKiss-Luc, a truncated mKiss-Luc plasmid, or the empty PGL4.10 backbone; ERα or empty pcDNA3.1; and TK-βgal as described above. To account for size differences of truncated promoter plasmids, transfection treatments were adjusted with the addition of an empty

PCS2 vector plasmid so that all cells were transfected with the same molar concentration of DNA. All cells were transfected for 6 hours. Following transfection, cells were serum-starved in serum-free DMEM for 12 hours. Cells were then hormone treated with vehicle or 1μM E2 in serum-free media for 24 hours.

μERE Assays

KTaV-3 cells were plated at 25,000 cells per well in charcoal-stripped DMEM. All cells were transfected for 24 hours. One day after plating, cells were transiently transfected with the following: either -4058/+455 mKiss-Luc, -1711/+455 mKiss-Luc, μERE mKiss-Luc plasmid, or the empty PGL4.10 backbone; ERα or empty pcDNA3.1; and TK-βgal as described above. To account for size differences of the -1711/+455 plasmid, transfection treatments were adjusted with the addition of an empty PCS2 vector plasmid. All cells were transfected for 24 hours, directly followed by a 48-hour hormone treatment with vehicle or 1 nM E2 in charcoal-stripped media.

RESULTS

Determining the structure of the mKiss-Luc plasmid

The mKiss-Luc plasmid contains 4058 base pairs upstream and 455 base pairs downstream of the *Kiss1* transcriptional start site (-4058/+455), and three putative estrogen response elements (ERE) have been identified in the upstream region (Fig. 2A). Based on UCSC Genome Browser, a *Kiss1* isoform contains an 84 base-pair noncoding exon located at -3291 to -3208 (21). To determine the structure of the mKiss-Luc plasmid, KTaV-3 cells were transfected with the mKiss-Luc plasmid. RNA was isolated from the cells and converted to cDNA. Polymerase chain reaction was performed on the cDNA, using a forward primer located in the putative noncoding exon and a reverse primer located in the luciferase reporter gene, using an annealing temperature gradient, and the products were run on a gel (Fig. 2B). If the putative noncoding exon was transcribed and spliced to the *Kiss1* exon 1, we would expect the PCR product to be 674 base pairs in length. Thus, Bands 1 and 2, roughly 650 and 500 base pairs in length, respectively, were chosen for sequencing (Fig. 2B). The Band 2 sequence aligns to a mouse bacterial artificial chromosome and is off-target from the mKiss-Luc plasmid. The Band 1 sequence aligns to 39 base pairs in the putative non-coding exon and 162 base pairs that span exon 1, suggesting that the noncoding exon was transcribed and spliced to exon 1. Therefore, the noncoding exon may be present in the upstream region of the mKiss-Luc plasmid. However, these data do not confirm a new location for the TSS, which would necessitate further analysis to determine. Thus, I will continue to refer to the mKiss-Luc plasmid as -4058/+455 based on the previously determined structure.

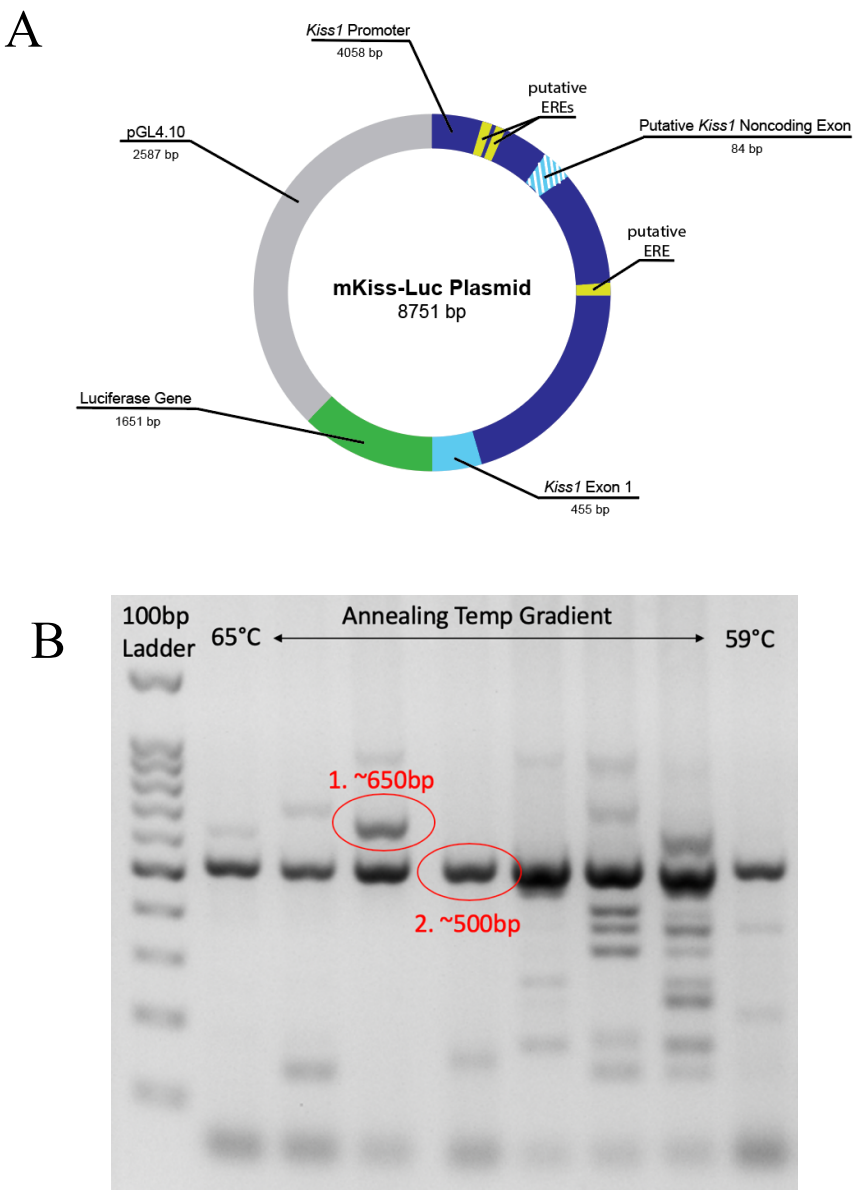


Figure 2. Structure of mKiss-Luc plasmid construct. A: A schematic of the mKiss-Luc plasmid, which contains 4058 base pairs upstream and 455 base pairs downstream of the *Kiss1* transcriptional start site (-4058/+455). Three putative estrogen response elements (ERE) and a putative noncoding exon have been identified in the upstream region. B: The mKiss-Luc mRNA product was isolated from KTaV-3 cells. After conversion to cDNA, polymerase chain reaction was performed using an annealing temperature gradient. Bands 1 and 2 were extracted from the gel and sequenced.

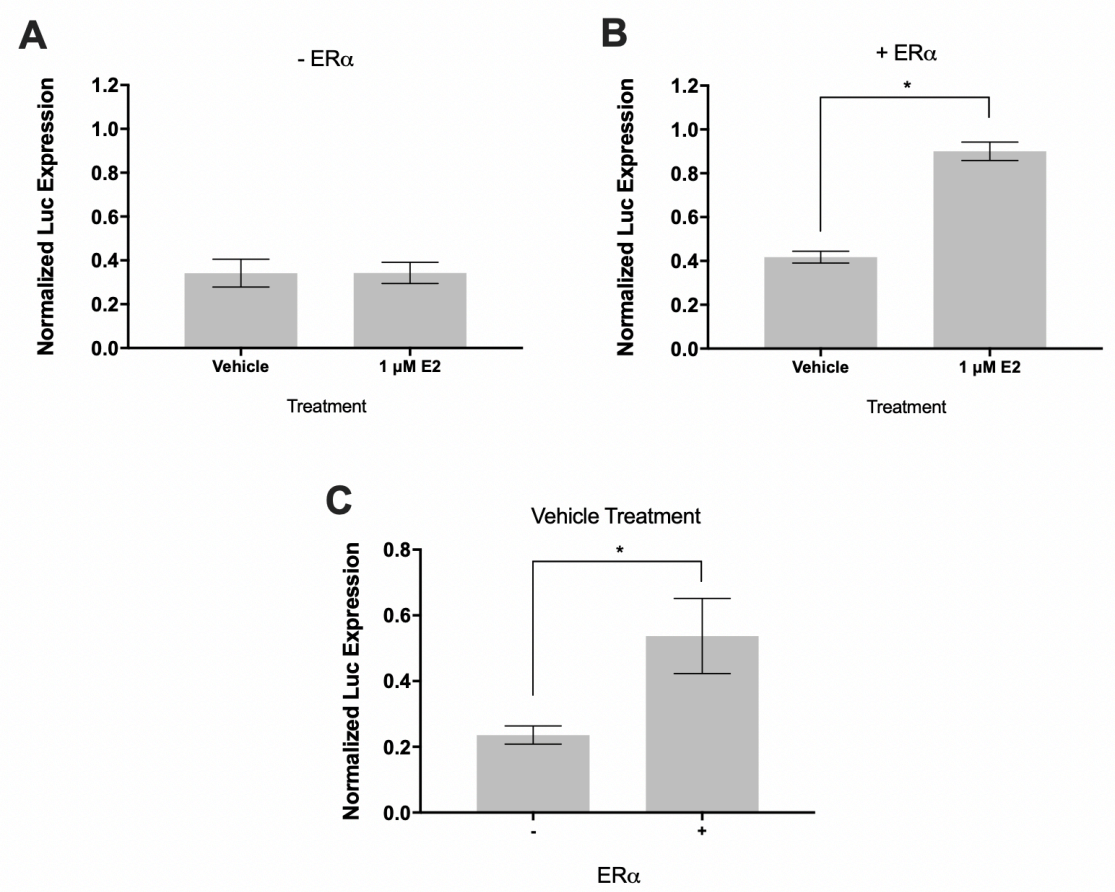


Figure 3. E2 treatment with ERα induces mKiss-Luc transcription in KTaV cells. KTaV-3 cells were co-transfected with full-length mKiss-Luc and ERα for 6 hours, then treated with 1 μM E2 or vehicle for 24 hours. A: Without ERα overexpression, E2 does not induce mKiss-Luc expression. B: With overexpression of ERα, E2 significantly increases mKiss-Luc transcription compared to the vehicle. C: ERα alone induces mKiss-Luc expression under vehicle treatment. For all experiments, luciferase expression is normalized to TK-βgal, technical triplicates were averaged and subsequently normalized to pGL4.10 backbone. Data was analyzed by unpaired Student's t-test ($p<0.05$), N=3, values represent mean, and error bars represent SEM.

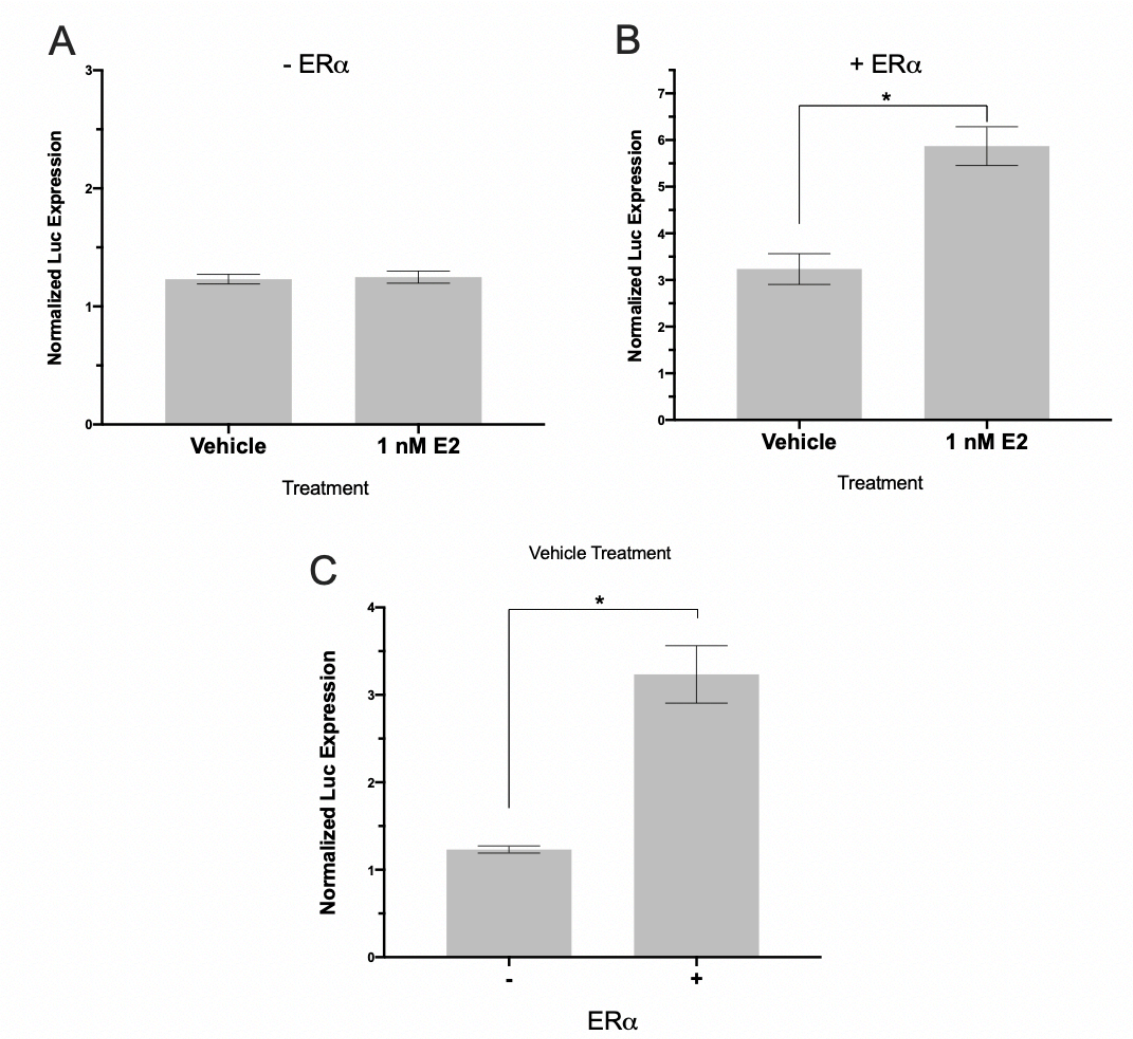


Figure 4. 48-hour E2 treatment induces robust mKiss-Luc expression in KTaV-3 cells. KTaV-3 cells were co-transfected with the -4058/+455 mKiss-Luc plasmid and ERα for 24 hours. The transfected cells were then treated with vehicle or 1 nM E2 for 48 hours. A: Without overexpression of ERα, 1 nM E2 does not alter mKiss-Luc expression. B: With the overexpression of ERα, 1 nM E2 results in robust induction of mKiss-Luc expression ($p<0.005$). C: Overexpression of ERα significantly increases mKiss-Luc expression ($p<0.005$). For all experiments, luciferase expression is normalized to TK-βgal, technical triplicates were averaged and subsequently normalized to pGL4.10 backbone, N=6 and error bars represent SEM. Statistical significance was determined by unpaired Student's t-test.

E2-ERα induces mKiss-Luc transcription in KTaV-3 cells

To confirm the action of E2 and ERα on mKiss-Luc expression, KTaV-3 cells were co-transfected with the full-length mKiss-Luc plasmid and ERα for 6 hours. The cells were then treated with ethanol vehicle of 1 μM E2 for 24 hours. In the absence of ERα overexpression, E2 does not induce mKiss-Luc expression (Fig. 3A). However, with the overexpression of ERα, E2 significantly increases mKiss-Luc expression compared to the vehicle treatment ($p<0.05$), confirming E2-ERα induction in the AVPV-derived cells *in vitro* (Fig. 3B). Notably, the presence of ERα alone can induce mKiss-Luc expression (Fig. 3C). All experiments testing the truncated mKiss-Luc plasmid constructs followed this transfection and hormone treatment protocol.

The same experiments confirming the action of E2 and ERα on mKiss-Luc expression were repeated using a 24-hour transfection and 48-hour hormone treatment protocol. In this protocol, cells were treated with a smaller concentration of 1 nM E2. Similarly, 1 nM E2 does not alter mKiss-Luc expression without overexpression of ERα ($p>0.05$), and overexpression of ERα without E2 treatment significantly increases mKiss-Luc expression ($p<0.005$) (Fig. 4). With the overexpression of ERα, 1 nM E2 results in robust induction of mKiss-Luc expression ($p<0.005$) (Fig. 4B). Because I found E2-ERα induction to be more robust using the longer transfection and longer hormone treatment protocol, subsequent experiments testing the μERE plasmids followed this protocol.

Truncations of the mKiss-Luc promoter

To determine broad regions of the *Kiss1* promoter that are necessary for the transcriptional effects of estradiol-bound ERα (E2 + ERα), the full-length mKiss-Luc plasmid was truncated from the 5' end using site-directed mutagenesis. Site-directed

mutagenesis resulted in four truncated reporter plasmids named after their length: -3877/+455, which contains all EREs intact; -3668/+455 and -2558/+455, which both delete regions containing two putative EREs; and -1711/+455, which deletes all three putative EREs (Fig. 5A). Additionally, to explore the structure of the mKiss-Luc plasmid given the presence of a putative noncoding exon, the putative intron was deleted using site-directed mutagenesis, resulting in a plasmid named after the deleted region: ∆-2706_-580/+455.

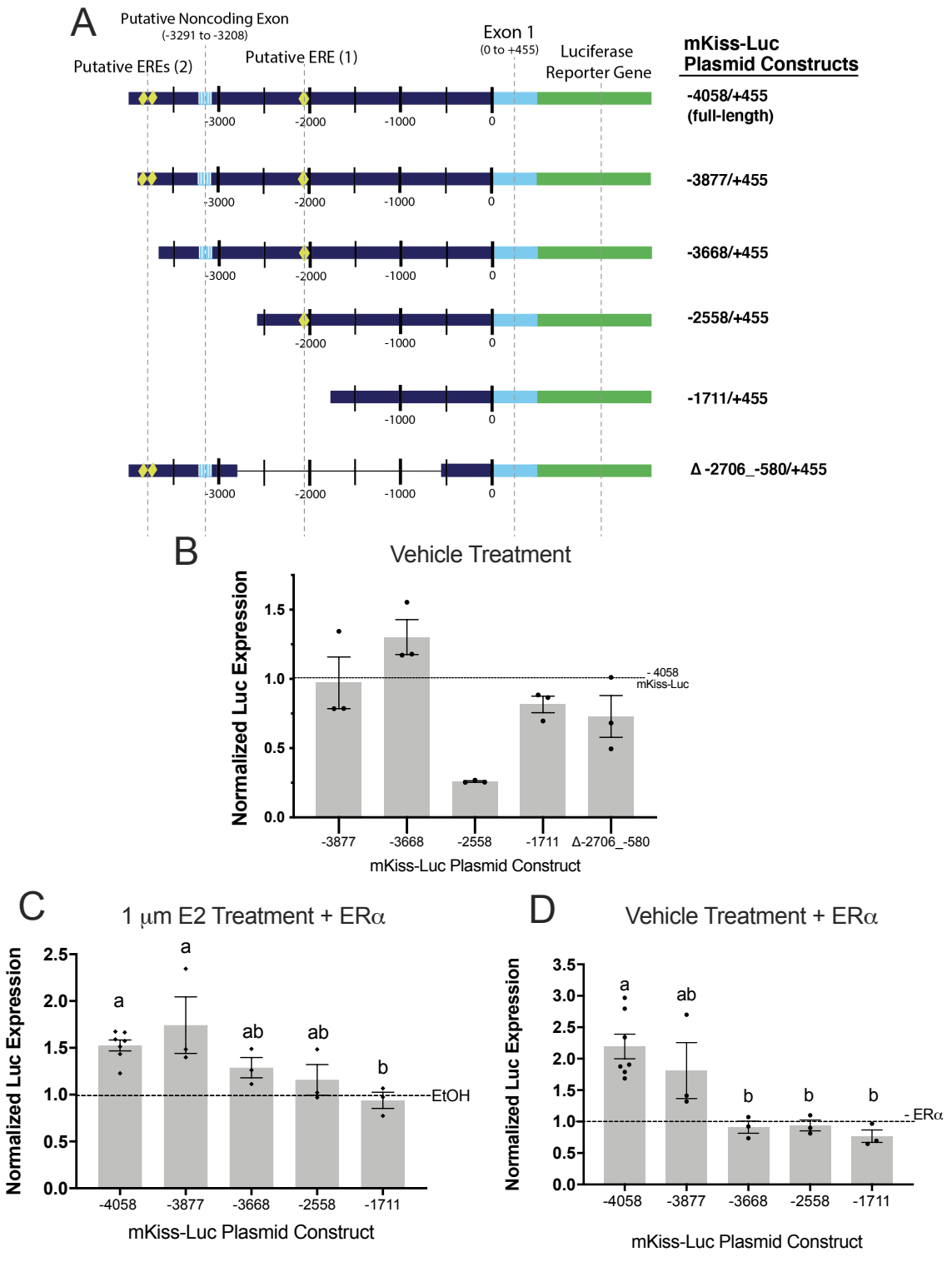
To measure mKiss-Luc transcription, KTaV-3 cells were co-transfected with the various reporter plasmids and ERα. First, basal mKiss-Luc expression under ethanol vehicle treatment, was examined. The basal expression of -2558/+455 appeared reduced compared to the -4058/+455 mKiss-Luc, which suggests the presence of a strong repressive element in the truncated plasmid (Fig. 5B). Basal expression is also moderately reduced in ∆-2706_-580/+455, suggesting that the putative intron contains important promoter elements (Fig. 5B). However, because the data varied greatly between experiments, we are unable to draw definitive conclusions.

The transfected KTaV-3 cells were also treated with E2 to investigate transcriptional regulation. As expected, E2 + ERα treatment increased the luciferase expression of the -4058/+455 mKiss-Luc (Fig. 5C). However, this E2-ERα induction was significantly lost in -1711/+455 ($p<0.05$), suggesting that the deleted region contains important elements for E2 regulation (Fig. 5C). Additionally, the E2-ERα induction in the -2558/+455 and -3668/+455 were weaker and more variable than the -4058/+455 mKiss-Luc (Fig. 5C). Notably, three EREs are deleted in the -1711/+455 construct, and two putative EREs are deleted in the -2558/+455 and -3668/+455 constructs (Fig. 5A). In

contrast, all three putative EREs are present in the -3877/+455 construct, which exhibits E2 + ERα induction that is not significantly different from the full-length mKiss-Luc reporter (Fig. 5C). These findings suggest that the regions containing three putative EREs are functionally important for E2 + ERα induction in the AVPV-derived cell line. Additionally, there may be an additive effect of E2 + ERα induction, as the region containing all three EREs results in stronger induction than the presence of only one ERE.

Finally, the response to ERα alone was examined in the truncated mKiss-Luc plasmids. Without E2 treatment, ERα can induce mKiss-Luc expression in the -4058/+455 mKiss-Luc (Fig. 5D). This ERα induction is significantly lost in -1711/+455, -2558/+455, and -3668/+455 ($p<0.05$), further supporting that the ERα mediated response is lost in these plasmids.

Figure 5. Basal mKiss-Luc expression and E2 induction is altered by deleting broad regions of the *Kiss1* promoter. A: Schematic of the mutated mKiss-Luc plasmid constructs. The full-length plasmid was truncated from the 5' end using site-directed mutagenesis, resulting in four truncated mKiss-Luc reporter plasmids named after their length from the TSS. From longest to shortest: -3877/+455, -3668/+455, -2558/+455, and -1711/+455. The ∆-2706_-580/+455 plasmid is named after the deletion of the putative intron, also achieved using site-directed mutagenesis. B: KTaV-3 cells were transfected mKissLuc plasmids of various lengths, then treated with vehicle. Luciferase expression for each plasmid construct was normalized to -4058/+455 mKiss-Luc expression. Basal mKiss-Luc expression appears to be reduced in -2558/+455. C: KTaV-3 cells were co-transfected with ERα and mKissLuc plasmids of various lengths, then treated with either vehicle or 1 μM E2. E2-induced expression was normalized to the vehicle-induced expression. E2 + ERα induces luciferase expression in the -4058/+455 mKissLuc. The truncated -1711/+455 plasmid resulted in a significant loss of E2-ERα induction. D: KTaV-3 cells were co-transfected with ERα and mKiss-Luc plasmids of various length, and treated with vehicle. Luciferase expression with overexpression of ERα was normalized to expression without overexpression of ERα. While ER alone induces -4058/+455 mKiss-Luc expression, this induction is significantly lost in the -1711, -2558, and -3668 plasmids. For all experiments, luciferase expression is normalized to TK-βgal, technical triplicates were averaged and subsequently normalized to pGL4.10 backbone. Data were analyzed by One-Way ANOVA and Post-Hoc Tukey's test ($p<0.05$), N=3-7, values represent mean, and error bars represent SEM.


A
Putative Noncoding Exon
(-3291 to -3208)
Putative EREs (2)
Putative ERE (1)
Exon 1
(0 to +455)
Luciferase
Reporter Gene
mKiss-Luc
Plasmid Constructs
-4058/+455
(full-length)
-3877/+455
-3668/+455
-2558/+455
-1711/+455
Δ -2706_-580/+455
-3000
-2000
-1000
0
B
Vehicle Treatment
Normalized Luc Expression
- 4058
mKiss-Luc
-3877
-3668
-2558
-1711
Δ-2706_-580
mKiss-Luc Plasmid Construct
C
1 μm E2 Treatment + ERα
EtOH
-4058
-3877
-3668
-2558
-1711
mKiss-Luc Plasmid Construct
D
Vehicle Treatment + ERα
- ERα
mKiss-Luc Plasmid Construct

Cis-mutations of putative EREs on the mKiss-Luc promoter

The mKiss-Luc plasmid contains three putative imperfect EREs on the *Kiss1* promoter (Fig. 6). To determine the functionality of the putative EREs in E2 + ERα transcriptional regulation, *cis*-mutations of each ERE sequence were created to disrupt ERα binding. This resulted in three mutated mKiss-Luc plasmid constructs, named after the mutated ERE: μERE a, μERE b, and μERE c (Fig. 7A). The KTaV-3 cells were co-transfected with ERα and the various mKiss-Luc plasmid constructs. Consistent with previous results, E2 + ERα induced expression of the -4058/+455 mKiss-Luc but had no effect on the truncated -1711/+455 plasmid, serving as positive and negative controls, respectively (Fig. 7). Preliminary results suggest that μERE a results in a weakened response to E2-ERα, compared to the -4058/+455 mKiss-Luc (Fig. 7B). E2 + ERα induction is significantly reduced in μERE b expression, suggesting that E2-ERα induction requires binding to the ERE b sequence (Fig. 7C). μERE c expression was not significantly different from full-length mKiss-Luc expression, suggesting that ERE c is not sufficient for E2-ERα induction in the KTaV-3 cells (Fig. 7D).

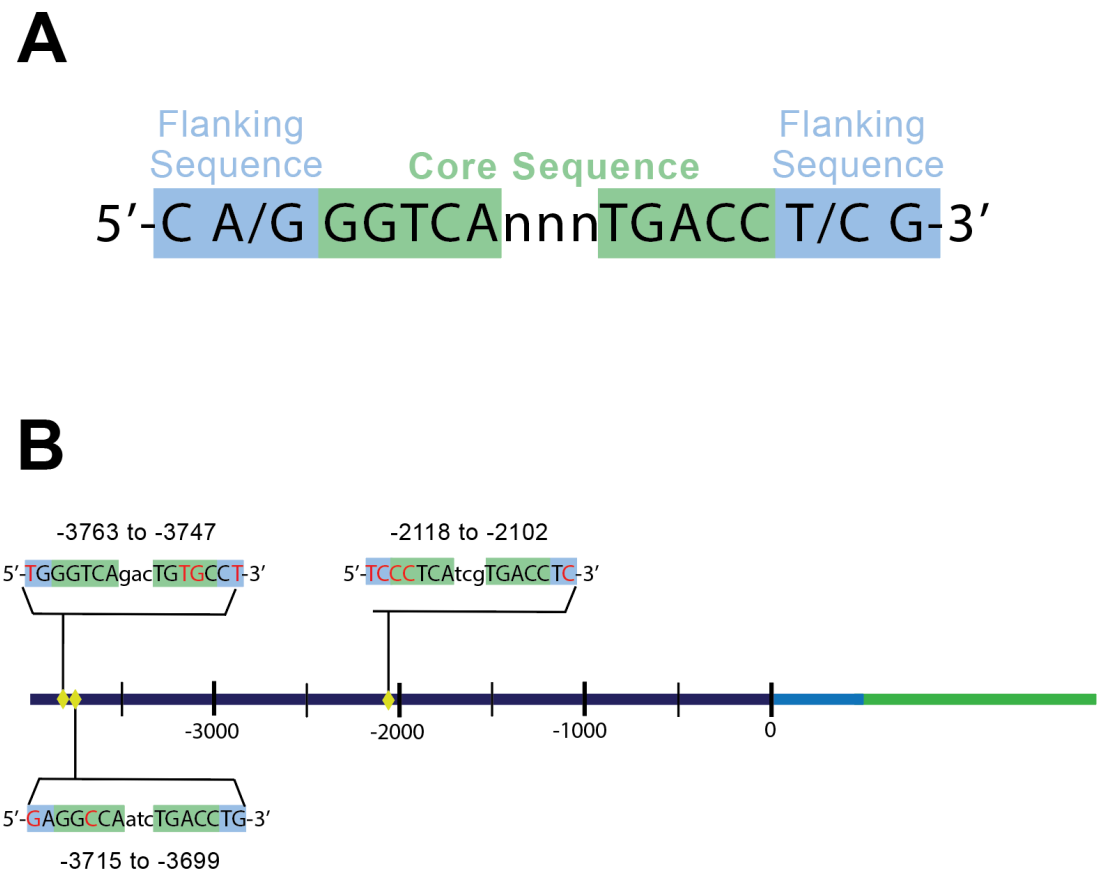
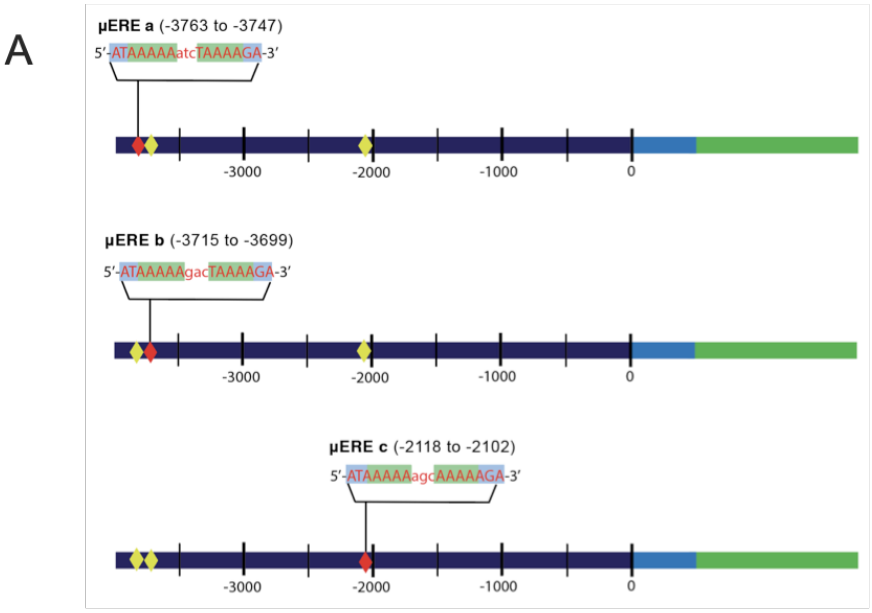
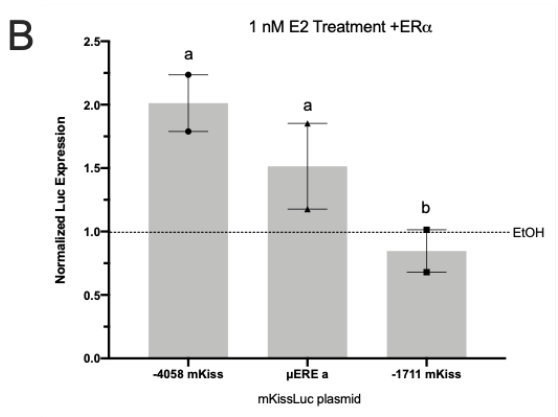
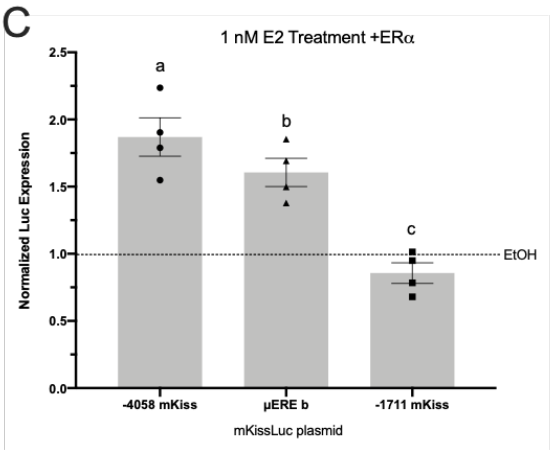


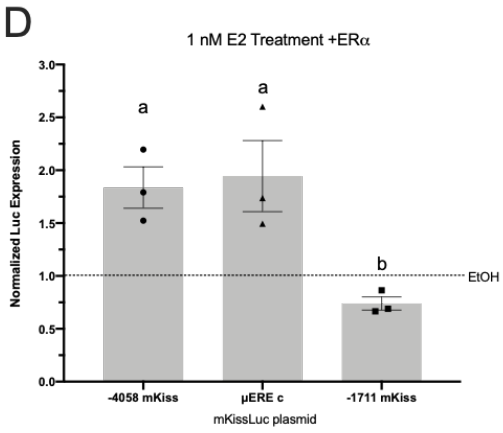
Figure 6. Schematic of estrogen response elements on *Kiss1* promoter. A: The ERE consensus sequence is a perfectly palindromic inverted repeats, shown in green. The flanking sequence, in blue, varies in functional EREs. B: The *Kiss1* promoter on the mKiss-Luc plasmid contains three putative imperfect EREs, which differ by one to two base pairs in the core sequence and one to three base pairs in the flanking sequence, depicted in red. The EREs are named from most distal: ERE a, ERE b, and ERE c.

Figure 7. ERE b contributes to E2+ERα induction of mKiss-Luc expression. A: Schematic of ERE *cis*-mutations on the mKiss-Luc plasmid, created using site-directed mutagenesis. The resulting plasmids are named after the mutated ERE: μERE a, μERE b, and μERE c. B,C,D: KTaV-3 cells were co-transfected with ERα and mKiss-Luc plasmid constructs, then treated with either vehicle or 1 nM E2. As expected, E2 induces expression of -4058/+455 mKiss-Luc, but this response is lost in the -1708/+455 plasmid. B: μERE a results in slight reduction of mKiss-Luc expression compared to the -4058/+455 mKiss-Luc, though not statistically significant (N=2). C: μERE b results in significantly reduced mKiss-Luc expression compared to the -4058/+455 mKiss-Luc (N=4). D: μERE c expression is not significantly different from the -4058/+455 mKiss-Luc (N=3). For each experiment, luciferase expression was normalized to βgal, data were compared by One-Way Blocked ANOVA and Post-Hoc Tukey's test where different letters denote significance ($p<0.05$). Values represent mean and error bars represent SEM.


A
μERE a (-3763 to -3747)
5′-ATAAAAAatcTAAAAGA-3′
-3000
-2000
-1000
0
μERE b (-3715 to -3699)
5′-ATAAAAAgacTAAAAGA-3′
-3000
-2000
-1000
0
μERE c (-2118 to -2102)
5′-ATAAAAAagcAAAAAGA-3′
-3000
-2000
-1000
0


B
1 nM E2 Treatment +ERα
Normalized Luc Expression
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
EtOH
-4058 mKiss
μERE a
-1711 mKiss
mKissLuc plasmid


C
1 nM E2 Treatment +ERα
Normalized Luc Expression
2.5
2.0
1.5
1.0
0.5
0.0
a
b
c
EtOH
-4058 mKiss
μERE b
-1711 mKiss
mKissLuc plasmid


D
1 nM E2 Treatment +ERα
Normalized Luc Expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
EtOH
-4058 mKiss
μERE c
-1711 mKiss
mKissLuc plasmid

DISCUSSION

Discerning the mechanism by which estrogen regulates *Kiss1* transcription is essential to understanding the control of female reproduction. The *Kiss1* promoter contains the sequence of three putative EREs, sites where ERα can bind to regulate transcription (22). In *in vivo* experiments, ERα with a mutated DNA-binding domain is unable to activate AVPV kisspeptin neurons, suggesting that the positive feedback of E2-ERα in AVPV kisspeptin neurons occurs through ERα binding to EREs (13). The present *in-vitro* studies have confirmed that E2 and ERα can robustly activate the *Kiss1* promoter in AVPV-derived cells, and one ERE sequence on the *Kiss1* promoter may contribute to this activation.

To contribute to the characterization of the *Kiss1* promoter, I sequenced the mRNA product of mKiss-Luc plasmid, revealing a putative noncoding exon located at -3291 to -3208 on the *Kiss1* promoter (Figure 1). I then investigated the intergenic region between the putative noncoding exon and the established *Kiss1* exon 1 by creating a deletion mutation of from -2706 to -580 on the mKiss-Luc plasmid. This plasmid, Δ-2706_-580/+455, appeared to have reduced basal mKiss-Luc expression compared to the -4058/+455 mKiss-Luc, suggesting the presence of promoter elements in this region (Fig. 4B). Further exploration is needed to fully characterize the *Kiss1* promoter, such as investigating *Kiss1* promoter elements, or perhaps other putative EREs, further upstream of what is contained in the mKiss-Luc plasmid.

E2 stimulates AVPV kisspeptin neurons which is necessary for the ovulatory surge. In mice, E2 has been shown to increase *Kiss1* mRNA in the AVPV regions (23). Additionally, previous work in our lab has shown that E2 activates the *Kiss1* promoter *in vitro* (17). Using this previously established protocol, I confirmed that E2 is capable of

inducing mKiss-Luc expression in KTaV-3 cells (Fig. 3). However, I also showed that a smaller concentration of E2 can robustly induce mKiss-Luc expression with a longer transient transfection and longer hormone treatment time (Fig. 6). On one hand, the robust response may be due to better transfection efficiency. On the other hand, AVPV kisspeptin activation is thought be coupled with circadian control (24). The length of E2 treatment may play a role in *Kiss1* promoter activation, and further work should explore the temporality of E2-ERα regulation.

To identify regions of the *Kiss1* promoter involved with E2 gene regulation, I compared the luciferase expression of the -4058/+455 mKiss-Luc plasmid to truncated mKiss-Luc plasmids. This revealed key regions of the *Kiss1* promoter that are necessary for E2 induction in AVPV-derived kisspeptin neurons. I found that the loss of the region containing all three putative EREs, demonstrated in -1711/+455, resulted in the complete loss of E2-ERα induction of luciferase expression in the KTaV-3 cell line (Fig. 4C). On the other hand, -3668/+455 and -2558/+455, which both contained the same single putative ERE, had a weaker E2-induced response compared to the full-length reporter (Fig. 4C). -3877/+455, which contained all three putative EREs, resulted in the same E2-ERα induction as the -4058/+455 mKiss-Luc (Fig. 4C). This suggests that the region from -3668 to -3877 is necessary for E2-ERα induction, and notably, contains two of the three putative EREs.

Furthermore, three of the truncated mKiss-Luc plasmids failed to induce luciferase expression following the overexpression of ERα, providing further evidence for key regulatory regions. ERα is able to stimulate -4058/+455 mKiss-Luc expression in the KTaV-3 cells without E2 treatment (Fig. 3). This is likely caused by overexpression

of ERα past physiological levels, resulting in excess ERα which can translocate into the nucleus and bind DNA without its ligand, E2. Interestingly, this induction by ERα is lost in the truncated plasmids. Overexpression of ERα does not induce mKiss-Luc expression in the -1711/+455, -2558/+455, -3668/+455 truncated plasmids, compared to the -4058/+455 mKiss-Luc (Fig. 4D). However, -3877/+455 expression is not significant different from the -4058/+455 mKiss-Luc (Fig. 4D). These data further support that ERα is no longer able to alter transcription through regulatory sites that are lost in the regions between -1711 and -3668 bp upstream of the TSS, however those regulatory sites are present in the -3877/+455 plasmid. While these data identify key regions on the *Kiss1* promoter for E2-ERα regulation, we cannot conclude whether ERα binds directly to the putative EREs or to other regulatory sites. Additionally, the truncations of the *Kiss1* promoter are broad, and may also delete important *Kiss1* promoter regions.

To look more specifically at the EREs, I created *cis*-mutations of the putative EREs in the mKiss-Luc plasmid to block ERα binding (Fig. 7). The E2-stimulated luciferase expression from the μERE c plasmid was the same as the -4058/+455 mKiss-Luc (Fig. 7D). μERE a had a weakened response to E2 compared to the -4058/+455 mKiss-Luc, though increased replicates are necessary to determine if ERE a contributes to E2-ERα promoter activation (Fig. 7B). μERE b had a significantly reduced response to E2-ERα compared to the -4058/+455 mKiss-Luc, suggesting that the ERE b sequence is necessary for E2-ERα transcriptional activation (Fig. 7C). Although the ERE c sequence is not sufficient for E2-ERα *Kiss1* promoter activation in KTaV-3 cells, the ERE b sequence constitutes a functional ERE, providing further support that E2+ERα acts through an ERE-dependent mechanism for positive feedback in AVPV kisspeptin

neurons. Moreover, μERE b does not result in the complete loss of E2-ERα mKiss-Luc induction, suggesting that the putative EREs may differ in regulatory strength such that one ERE is not sufficient for the entire mKiss-Luc induction. There are two possible explanations: First, the regulatory power of EREs may be additive. While this mechanism is not completely clear, studies indicate that multiple ERα may act in synergy when binding to EREs in close proximity, enhancing the transcriptional effect of ERα (25,26). Second, the three putative EREs on the *Kiss1* promoter all differ in sequence, suggesting sequence-dependent regulatory strength. Experiments comparing the consensus ERE sequence with imperfect ERE sequences show that variations in the sequence result in different DNA affinity and transcriptional activity of ERα (27,28). While this mechanism is unclear, ERα binding to imperfect ERE sequences results in conformational changes to the receptor, which may create new sites for cofactors to bind ERα and modulate the transcriptional effect (9,27). The additive power and sequence specificity can be explored by creating *cis*-mutations of the putative EREs in combination.

There is also a possibility that the *cis*-mutations do not actually prevent ERα binding. An electrophoretic mobility shift assay, which determines protein-DNA interactions, would not only confirm the loss of ERα binding in these *cis*-mutations, but also provide further evidence for an ERE-dependent mechanism on the wildtype promoter.

Finally, it is possible that the immortalized KTaV-3 cell line, while useful for directly studying the *Kiss1* promoter sequence, does not completely recapitulate a physiological setting. That is, other cell-specific factors that are necessary for E2 regulation of *Kiss1* expression *in-vivo* may not be expressed *in-vitro*. Comparing RNA

sequencing between the KTaV-3 cell line and the AVPV region of female mice after estradiol treatment may reveal differences in transcription factor expression, providing direction for investigation in E2-ERα regulation of the *Kiss1* promoter.

Kisspeptin neurons are critical regulators of reproduction, and this work further contributes to characterization of the *Kiss1* gene by identifying one ERE that may contribute to E2 regulation. E2-ERα regulation of *Kiss1* in AVPV kisspeptin neurons is necessary for ovulation, a critical event in female reproduction, and elucidating this molecular mechanism is foundational for improving diagnoses and advancing therapeutics for patients who suffer from reproductive disorders.

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