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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Hormonal Interactions in Progesterone Regulation of Gonadotropin

Gene Expression

A thesis submitted in partial satisfaction of the requirements for the

Degree Master of Science

In

Biology

by

Yasmin Ghochani

Committee in Charge:

Professor Pamela L. Mellon, Chair
Professor Cornelis Murre, Co-chair
Professor Chris Armour

2009

The Thesis of Yasmin Ghochani 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

2009

This work is dedicated to my Mom, and sister for their continuous

and tremendous love and support. Your enormous strength and

encouragement empowers me as I face every journey that will follow this one.

Thank you and I love you!

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LIST OF ABBREVIATIONS

α-GSU   alpha glycoprotein subunit

AP-1   activating protein 1

ARE   Activin responsive element

β-gal   β-galactosidase

bp   base pair

CTE   C-terminal extension

DBD   DNA-binding domain

Egr-1   early growth response-1

ER   estrogen receptor

ERK   extracellular regulated kinase

FSH   follicle-stimulating hormone

GnRHR   gonadotropin-releasing hormone receptor

GSE   gonadotrope-specific element

HPG   hypothalamic-pituitary-gonadal

HRE   Hormone responsive element

JNK   c-Jun N-terminal kinase

LBD	ligand binding domain
LH	luteinizing hormone
luc	luciferase
MAPK	mitogen activated protein kinase
mRNA	messenger ribonucleic acid
NR	Nuclear receptor
Otx-1	orthodenticle homeobox 1
PKC	protein kinase C
PR	progesterone receptor
PRCE	Progesterone receptor conditional excision
PRE	Progesterone responsive element
PRKO	Progesterone receptor Knockout
Ptx-1	pituitary homeobox 1
SBE	Smad binding element
SF-1	steroidogenic factor 1
SR	steroid receptor
TK	thymidine kinase

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Chapter 4, Figure 4-1, is a reprint of Figure 5A as it appears in Progesterone Inhibits Basal and Gonadotropin-Releasing Hormone Induction of Luteinizing Hormone β-Subunit Gene Expression 2009. Thackray VG, Hunnicutt JL, Memon AK, Ghochani Y, Mellon PL, Endocrinology 150:2395-2403. The author of the thesis was an author of this paper, and conducted the experiment resulting in that figure in collaboration with the other authors.

ABSTRACT OF THE THESIS

Hormonal Interactions in Progesterone Regulation of Gonadotropin

Gene Expression

by

Yasmin Ghochani

Master of Science in Biology

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Professor Pamela L. Mellon, Chair

Professor Cornelis Murre, Co-chair

The differential regulation of gonadotropin secretion is integral to a properly functioning reproductive system. Transcriptional regulation and secretion of the two gonadotropins diverge during late proestrus and early estrus when LH levels decline with the termination of the preovulatory gonadotropin surge, while FSH remains elevated. Gonadal steroid hormone feedback is a crucial component of the control of gonadotropin synthesis in the pituitary gonadotrope, and progestins may be of particular importance for the secondary FSH surge. In the current study, we have focused on delineating the mechanisms of progestin regulation of the gonadotropin β-subunits. Within the murine FSHβ promoter, our studies demonstrated that a FOXL2 element at -350 and a nearby Smad half-site are necessary for both progestin

responsiveness and synergy between activin and progestin. In the context of the -300 rat LHβ promoter, we found that the -200/-150bp region contributes to basal gene expression, most likely by binding transcriptional activators. Additionally, we contributed to finer mapping of progesterone suppression of basal and GnRH-induced LHβ gene expression by identifying a critical segment at the -300/-280bp region of the promoter. Finally, to study the feedback regulation of progesterone in gonadotrope cells *in vivo*, we created a gonadotrope-specific PRKO: the $PR^{KOLacZ/Flox}$/LHβ-Cre mouse line. Though, our studies did not show any significant impairment of fertility, possibly due to genetic penetrance or mosaicism issues, they serve as preliminary data indicating trends towards lower reproductive function, such as lower number of litters and pups, as well as lower levels of circulating hormones.

I

Introduction

<u>The Hypothalamic-Pituitary-Gonadal Axis</u>

The hypothalamic-pituitary-gonadal (HPG) axis, consisting of the hypothalamus, anterior pituitary, and the gonads, plays an essential role in regulating mammalian reproduction, including the menstrual cycle, pregnancy, postpartum, and menopause, as well as fetal and pubertal development. Gonadotropin-releasing hormone (GnRH) is secreted in a pulsatile manner from specific neurons in the hypothalamus into the hypophyseal portal system (1). The GnRH receptor (GnRHR), a member of the rhodopsin receptor family, is a G-protein coupled (GPCR), seven-transmembrane receptor (2-5). The binding of GnRH to its receptor located on the surface of gonadotrope cells in the anterior pituitary results in the transcription and secretion of the gonadotropins, follicle- stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH are heterodimeric glycoproteins with a common α-glycoprotein subunit (α-GSU), and unique β subunits (LHβ and FSHβ) that confer the biological specificity. Synthesis of the β subunits is also the rate-limiting step for production of the mature hormones (6, 7). The gonadotropins, secreted into the systemic circulation, act on the gonads to regulate reproductive function. FSH is important for ovarian folliculogenesis prior to the antral stage, and sperm production in the testes, whereas LH is necessary for

steroidogenesis and ovulation (8). Feedback of gonadal steroid hormones such as estrogen, progesterone, and testosterone, in addition to peptide hormones such as activin, inhibin, and follistatin to the anterior pituitary and hypothalamus further regulate GnRH, FSH and LH synthesis and secretion (9, 10).

The Anterior Pituitary

The pyramidal ectodermal cells of the Rathke's Pouch give rise to the anterior pituitary gland (11, 12), which is composed of five different endocrine cell populations: the corticotropes, thyrotropes, gonadotropes, somatotropes, and lactotropes. The six peptide hormones synthesized and secreted by these different cell populations are adenocorticotropic hormone released by the corticotropes, thyroid-stimulating hormone by thyrotropes, LH and FSH by gonadotropes, growth hormone by somatotropes, and prolactin by lactotropes, which altogether play critical roles in growth, metabolism, fetal development, reproduction, development and nervous system function (11).

The gonadotrope cell population comprises only 10-15% of the anterior pituitary, thus it is difficult to investigate its function *in vivo* (11). The Mellon laboratory, however, has created immortalized cell lines derived from transgenic mice that developed pituitary tumors due to targeted oncogenesis (13). The various available immortalized cell lines provide us with tools to

study the gonadotropes at different stages of development. The αT3-1 gonadotrope-derived cell line expresses GnRHR and αGSU, representing immature gonadotropes, while the LβT2 cells have been shown to endogenously express FSHβ and LHβ along with GnRHR, αGSU, activin and activin receptors, and various steroid receptors including the progesterone receptor (PR), and thus representing mature gonadotrope cells. The LβT2 cell line, therefore, was used for the investigations of hormonal regulation of FSHβ and LHβ gene expression presented in this study.

Differential Regulation of Gonadotropin Transcription During The Rodent Reproductive Cycle

The pituitary gonadotropins are differentially regulated to allow for normal mammalian reproductive function. The rodent reproductive/estrous cycle is characterized by four phases: proestrus, estrus, metestrus (or diestrus I) and diestrus (or diestrus II) (14) with ovulation taking place taking place during the period from the beginning of the proestrus to the end of estrus (15).

The LHβ and FSHβ genes are expressed in correlation with LH and FSH levels in the blood. In the afternoon of proestrus, LHβ and FSHβ transcription is increased by three fold and four fold, respectively (16-18), corresponding to LH and FSH surges, triggered by GnRH, to allow for ovulation of the mature follicle in response to LH (19). Furthermore, FSHβ

transcription is increased by three fold during estrus (18) allowing for an independent secondary FSH surge that leads to follicular recruitment for the following cycle (7, 20-23).

Multiple mechanisms have been proposed to provide for the distinct regulation of LHβ and FSHβ transcription. GnRH pulsatile secretion may contribute to the differential regulation of FSH and LH (24). Furthermore, feedback regulation by the activin/follistatin/inhibin system is thought to be important for differential LHβ and FSHβ synthesis. An increase in activin levels during estrus, due to lower follistatin and/or inhibin, is thought to selectively favor FSHβ synthesis (20, 21). Additionally, steroid hormones, such as progesterone, testosterone, and glucocorticoids have all been shown to induce FSHβ but inhibit LHβ expression (10, 25). Thus the differential expression of the gonadotropins may be due to hormonal interactions in the feedback mechanisms that regulate their synthesis. Consequently, some of these hormonal interactions in progesterone regulation of gonadotropin transcription were investigated in this study.

The Gonadotropins

Absence of the LHβ gene has been shown to result in hypogonadism and infertility in both males and females (26). Furthermore, pubertal development is inhibited in humans with LHβ mutations and they are infertile

(27). It has been shown that folliculogenesis in female mice deficient in FSHβ is inhibited, while male mice are fertile but have impaired reproductive function (28). The human FSHβ gene is also critical for reproductive function in both males and females, where mutations result in absent or incomplete pubertal development in women, normal pubertal development but azoospermia in men, and infertility in both women and men (29).

GnRH Regulation of LHβ and FSHβ Gene Expression

The GnRH signaling pathway is a critical regulator of transcription, synthesis, and secretion of the pituitary gonadotropins, and is thus essential for normal reproductive function. The GnRHR is a high affinity, seven transmembrane domain receptor, which lacks the carboxy-terminal cytoplasmic domain (2), important for GPCR cytosolic regulatory protein coupling and desensitization (30), causing GnRH signal transduction to be relatively slower than other GPCRs. The GnRHR Gα-proteins expressed in the anterior pituitary gonadotrope cells are $G\alpha_q$ and $G\alpha_{11}$ (31). Upon binding of GnRH to its receptor and activation of $G\alpha_{q/11}$, phospholipase Cβ is activated, allowing for Ca^{++} release from intracellular stores to increase the activity of the protein kinase C (PKC), and calcium/calmodulin kinase II (32, 33). GnRHR signaling also leads to activation of mitogen-activated protein kinases (MAPK), such as extracellular regulated kinase (ERK1/2), c-Jun N-terminal kinase

(JNK), and p38MAPK (32, 34, 35).

There are many conflicting reports regarding the GnRH signal transduction pathway that leads to induction of LHβ transcription. It is thought that LHβ gene expression by GnRHR proceeds through PKC signaling (36). However, involvement of calcium alone or together with various MAPK proteins, such as p38, JNK, and ERK1/2 in LHβ induction is unclear and debated among various studies (37-40).

Induction of the early growth response-1 (Egr-1) transcription factor by GnRH, as well as the basal factors, steroidogenic factor-1 (SF-1), ,pituitary homeobox 1 (Ptx-1), and/or orthodenticle homeobox 1 (Otx-1) in the proximal LHβ promoter have been shown to be important for GnRH induction of LHβ transcription (41-44). Interactions of the transcription factors binding the proximal promoter sites along with more distal Sp1 binding sites (45), and an overlapping CArG element, important for pulsatile GnRH induction (41), elicit basal promoter activity as well as GnRH induced LHβ transcription (41, 42, 46, 47).

It has been shown that serum FSH levels are reduced 60-90% in mice lacking GnRH, leading to the conclusion that GnRH regulates FSH gene expression (48). Again, there are conflicting reports regarding Ca^{++} involvement in the GnRH signal transduction pathway leading to induction of FSHβ transcription. A study by Vasilyev *et al.* reported necessity of Ca^{++} (49),

while another study reported its lack of involvement (50). GnRH regulation of FSHβ gene expression, similar to LHβ transcriptional induction, occurs through the PKC and MAPK signaling pathways (49, 51, 52), by GnRH induction of a variety of intermediate, immediate-early genes, such as Fos isoforms (c-Fos, FosB, Fra-1 and Fra-2) and Jun isoforms (c-Jun, JunB and JunD), that heterodimerize to create activator protein-1 (AP-1). AP-1 is thought to interact with species-specific basal factors leading to FSHβ basal levels, such as the AP-1 half-site at the -76/-69 base pairs (bp) upstream of the 5' transcriptional start site on murine proximal FSHβ promoter that is juxtaposed to a CCAAT box binding NF-Y (51). Recently, it was shown that GnRH induction of a heterodimeric AP-1, comprised of c-Fos, and JunB, through a MAPK pathway, leads to induction of the murine FSHβ gene expression (32, 51).

Activin Regulation of FSHβ Transcription

Activin strongly induces FSHβ expression in gonadotrope cells (53). Activin, is composed of a homodimer of two β subunits, with two critical isoforms, A and B. Activin binding to the type II receptor, leads to heterodimerization and phosphorylation of the type I receptor (54). Subsequently the type I receptor phosphorylates Smad2 and Smad3, which bind to Smad4 and translocate into the nucleus, to induce transcription of

target genes such as FSHβ (54-56). The FSHβ promoter contains three sites that bind Smad proteins (activin-response elements, AREs, or Smad binding elements, SBEs), that are recruited to the promoter via activin-induced TGFβ receptors. Inhibin (a heterodimer of one of the activin subunits and a unique α subunit) (57) and follistatin (an activin-binding protein) (58) inhibit activin response in an autocrine and paracrine fashion.

Smad3 appears to be the limiting factor in activin induction of murine FSHβ (59, 60). The -267 Smad Binding Element (SBE) in the murine FSHβ promoter is a consensus SBE comprised of a palindrome sequence GTCTAGAC (59, 61, 62). Two other Activin Response Elements (ARE; at - 120, and -153 bp), containing a Smad half-site AGAC, are critical for activin induction in all species examined (63). One of these AREs is bound by Pbx and/or Prep, leading to Smad protein interaction, and thus allowing tethering of the activated Smads to the promoter and higher affinity binding (63, 64). Furthermore, the TAK1 signal transduction pathway has also been implicated in activin induction of the FSHβ gene expression (65), though its mechanism is much less studied.

Progesterone Regulation of LHβ and FSHβ Gene Expression

Progesterone activates the progesterone receptor (PR), which is critical for signaling pathways that facilitate sexual behavior, hypothalamic GnRH

secretion, and release of preovulatory gonadotropin surges (66). Progestins, like other steroid hormones, are lipophilic molecules that can readily cross the plasma membrane and bind their receptors located predominantly in the nucleus. There are two isoforms of the progesterone receptor (PR): PRA and PRB, both encoded by the same gene, in many species including humans and rodents, under transcriptional control of distinct, independently regulated, estrogen inducible promoters. PRA lacks the 128-165 amino acids at the N-terminus, which is thought to be involved in transactivational function in PRB, making PRB a specific transcriptional regulator of target genes not activated by PRA (67-69). Furthermore, PRB is a stronger regulator of many promoters that are responsive to both isoforms (70).

PR, similar to other steroid/thyroid receptors, has a highly variable N-terminal region that mediates its activation functions, a central DNA binding domain (DBD) comprised of two zinc fingers, and a conserved ligand binding domain at its carboxyl-terminus (LBD) (71). The inactive receptor exits as a complex of molecular chaperones including heat shock proteins, which upon ligand binding, dissociate from the receptor to allow the DBD association with hormone responsive elements (HREs), composed of two inverted sequences separated by three nonspecific nucleotides, on the target gene's promoter region, thus causing transcriptional regulation of the target gene. PR, being a 3-keto steroid receptor (SR), binds HREs, with a consensus sequence: GGTACA-N_3-TGTTCT, as a homodimer (71-74). DNA binding regulation of

target gene expression by PR also involves coactivator proteins that act as bridges between the transcription initiation complex and PR (69, 72, 75, 76). Some coactivator molecules identified are members of the p160/SRC family (SRC1, SRC2), CBP, p300, and RAP250. Others such as SHP may have inhibitory functions (77-79).

PR can also be activated indirectly, without ligand binding, by pathway cross-talk with signal transduction pathways of some plasma membrane receptors (80). Furthermore, It has been shown that some SRs can regulate gene expression without direct DNA binding through interacting with other transcription factors such as AP-1 (81, 82).

Progesterone is a component of both stimulatory and inhibitory ovarian feedback regulation of gonadotropin secretion. Treatment with progesterone has been shown to block GnRH stimulation and LH surges by estrogen (83-86); however, in animals primed by estrogen, its effects were the opposite, leading to amplification and temporal advancement of the gonadotropin surges (87). Furthermore, in ovariectomized animals, progesterone replacement has been shown to lead to suppression of elevated LH secretion (88-90) and increases GnRH pulse frequency (91, 92), most likely demonstrating its action on basal gonadotropin secretion.

PR functions within the HPG axis are numerous. Using PR null mutant (PRKO) mice, many functions have been revealed. Progesterone has been shown to amplify the stimulatory effects of estrogen, affecting female sexual

and estrous behavior, including lordosis, with PRKO mice being unresponsive to effects of progesterone on sexual behavior (93). Positive and negative feedback of progestins along with the other ovarian steroid hormone, estrogen, regulate gonadotropin gene expression and secretion (66). During the rodent estrous cycle, estrogen levels begin to increase at metestrus, reaching peak levels during proestrus and returning to baseline at estrus (94). Furthermore, progesterone levels increase during metestrus and diestrus, and decrease afterwards (95, 96). Autoradiography and in situ hybridization studies have revealed that nearly all PR within the anterior pituitary gland is expressed in the gonadotrope cells (97). PR in the anterior pituitary and many other brain regions is strongly induced by estrogen (98, 99). PRKO female rodents are infertile and display severe reproductive defects, including impaired mammary gland development, sexual function, thymic function, uterine hyperplasia, and an inability to ovulate (93). Additionally, they do not experience an elevation of LH and FSH levels prior to ovulation (100), and they have elevated basal LH levels (100). Furthermore, in PRKO mice LH surges are not only absent in intact proestrus animals but also in ovariectomized mice treated with steroid (101). It is important to note that results obtained from the PRKO mice do not distinguish between potential PR inhibitions of GnRH secretion in surges versus inhibition of GnRH signal transduction in pituitary gonadotropes. Furthermore, the expression of many genes involved in reproductive function are regulated, in part, by PR action,

ranging from effects on neurotransmitters, receptors, G-proteins, components of secondary messenger pathways, and many other signaling molecules (102).

Summary

The precise temporal and spatial regulation of the HPG axis is essential for normal reproductive function. In this study, mechanisms of progesterone transcriptional regulation of FSHβ and LHβ in anterior pituitary gonadotrope cells were studied, using the immortalized gonadotrope-derived LβT2 cell line. The FSHβ promoter is synergistically induced by activin and progestin co-treatment (103), discussed in detail in Chapter III. Furthermore, progesterone has been shown to suppress basal and GnRH-induced LHβ subunit gene expression, through the action of two 50bp repressive elements within the -300/-150 bp region of the LHβ promoter (104), discussed in detail in Chapter IV.

In our studies, we investigated the regulatory regions on the murine FSHβ promoter involved in the synergistic induction of FSHβ gene expression by progesterone and activin co-treatment. Initially, we demonstrated that low levels of exogenous PR, which may mimic physiological levels, are sufficient for progestin responsiveness on the murine FSHβ promoter. We then revealed the importance of previously identified HREs for both progestin induction of

FSHβ and synergy between progestins and activin. We also confirmed, previously reported data on the necessity of the -381 HRE for progestin and synergistic induction of FSHβ gene expression. Furthermore, we showed that disruption of single SBE sites on the murine FSHβ promoter prevents cross-talk between activin and progestins, indicating that the activin response, and consequently the synergistic induction of the promoter by activin and progestin require the binding of factors to the multiple SBEs in the FSHβ promoter. Additionally, the -267 SBE was shown to be of particular importance for this synergy, since its disruption led to a substantial decrease in the induction of FSHβ gene expression.

We then set out to further study the region between the -381 HRE and the -267 SBE. We revealed that a 21 bp deletion, from -359/-339 bp on the murine FSHβ promoter, affected activin, progestin, and the synergistic induction of FSHβ gene expression dramatically, though, as the -381 HRE and the -267 SBE were brought closer together, some of the synergy between activin and progestin was rescued. On closer examination, it was recognized that the 21bp deletion contained a Smad half-site at -356 bp, a newly-characterized FOXL2 site at -350 bp, and one bp of an SF1 site at 339 bp. Individual mutation of the complete SF1 site did not affect activin, progestin, or their synergistic induction of the murine FSHβ expression, indicating that the SF-1 site does not play a significant role. However, our results indicate that, in addition to a role in the activin response, the FOXL2 and the nearby Smad

half-site are necessary for progestin responsiveness and synergy between activin and progestin on the FSHβ promoter.

We then investigated the regulation of LHβ gene expression by progesterone and GnRH interactions. Our studies revealed that a conserved lysine on the murine PR DBD is not required for progestin suppression of LHβ gene expression and most likely, there are other residues in the DBD that are important for tethering interactions remaining to be identified. In finer mapping and characterization of the repressive elements, we revealed that in the context of the -300 rat LHβ promoter, the -200/-150bp region contributes to basal gene expression, most likely by binding specific transcriptional activators. Furthermore, only the -300/-280 bp region, within the -300/-250 bp repressive element, was shown to be important for progestin suppression of the LHβ promoter.

In addition, our studies demonstrated that the two 50 bp repressive elements together, upstream of a TK-luc heterologous promoter are sufficient for progestin suppression. The -300/-250 bp region (but not the -200/-150 bp region) alone is also sufficient for progestin suppression of basal induction. Twenty bp segments of this region, subcloned upstream of the TK-luc reporter, also revealed that the -300/-280 bp region was the only segment sufficient to significantly suppress basal induction due to progestin treatment, confirming the data obtained in the context of the -300 rat LHβ promoter. In conclusion, our studies contributed to the finer mapping of the progesterone suppression

of basal and GnRH-induced LHβ gene expression by identifying a critical segment at the -300/-280bp region of the promoter.

To study the feedback regulation of progesterone only in gonadotrope cells *in vivo*, we created a gonadotrope-specific PRKO: the PR$^{KOLacZ/Flox}$/LHβ-Cre mouse line (105). Our studies did not show any significant impairment of fertility, possibly due to genetic penetrance issues, but serve as preliminary data indicating trends towards lower reproductive function in the gonadotrope-specific knockout mice, such as lower number of litters and pups, as well as lower levels of circulating hormones.

Materials and Methods

Hormones

Promegestone (R5020) was purchased from NEN Life Sciences (Boston, MA), activin from Calbiochem (La Jolla, CA), and GnRH from Sigma-Aldrich (St. Louis, MO).

Plasmid Constructs

The mouse FSHβ promoter was kindly provided by Malcolm Low. The construction of the -1000FSHβluc reporter plasmid was described previously (25). The -985 ovine FSHβluc reporter plasmid has been previously described (106). The -1028/+7 human FSHβluc reporter plasmid, in pGL3, was generously provided by Daniel Bernard. The 1.8 rat LHβ luciferase reporter, in pGL3, was kindly donated by Dr. Mark Lawson. The 1294 PR antibody (Ab), and Wild-type (WT) mouse and human PRB, both in pcDNA-I, were generously provided by Dean Edwards.

Mutagenesis

The QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to generate mutations in mouse FSHβ, human FSHβ and rat LHβ promoter plasmids. Generation of the -139 HRE, -197 HRE, -230 HRE, -273 HRE, and -381 HRE mutants in the mouse FSHβluc reporter plasmid was described previously (25), along with the -120 SBE, -153 SBE, -267 SBE, and 3xSBE mutations (103). The sequences of the mutated promoter fragments were confirmed by dideoxyribonucleotide sequencing performed by the DNA Sequencing Shared Resources, UCSD Cancer Center. Mutagenesis primers are listed in Table 2-1.

Cell Culture and Transient Transfections

All cell culture and transient transfection experiments were performed using the LβT2 cell line. The cells were maintained in 10 cm plates in DMEM (Dulbecco's Modification of Eagles Medium) from Mediatech Inc., (Herndon, VA) with 10% FBS (Fetal Bovine Serum) from Omega Scientific, Inc., (Tarzana, CA) and penicillin/streptomycin antibiotics (Gibco/Invitrogen, Grand Island, N.Y.) at $37^{o}C$ and 5% CO_2. 1X Trypsin-EDTA (Sigma-Aldrich, St. Louis, MO) was used in passing of the cells. The cells were split $3X10^5$ cells/well into 12-well plates, and were transfected 18 hours later, using Fugene 6 reagent (Roche Molecular Biochemical, Indianapolis, IN) following

the manufacturer's instructions. For all experiments, the cells were transfected with 400 ng of the promoter plasmid, 10 or 100 ng of mouse PRB (unless otherwise stated, i.e. 10 ng for FSHβ and 100 ng for LHβ experiments), and, to control for transfection efficiency, 200 ng of a β-galactosidase reporter plasmid that is driven by the Herpes Virus thymidine kinase promoter (TK). The cells were switched to serum-free DMEM containing 0.1% BSA, 5 mg/L transferrin, and 50 mM sodium selenite 6 hours after transfection.

The cells were treated with various hormones after overnight starvation in the serum-free media. Vehicle control for R5020 was 0.1% ethanol and that for activin and GnRH was 0.1% BSA. For experiments with the FSHβ promoter, the cells were treated with 0.1% ethanol, and 10 ng/ml activin (unless stated otherwise) for 24 hours; 0.1% ethanol and 10^{-8} M GnRH for 6 hours; 10^{-9} M R5020 and 0.1% BSA for 24 hours; or the indicated hormone combinations at the above concentrations and time period. For the LHβ promoter, the cells were treated for 6 hours with 0.1% ethanol and 0.1% BSA; 0.1% ethanol and 10^{-8} M GnRH; 10^{-7} M R5020 and 0.1% BSA; or both 10^{-7} M R5020 and 10^{-8} M GnRH.

Luciferase and β-galactosidase Assays

Following the hormone treatment, the cells were washed with 1X phosphate buffered saline (PBS), followed by lysis with 0.1 M K-phosphate

buffer pH 7.8 containing 0.2% Triton X-100. The lysed cells (30 µl) were then assayed for luciferase (luc) activity using a buffer containing 100 mM Tris-HCl (pH 7.8), 15 mM $MgSO_4$, 10 mM ATP, and 65 µM luciferin. β-Galactosidase (β-gal) activity was assayed, with 15 µl of the lysed cell extracts, using the Tropix Galacto-light assay (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. Both assays were measured using a Veritas Microplated Luminomiter using an EG&G Berthold Microplate Luminometer (PerkinElmer Corp., Norwalk, CT).

Nuclear Extracts

Nuclear extracts were prepared from LβT2 cells, untreated or treated for 2.5 hours with 0.1% BSA or 10^{-8} M GnRH following overnight starvation in serum-free media, as previously described (107) by allowing the cells to swell in a hypotonic buffer (20 mM Tris pH7.4, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM PMSF in isopropanol, protease inhibitor cocktail from Sigma-Aldrich), passing them through a $25^5/_8$ G needle 3 times, spinning down the nuclear material, resuspending and incubating the pellet 30 minutes in a hypertonic buffer (20 mM Hepes pH 7.9, 20% Glycerol, 420 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM PMSF in isopropanol, and protease inhibitor from Sigma-Aldrich), centrifuging, and freezing of aliquots of the supernatant

(nuclear extracts) for use in *Electrophoretic Mobility shift assay* (*EMSA*) experiments.

EMSA

Purification of recombinant PR from Sf9 insect whole cell extracts was described previously (108). Oligonucleotides obtained from Integrated DNA Technologies were annealed, then labeled with γ^{32}P ATP using T4 Polynucleotide Kinase from New England Biolabs, Inc., (Beverly, MA), and column purified using Micro Bio-Spin Chromatography Columns (Bio-Rad Laboratories, Inc., Hercules, CA), with both reagents being used according to the manufacturer's protocol. Purified recombinant PR, and/or LβT2 nuclear extracts were incubated with 2 fmol of ^{32}P-labeled oligonucleotide at 4 C for 30 min in 20 μl binding reactions containing DNA-binding buffer (10 mM HEPES (pH 7.8), 50 mM KCl, 0.5 mM MgCl2, 0.1% Nonidet P-40, 1 mM dithiothreitol, 2 μg polydeoxyinosinic deoxycytidylic acid, and 10% glycerol).

Following incubation, the reactions were run on a 5% polyacrylamide gel (30:1 acrylamide-bisacrylamide) containing 2.5% glycerol in a 0.5X Tris-acetate-EDTA buffer. The 1294 PR mouse monoclonal Ab was used to supershift PR and mouse IgG was used as a control for nonspecific binding. A 1000X excess of consensus PRE was used for competition. Oligonucleotides used in EMSA are listed in Table 2-2.

Genotyping of Gonadotrope- Specific PRKO mice

The breeding resulting in the birth of female PR$^{KO/Flox}$/Cre+/- mice is outlined in Figure 5-1. Mice were maintained in a temperature controlled (22°C) room, with a 12-hour light, 12-hour dark photocycle, and fed rodent chow and fresh water, *ad libitum*. All procedures were in accordance with an approved Animal Care and Use Protocol (S00261) on file with the UCSD Institutional Animal Care and Use Committee.

Generation of the gonadotrope-specific Cre transgenic mouse (LHβ-Cre) capable of ablating floxed genes in mature pituitary gonadotropes was described previously (105). To evaluate the existence of the heterozygous PR^{LacZ} knockin allele (109), and the LHβ-Cre allele, Accupower PCR PreMix (Bioneer, Daejeon, Korea) was used for polymerase chain reaction (PCR) genotyping of the mice (all genotyping primer sets are shown in Table 2-3). PCR cycling conditions for the Cre genotyping reaction was as follows: initial denaturation (98°C, 5 min), product amplification (30 cycles of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C) with a final extension at 72°C for 10 min. The expected Cre band (500 bp) was resolved on a 2% agarose gel. PCR cycling conditions for genotyping of heterozygous PR^{LacZ} (PRLacZ, PR 1 and PR 2 primers used) was as follows: initial denaturation (98°C for 5 min), product amplification (40 cycles of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C) with a final extension at 72°C for 10 min. The expected PR wild-type (wt) band at 590 bp, and the LacZ knockin band at 148 bp were resolved by gel

electrophoresis. The mice were crossed to create homozygous mice carrying the PR conditional excision (PRCE) (110) allele (PR$^{Flox/Flox}$) and thus all mice were expected to carry the heterozygous flox allele. PCR cycling conditions for genotyping of heterozygous PR^{Flox} (Table 2-3: PR-flox ABCD primers (110)) allele was as follows: initial denaturation (94°C, 2 min), product amplification (35 cycles of 94°C, 20 sec, 59°C, 20 sec, 68°C, 40 sec) with a final extension at 68°C for 5 min. The expected PR WT band was 160 bp, whereas the flox allele was 210 bp.

Based on sequence information obtained for the PR genomic DNA and the reported location of the loxP sites flanking the PR exon 1 (110) primers were designed (M. Brayman; Table 2-3) to evaluate Cre recombination in the pituitary, leading to the gonadotrope-specific PRKO mice. The primers PR-flox J and K flank the loxP sites in PRflox, and PR-flox O is within the excised region, leading to product sizes: JO at 571 bp and JK at 263 bp with PCR cycling conditions as the following: initial denaturation (95°C, 5 min), product amplification (29 cycles of 95°C, 30 sec, 59°C, 30 sec, 72°C, 30 sec) with a final extension at 72°C for 5 min.

Vaginal Opening and Estrous Cycles

The opening of the vaginal orifice, marking the onset of puberty, was checked every day at 1:00 PM, starting 21 days after birth, by observation and

light touch (gloved finger) of the vaginal opening, without manipulation by a probe. Vaginal smears were taken every day at 1:00 PM from female mice, 10-12 weeks old, and cytology was used to identify the phase of the estrous cycle for 10 consecutive days. Smears were collected by flushing the vagina with approximate 50 µl of tap water. The samples were applied to slides and allowed to sit and dry for approximately 1 hour. The slides were then stained with 0.1% aqueous Methylene blue for 5 minutes and allowed to dry for 1 hour. Stained slides were observed using a light microscope. During diestrus, vaginal smear cytology consists of predominantly leukocytes (small round cells). Proestrus is indicated by the presence of mostly nucleated epithelial cells. Estrus is indicated by mostly anucleated, cornified epithelial cells. Smears taken at metestrus have all cell types present in approximately equal proportions (94).

Statistical Analysis

The transient transfection experiments were performed in triplicate, and each experiment was repeated independently at least three times (unless otherwise specified). The data were normalized for transfection efficiency by presenting the luc assay activity relative to β-gal activity, and relative to the empty pGL3 plasmid (control for hormone effects on the vector DNA). The data were analyzed by one-way ANOVA, followed by *post hoc* comparisons

with the Tukey-Kramer honestly significant difference test (HSD) or two-way ANOVA representing a significant synergistic interaction. In the figures, the *error bars* represent SEM. Normal or Box Cox Transformed ratios for each promoter construct in each cell type were compared, and in all analyses, $P \leq$ 0.05 was considered significant. The gonadotrope-specific PR knockout analyses were assessed for significant difference from control using Student's *t*-test.

Table 2-1: Mutant FSHβ Promoter and PR Gene Sequences
(All bases that were changed are in bold font.)

Human FSHβ Promoter Mutants

H-381StrongMut	5'-TTTGTTTCTTCCTTCACAG**TG**TT**CA**ATATGCTCTTGGAGCAATTT-3'
H-267Insert	5'-AAAGATACAAAAGAAAA**GTCTAGAC**TCTGGAGTCACAATTAATT-3'

Mouse FSHβ Promoter Mutants (Kindly provided by P. Corpuz)

FoxL2	5'-ATCAATTAAGACATATT**AAAAA**TTACCTTCGCAATGGAGCCAAAG-3'
Smad	5'-TTCTTGGATCAATTAA**TTT**ATATTTTGGTTTACCTTCGCAATGG-3'
SF-1	5'-ATCAATTAAGACATATTTTGGTTTA**AAAA**CGCAATGGAGCCAAAG-3'
Smad+FoxL2	5'-ATCAATTAA**TTT**ATATT**AAAAA**TTACCTTCGCAATGGAGCCAAAG-3'
FoxL2+SF-1	5'-ATCAATTAAGACATATTAAAAATTAAAAACGCAATGGAGCCAAAG-3'

PR Mutants

PR DBD-CTE K581A	5'-TGCTTACCTGTGGGAGCTGC**GCG**GTCTTCTTTAAGAGGGCAATG-3'

Table 2-2: EMSA

LHβ -225/-191	5'-TACCTGTTCCCTGTGTTCCCAATGTCAGTTAAGCT-3'
LHβ -225/-191 PRE mutant	5'-TACCT**C**TT**G**CCTGT**C**TT**G**CCAATGTCAGTTAAGCT-3'
LHβ -300/-250	5'-ACCATCGGAGTGGGTCTGACTGAAGTTCATTCCAGCATCCT AGGGCCAGC-3'
LHβ -250/-200	5'-TAGCAGCCTGCAGAGTTCTCCCCTTTACCTGTTCCCTGTG TTCCCAATGT-3'
LHβ -200/-150	5'-CAGTTAAGCTCAGGCACCTGGGCTGAGTGTGAGGCCAATT CACTGAGACA-3'
FSHβ -99/-64	5'-CTTTCAGCAGGCTTTATGTTGGTATTGGTCATGTTA-3'

Table 2-3: Genotyping Primer Sequences

LHβ Cre Forward	5'- GCATTACCGGTCGTAGCAACGAGTG -3'
LHβ Cre Reverse	5'- GAACGCTAGAGCCTGTTTTGCACGTTC -3'
PRlacZ	5'-CTTCACCCACCGGTACCTTACGCTTC-3'
PR 1	5'-TAGACAGTGTCTTAGACTCGTTGTTG-3'
PR 2	5'-GATGGGCACATGGATGAAATC-3'
PR-flox A	5'-TGTGCACTTTTGGAGGCAAG-3'
PR-flox B	5'-GTGGAGGCTTCTGGACAGT-3'
PR-flox C	5'-TAAAGCGCATGCTCCAGAC-3'
PR-flox D	5'-TGATTTTGCCTTTGGCAGATG-3'
PR-flox J	5'- GGAATGTGTGCACTTTTGGA-3'
PR-flox K	5'-GTGGGGAAAATGCTCTTGAA-3'
PR-flox O	5'- GTCCACTCTCAAGCCCAGTC-3'

III

Identification of Novel Regulatory Regions within the FSHβ Promoter Involved in the Synergistic Induction of FSHβ Gene Expression by Progestins and Activin

<u>**Introduction**</u>

In recent years, Schwartz and colleagues have provided evidence that PR activation may be important in stimulating the secondary FSH surge (111). They have revealed that PR antagonism on proestrus attenuates the FSH surge on estrous, regardless of serum inhibin levels decreasing (111).

The dimeric protein, activin, a member of the TGFβ family of signaling molecules, also selectively and potently stimulates FSH synthesis and secretion in a paracrine manner. Using primary pituitary cell cultures, Szabo *et al.* demonstrated that activin may play a role in PR-mediated stimulation of secondary FSH surges and that cross-talk between the two signal transduction pathways may also be important (112). Furthermore, Miyake *et al*. (1993) have shown that FSH levels increase in primary pituitary cells due to activin and progesterone co-treatment (113). Other studies reveal that progesterone stimulation of FSH secretion is prevented by inhibin (114) and follistatin (115). Smad transcription factors have also been shown to interact directly with various steroid receptors (103, 116-120). The molecular mechanisms that

mediate cross-talk between activin and progesterone signaling pathways involved in the regulation of FSHβ gene expression have been investigated recently and are the focus of our studies presented in this chapter.

Recently, our lab showed that the FSHβ promoter is induced synergistically in LβT2 cells after activin and progesterone hormone co-treatment (103). It was demonstrated that gonadotrope cells are sufficient for synergy between activin and progestins. and that this synergistic interaction occurs directly on the FSHβ promoter. Additionally, both Smad and PR signaling as well as DNA binding to the FSHβ promoter were required for the synergy (103). In the study presented, the molecular mechanism of FSHβ transcriptional regulation by activin and progesterone was further investigated.

Low Levels of Exogenous PR are Sufficient for Progestin Responsiveness on the FSHβ Promoter

Although the immortalized LβT2 gonadotrope-derived cell line has been shown to express PR (25, 121), the endogenous levels may be low because progestin responsiveness on the FSHβ promoter is less than 2 fold (25). Thus, to amplify the hormone response, exogenous PR was transfected into the cells. 100 ng of transfected PR resulted in a significant progestin response on the FSHβ promoter (25), and as more PR was added, the response increased without reaching saturation. Since the progestin response was receptor-dependant and the receptor was limiting in the cell, decreasing amounts of PR was then transfected into LβT2 cells in order to determine whether lower amounts of PR, which may mimic physiological levels were sufficient for progestin induction of the FSHβ promoter (Figure 3-1). At 100 ng/ml, the response to R5020 (a synthetic progesterone analog) on the FSHβ promoter was approximately 27 fold; 50ng/ml 19 fold; 25 ng/ml 17 fold; 10 ng/ml 10 fold; 5 ng/ml 4.5 fold. Since 10 ng/ml of PR gave a robust induction, all subsequent experiments were performed with this amount of receptor.
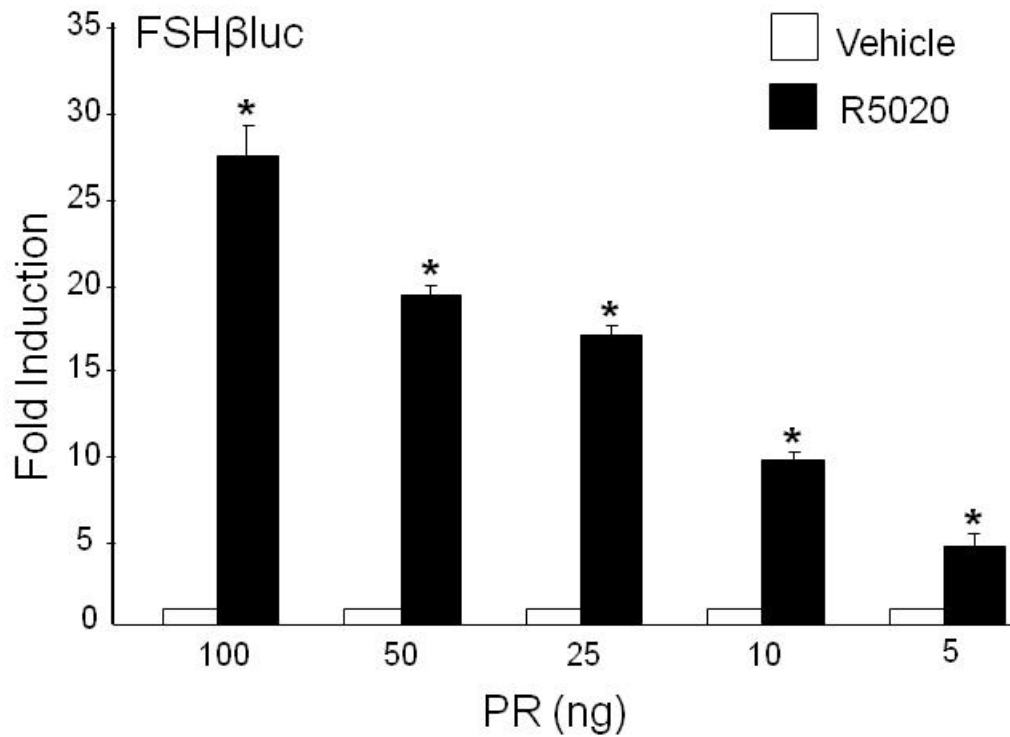
Figure 3-1: 10 ng of PR is sufficient for progestin responsiveness on the FSHβ promoter

The murine -1000FSHβluc reporter was transiently transfected into LβT2 cells along with an expression vector containing murine PR at 100-5 ng/well, as indicated. After overnight starvation in serum-free media, the cells were treated for 24h with vehicle or 10^{-9} M R5020. The results represent the mean ± SEM of at least three experiments performed in triplicate. *, Significant difference from the vehicle-treated control.

GnRH and Progestin Signaling Pathways Do Not Synergize on the FSHβ Promoter

Recently, it has been shown that activin and GnRH synergistically induce rodent FSHβ gene expression (59, 122). FSHβ is also induced in LβT2 cells after activin and steroid hormone co-treatment (103, 123). It was of interest, therefore, to investigate whether GnRH and progestin signaling pathways interact to mediate FSHβ gene expression. As shown in Figure 3-2A, GnRH induced the FSHβ promoter 2.9 fold and R5020 8.4 fold as shown previously. However, GnRH and R5020 co-treatment only upregulated FSHβ gene expression 9 fold, indicating that they do not interact to regulate the FSHβ promoter. Since activin, GnRH and progesterone are all present during the menstrual/estrous cycle and regulate FSHβ gene expression, experiments were carried out to analyze FSHβ induction in the presence of all three hormones (Figure 3-2B). As shown previously, activin and GnRH as well as activin and progestin synergistically enhanced FSHβ transcription. In addition, GnRH significantly reduced the synergistic induction of FSHβ by activin and R5020 co-treatment.
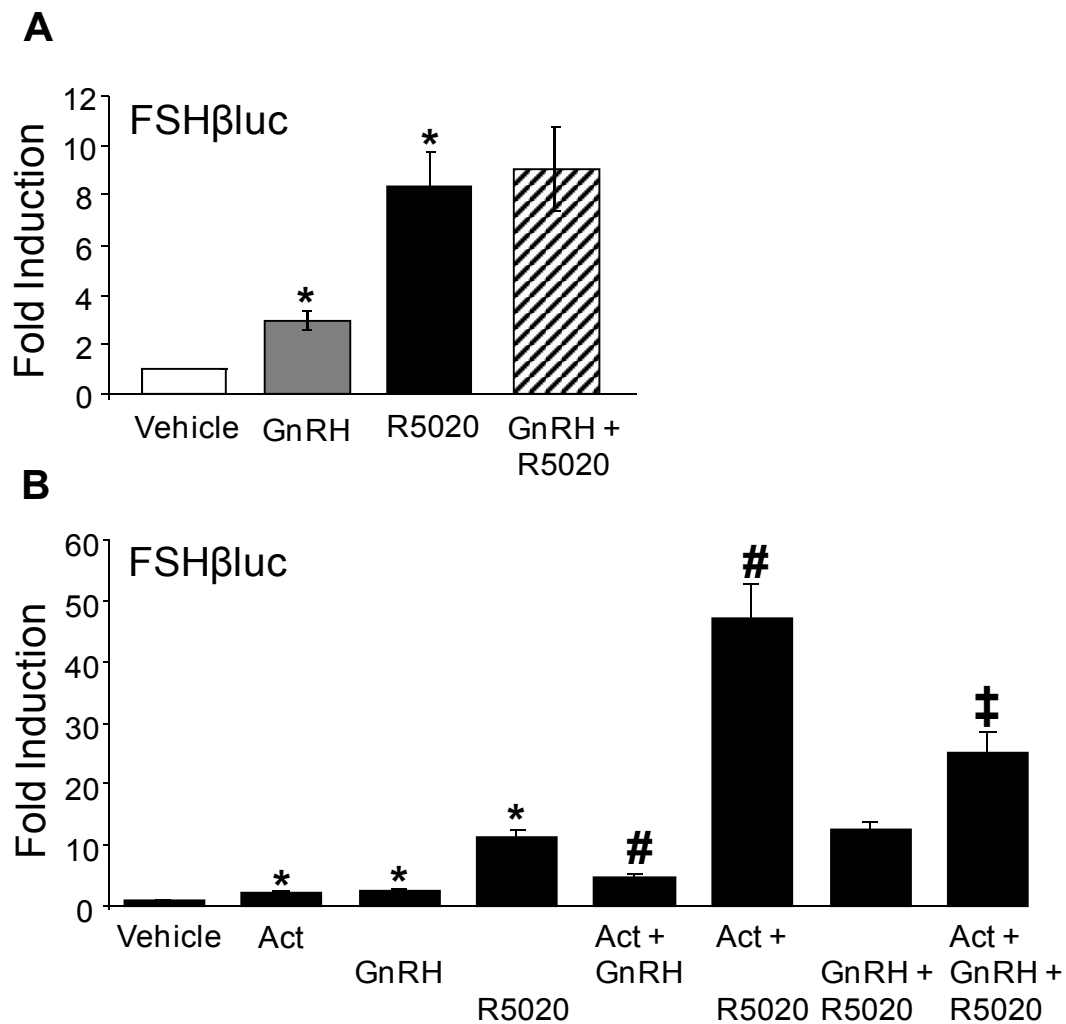
Figure 3-2: GnRH and R5020 do not synergistically regulate FSHβ gene expression, but GnRH reduces the synergistic induction of FSHβ by activin and R5020 co-treatment

The -1000FSHβluc was transiently transfected into LβT2 cells along with 10 ng of PR. After overnight starvation in serum-free media, the cells were treated for 24h with the indicated hormones. (A) The cells were treated with vehicle, 10nM GnRH, 10^{-9} M R5020, or both for 24 hours. (B) The cells were treated with vehicle, 10ng/ml activin (Act), 10nM GnRH, 10^{-9} M R5020 individually, or with the indicated combinations. The results represent the mean ± SEM of at least three experiments performed in triplicate. *, Significant difference from the vehicle-treated control; #, significant interaction as defined by a two-way ANOVA; ‡ significant difference from Act and R5020 as defined by Student's t-test ($P<0.05$).

Progestin Responsiveness and Synergy with Activin Is Not Conserved On the Ovine and Human FSHβ Promoters

A reporter gene containing the proximal ovine FSHβ gene promoter was shown to respond to progesterone treatment via six putative PREs in ovine primary pituitary cultures (124). Activin has been shown to regulate ovine FSHβ gene expression by Smad2/3 and TAK1- dependent signaling pathways (65, 125). Data regarding transcriptional regulation of the human FSHβ gene is limited. One polymorphism in the human FSHβ promoter mapped to a putative PRE element, suggesting that there may be conservation of regulatory mechanisms for this gene's expression among mammals (126). Furthermore, GnRH and activin synergistically induced human FSHβ transcription through a high affinity AP-1 site on the human promoter and Smad dependent signaling pathways (127). To determine whether the ovine and human promoters respond to progestins, or synergistically to progestins and activin, murine -1000, ovine -985, or human -1028/+7 FSHβluc reporter constructs were transiently transfected into LβT2 cells along with mouse PR (Figure 3-3A). In contrast to the murine promoter which was induced 16.4 fold by progestin treatment and 83.4 fold by activin and progestins, the ovine and the human FSHβ promoters did not exhibit progestin responsiveness alone or in the presence of activin. To confirm that the lack of a synergistic response was not due to low levels of exogenous activin, 50ng/ml of activin was used for all these experiments. Furthermore,

human PR was also transfected to determine if progestin responsiveness and a synergistic induction on the human FSHβ promoter required the human PR, and as shown in Figure 3-3B, there was also no response with human PR.

The -381 HRE On the Human FSHβ Promoter Is Not Sufficient For Progestin Induction or Synergy

An HRE at -381 bp upstream of the transcriptional start site on the FSHβ promoter has been shown to be necessary for progestin and synergistic induction of FSHβ gene expression, since mutating it reduced the response to R5020 to approximately 26% of the wild-type murine FSHβ promoter, and synergy between progestin and activin was decreased by 73% (25). Given these results, it was concluded that the -381 HRE plays a prominent role in progestin responsiveness (103). The human FSHβ promoter region contains a putative half site similar to the murine -381 HRE at -395 upstream of the start site (25). We therefore investigated whether addition of the murine -381 HRE, -267 SBE, or both would be sufficient to restore progestin responsiveness or synergy between progestins and activin in LβT2 cells. With the addition of the -267 SBE site, the human FSHβ promoter responded to activin 3 fold greater than the WT human promoter. On the other hand, neither the progestin response nor synergy with activin and R5020 co-treatment were rescued by the addition of the -381 HRE. Thus, this experiment indicated that the -381

HRE is not sufficient for progestin responsiveness on the human FSHβ promoter. Furthermore, although the -267 SBE could restore substantial activin responsiveness to the human promoter, it was unable to restore activin and progestin synergy, indicating that progestin responsiveness is also required for the synergy.
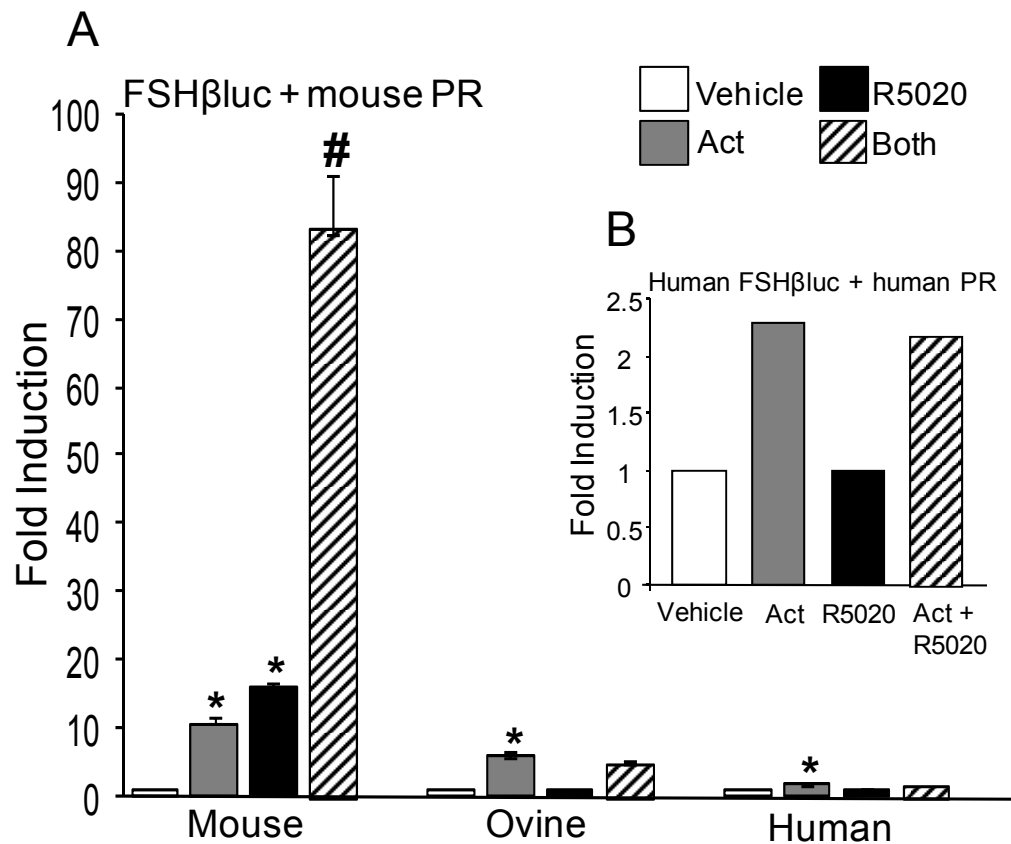
Figure 3-3: Progestin responsiveness and synergy between progestins and activin is not conserved on the ovine and human FSHβ promoters

(A) The murine -1000, ovine -985, or human -1028/+7 FSHβluc reporter constructs were transiently transfected into LβT2 cells along with 10 ng mouse PR. (B) The human FSHβluc reporter was transfected along with 10 ng human PR was transfected into LβT2 cells; the figure corresponds to one experiment performed in triplicate. After overnight starvation in serum-free media, the cells were treated for 24h with vehicle, 50ng/ml activin, 10^{-9} M R5020, or both. The results represent the mean ± SEM of at least three experiments performed in triplicate. *, Significant difference from the vehicle-treated control; #, significant interaction as defined by a two-way ANOVA.
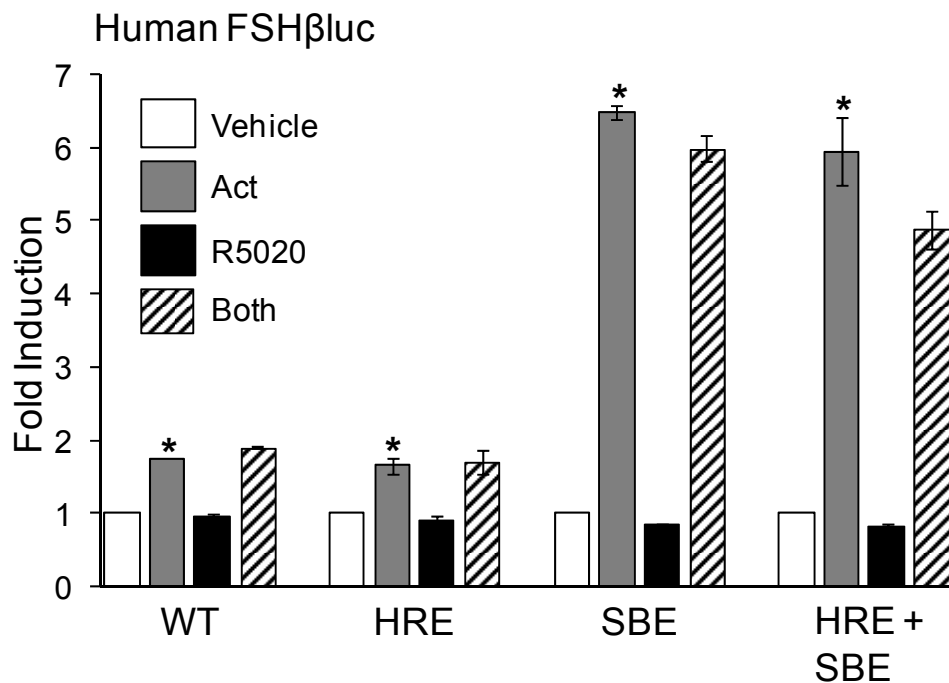
Figure 3-4: Addition of the murine -381 HRE, -267 SBE, or both to the human FSHβ promoter was not sufficient for R5020 induction or synergy between activin and R5020

The WT human -1028/+7 FSHβluc reporter or mutants containing the murine -381 HRE, -267 SBE or both were transiently transfected into LβT2 cells, as indicated along with PR. After overnight starvation in serum-free media, the cells were treated for 24h with vehicle, 50ng/ml activin, 10^{-9} M R5020, or both. The results represent the mean ± SEM of two experiments performed in triplicate. *, Significant difference from the vehicle-treated control; #, significant interaction as defined by a two-way ANOVA.

Previously Identified HREs Are Important for Both Progestin Induction of FSHβ and Synergy Between Progestins and Activin

The murine -1000 FSHβ promoter containing mutations at the -139, -197, -230, -273, and -381 HREs were previously studied for their responsiveness to progestins (25), and the -381 HRE mutant was examined for its response to activin, progestins, and co-treatment (103). The WT -1000 FSHβ promoter reporter and mutations in the above mentioned HRE sites were then tested for responsiveness to activin, progestins, and both with limiting PR conditions (10ng) to determine the importance of the sites under more physiological conditions (Figure 3-5). The -139, -197, -230, and -273 HRE mutants showed a decrease in progestin responsiveness by approximately 49%, 41%, 68%, and 64% of the WT R5020 induction, respectively. The -197 HRE mutation increased the activin response by approximately 57%, which may be due to the creation of an activin responsive element as an unintended result of the mutation or disruption of an activin repressive element. None of the other mutated promoters showed any significant difference in their activin response. The synergistic induction of the mutated promoters due to activin and R5020 co-treatment was reduced by 32%, 62%, 73%, and 64% for the -139, -197, -230, and -273 HRE mutants, respectively. The only HRE mutation that led to a complete lack of response to R5020, and complete disappearance of any synergistic induction of the promoter was the -381 HRE mutation, confirming the importance of this site for

progestin responsiveness on the FSHβ promoter as well as for the synergy between activin and progestin.
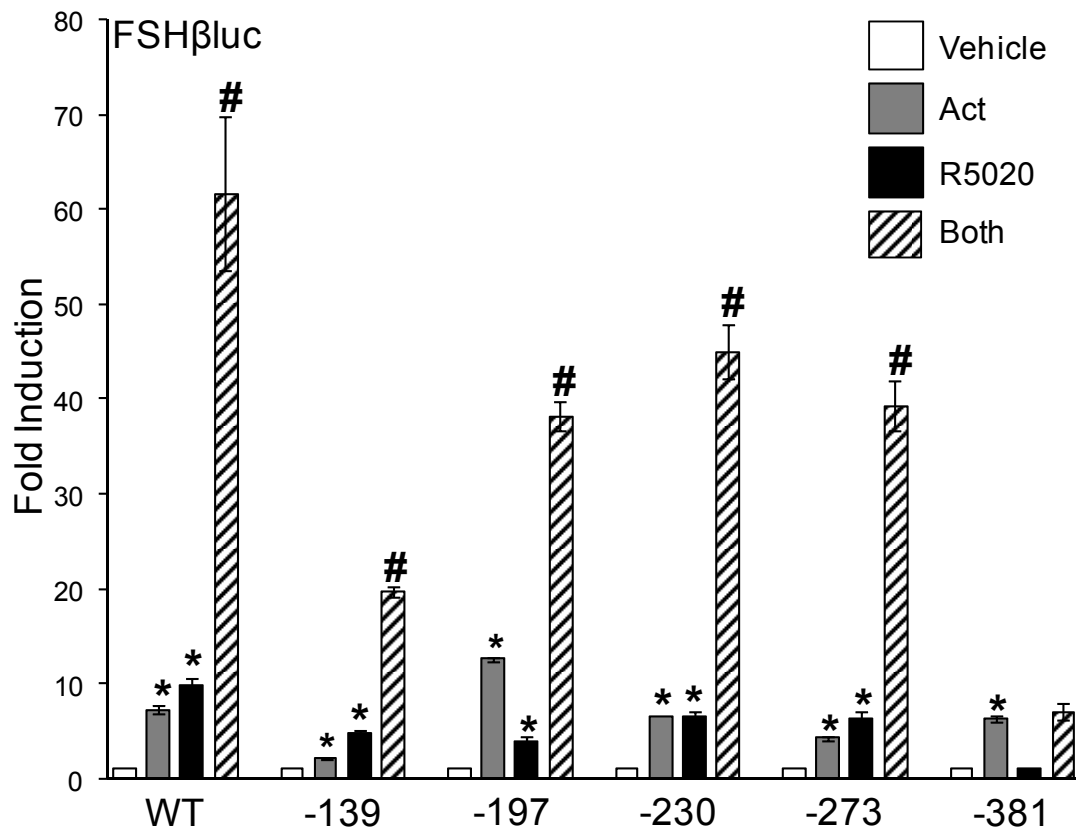
Figure 3-5: HREs other than the -381 are also necessary for full progestin responsiveness

The wild-type murine -1000FSHβluc reporter and mutants were transiently transfected into LβT2 cells, as indicated along with 10ng PR. After overnight starvation in serum-free media, the cells were treated for 24h with vehicle, 10ng/ml activin, 10^{-9} M R5020, or both. The results represent the mean ± SEM of at least three experiments performed in triplicate. *, Significant difference from the vehicle-treated control; #, significant interaction as defined by a two-way ANOVA.

Disruption of the Smad Binding Elements Prevents Cross-talk Between Activin and Progestins

The expression of a luciferase reporter containing three mutated SBEs at -120, -153, and -267, the 3xSBE (*Materials and Methods*) in the murine -1000 FSHβ proximal promoter was previously studied for its responsiveness to activin, progestin, or both (103). Here, the WT murine -1000 FSHβluc and mutants of the SBEs above were tested for responsiveness to activin, progestin, and both with limiting PR conditions (10ng) to determine the importance of the sites under more physiological conditions (Figure 3-6). The -120, -153, and -267 SBE mutants showed a decrease in progestin responsiveness by approximately 35%, 40%, and 13% of the WT activin induction, respectively. The -153 SBE mutation also decreased the R5020 response by 65%. None of the other mutated promoters showed any significant difference in their progestin response. The synergistic induction of the mutated promoters due to activin and R5020 co-treatment was reduced to 46%, 53%, and 25% for the -120, -153, and -267 SBE mutants, respectively. The 3xSBE mutation led to a complete lack of response to activin, and complete disappearance of any synergistic induction of the promoter. These results indicate that the activin response and consequently the synergistic induction of the promoter by activin and progestin require the binding of factors to the multiple SBEs in the FSHβ promoter. The -267 SBE appears to be of

particular importance for the synergy since its disruption led to a substantial decrease in the induction of FSHβ gene expression.
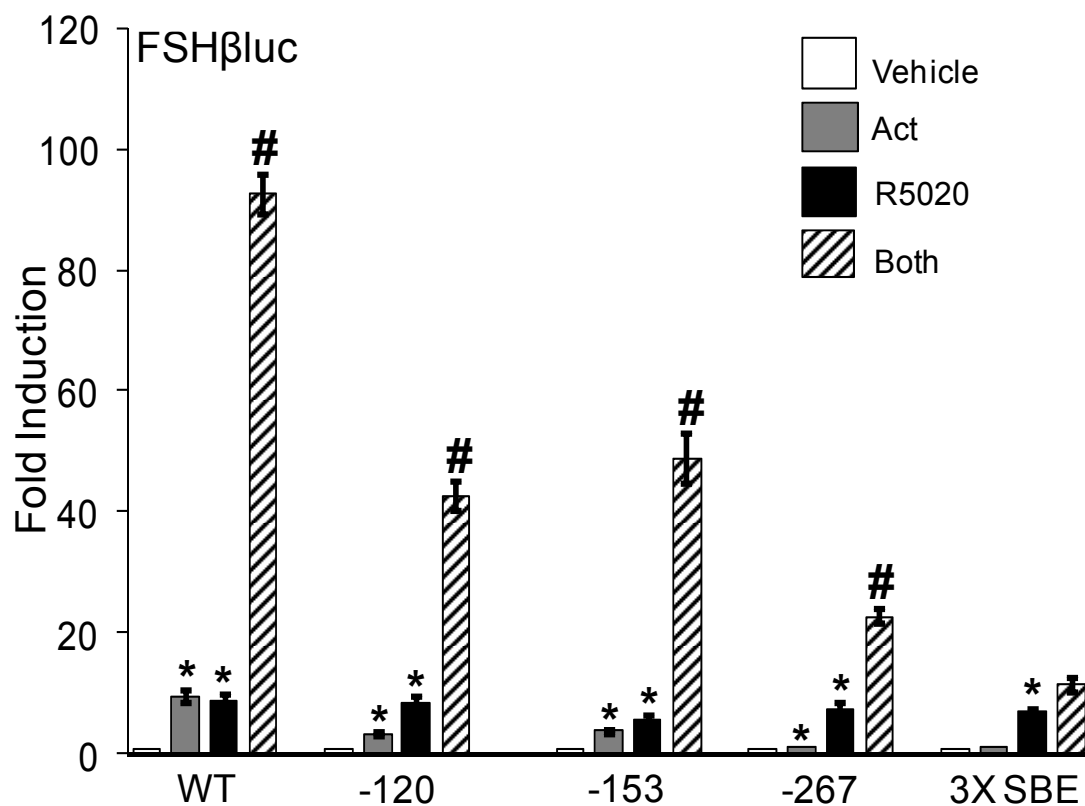
Figure 3-6: Disruption of the Smad Binding Elements prevents cross-talk between activin and progestins

The WT -1000FSHβluc reporter or mutants of the indicated SBEs were transiently transfected into LβT2 cells with PR. After overnight starvation in serum-free media, the cells were treated for 24h with vehicle, 10ng/ml activin, 10^{-9} M R5020, or both. The results represent the mean ± SEM of at least three experiments performed in triplicate. *, Significant difference from the vehicle-treated control; #, significant interaction as defined by a two-way ANOVA.

The Region Between the -381 HRE and the -267 SBE Contains a FOXL2 Site and a Smad Half-Site Both Important for Progestin Responsiveness and Synergy

As both the -381 HRE and the -267 SBE are particularly important for the synergistic interaction between progestins and activin on the FSHβ promoter, bringing the two sites closer together on the promoter may provide insight regarding the contributions of spacing and/or other factors in that region that be important for the synergy between activin and progesterone. As shown in the schematic in Figure 3-7, four deletions of approximately 8 (85bp), 6 (63bp), 4 (42bp), or 2 (21bp) DNA helical turns were created in the murine proximal FSHβ promoter between the -381 HRE and the -267 SBE. All the deletions started at -359 bp upstream of the transcriptional start site. The activin response of the 21 bp, 42 bp, 63 bp, and 85 bp deletions were reduced to 54%, 57%, 53%, and 62% of the WT promoter response, respectively. The progestin response was reduced to 44% and 70% of the WT promoter response for the 21bp, and 85bp deletion mutation reporters, respectively, while the 42 and 63 bp deletions did not affect the R5020 response. The synergy was reduced to 19%, 35%, 39%, and 56% of the WT promoter synergistic induction, for the 21 bp, 42 bp, 63 bp, and 85 bp deletions, respectively. The 21 bp deletion affected the activin, progestin and the synergistic induction of FSHβ gene expression most dramatically. It is interesting to note that, as the -381 HRE and the -267 SBE were brought

closer together, some of the synergy between activin and progestin was rescued.

Since it appeared from the above experiment that the region between the -381 HRE and the -267 SBE contains elements that are important for hormonal regulation of the promoter, we then undertook a more systematic analysis of this region. 10 bp block deletions from -370 to -271 in the FSHβ proximal promoter region were created, and their responsiveness to activin, progestin, or co-treatment was measured. The -370/-361 deletion, overlapped the last 4 bps of the 3' end of the -381 HRE, and thus led to the reduction of R5020 responsiveness and synergy by 15% and 17% of the WT reporter expression. The deletions at -360/-351, -350/-341, and -320/-311 all resulted in significant reductions in the induction of FSHβ gene expression by activin (83%, 78%, and 90% of WT induction, respectively), R5020 (49%, 70%, 63% of WT induction, respectively) and co-treatment, (44%, 44% and 39% of WT induction, respectively).

On closer examination of the 10 bp deletions through -360 to -341, it was recognized that a Smad half-site at -356 bp, a newly-characterized FOXL2 site at -350 bp (D. Coss, personal communication), and one bp of an SF1 site at -339 bp (Figure 3-10) were deleted in the 21 bp deletion in Figure 3-8.. The complete SF1 site was deleted in the -340/-330 block deletion (Figure 3-9) and it did not have an effect on activin, progestin, or their synergistic induction of the murine FSHβ expression, indicating that the SF-1

site does not play a significant role. Specific mutations in each of the above mentioned sites and double mutants were created (P. Corpuz; *Materials and Methods,* Table 2-3), and their expression was measured to understand the effects of these sites on hormonal induction of the FSHβ promoter. As expected from the deletion studies, the SF1 mutation did not significantly alter FSHβ induction by activin, R5020, or both. The FOXL2 mutation, on the other hand, reduced activin response to 82%, progestin response to 42% and synergistic induction to 30% of the WT promoter. The FOXL2/SF1 double mutant did not significantly differ in any of the responses from the FOXL2 mutated reporter, again indicating that the SF1 site does not play a role. The Smad half-site mutation reduced activin response to 89%, progestin response to 47% and synergistic induction to 51% of the WT promoter induction. The FOXL2/Smad half-site double mutation reduced activin response to 85% and the progestin response to 18% of the WT promoter. The synergistic induction of the FOXL2/Smad half-site double mutant was very significantly reduced by to 12% of the WT synergy. Thus, the results indicate that, in addition to a role in the activin response, the FOXL2 and the nearby Smad half-site are necessary for progestin responsiveness and synergy between activin and progestin on the FSHβ promoter.
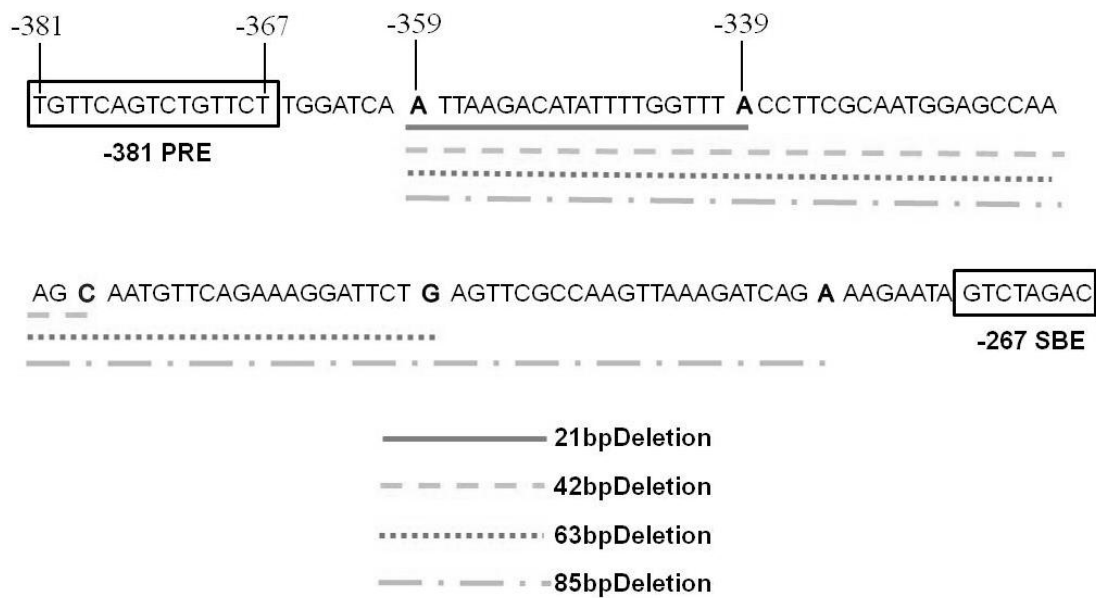
Figure 3-7: Schematic of deletions made between the -381 HRE and the -267 SBE in the murine -1000 FSHβ promoter

The deletion mutations were generated as described in the *Material and Methods* section.
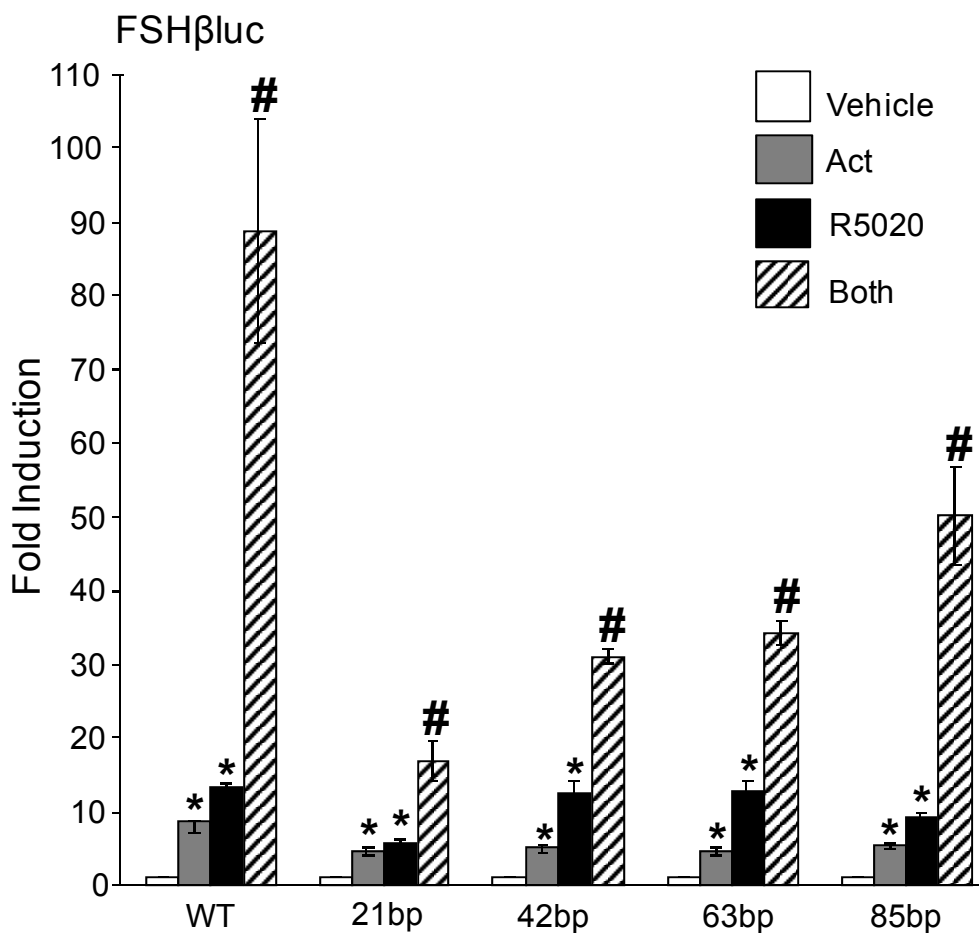
Figure 3-8: Deletions in the region between the -381HRE and the -267SBE identify a 21 bp region affecting activin, progestin, and synergistic induction of the FSHβ gene expression

The WT murine -1000FSHβluc reporter construct or mutants with the indicated deletions were transiently transfected into LβT2 cells with PR. After overnight starvation in serum-free media, the cells were treated for 24h with vehicle, 10ng/ml activin, 10^{-9} M R5020, or both. The results represent the mean ± SEM of at least three experiments performed in triplicate. *, Significant difference from the vehicle-treated control; #, significant interaction as defined by a two-way ANOVA.
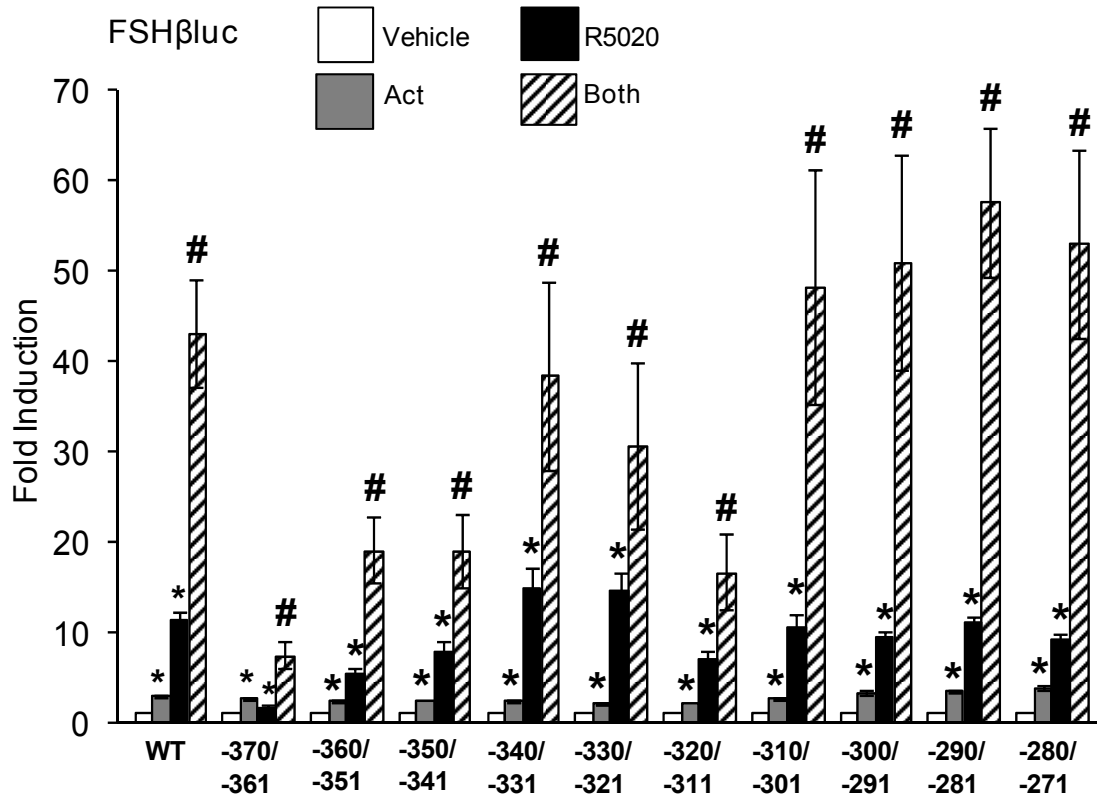
Figure 3-9: 10 bp block deletions between the -381 HRE and the -267 SBE reveal important regions affecting activin and progesterone induction of FSHβ individually and synergistically

The WT -1000FSHβluc reporter or mutants with the indicated 10bp deletions were transiently transfected into LβT2 cells with PR. After overnight starvation in serum-free media, the cells were treated for 24h with vehicle, 10ng/ml activin, 10^{-9} M R5020, or both. The results represent the mean ± SEM of at least three experiments performed in triplicate. *, Significant difference from the vehicle-treated control; #, significant interaction as defined by a two-way ANOVA.
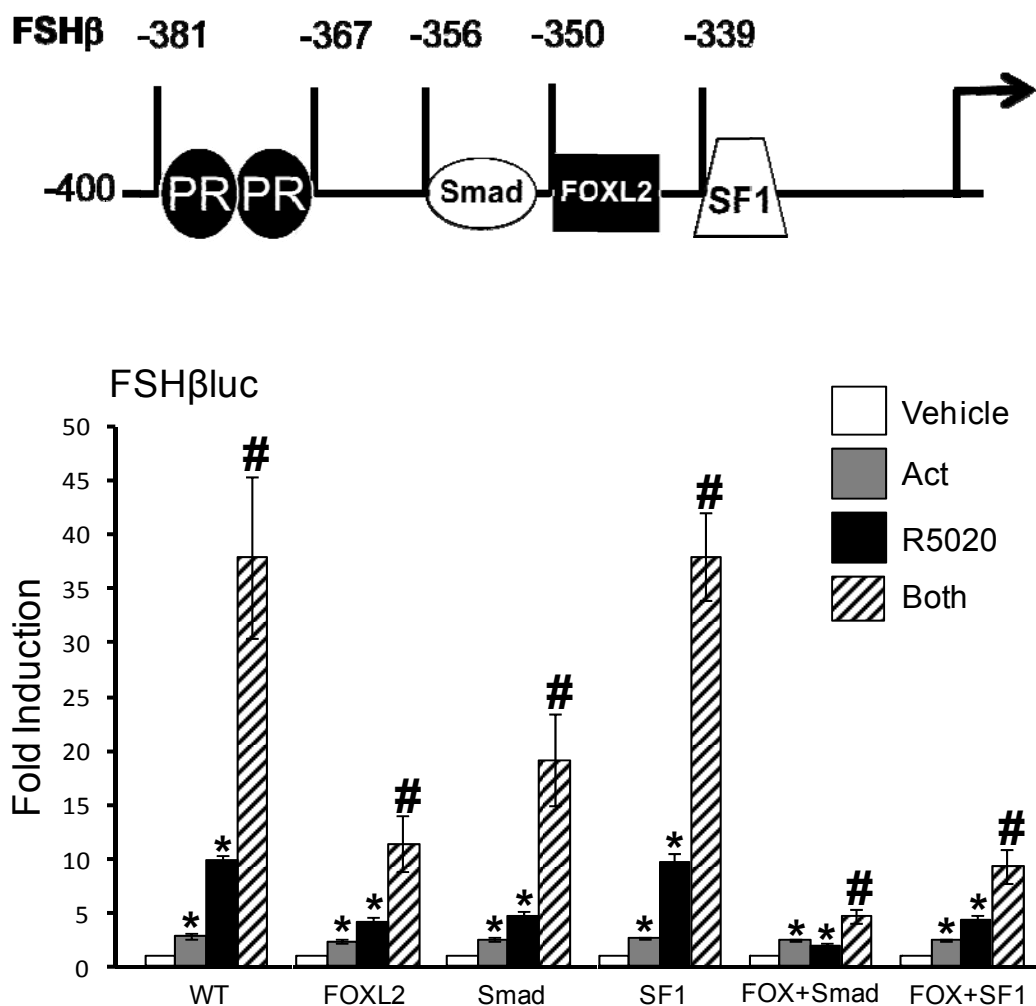
Figure 3-10: FOXL2 site at -350/-342 of the murine FSHβ promoter and nearby Smad half-site are necessary for activin and progestin induction of FSHβ individually and synergistically

The WT -1000FSHβluc reporter or specific mutants as indicated above were transiently transfected into LβT2 cells with PR. After overnight starvation in serum-free media, the cells were treated for 24h with vehicle, 10ng/ml activin, 10^{-9} M R5020, or both. The results represent the mean ± SEM of at least three experiments performed in triplicate. *, Significant difference from the vehicle-treated control; #, significant interaction as defined by a two-way ANOVA.

IV

Characterization of LHβ Promoter Regions Important for Progesterone Suppression of Basal and GnRH-Induced LHβ Gene Expression

<u>Introduction</u>

Although progesterone has been shown to reduce serum LH levels, some studies have reported no progesterone effect on either LHβ mRNA levels in ovariectomized, estrogen-treated rats (128), or on GnRH-induced LHβ gene transcription in LβT2 cells (129). It is possible that the stimulatory effect of estrogen on LHβ gene expression may have obscured the progesterone suppression in these earlier studies. LHβ mRNA levels decrease during estrus as progesterone levels reach their maximum, thus suggesting a potential role for progesterone in inhibiting the stimulatory effects of GnRH on the LHβ gene expression.

Our lab recently demonstrated that progesterone is able to suppress both basal and GnRH-induced transcription of LHβ gene expression in LβT2 cells, with exogenous expression of PR (25, 103). The suppression mapped to a region between -300 to -150 of the LHβ promoter (104). This region was shown to be both necessary and sufficient since its deletion abolished the progesterone suppression, and a heterologous promoter including this region could be suppressed by progesterone. Furthermore, chromatin immune-

precipitation (ChIP) analysis revealed that PR was recruited to the LHβ promoter. However, gel-shift assays did not detect direct DNA binding of PR, though the PR DBD was shown to be necessary for the progestin suppression of LHβ. Therefore, it is hypothesized that the suppression is mediated by indirect binding of PR or tethering to the LHβ promoter. Our studies presented in this chapter aim to further characterize the repressive elements on the LHβ promoter.

Recombinant PR Binds at -225/-191 and Mutations of this Site Eliminate PR DNA Binding

Recently, it was shown that PR binding to the repressive region at -300/ -150 was not critical for LHβ suppression of basal transcription and GnRH-induced LHβ gene expression in gonadotrope cells (104). As shown in Figure 4-1, recombinant PR is able to bind the previously identified -225/-191 PRE. Mutations in this PRE were used in transient transfection studies and it was shown that this PRE is not necessary for progesterone repression of basal or GnRH-induced LHβ transcription (104). It was, therefore, important to demonstrate that PR does not in fact bind this PRE *cis* mutation, where G and C residues important for high-affinity DNA binding were mutated (V. Thackray; *Materials and Methods*: Table 2-2) shown in Figure 4-1: lane 2, and that the lack of functionality of this site observed in the transient transfection experiments is not due to PR still being able to bind the site despite the mutation.

Mutation of a Conserved Lysine in the PR DBD Does Not Eliminate the Progestin Effect on LHβ Gene Expression

PR does not appear to bind to the LHβ promoter directly, although the PR DBD is required for progestin suppression of both basal and GnRH-induced LHβ transcription (104). Therefore, it is hypothesized that PR is

recruited to the promoter via tethering to other transcription factors. Nuclear receptors are thought to use co-activator complexes that provide bridging mechanisms to allow the assembly of the basal transcription apparatus (130, 131), and some co-activator interactions have been shown to occur through the C-terminal extension (CTE) of the NR DBD that may not involve direct DNA binding (132). It has also been shown that ER-mediated transcriptional activity through non-classical AP-1 pathways does not require direct DNA binding but does require binding domains known to alter co-activator interactions (133), with one example of such interactions occurring through a highly conserved lysine in the CTE of the estrogen receptor (ER) (134). Mutation of this lysine (Figure 4-2: K581A mutation schematic) in mouse PR, however, did not affect progestin suppression of basal and GnRH-induced LHβ gene expression in LβT2 cells (Figure 4-2). Thus, it can be concluded that this particular lysine is not required for progestin suppression of LHβ gene expression and most likely, there are other residues in the DBD important for tethering interactions that remain to be identified.
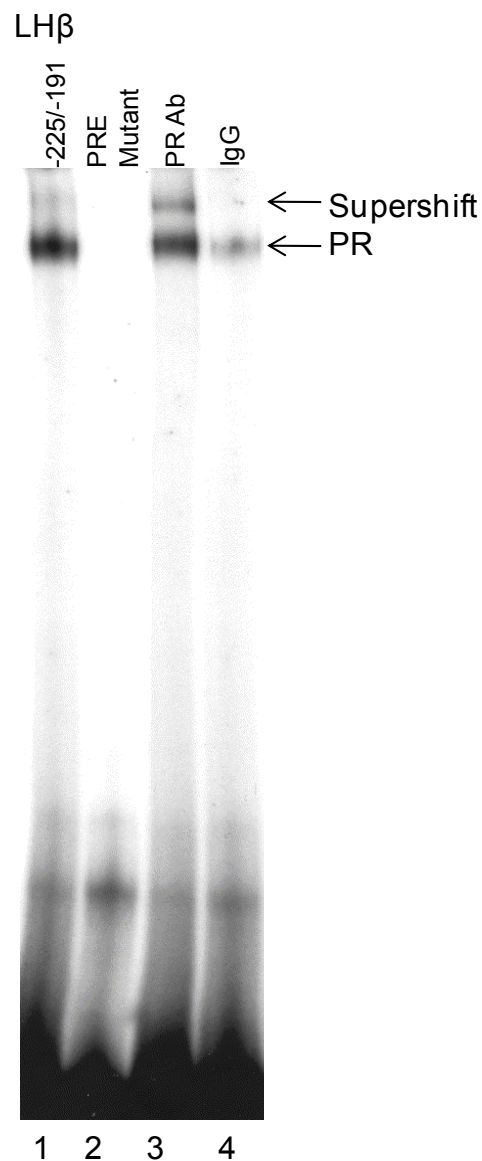
Figure 4-1: Mutation at the putative -221/-207 PRE eliminates PR binding (104)

Purified recombinant PR was incubated with a WT (lane1) or PRE mutant (containing a *cis* mutation where G and C residues important for high-affinity DNA binding were mutated; lane 2) -225/-191 LHβ probe and tested for complex formation in EMSA. PR antibody (PR Ab, lane 3) or nonspecific IgG control antibody (IgG, lane 4) were added to the binding reaction as shown. PR binding and the antibody supershift are indicated.
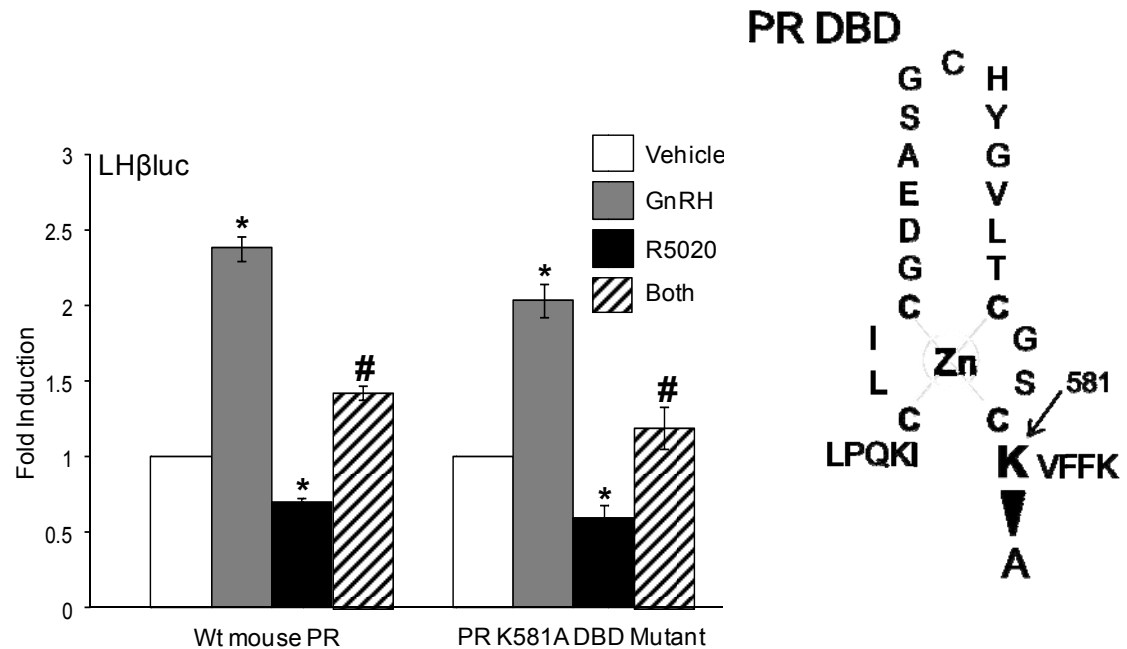
Figure 4-2: Mutation of a conserved lysine in the PR DBD does not alter progestin inhibition of basal and GnRH-induced LHβ gene expression

LβT2 cells were transfected with a 1.8 kb rat LHβ promoter-luc reporter construct and 100 ng of a expression vector containing WT PR or a K581A DBD mutant PR, which allows DNA-binding but not tethering to other transcription factors such as AP-1. After overnight starvation in serum-free media, the cells were treated for 6 h with vehicle, 10^{-8} M GnRH, 10^{-7} M R5020, or both as indicated. Results represent the mean ± SEM of at least three independent experiments performed in triplicate and are presented as fold induction of hormone treatment relative to the vehicle control. *, Significant difference from the vehicle-treated control; #, significant interaction as defined by a two-way ANOVA.

Recombinant PR and/or GnRH Treatment Do Not Change Protein Complex Formation in the -300/-250 and -250/-150 Repressive Regions

The -300/-150 region of the rat LHβ promoter region was divided into three 50 bp regions and tested for complex formation in EMSA with increasing amounts of recombinant PR (Figure 4-3) or vehicle- vs. GnRH-treated nuclear extracts (Figure 4-4) in the binding reactions. Two of the 50 bp regions from -300 to -250 and -200 to -150 have been characterized as necessary repressive elements for progesterone suppression of the rat LHβ promoter (104). The -250/-200 probe was used as a control in both EMSA experiments. The addition of recombinant PR to LβT2 nuclear extracts in the EMSA binding reactions with either of the 50 bp repressive elements does not appear to lead to complex shifts, complex eliminations or direct PR binding to the DNA (PR binding seen in control region; Figure 4-3). However, as indicated in Figure 4-3, there are many protein complexes formed on each of the 50 bp probes, which likely complicated our analysis. A similar EMSA experiment was also performed with GnRH-treated LβT2 nuclear extracts and essentially the same number of complexes was seen with no apparent changes due to increasing amounts of recombinant PR (data not shown).

In Figure 4-4, vehicle versus GnRH-treated nuclear extracts were incubated with all three 50 bp probes to analyze complex formations in the presence of GnRH. GnRH treatment was effective (Figure 4-4A) since an AP1 complex formation was observed on a previously identified FSHβ proximal

promoter region (51). However, GnRH treatment did not appear to affect any of the complexes formed with vehicle-treated nuclear extracts on either of the 50 bp repressive elements. It is important to note that these figures are representative of many EMSA experiments and although small variations were observed, none were consistent throughout all of the experiments. These results indicated that further mapping and analysis of the two 50 bp repressive elements was necessary to identify specific factors and/or sites required for progestin suppression of LHβ gene expression.
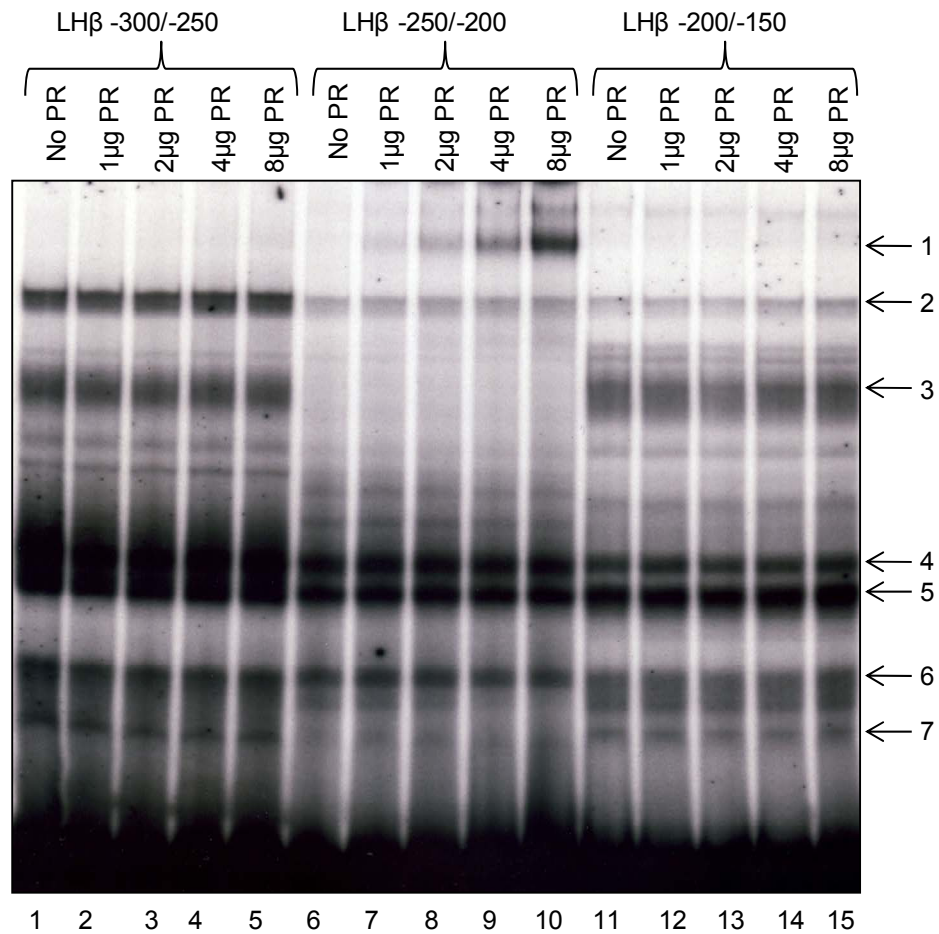
Figure 4-3: PR does not change protein complex formation on the -300/-250 and -200/-150 regions

Nuclear extracts from LβT2 cells were incubated with increasing amounts of purified, recombinant PR and -300/-250 (lanes 1-5), -250/-200 (lanes 6-10), or -200/-159 (lanes 11-15) probes from the rat LHβ promoter and tested for complex formation in EMSA. Seven complexes are indicated with complex 1 showing PR binding to the -200/-250 probe.
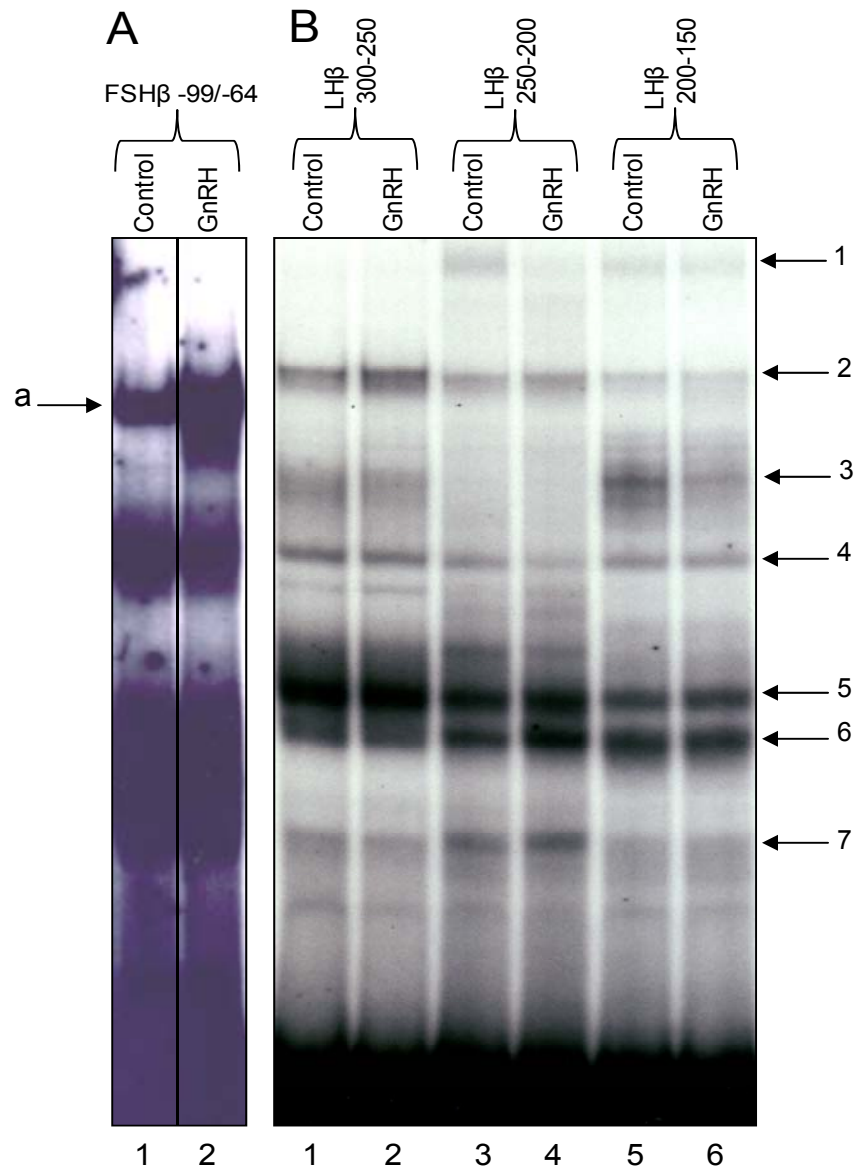
Figure 4-4: GnRH does not change protein complex formation on the -300/-250 and -200/-150 regions

(A) Vehicle-treated control (lane 1: A) and GnRH-treated (lane 2: A) LβT2 nuclear extracts were incubated with mouse FSHβ -99/-64 probe in EMSA. The GnRH induced AP1 binding (complex a; lane2: A) to the FSHβ probe indicates that GnRH treatment of the cells was effective. (B) Vehicle-treated control and GnRH-treated LβT2 cells were incubated with rat LHβ -300/-250 (lanes 1-2), -250/-200 (lanes 3-4), or -200/-159 (lanes 5-6) probes and tested for complex formation in EMSA. GnRH does not appear to change any complexes on each of the 50 bps.

Mapping of the 50bp repressive elements

To further understand the mechanism behind progesterone suppression of basal and GnRH-induced LHβ gene expression, 20 bp deletions (5 bp overlap) were created in the -300/-250 and -200/-150 bp regions of the -300 rat LHβ promoter region (along with 50 bp deletion of the two segments themselves), and transient transfection studies with LβT2 cells were carried out. The basal transcription activity in the absence of hormone treatment (Figure 4-5) did not significantly decrease from WT induction for the -300/-250 bp region or any of the 20 bp deletions within that region. However, the -200/-150 bp deletion and the 20 bp deletions within that region all showed a significant decrease to 33%, 53%, 62% and 48% of WT induction, for the -200/-150, -200/-180, -185/-165, and -170/-150 deletions, respectively. This indicates that, in the context of the -300 rat LHβ promoter construct, the -200/-150bp region contributes to basal gene expression, most likely by binding specific transcriptional activators.

As shown in Figure 4-6, all the deletion mutations, however, were able to allow progestin suppression except for the -200/-150 and -170/-150bp deletion mutations. In the context of the -300 rat LHβ promoter construct, either of the 50 bp deletions abrogated progestin suppression of the GnRH induction, indicating that both of these repressive elements are necessary for progestin suppression of the GnRH-induced LHβ transcription, as was shown previously using a -500 LHβ promoter-reporter construct (104). In addition, all

of the constructs containing deletions within the -200/-150 bp region lacked synergistic interactions between GnRH and progestins. Within the -300/-250 bp region, the constructs containing the -285/-265 and the -270/-250 bp deletions retained progestin suppression of the GnRH response indicating that they are not important for progestin suppression of the LHβ promoter. The -300/-280bp region however, was shown to be necessary for the suppression of the GnRH-induced LHβ gene expression.

To assess whether the two 50bp repressive elements, -300/-250 and -200/-150, in the LHβ promoter are sufficient for suppression by progestins, together (without the -250/-200 bp region) and individually, the response of these elements on a heterologous TK-luc reporter was tested (Figure 4-7A). It was previously shown that the -300/-150 bp region (upstream of a TK-luc reporter) was sufficient for progestin suppression (104). The deletion of the -250/-200 bp region did not affect progestin suppression, indicating that this region (which contained a putative PRE) is not necessary for the progestin suppression. The -300/-250 bp region (but not the -200/-150 bp region) alone was also sufficient for progestin suppression of basal induction as shown in Figure 4-7A. Due to the sufficiency of the -300/-250bp region, 20 bp segments of this region were subcloned upstream of the TK-luc reporter and their ability in suppressing induction due to progestin treatment was tested. As shown in Figure 4-7B, the -300/-280 bp region was the only segment sufficient to

significantly suppress basal induction due to progestin treatment, thus correlating to the results obtained in the -300 rat LHβ promoter.
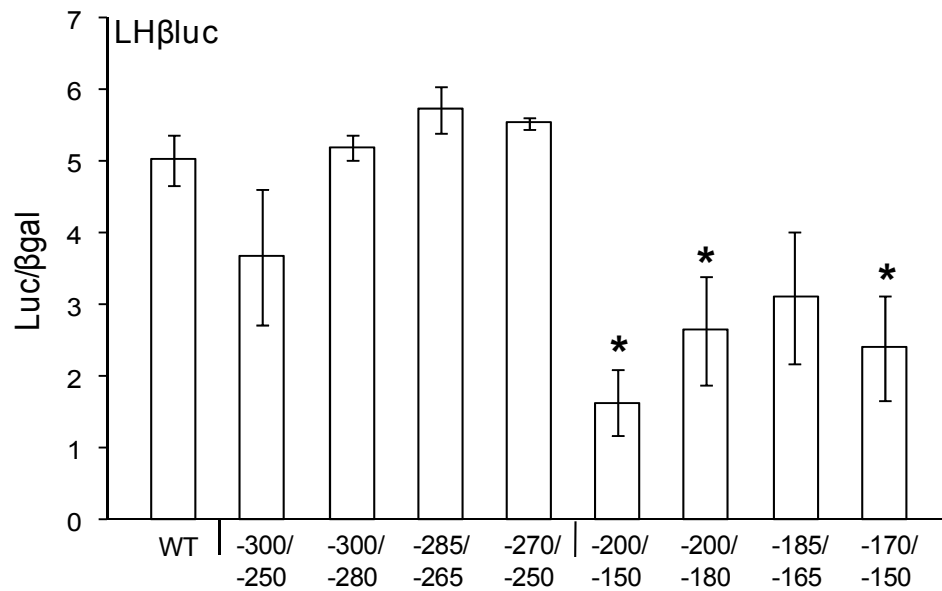
Figure 4-5: Basal suppression of LHβ gene expression maps to -200/-150 region of the LHβ promoter

LβT2 cells were transiently transfected with 100 ng PR, and either WT -300 rat LHβ promoter-luc reporter construct or mutants containing the indicated 50bp or 10bp block deletions to compare basal transcriptional activity in the absence of hormone treatment. Results represent the mean ± SEM of at least three independent experiments performed in triplicate and are presented as luc/βgal normalized to PGL3 empty vector for basal gene expression. *, Significant difference from WT LHβ basal induction.
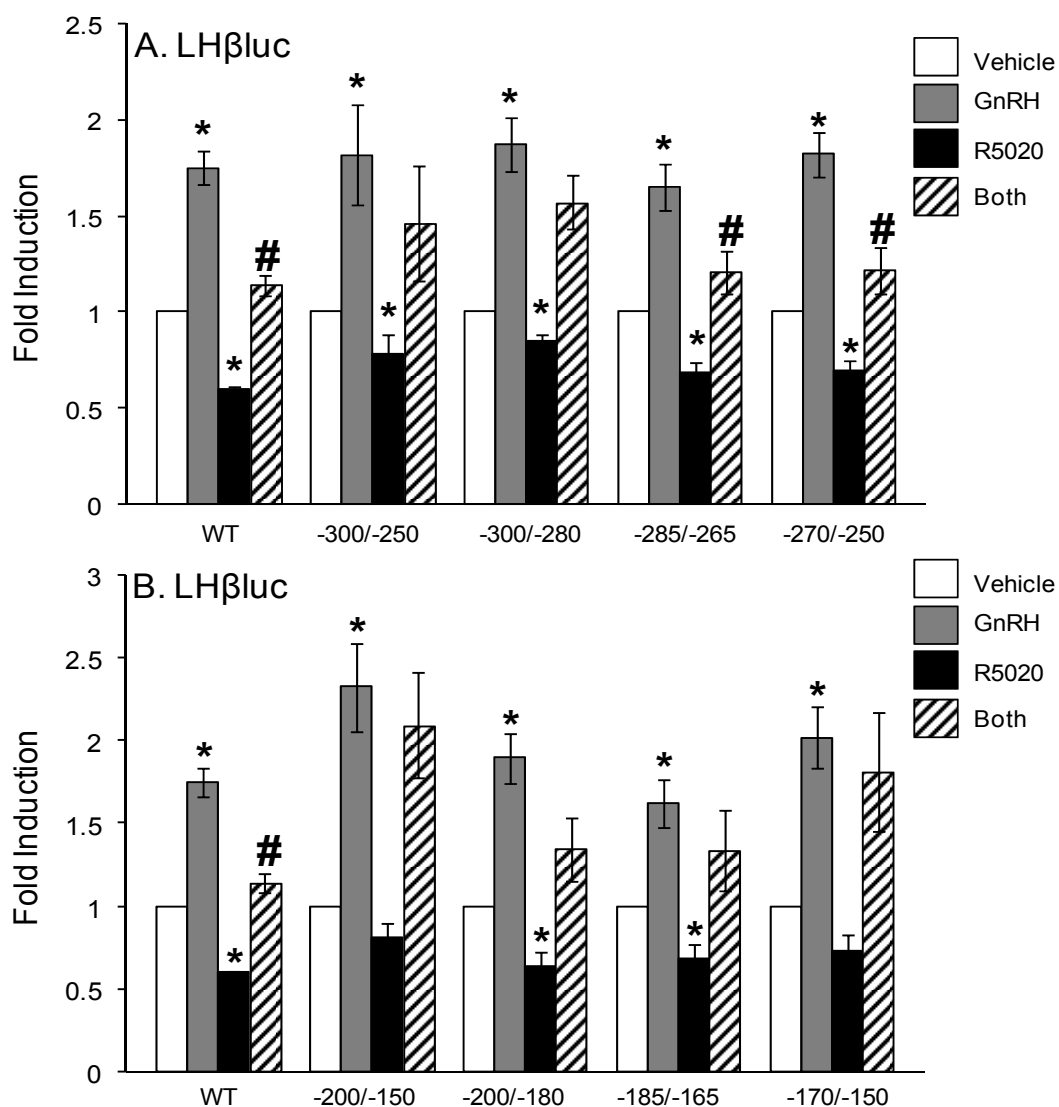
Figure 4-6: Mapping regions of the LHβ promoter for progesterone suppression of basal and GnRH-induced LHβ gene expression

LβT2 cells were transiently transfected with 100ng PR, and either WT -300 rat LHβ subunit promoter-luc reporter construct or its mutated form containing the indicated 50 bp or 10bp block deletions ((A) -300/-250 mutated segment; (B) -200/-150 mutated segment). After overnight starvation in serum-free media, the cells were treated for 6 h with vehicle, 10^{-8} M GnRH, 10^{-7} M R5020, or both as indicated. Results represent the mean ± SEM of at least three independent experiments performed in triplicate and are presented as fold induction of hormone treatment relative to the vehicle control. *, Significant difference from the vehicle-treated control; #, significant interaction as defined by a two-way ANOVA.
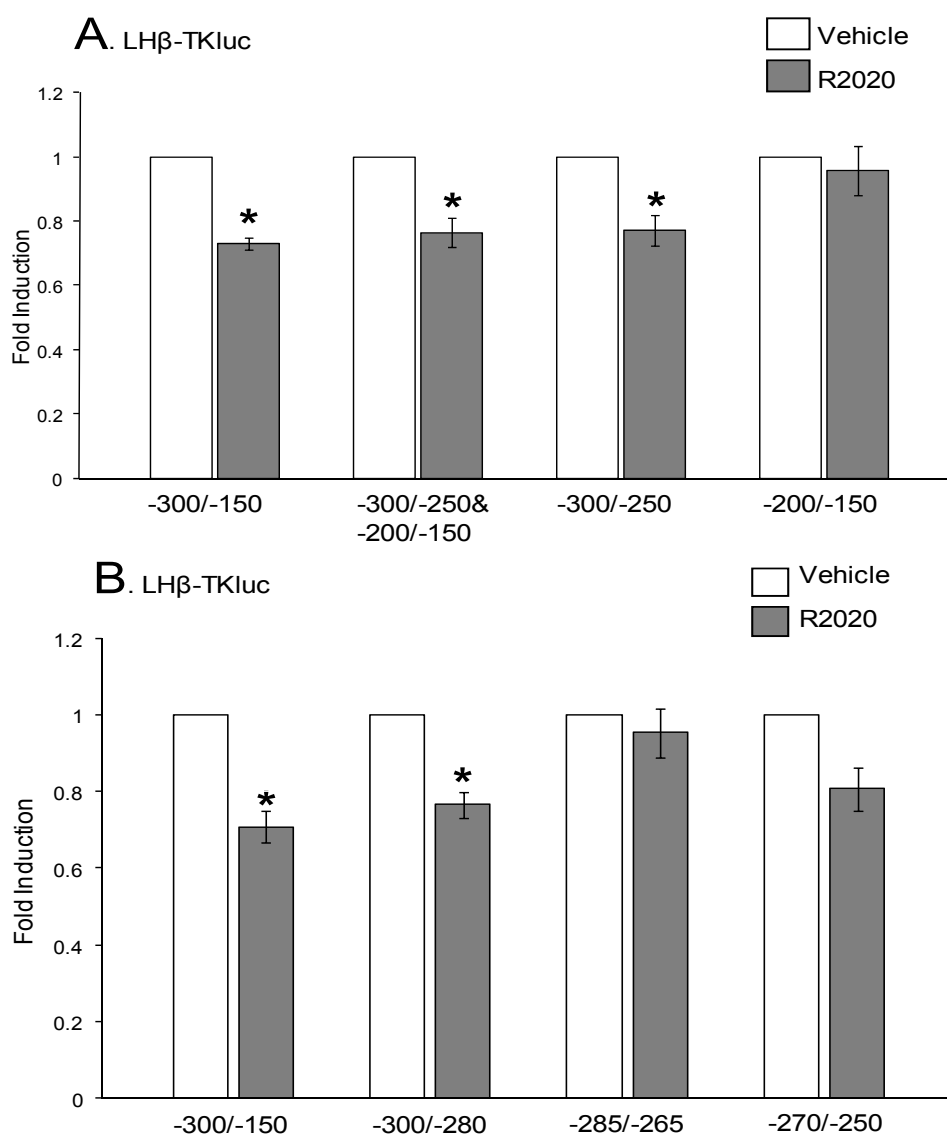
Figure 4-7: The -300/-280 region of LHβ promoter is sufficient for suppression by progesterone

LβT2 cells were transiently transfected with 100 ng PR, and either a TK-luc reporter plasmid containing the rat LHβ -300/-150 repressive element, or various segments within that region as indicated ((A) -300/-250 segment of interest; (B) -200/-150 segment of interest). After overnight starvation in serum-free media, the cells were treated for 6 h with vehicle, 10^{-8} M GnRH, 10^{-7} M R5020, or both as indicated. Results represent the mean ± SEM of at least three independent experiments performed in triplicate and are presented as fold induction of hormone treatment relative to the vehicle control. *, Significant difference from the vehicle-treated control.

Chapter 4, Figure 4-1, is a reprint of Figure 5A as it appears in Progesterone Inhibits Basal and Gonadotropin-Releasing Hormone Induction of Luteinizing Hormone β-Subunit Gene Expression 2009. Thackray VG, Hunnicutt JL, Memon AK, Ghochani Y, Mellon PL, Endocrinology 150:2395-2403. The author of the thesis was an author of this paper, and conducted the experiment resulting in that figure in collaboration with the other authors.

V

Gonadotrope-Specific PR Knockout Mice

Introduction

The many physiological actions of progesterone in various target tissues are reviewed in (135). Studies of homozygous PRKO mice have revealed the critical *in vivo* role of PR in normal development, and ovarian, uterine, brain, pituitary, and mammary gland functions (93, 100). Most importantly, the PRKO female mice are infertile and display severe reproductive defects. The severity of reproductive defects experienced by the PRKO mice makes it difficult to study the mechanism(s) of action of progesterone in a specific target tissue such as its stimulatory and inhibitory feedback effects on pituitary gonadotropins, and investigation of how such feedback is necessary for normal reproductive function. Furthermore, the PRKO mice do not experience an elevation of LH and FSH levels prior to ovulation (100), and they have elevated basal LH levels (100). As discussed previously, the obtained results from the PRKO mice do not distinguish between potential PR inhibitions of GnRH secretion in surges versus GnRH signal transduction in pituitary gonadotropes. Therefore, a gonadotrope-specific PRKO mouse is very useful in assessment of the PR action in GnRH signal transduction in pituitary gonadotropes.

The site-specific DNA recombinase Cre system is a tool used for controlling gene expression in a tissue-specific manner in mice (136). Cre-recombinase is an enzyme that facilitates or catalyzes DNA modification between two recognition sites. Thus Cre mediates the recombination of two directly repeated target loxP sites to a single loxP site, with concurrent excision of the DNA region flanked by the loxP sites (the "floxed" DNA) that are recognized by the particular recombinase (137, 138). This technology was used in the generation of gonadotrope-specific PRKO mouse line as discussed in this chapter.

Generation of PRKO in Mouse Pituitary Gonadotrope: The PR$^{KOLacZ/Flox}$/ LHβ-Cre+ Mouse Line

The mating scheme to generate gonadotrope-specific PRKO mice is shown in Figure 5-1. In previous studies, the transgenic LHβ-Cre mouse line has been shown to express the Cre-recombinase in gonadotrope cells expressing the LHβ-subunit gene, where it is capable of removing floxed genes in mature pituitary gonadotropes (105). A heterozygous PR^{KOLacz} (PR^{KO}) knockin mouse (lacZ reporter encoding β-gal is knocked into exon 1 of the murine PR gene) was previously used to study spatiotemporal expression of PR in the mammary gland (109). This heterozygous PR^{KOLacz} Knockin mouse line was crossed to the mouse line carrying the gonadotrope specific LHβ-Cre transgene. The resulting female PR$^{KOLacZ/WT}$/LHβ-Cre+/- mice were crossed to a homozygous mouse line carrying a PR conditional excision allele (PRCE) where exon 1 of the nuclear receptor is flanked by loxP sites (110). The heterozygous PR^{KOLacz} mice were used as the genetic background for the gonadotrope specific PRKO line, since the PR haplosufficiency was assumed in all tissues where PR functions, but in the gonadotrope cells of the anterior pituitary, the LHβ-Cre genotype would excise the remaining genetic copy of PR, creating the null allele and condition KO in the pituitary alone. The resulting female mice carrying PR$^{KOLacZ/Flox}$/LHβ-Cre+ (mutant) or LHβ-Cre- (control) progeny were analyzed for onset of puberty, reproductive cycle abnormalities, fertility, and circulating hormone levels.

Genotyping PR^KOLacZ/Flox/LHβ-Cre+/- mice and the pituitary gonadotrope Cre recombination leading to excision of the PR floxed allele

Primers (*Materials and Methods:* Table 2-3) designed to detect the LHβ-Cre, the PR^WT/LacZ, and PR^WT/Flox, and the floxed PR^Flox alleles were used to confirm the genotype of the mice as shown in Figure 5-2.

PCR reactions were carried out (Figure 5-2) to detect the heterologous PR^LacZ allele (lanes 1-2; PR^wt: 590; PR^LacZ: 148 bp), the heterologous PR^Flox allele (lanes 3-4; PR^wt: 160; PR^Flox: 210 bp) and the LHβ-Cre allele in a control (Cre-) and a mutant (Cre+; boxed) mouse (lanes 5-6; Cre+: 500 bp). Primers to detect the excision of the floxed PR allele exon 1 in the pituitary by the Cre-recombinase were also designed (M. Brayman; *Materials and Methods:* Table 2-3) according to the PRCE transgenic mouse description (110). The specific recombination of the floxed PR^Flox allele in the pituitary, and not the toe DNA preparations, of the mutant mouse is also shown (lanes 7-10). A band of 571 bp for the PR^wt allele (JO primers: J flanking the loxP sites, and O within the excision site) and a boxed band of 263 bp for the floxed PR^Flox allele (JK primers both flanking the loxP sites) is present in the LHβ-Cre+ mutant mouse pituitary DNA sample (lane 10). The floxed band is not present in the pituitary sample of the LHβ-Cre- control mouse (lane 9) or in toe DNA preparations of either mice (lanes 7-8), showing pituitary-specific recombination. The same floxed allele band can be seen in the positive DNA control from the hypothalamus of another mouse line: the PR^KOLacZ/Flox/Syn-Cre+ mouse

(Figure 5-2 lane 12) and is missing in the DNA from the hypothalamus of a PR$^{KOLacZ/Flox}$/Syn-Cre- mouse (Figure 5-2: lane 11). This result indicated that the LHβ-Cre was able to specifically recombine the floxed allele in the pituitary.
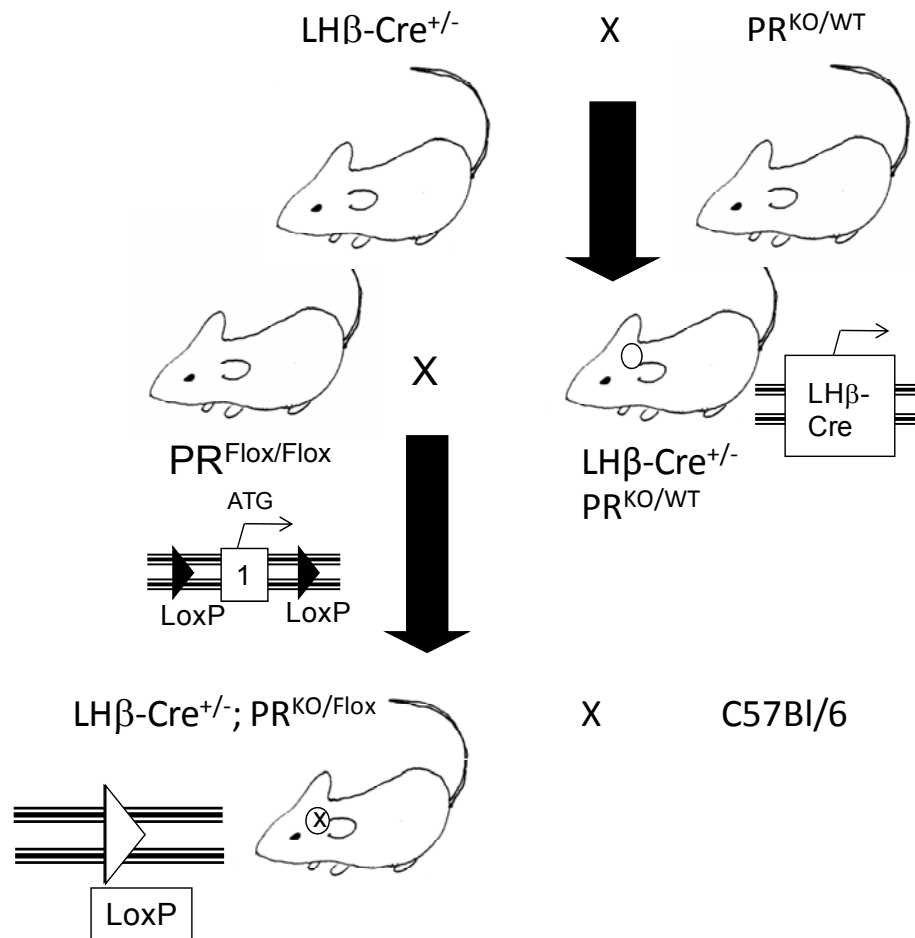
Figure 5-1: Scheme to generate mouse line with gonadotrope-specific PRKO

By crossing gonadotrope-specific LHβ-Cre mice with a line of heterozygous PR^{KOLacz} mice, then crossing the double transgenic with a line of mice carrying the floxed PR gene, female PR$^{KOLacZ/Flox}$/LHβ-Cre+ (mutant) or Cre- (control) progeny were obtained.
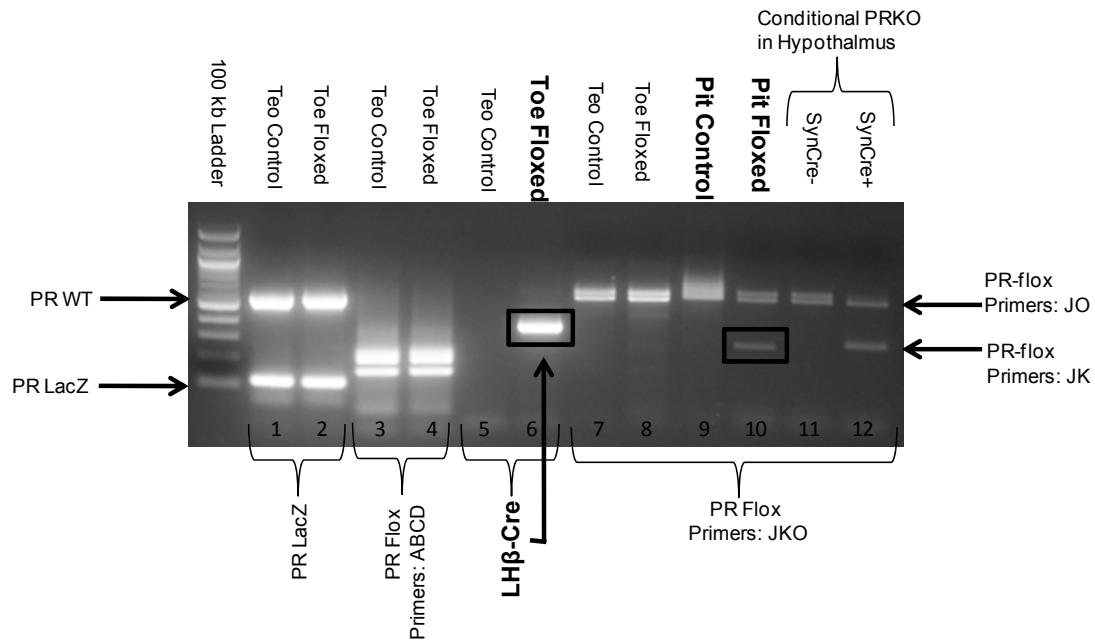
Figure 5-2: Confirming the gonadotrope-specific PRKO

PCR reactions were carried out to detect the heterologous PR^{LacZ} allele (lanes 1-2; PR^{wt}: 590; PR^{LacZ}: 148 bp), the heterologous PR^{Flox} allele (lanes 3-4; PR^{wt}: 160; PR^{Flox}: 210 bp) and the LHβ-Cre allele in a control (Cre-) and a mutant (Cre+; boxed) mouse (lanes 5-6; Cre+: 500 bp. The specific recombination of the floxed PR^{Flox} allele in the pituitary (not toe DNA samples) of the mutant mouse is also shown (lanes 7-10). A band of 571 bp for the PR^{wt} allele and a boxed band of 263 bp for the floxed PR^{Flox} allele is present in the LHβ-Cre+ mutant mouse pituitary DNA sample (lane 10). The floxed band is not present in the pituitary sample of the LHβ-Cre- control mouse (lane 9) or in toe DNA preparations of either mice (lanes 7-8). The floxed allele is present in the positive DNA control from the hypothalamus of a PRF/SynCre+ mouse (lane 11) and is absent in the DNA from the hypothalamus of a PRF/SynCre- mouse (lane 12).

Onset of Puberty and Length of the Reproductive Cycle Are Not Significantly Different For gonadotrope-specific PRKO vs. Control Mice

The onset of puberty in mice was measured as the opening day of the vaginal orifice. As shown in Figure 5-3A, the average day of vaginal opening between the control and gonadotrope-specific PRKO mice was not significantly different, averaging at about 24 days after birth; indicating that the gonadotrope-specific PRKO mice have a similar onset of puberty as the heterozygous PR^{lacz} mice.

The length of time in days for a control or mutant mouse to go through all phases of one reproductive or estrous cycle: diestrus, proestrus, estrus, and metestrus, was assessed by the type of cells present in vaginal smears (94) of 10-12 week-old female mice (four controls and five mutants), with ovulation occurring from proestrus to end of estrus (15). As shown in Figure 5-3B, the length of the estrus cycle was not significantly different between the two groups, although the average length was reduced by half a day for the mutant group of mice.
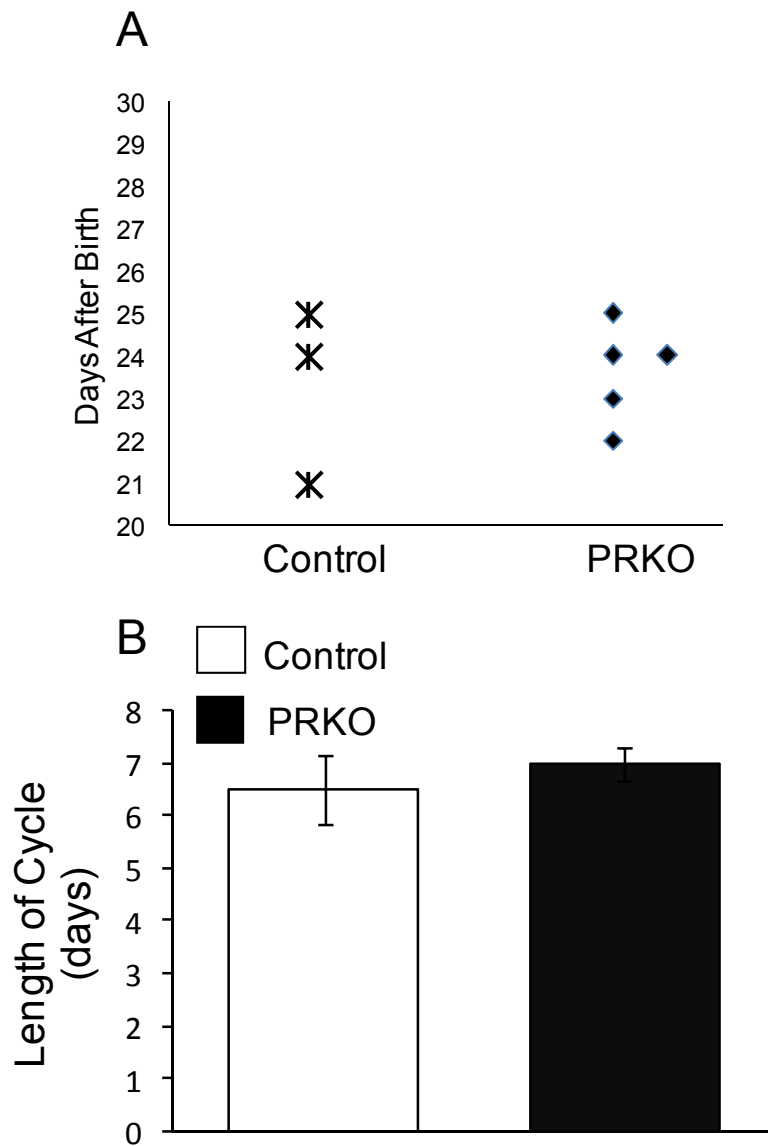
Figure 5-3: Day of vaginal opening marking the onset of puberty, and the length of estrous cycle is not significantly different for PRKO vs. control mice

(A) The vaginal opening of 3 control and 5 mutant mice was examined starting 21 days after birth. (B) The length of time in days for 4 control and 5 mutant mice to go through one reproductive or estrous cycle was assessed by the cell types obtained in vaginal smears.

Cumulative Number of Litters, and Number of Pups/Month Are Not Significantly Different For Gonadotrope-Specific PRKO vs. Control Mice

Eight-week old female $PR^{KO/Flox}$ / Cre+ (mutant: N=10) or Cre- (control: N=9) mice were crossed to eight-week old male C57Bl/6 mice and were analyzed in a fertility assessment experiment for 6 months. The cumulative number of litters produced by the 9 control mice and 10 mutant mouse groups, shown in Figure 5-4, was not significantly different. However, a subgroup of 2 mice, plotted separately, never had a litter or only produced two litters in the four month period, indicating that there may be a penetrance issue for the mutant mice that did not exhibit any significant phenotype. Many studies have reported such genetic penetrance issues. For example, one mutation can exhibit strikingly different phenotypes on different genetic backgrounds, potentially due to different alleles at modifying loci in various inbred strains (139). In addition, the number of the pups produced by the two groups was only significantly different for the first month they were in the fertility assessment (Figure 5-5), though on average the mutant mice had a smaller number of pups/month in the other months they were analyzed in the fertility assessment.
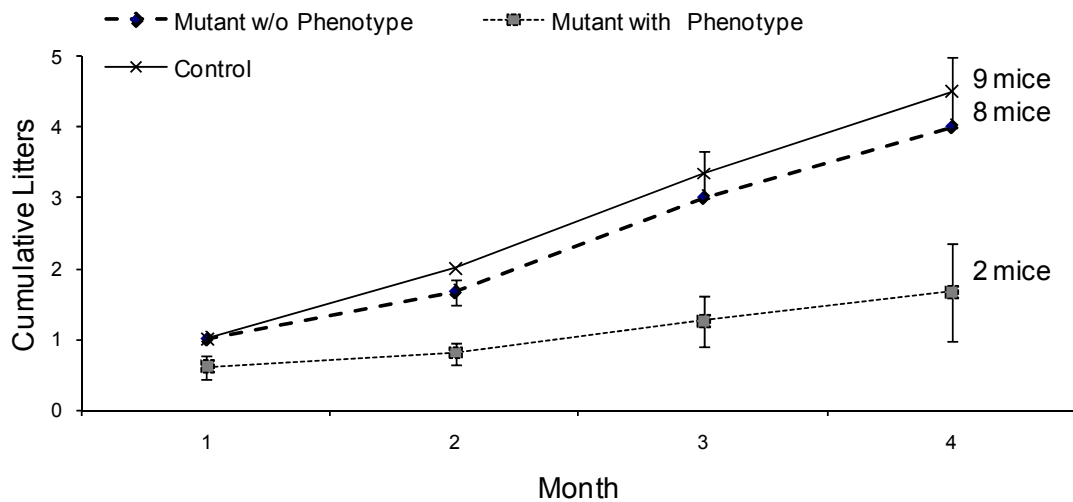
Figure 5-4: Cumulative litters of Control vs. PRKO mice for 4 months

Eight-week old female PR$^{KO/Flox}$ / Cre+ (mutant: N=10) or Cre- (control: N=9) mice were crossed to eight-week old male C57Bl/6 mice and the number of litters they produced/month was recorded. The cumulative number of litters produced for both groups is shown above. A subgroup of 2 mice, plotted separately, never had a litter or only produced two litters in the four month period.
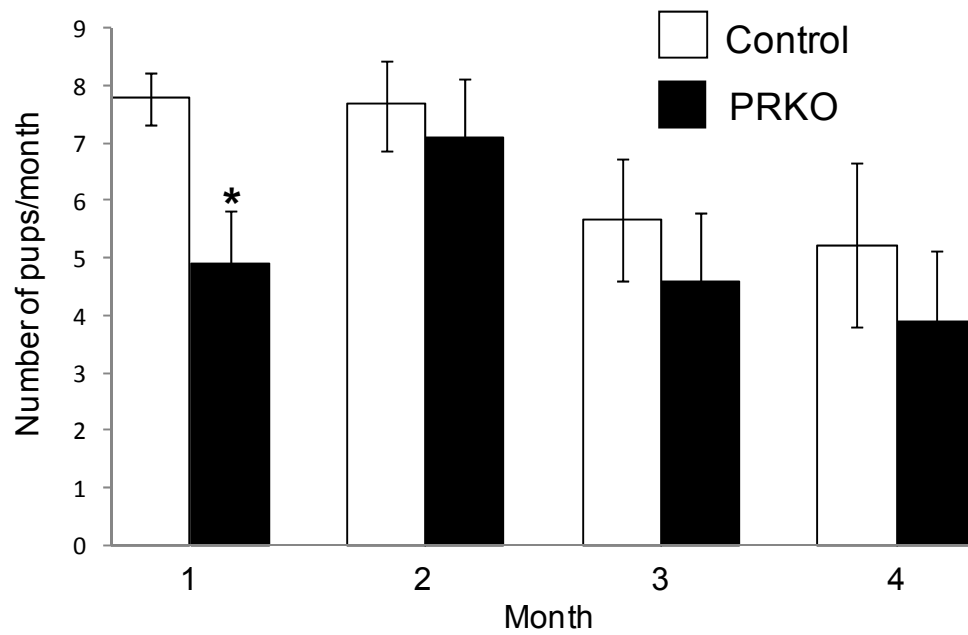
Figure 5-5: Number of pups/month for control vs. PRKO mice

Eight-week old female PR$^{KO/Flox}$ / Cre+ (mutant: N=10) or Cre- (control: N=9) mice were crossed to eight-week old male C57Bl/6 mice and the number of the pups they produced/month were recorded and plotted above.

Circulating FSH, LH and Progesterone Hormone Levels Are Not Significantly Different For Gonadotrope-Specific PRKO vs. Control Mice

The control (N=9) and mutant (N-10) mice were sacrificed at age of approximately 10 months old (during metestrus) and their systemic blood was collected and assayed for FSH, LH and progesterone levels at the University of Virginia Center for Research in Reproductive Ligand Assay and Analysis Core. Similar to the data collected for number of litters and pups, none of the blood assays showed a significant difference in the hormone levels between the two groups, although on average the mutant mice had lower levels of circulating hormone for all three assays (Figure 5-6).
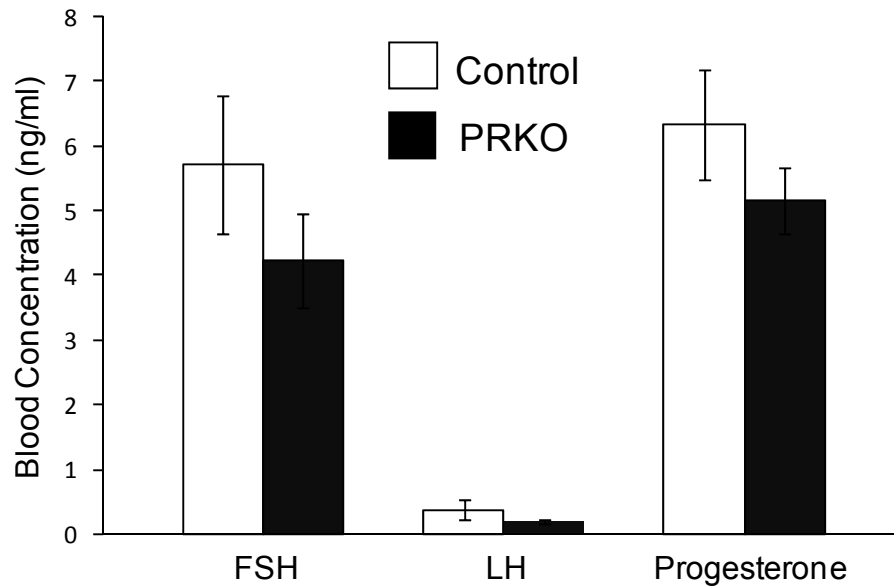
Figure 5-6: Blood Assays for control vs. PRKO mice at Metestrus

Eight-week old female PR$^{KO/Flox}$ / Cre+ (mutant: N=10) or Cre- (control: N=9) mice were crossed to eight-week old male C57Bl/6 mice and were analyzed in a fertility assessment for 6 months. The control and mutant mice were sacrificed at the age of approximately 10 months old and their systemic blood was collected and assayed for FSH, LH and progesterone levels.

VI

Discussion

The precise and differential regulation of gonadotropin secretion is integral to the proper functioning of the HPG axis and normal reproduction. Transcriptional regulation and secretion of the two gonadotropins diverge during late proestrus and early estrus when LH levels decline with the termination of the preovulatory gonadotropin surge, while FSH remains elevated. Gonadal steroid hormone feedback is a crucial component of the control of gonadotropin synthesis in the pituitary gonadotrope, and progestins may be of particular importance for the secondary FSH surge. In the current study we have focused on delineating mechanisms of progestin regulation of the gonadotropin β subunits.

Precise regulation of FSH levels is crucial for the menstrual or estrous cycle. Both circulating FSH levels and β-subunit mRNA in the pituitary normally fluctuate 4 folds during the cycle (16, 18). In our study (Chapter 3), we characterized specific molecular mechanisms of hormonal interactions in induction of murine FSHβ gene expression by progestins in the LβT2 cells.

A concern from various previous studies carried out in our laboratory was the high levels of exogenous PR that were transfected in the LβT2 cells in transient transfection studies on an FSHβ-luc reporter. Various studies have demonstrated that regulation of PR, and not circulating progesterone levels, is

the regulatory mechanism of progesterone action. For example, in studying lordosis sexual behavior in rats, it has been shown that PR levels are downregulated due to high estrogen and progesterone induction of the receptor, and successive progesterone treatments are ineffective on PR or the behavior due to the limiting receptor amounts (140). Since progestin response in LβT2 cells is receptor-dependant and the receptor is limiting in the cell (25), lower amounts of PR, which are thought to mimic physiological levels more accurately were shown, in our studies, to be sufficient for progestin induction of the FSHβ promoter (Figure 3-1). Therefore, all of our subsequent transient transfection studies with the FSHβ-luc reporter were carried out with limiting PR conditions.

FSHβ gene transcription is induced independently and synergistically by GnRH and activin (3, 53, 122, 141). Progestins have also been shown to stimulate FSHβ transcription alone, and through synergistic interaction by activin via Smad protein signaling (25, 103). Therefore, both GnRH and progestins are able to interact with various components of the activin signaling pathway, leading to the hypothesis that there may be cross-talk between their signaling pathways as well. Furthermore, steroid receptors have been shown to interact with transcription factors such as AP-1, which is also induced and recruited to the FSHβ promoter by GnRH (51, 142-144). However, we demonstrated that there is no significant interaction between GnRH and progestins on the FSHβ promoter in LβT2 cells (Figure 3-2A). This is

particularly interesting since cross-talk at the PR has been implicated as critical to the GnRH self-priming mechanism, i.e. enhanced LH secretion, in pituitary gonadotropes (145, 146), indicating that such mechanistic interactions may be important for LH regulatory and/or secretory pathways but not for FSHβ transcriptional regulation. It remains to be determined whether GnRH in presence of other steroid hormones such as androgens and glucocorticoids modulate FSHβ gene expression.

For differential regulation of the gonadotropins and the secondary FSH surge, various combinatorial mechanisms have been suggested, where the interactions among multiple hormonal signaling pathways in the pituitary gonadotrope are probably responsible for regulating FSH synthesis (103). In investigating such combinatorial interactions among activin, GnRH, and progestins, we first confirmed the synergistic interactions among activin and GnRH or progestins, previously reported on the FSHβ promoter (103, 122). Secondly, we unmasked a repressive action of GnRH on the synergistic induction of FSHβ promoter by activin and progestin co-treatment (Figure 3-2B). The GnRH repressive effect on the synergistic induction of the FSHβ may occur due to a limiting factor in the signaling pathways. Smad proteins, for example are needed for both activin and GnRH, and activin and progesterone interactions (122, Thackray, 2008 #5461). Thus common proteins such as Smads may act as limiting factors in obtaining maximal synergistic induction due to activin and progesterone co-treatment, when GnRH is present.

The synergistic interaction of activin and progestins has been shown to involve a direct interaction of activin and progestin signaling pathways on the proximal FSHβ promoter (103), providing a potential model for production of the secondary FSH surge. Activin may function as a tonic hormone to maintain basal FSHβ levels, whereas progestins synergistically interact with activin to induce FSHβ mRNA levels and produce the secondary surge (103). Considering the importance of activin and progestin interaction according to the above model, we further investigated the molecular mechanisms responsible for the activin and progestin synergistic induction of the FSHβ promoter.

Since the extent of steroid hormone response conveyed by a single HRE is often weak, multiple HREs are often found in close proximity in the promoters of steroid-responsive genes (147). Six HREs were characterized and shown to bind PR in rat and ovine promoters (124, 148). Similar HREs were characterized in the murine FSHβ promoter at -139, -197, -230, -273, and -381 bps upstream of the transcriptional start site (25). The -381 HRE, a direct repeat element as opposed to a classical palindomic inverted repeat, was shown previously to be necessary for progesterone response, and for synergistic induction of the promoter by activin and progestin co-treatment (25, 103). We examined the relative importance of the other HREs for progesterone response alone and in synergistic interaction, both under limiting PR conditions. We demonstrated the importance of the other HREs in playing

a supporting role in allowing maximal responsiveness. The synergistic induction of the murine FSHβ promoter and progestin response were both reduced due to mutations in the other four HREs. Therefore, all the HREs are necessary for maximal synergistic induction of the promoter. Furthermore, we confirmed that the only HRE mutation that led to a complete lack of response to progestins, and complete disappearance of any synergistic induction of the promoter was the -381 HRE mutation, confirming the importance of this site for progestin responsiveness on the FSHβ promoter as well as for the synergy between activin and progestin (Figure 3-5).

Activin signaling required for the synergy has been shown to occur through a Smad-dependent mechanism, with Smad proteins being necessary and sufficient for the synergy (104). Furthermore, it was shown that the Smad binding to the FSHβ promoter at SBEs was necessary for the synergistic interaction between activin and progestins. In our study, we revealed that disruption of any single Smad binding element reduces cross-talk between activin and progestins and does not allow for maximal synergistic induction of the promoter (Figure 3-6). As was shown previously, the 3xSBE mutation led to a complete lack of response to activin, and complete disappearance of any synergistic induction of the promoter (103). The reduced induction seen with individual mutations at the -120, -153, and -267 SBE sites, under PR limiting conditions, indicate that activin response and consequently the synergistic induction of the promoter by activin and progestin require the binding of factors

to the multiple SBEs in the FSHβ promoter. The -267 SBE appears to be of particular importance for the synergy since its disruption led to a substantial decrease in the induction of FSHβ gene expression.

All the HREs previously characterized in the murine FSHβ promoter have a degree of conservation across multiple species, including ovine and human, suggesting that they may play roles in steroid regulation of the other mammalian species (25). Furthermore, GnRH and activin are shown to synergistically induce the human FSHβ transcription through a high affinity AP-1 site on the human promoter and Smad dependent signaling pathways (127). It is important to note that FSHβ mRNA levels have been shown to decrease after progestin treatment in ovine mixed pituitary cell cultures (149). We investigated the ovine and human promoter responsiveness to progestins, or existence of synergistic interactions among progestins and activin (Figure 3-3A).

We showed that neither of the reporters were progestin responsive or led to any synergistic induction of the promoter. One possibility is that the response seen in the murine FSHβ promoter, as compared with sheep and human, differ due to species-specific steroid effects on the FSHβ promoter. For instance, the -381 HRE, essential for hormone responsiveness on the murine FSHβ promoter, is not present in sheep, and only exists as a half site in human. Furthermore, there may be factors present in sheep and human

gonadotropes that promote the induction that are lacking in the mouse-derived LβT2 cells.

Although activin directly stimulates the murine FSHβ gene expression through a consensus 8 bp -267 SBE, the human FSHβ promoter is relatively insensitive to the direct effects of activin and lacks this SBE (60). In order to obtain progestin responsiveness and synergistic induction of the human FSHβ promoter, we added a complete mouse -381 HRE site, a -267 SBE site or both to the human promoter (Figure 3-4). However, the -381 HRE was not sufficient for progestin response or synergistic induction on the human FSHβ promoter. The human promoter was originally not as activin responsive as the mouse, and the -267 site restored substantial activin responsiveness to the human promoter, though it was unable to restore activin and progestin synergy, indicating that progestin responsiveness is also required for the synergy.

Since we showed the necessity of both functional HREs and SBEs for the synergistic induction to occur on the murine FSHβ promoter, the contribution of other transcription factors between the critical -381 HRE and the -267 SBE were analyzed (Figures 3-7 & 3-8). The 21 bp deletion affected the activin, progestin and the synergistic induction of FSHβ gene expression most dramatically, indicating that the region contains elements that are important for hormonal regulation of the promoter (Figure 3-8). The fact that synergy was rescued when the -381 HRE and the -267 SBE were brought even closer together indicates that the region between the two hormone

response elements may also be important for stabilizing PR and Smad protein interactions. To better investigate the regions responsible for the dramatic reduction in activin, progestin, and synergistic induction of the promoter, we used a more systematic approach throughout the region between the -381 HRE and the -267 SBE, by creating 10 bp deletion mutations. The -370/-361 deletion overlapped the last 4 bps of the 3' end of the -381 HRE, and thus, expectedly, led to substantial reduction in progestin responsiveness and synergy. The deletions at -360/-351, -350/-341, and -320/-311 all resulted in significant reductions in the induction of FSHβ gene expression by activin, progestin, and their co-treatment. Investigation of the factor(s) present at the -320/-311 bp region was left for future study, and we focused our studies on the -360/-341 bp region.

On closer examination of the 10 bp deletions through -360 to -341, it was recognized that a Smad half-site at -356 bp, a newly-characterized FOXL2 site at -350 bp (D. Coss, personal communication), and one bp of an SF1 site at -339 bp (Figure 3-10) were deleted in the 21 bp deletion. However, the complete SF1 site, deleted in the -340/-330 block deletion (Figure 3-9) did not have an effect on activin, progestin, or their synergistic induction of the murine FSHβ expression, indicating that the SF-1 site does not play a significant role. Our studies revealed, using specific mutations in each of the above mentioned sites and double mutants, that the FOXL2 and the nearby

Smad half-site are necessary for progestin responsiveness and synergy between activin and progestin on the FSHβ promoter.

Complementing our data, very recently, it was revealed that the forkhead factor, FOXL2 within -145/-185 bp region, allows for activin induction of porcine FSHβ promoter, which like the human, lacks the -267 SBE. Interestingly the same FOXL2 site in the human promoter was not shown to bind with high affinity leading to the conclusion that FOXL2 is necessary for activin response of the promoters. In the mouse promoter, more proximal FOXL2 sites, than that examined in our studies, were identified, which were not necessary for activin response of the promoter (150). It is important to note that our data also points to the importance of the FOXL2 site for progestin and synergistic induction of the promoter, though mechanisms of interaction remain to be further studied. FOXL2 has been shown to interact with Smad proteins (ref) but it remains to be determined if FOXL2 can directly interact with PR. Furthermore, we have shown repeatedly that responsiveness of the FSHβ promoter to both activin and progestin is required for their synergistic induction of the promoter. In our studies of the human promoter, we were able to rescue activin induction by addition of the consensus SBE site. However, that proved insufficient for any synergistic induction of the promoter even in the presence of the -381 HRE. Therefore, it may be that the human promoter requires a high affinity FOXL2 site and the -381 HRE to facilitate progestin and synergistic induction of the promoter, which will be investigated in the future.

To further analyze progestin differential regulation of the gonadotropins, our studies shifted to focus on the mechanism of regulation of LHβ gene expression (Chapter 4). The precise regulation of LH is essential to normal reproductive function. Reduced levels of LH result in infertility due to defective gamete development or hypogonadism (9), whereas increased levels has been shown to result in precocious puberty (151), polycystic ovaries, and ovarian tumors (128), pituitary adenomas (151, 152), and infertility due to anovulation (128). It was recently demonstrated that progestins can suppress both basal transcription and GnRH induction of the LHβ gene in gonadotrope cells through classical PR pathways directly involving the LHβ promoter (104). These findings complement the physiological data showing that during the luteal phase of the menstrual cycle, progesterone levels are high, and LH levels are low compared to concentrations during the follicular phase, potentially as a consequence of the high progesterone (153-155). Our studies reported in Chapter 4 complement the recent studies by Thackray *et al.* to further elucidate the mechanism of progestin regulation of the rat LHβ promoter.

Progestin suppression of LHβ transcription is shown to require hormone-bound PRB containing its DBD and a region from -300 to -150 of the promoter (104). Our studies demonstrated that PR is able to bind a putative PRE at -225/-191 in EMSA, and mutations of this site eliminate the PR DNA binding (Figure 4-1). However, the mutations of the site or deletion of a 50 bp

region from -250/-200 that encompassed this element were shown to not relieve progesterone suppression of LHβ transcription, suggesting that direct DNA binding by PR is not necessary for this effect (104). Therefore two 50 bp regions from -300 to -250 and -200 to -150 were characterized as necessary repressive elements for progesterone suppression of the rat LHβ promoter (104).

In investigating the necessity of the PR DBD, we examined a residue that was reported to be necessary for "tethering" or binding to other transcription factor(s) for other steroid hormones (Figure 4-2). It has been shown that ER-mediated transcriptional activity through non-classical AP-1 pathways does not require direct DNA binding but does require binding domains known to alter co-activator interactions (133), with one example of such interactions occurring through a highly conserved lysine in the CTE of the estrogen receptor (ER) (134). Our studies revealed that this conserved lysine on the murine PR DBD is not required for progestin suppression of LHβ gene expression and most likely, there are other residues in the DBD that are important for tethering interactions, which remain to be identified.

We were also interested in examining complex shifts, formations, and/or eliminations in EMSA due to recombinant PR and/or GnRH treatment. Our studies showed that recombinant PR and/or GnRH treatment did not change protein complex formation in the -300/-250 and -250/-150 repressive regions (Figure 4-3 & 4-4). However, there were many complexes detected in

the EMSA experiments, indicating that further mapping and analysis of the two 50 bp repressive elements was necessary to identify specific factors and/or sites required for progestin suppression of LHβ gene expression.

Our finer mapping and characterization of the repressive elements revealed that in the context of the -300 rat LHβ promoter construct, the -200/-150bp region contributes to basal gene expression, most likely by binding specific transcriptional activators (Figure 4-5). Furthermore, only the -300/-280 bp region, within the -300/-250 bp repressive element, was shown to be important for progestin suppression of the LHβ promoter (Figure 4-6A). Our studies demonstrated that the two 50 bp repressive elements together, upstream of a TK-luc heterologous promoter (Figure 4-7) are sufficient for progestin suppression. The -300/-250 bp region (but not the -200/-150 bp region) alone is also sufficient for progestin suppression of basal induction. 20 bp segments of this region, subcloned upstream of the TK-luc reporter, also revealed that the -300/-280 bp region was the only segment sufficient to significantly suppress basal induction due to progestin treatment, confirming the data obtained in the context of the -300 rat LHβ promoter. In conclusion, our studies contributed to the finer mapping of the progesterone basal and GnRH-induced suppression of the LHβ gene expression, identifying a critical segment at the -300/-280 bp region of the promoter. Additional experiments will be necessary to define the cis regulatory elements and transcription factors that play a role in the regulation of LHβ gene expression by ligand-

bound PR. Altogether, our work contributed to further characterization of progesterone action on LHβ transcription in the pituitary.

Transgenic mouse models have provided much insight into the functions, mechanisms, and regulation of various components within the HPG axis. Much of the data obtained from such models have been discussed in this report to provide physiological explanations for the molecular mechanistic phenomena observed. In our studies described in Chapter 5, we examined progesterone regulation of the gonadotropin genes in the anterior pituitary to better understand the physiological role(s) it plays in the maintenance of reproductive fitness. Selective ablation of the PR in the gonadotrope cells was performed using the Cre-LoxP system in mice (Figure 5-1), in the hopes that the gonadotrope-specific knockout would determine whether PR is necessary for gonadotropin regulation or whether there is a more dominant steroid regulation of the gonadotrope through afferent communication with the GnRH neuron or complex interactions with other regulators of the HPG axis.

For our study of the feedback regulation of progesterone only on the anterior pituitary gonadotropins *in vivo*, the PRKO/Flox/Cre+ mouse line was created. Our studies did not, however, show any significant impairment of fertility, possibly due to genetic penetrance and mosaicism issues, but can serve as preliminary data suggesting trends in lower reproductive functions, such as lower number of litters and/or pups, and lower levels of circulating FSH, LH and progesterone for the gonadotrope-specific PRKO mice.

Mosaicism issues could have been a factor that contributed to lack of significant phenotype in our gonadotrope-specific PRKO mice potentially due to low LHβ-Cre expression in some of the cells (156). It is possible to create our gonadotrope-specific PRKO mouse line using the α-GSU-Cre transgenic mouse line. This mouse has been used to successfully ablate expression in the pituitary; for instance in the pituitary-specific ERα knockout; ERα$^{flox/flox}$ α-GSU^{cre} mouse line (157).

Furthermore, as seen in Figure 5-1, the gonadotrope-specific PRKO mice were created on the background of heterozygous PR^{LacZ} knockin mice. Throughout our experiments, we recognized that this background, i.e. our control mice were obese and ceased to have litters early, making it difficult to assess the impairments, if any, due to the lack of PR in the pituitary gonadotropes. Additionally, the PR^{LacZ} background may have contributed to genetic penetrance issues, which are reported by various studies. For instance one mutation can exhibit strikingly different phenotypes on different genetic backgrounds, potentially due to different alleles at modifying loci in various inbred strains (139). Thus a different background such as a homozygous PRflox mouse line may be more insightful in studying reproductive function of progesterone in the pituitary gonadotropes. Such studies on different backgrounds and/or other variations ensuring better results remain to be carried out in future experiments.

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