Ligand-independent activation of steroid hormone receptors by gonadotropin-releasing hormone

by

Junling Chen

Bachelor of Medicine, Xi'an Medical University, 1993
Master of Science, University of Edinburgh, 2000

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Abstract

Nuclear receptors including estrogen receptors (ERs) and progesterone receptors (PRs) are activated by their ligands as well as by signaling pathways in response to peptide hormones and growth factors. In gonadotrophs, gonadotropin releasing hormones (GnRHs) act *via* the GnRH receptor (GnRHR). Both GnRH-I and GnRH-II activate an estrogen response element (ERE)-driven luciferase reporter gene in LβT2 mouse pituitary cells, and GnRH-I is more potent in this regard. The ERα is phosphorylated at Ser^{118} in the nucleus and at Ser^{167} in both nucleus and cytoplasm after GnRH treatments, and this coincides with increased ERα binding to its co-activator, the P300/CBP-associated factor (PCAF). Most importantly, both GnRH subtypes robustly up-regulate expression of the immediate early response gene, *Fosb*, while co-treatment with ERα siRNA or PCAF siRNA attenuates this effect. This appears to occur at the transcriptional level because co-recruitment of ERα and PCAF to an ERE within the endogenous *Fosb* promoter is increased by GnRH treatments, as shown by chromatin immunoprecipitation assays. Furthermore, cross-talk between GnRH-I and PR accentuates gonadotropin production. GnRH-I activates a progesterone response element (PRE)-driven luciferase reporter gene and gonadotropin α subunit (*Gsuα*) gene expression in two mouse gonadotroph cell lines, αT3-1 and LβT2. Up-regulation of the PRE-luciferase reporter gene by GnRH-I is attenuated by pre-treatment with protein kinase A (H89) and protein kinase C (GF109203X) inhibitors, while only GF109203X inhibits GnRH-I-induced *Gsuα* mRNA levels. In both cell lines within the same time-frame, knockdown of PR levels by siRNA reduces GnRH-I activation of *Gsuα* mRNA levels by approximately 40%. Both GnRH-I and GnRH-II also increase mouse *Gnrhr*-luciferase promoter activity and this is significantly reduced by knockdown of PR in LβT2 cells. We conclude that the effects of GnRH-I on *Fosb*

and *Gsuα* expression, as well as mouse *Gnrhr* promoter activity in mouse gonadotrophs are mediated by ligand-independent activation of ERα and PR. These ligand-independent effects of GnRHs on steroid hormone receptor function may influence the magnitude of changes in the expression of specific genes in the pituitary during the mouse estrous cycle, which in this context may serve as a model in the human menstrual cycle.

Preface

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Contributions

An BS and I designed and performed the experiments. I drafted the manuscript. Leung PC, Hammond GL and Cheng L were responsible for supervision of this work. Hammond GL and Leung PC critically revised the manuscript. All authors read and approved the final manuscript.

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receptor contributes to gonadotropin alpha-subunit expression in mouse gonadotrophs. Endocrinology 151:1204-1211.

Contributions

An BS, So WK and I participated in the design and performance of the study, as well as the discussion of the results. I drafted the manuscript. Leung PC, Hammond GL and Cheng L were responsible for supervision of this work. Hammond GL and Leung PC critically revised the manuscript. All authors read and approved the final manuscript. An BS and I contributed equally.

Presentation

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List of Abbreviations

AF	Transactivation function
AP-1	Activator protein-1
Bp	Base pair(s)
cAMP	Cyclic adenosine 5'-monophosphate
CBP	cAMP response element-binding protein-binding protein
CDK	Cyclin-dependent kinase
C/EBPβ	CCAAT/enhancer binding protein β
ChIP	Chromatin immunoprecipitation
Cpm	Counts per minute
CREB	CAMP-response element binding protein
DBD	DNA binding domain
E_2	Estradiol
EGF	Epidermal growth factor
Egr1	Early growth response 1
ER	Estrogen receptor
ERE	Estrogen-response element
ERK	Extracellular signal regulated protein kinase
FBS	Fetal bovine serum
FSH	Follicle stumulating hormone
FSHB	FSH beta
GAP	Gonadotropin-releasing hormone-associated peptide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GnRH	Gonadotropin-releasing hormone
GnRHR	GnRH receptor
GPCR	G protein-coupled receptor
GSUα	Gonadotropin α subunit
GR	Glucocorticoid receptor
GRIP-1	Glucocorticoid receptor interacting protein 1
hCG	Human chorionic gonadotropin
Hsp	Heat shock protein

IGF-I	Insulin-like growth factor I
JNK	c-Jun N-terminal protein kinase
Kb	Kilo base pairs
LBD	Ligand binding domain
LH	Luteinizing hormone
LHB	LH beta
MAPK	Mitogen-activated protein kinase
M-MLV	Moloney murine leukemia virus
NFκB	Nuclear factor κB
P4	Progesterone
PCAF	P300/CBP-associated factor
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PR	Progesterone receptor
PRE	Progesterone-response element
RAR/RXR	Retinoic acid receptor/retinoid X receptor
RSK	Ribosomal S6 kinase
RSV	Rous sarcoma virus
SRC	Steroid receptor coactivator
STAT	Signal transducer and activator of transcription
TGFα	Transforming growth factor alpha
TM	Transmembrane helices
UTR	Untranslated regions

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Chapter 1 Introduction

1.1 Overview

In the reproductive system, numerous hormones and signaling pathways interact to regulate the hypothalamic-pituitary-gonadal axis. One of the master hormones in this regard is gonadotropin releasing hormone-I (GnRH-I), which is released from neurons within the hypothalamus in a pulsatile manner. The main target of hypothalamic GnRH-I is the anterior pituitary. After binding to the GnRH receptor (GnRHR) on the surface of gonadotrophs, GnRH-I promotes the production and release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). These gonadotropins are members of the glycoprotein hormone family which comprise a common gonadotropin α subunit (GSUα) and unique β subunits (1). The gonadotropins play a essential role in folliculogenesis, steroidogenesis, and ovulation in the gonads (2-4). Steroid hormones from the gonads, including estradiol (E_2) and progesterone (P4), in turn feedback at the level of the hypothalamus and pituitary to modulate the expression and secretion of GnRH and the gonadotropins (Figure 1.1) (5).

Changes in plasma hormone levels during the human menstrual cycle, which is commonly divided into the follicular phase, ovulation, and the luteal phase, are the result of a highly synchronized regulation of the hypothalamic-pituitary-ovary axis. Between the luteal and follicular phase transition, there is a small increase in serum FSH concentrations 2-4 days before the inception of the menstruation (6). In the early follicular phase the FSH level maintains improved until the midfollicular phase, during which decreases to a basal level (7). The dominant follicle is selected in this period. The intercycle FSH rise is regulated by a considerable reduction in the plasma E_2 and P4 levels during the late luteal phase (8); this

decline also reduces the negative feedback effect of these steroid hormones in the early and midluteal phase.

The rise of plasma FSH concentrations in the transition from the luteal to follicular phase causes a cohort of ovarian follicles to develop. Usually only one follicle in which the granulosa cells with the lowest FSH threshold and the highest sensitivity to FSH becomes the first in the cohort to secrete E_2 (9). The E_2 concentration then feeds back to repress FSH secretion *via* the hypothalamic-pituitary axis. Therefore, FSH levels decline to a concentration insufficient to sustain the development of other follicles with relatively higher FSH thresholds. E_2 negatively feeds back on GnRH-I and LH secretion during most phases of the menstrual cycle (10), but E_2 is also the principal regulator that provides the positive feedback required to sensitize the pituitary to GnRH. This positive feedback is essential for triggering the LH surge by E_2 when a certain plasma level is achieved and maintains for a particular duration (11, 12). Although the extent to which P4 participates in the positive feedback effect before ovulation is less clear, studies have indicated that the GnRH self-priming effect and the onset of LH surge prompted by E_2 depend on the activation of progesterone receptor (PR) in ovariectomized *Pr* knockout mice administered with E_2 (13). The LH surge results in the resumption of meiosis of the oocyte, ovulation and granulosa cell luteinization. In the luteal phase, the elevated P4 level induces the production of E_2. The augmented plasma level of P4 first inhibits the pulsatile secretion of GnRH-I and LH and then precludes the elevated E_2 plasma levels induced GnRH-I and LH surges (14-18). Consequently, the levels of FSH and LH decrease quickly over time, and the corpus luteum subsequently undergoes atrophy. With the decrease levels of P4, menstruation occurs and the next cycle begins (19).

Mouse and humans share certain similarities in their reproductive systems, including the

hormone synthesis and release from the hypothalamic-pituitary-gonadal axis. One significant difference is that mouse has an estrous cycle of 4 days, which is divided into diestrus, proestrus, estrus and metestrus phase. In the proestrus phase, there is an estrogen peak, followed by a progesterone, FSH and LH surge. Ovulation occurs during the estrus phase.

Steroid hormone receptors are of critical importance in the reproductive system. In addition to being activated by their ligands, steroid hormone receptors, including estrogen receptor (ER) and PR, are activated by growth factors as well as by GnRH-I. Ligand-independent activation of ERα and PR by GnRH has been found in mouse gonadotrophs. As observed in mouse αT3-1 pituitary cells, GnRH-I works in concert with E_2 to influence the timing and/or magnitude of the ERα-mediated effects on gene expression (20); but the detailed mechanisms remain elusive. Furthermore, GSUα protein levels are impacted by the ligand-independent activation of PR by GnRH-I and GnRH-II in the αT3-1 cell line (21). More direct evidence that the transactivation of PR by GnRH-I contributes to *Gsuα* gene expression is lacking. In addition, it is not known whether cross talk between GnRH and PR contributes to the expression of other genes in the pituitary, such as the *Gnrhr,* which possesses a PRE and AP-1 binding site in its promoter. In this project, the molecular mechanisms responsible for the ligand-independent activation of ERα and PR by GnRH have been studied, especially in relation to induction of their endogenous genes.

1.2 GnRH and the GnRH receptor

1.2.1 GnRH-I and GnRH-II

There are more than 20 naturally occurring GnRHs sharing 10-50% amino acid identity

across vertebrate species. The majority of vertebrates contain minimum two, but normally three GnRH subtypes, which vary in the amino acid sequence, tissue localization, and embryonic derivation (3, 22). On the basis of evidence from high performance liquid chromatography (23), immunohistochemistry (24-26), northern blots (27), and *in situ* hybridization (28), at least two GnRH subtypes, GnRH-I and GnRH-II have been ascertained in the central nervous system in mammals. The preprohormone encoded by GnRHs comprises a signal sequence, a ten-amino acid GnRH peptide, a conserved proteolytic site (Gly-Lys-Arg), followed by a GnRH-associated peptide (GAP) (29).

GnRH-I is preserved all over the evolution from invertebrates to vertebrates, as demonstrated by the 60% of shared identity between tunicates and mammals (30). The human *GnRH-I* gene is pinpointed on chromosome 8p11.2-p21 (31) and comprises four exons disconnected by three introns. The genomic structures have been intensively studied and reviewed (Figure 1.2) (32-34). Exon 1 of the gene contains a 61 base pairs (bp) 5′-untranslated region (UTR), which is represented in the mRNA expressed in the brain. Exon 2 codes the signal sequence, followed by the ten amino acid GnRH peptide, the conserved proteolytic site (Gly-Lys-Arg), and the beginning eleven GAP residues. Exon 3 encodes the subsequent thirty-two GAP residues. The last exon codes for the rest GAP residues, the translation stop codon, and the complete 3′-UTR (32, 35). The amino acid sequence of mammalian GnRH-I is as follows: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2 (36, 37).

In human brain, GnRH-I neurons are located in the preoptic area and basal hypothalamus. Other regions in the brain have also been identified including the septal region, anterior olfactory area, the cortical and medial amygdaloid nuclei (38). Immunocytochemical studies have found the localization of GnRH-I fibers in the median eminence and infundibular stalk.

They are also detectable in substantial projections to the neurohypophysis (39, 40). *GnRH-I* mRNA is localized in the normal pituitary and in several types of pituitary adenomas (41, 42). In humans and other vertebrates, GnRH-I represents a fundamental neuroendocrine connection between the central nervous system and the reproductive system. Alterations in the frequency and amplitude of GnRH-I release from the hypothalamus in response to variations in steroid hormones sequentially alter the synthesis and secretion of both FSH and LH (43). Generally, rapid GnRH pulses, which occur in the follicular phase, favor LH secretion, and slow GnRH pulses, which occur in the luteal phase, favor FSH secretion. LH is released in a pulsatile manner, and every episode is consistent with one GnRH-I pulse. In contrast, the release pattern of FSH possesses both pulsatile and constitutive constituents, with approximately two-thirds of the total pulses successfully matching the GnRH-I pulses as observed in sheep (44).

Other than its well-documented endocrine function, GnRH-I is also a potential autocrine and paracrine factor in several tissues outside of pituitary including the ovary, uterus and placenta (45-48). Since the discovery of GnRH-I, several GnRH-I agonists with enhanced biological potency, and GnRH-I antagonists have been discovered and comprehensively investigated (49). Some regimens have been applied for the treatment of several gynecological endocrine diseases and in controlled ovarian hyperstimulation for assisted reproductive techniques (50, 51).

GnRH-II is the most conserved GnRH subtype because it is found in members of every vertebrate class (52-55) and shares 100% identity between birds and mammals (31). Initially discovered in chicken and commonly known as GnRH-II, the gene for human *GnRH-II* is discovered on chromosome 20p13 by fluorescence *in situ* hybridization (27) and comprises four exons interrupted by three introns (Figure 1.2) (34). Compared to *GnRH-I* (5.1 kilo base

pairs (kb)), the human *GnRH-II* gene is notably small (2.1 kb) due to the comparatively shorter introns. The length of the individual preprohormone elements of the two subtypes of GnRH in human is comparable, with the exception that GnRH-II has a 50% longer GAP compared to GnRH-I. A comparable discrepancy in the GAP has been found in tree shrew, indicating that this might be a feature among GnRH-II precursors in some mammals (35, 36, 56, 57). The sequence of GnRH-II is pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH2, and it only varies from that of GnRH-I at positions 5, 7, and 8 (27, 36).

GnRH-II localizes among the reproductive system and other systems. The most important difference in the tissue distribution of the two GnRH subtypes is that the expression of GnRH-II is at considerably excessive levels outside the brain (27). In the human central nervous system, immunopositive GnRH-II signals occur in the midbrain and limbic structures, where they have a crucial function in the behavioral aspects of reproduction (58, 59). Comparing with GnRH-I, GnRH-II is more efficient in accelerating reproductive behavior in female sparrow (60), although its mechanism of action is unknown. In certain human neuronal cell lines, both expression of GnRH-I and GnRH-II is validated at the mRNA and protein levels, but there is approximately ten- to one hundred-fold higher amount of GnRH-I than that of GnRH-II (61). In monkeys, similar to GnRH-I, GnRH-II also localizes to the supraoptic, paraventricular, arcuate nucleus and pituitary stalk area (28, 62), where it regulates gonadotropin secretion. In rhesus monkeys, GnRH-II effectively stimulates gonadotropin release *in vivo*, a step mediated *via* the type I GnRHR; specifically, treatment with GnRH-II elevates plasma LH concentrations. Although a single treatment of GnRH-II fails to affect plasma FSH concentrations, a significant increase is achieved after repeated exposures. Antide, a type I GnRHR antagonist, completely blocks GnRH-II-induced LH release (59). Furthermore, in male sheep, GnRH-II prefers FSH release (63). Both GnRH-I

and GnRH-II have also been localized in the pituitary of various teleost fish species in which they regulate gonadotropins and growth hormone (36). In the ram, treatments with two type I GnRHR-specific antagonists block GnRH-II-stimulated gonadotropin secretion; however, the concurrent treatment with GnRH-II fails to change GnRH-I-stimulated LH release (64). Thus, whether GnRH-I and GnRH-II elicit different effects on gonadotropin synthesis and secretion remains elusive, but this may be related to their different patterns of pulsatile secretion, concentration or the effects on intracellular signaling pathways (65). Compared to GnRH-I, GnRH-II is less vulnerable to peptidase and more stable, which improves its tissue bioavailability. Such characteristics have important implications for GnRH-II functions, particularly in hormone regulation of the reproductive system, and may result in the clarification of innovative paracrine physiological functions of GnRH-II (66). Currently, there is a lack of information on GnRH-II secretion profiles. Further studies are required to assess the effects of different GnRH subtypes on the induction of gonadotropin subunit genes, as well as other genes that are essential for pituitary function.

GnRH-II has also been identified in other reproductive tissues including the endometrium (67), ovarian surface epithelial cells (68), granulosa cells (69), breast tissue (70), as well as ovarian epithelial and breast tumors (68, 70). In addition, GnRH-II plays an important role in ovarian function and implantation in humans. It is uncertain whether in these tissues the responsiveness induced by GnRH-II is significantly different from that elicited by GnRH-I. In addition to the reproductive tissues, *GnRH-II* mRNA has been localized in human kidney, bone marrow, and prostate. Compared with the central nervous system, kidney contains around 30-fold higher *GnRH-II* mRNA levels, and the prostate and bone marrow possess approximately 4-fold higher levels (27).

1.2.2 GnRH receptor

GnRHR belongs to the G protein-coupled receptor (GPCR) family, the structures of which are characterized by seven transmembrane helices associated with continuous intracellular and extracellular loops, as well as an external N-terminal domain. The external domains and the transmembrane regions create the area for the binding of the ligand, and the intracellular fractions are responsible for communications with intracellular G proteins, as well as other regulatory proteins (71, 72). The mammalian genome contains two subtypes of *GnRHRs*, *type I* and *type II GnRHR*, located on chromosome 4 and 1 in human, respectively. The *type I GnRHR* genes from the human (73), mouse (74), rat (75), pig (76) and sheep (77) have been characterized, and exhibit extensive sequence similarity in the coding areas. Their structural organization consists of 3 exons interrupted by 2 introns which have been extensively studied and reviewed (Figure 1.3) (34, 78). Although the borders between exon and intron are preserved among different species, the sizes of the introns, the length and sequence of the 5′-and 3′-UTRs are different in the genes. The first exon codes type I GnRHR amino-terminal domain, transmembrane helices (TM) 1 to 3 and the first section of TM 4. The second exon contains the other section of TM 4 and TM 5. The third exon consists of TM 6 and 7 (79). The *type II GnRHR* gene exhibits the similar exon and intron construction as *type I GnRHR*, with the exception that the third exon encodes an intracellular carboxyl terminal tail, which is deficient in *type I GnRHR*. In the second exon of the human *type II GnRHR* gene, a premature stop codon (UAA) is identified which indicates products of this gene are unfunctional. A second locus possessing a pseudogene for human *type II GnRHR* is localized on chromosome 14. The chimpanzee *type II GnRHR* gene also contains a premature stop codon (58). A fully functional *type II GnRHR* gene, however, is ascertained in

other mammalian genomes including lower primates, for instance, the marmoset monkey (63), African green monkey and rhesus monkey (80). In nonprimate mammals, the *type II GnRHR* gene potentially encodes functional protein in pigs and dogs, whereas not in cows and sheep. In mouse genome, there is no *type II Gnrhr* gene (65), whereas rat genome contains a gene remnant (81). Thus in the following content *Gnrhr* refers to *type I Gnrhr* in mouse.

Since there is no functional type II GnRHR protein in the mouse or human, both GnRH-I and GnRH-II function through the type I GnRHR in these species (58). The intracellular tail in many GPCRs has a fundamental regulatory function in ligand-stimulated receptor signaling, desensitisation, and trafficking (82). Noticeably, comparing with other GPCR members, the type I GnRHR is special due to its total lack of a cytoplasmic carboxyl terminal tail (54, 83-86), and it does not exhibit rapid desensitisation (87). In the human type I GnRHR, several amino acid residues have been ascertained to have important function. In particular, in the third cytoplasmic loop Ala is vital for G protein coupling and GnRHR internalization (88). Some other amino acids have been found to be responsible for binding of the ligand (89-93). The species-specific Lys residue is a significant determinant of expression and internalization (94). The mammalian type I GnRHR has approximately ten-fold binding affinity for GnRH-I other than GnRH-II (95). On the other hand, the type II GnRHR has around four hundred-fold preference for GnRH-II comparing with GnRH-I (63, 80, 96).

The distribution of immunoreactive type I GnRHR has been studied in normal pituitary and human pituitary tumors. In normal anterior pituitary, type I GnRHR is localized in gonadotrophs, thyrotrophs and somatotrophs. It is co-localized with GSUα, FSH beta (FSHB) and LH beta (LHB) in gonadotrophs, thyroid-stimulating hormone beta in thyrotrophs, and growth hormone in somatotrophs (97). In accordance with its mRNA expression profile in

pituitary tumors, immunoreactive type I GnRHR has been detected in adenomas derived gonadotrophs and somatotrophs (42, 97).

In the pituitary, the type I GnRHR cascade has been comprehensively studied due to the importance of GnRH-I in the modulation of synthesis and secretion of LH and FSH. The requirement for type I GnRHR in the regulation of gonadotrophin is highlighted by the fact that in the human, type I GnRHR mutations cause hypogonadotrophic hypogonadism which has the clinical symptoms of sexual development delay, apulsatile or low levels of gonadotrophin, and low levels of steroid hormone (3, 98). This mutation results in majority mislocalised GnRHR proteins, that display changed membrane trafficking (99) and endoplasmic reticulum preservation, which may be restored to function by pharmacological chaperones (100).

Apart from the pituitary, the localization of *type I GnRHR* mRNA has been identified in other normal tissues as well as malignant cells outside of brain, including ovary, uterus, breast, placenta and prostate (101). GnRH-I and GnRH-II exhibit other functions after binding with GnRHR such as inhibition of cell proliferation in some cell types, including cancer cells (86, 102-109).

1.2.3 Signal transduction mechanism of type I GnRHR

In the anterior pituitary, following binding to the receptor, GnRH-I activates the protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways; all of these pathways are indirectly involved in regulating gonadotropin subunit genes *via* mechanisms that are not well defined (110-116). Binding of GnRH-I to the type I GnRHR causes the coupling of the Gαq/11 proteins and subsequently stimulation of phospholipase Cβ (117). This hydrolyzes

phosphatidylinositol 4,5-bisphosphate to diacylglycerol and (1,4,5) inositol trisphosphate that, in turn, activates conventional PKC isoforms and mobilizes cytoplasmic calcium, respectively. In terms of PKC isoforms, α and βII have been discovered in gonadotroph cells (118-121).

GnRH-I also activates PKC-dependent and -independent MAPK cascades that influence gene expression (115, 122-126). Type I GnRHR signaling activates four kinase cascades, containing the extracellular signal regulated protein kinase (ERK) (122, 123, 125, 127-131), the c-Jun N-terminal protein kinase (JNK) (4, 115, 129, 132, 133), p38 MAPK (123), and the big MAPK (115); these subsequently induce expression of several genes. The activation of ERKs by GnRH-I has been widely recognized. In αT3-1 cells, the GnRH-I pulse pattern affects the responsiveness of ERKs to GnRH-I such that continuous exposure of GnRH-I stimulates short-term ERK activity, while pulsatile GnRH-I results in prolonged activation of ERK activity (128). Stimulation of ERK takes place mainly through PKC pathway in αT3-1 cell line (129, 133). In LβT2 cells, the binding of type I GnRHR by GnRH-I leads to activation of ERK and induction of *c-fos, Gsuα, Lhb,* and *Fshb* gene expression (113, 115, 121, 134). The JNK signaling cascade is another fundamental MAPK signaling participated in GnRH-I pathway in the αT3-1 pituitary cells. GnRH-I treatment results in a significant enhancement in JNK signaling, surpassing the sequential activation of PKC, c-Src, CDC42 (Rac), and MEKK1 (115, 129). A JNK cascade regulates both basal and GnRH-I-increased rat *Lhb* promoter activity (115, 132). Nevertheless, other reports have indicated that JNK activation is mainly PKC-independent and mediated by elevated intracellular calcium (132, 133). The PKC-independent pathways may due to the distinct circumstances under which the PKC-dependent pathway is changed. In the fish, tilapia, GnRH-I also regulates LH and FSH secretion through divergent signaling *via* receptor binding. In this species, the expression of

the *gsuα* and *lhb* subunit mRNAs is increased by ERK activation, while the induction of *fshb* is mediated by the cyclic adenosine 5′-monophosphate (cAMP)-PKA signaling cascades (135). Another study has found that PI3K is engaged in the negative adjustment of the *Gsuα* and *Fshb* subunit gene expression and the cell proliferation in LβT2 cells (116).

1.2.4 Regulation of type I GnRHR

The sensitivity of GnRH-I in gonadotrophs relies on the amount of GnRHR on the surface of the cells, and this is partially regulated at the transcriptional step (136, 137). Several hormones including GnRH-I itself (123, 138-141), melatonin (142), steroid hormones (143-145), activin (31), human chorionic gonadotropin (hCG) (146, 147), intracellular signaling pathways (84, 148, 149) and transcription factors (150-152) regulate mammalian *type I GnRHR* transcription in different tissues from various species. In the pituitary, GnRH-I robustly regulates *type I Gnrhr* expression in the way that lower dosages of GnRH-I increase *Gnrhr* expression, while higher dosages decrease *Gnrhr* levels in rodents (140, 141). The frequencies of GnRH-I pulse influence the degree of increase, and highest enhancement is reached at a thirty-minute frequency in rat primary pituitary cells (153). In αT3-1 cells, the stimulation of MAPK signalings by GnRH-I influences *type I Gnrhr* gene expression. Activation of the JNK pathway by GnRH-I stimulates activator protein-1 (AP-1), which interacts with the AP-1 element in the *type I Gnrhr* promoter and enhances endogenous *type I Gnrhr* gene expression (4). It has been discovered that GnRH-I has a biphasic effect on *type I GnRHR* expression in human cells such as granulosa luteal cells, normal ovarian surface epithelium, ovarian cancer, and peripheral blood mononuclear cells (69, 124, 154, 155). Inhibition of *type I GnRHR* mRNA levels by GnRH-II irrespective of the concentration used has been reported in human granulosa luteal cells (69).

The steroid hormone P4 directly inhibits t*ype I GnRHR* expression in the pituitary (156). Intriguingly, P4 displays a dual function in regulating human *type I GnRHR* gene transcription such that it suppresses the *type I GnRHR* promoter in gonadotrophs but has stimulatory effects in placental cells (157). Due to the critical significance of GnRHRs in reproductive system and the prevalent usage of GnRH analogues and antagonist in endocrinology and malignant therapy, it is fundamental to clarify both the physiological and therapeutic roles of GnRH and its receptor.

In summary, GnRH-I and GnRH-II regulate the synthesis and secretion of gonadotropin in the pituitary after binding with their receptor, type I GnRHR, both in mouse and humans. GnRH activates several signaling pathways, which are indispensable for the gonadotropin subunit genes expression. The functions of GnRH depend on the amount of type I GnRHR at the gonadotroph cell surface, and this is regulated by several hormones and factors including the GnRH and P4.

1.3 Estrogen receptor α

1.3.1 Estrogen receptor

Estrogens modulate many physiological functions including cell growth, development, and tissue-specific gene expression in the reproductive, skeletal and central nervous systems. Estrogens are a group of compounds and the major estrogens in human are E_2, estriol, and estrone. Among them, E_2 is the most important physiological ligand of the ER. E_2 is produced primarily by follicles and the corpus luteum in the ovaries. In the menstrual cycle, under the stimulation of FSH and LH, there are systematic changes in the level of E_2 with

one surge occurring at 24-36 hours before ovulation and the other during the middle luteal phase. Feedback regulation of the hypothalamus and pituitary by estrogen is especially essential during the menstrual cycle.

In the physiology and pathophysiology of many tissues, E_2 exerts critical roles by interacting with ER, which belongs to nuclear receptor superfamily. ERα and ERβ are two recognized subtypes of ER, which are coded by different genes and distinguished in construction, function, and tissue localization (84, 158, 159). In humans, the ERα gene has been mapped to chromosomal locus 6q25.1 (160), whereas ERβ gene is located on 14q22-24 (161). Both ER subtypes are composed of six domains. In details, the N-terminal A/B domain exhibits 17% amino acid sequence identity between ERα and ERβ. ERα contains a ligand-independent transactivation function 1 (AF-1) in this region. The central C region is the DNA-binding domain (DBD), and it possesses two zinc finger structures crucial for connecting to estrogen response elements (EREs) in DNA sequences. This structure is considered to be the hallmark of the nuclear receptor superfamily (162, 163). In addition, the DBD mediates receptor dimerization. Given their highly conserved amino acid similarity (96%) within domain C, ERα and ERβ should bind to the same ERE. The D domain possesses a nuclear localization signal. There are several functions in the carboxyl-terminal E/F domain involving binding to the ligand, dimerization, as well as ligand-dependent transactivation function 2 (AF-2), comprising a shallow hydrophobic groove made up by residues among helices H3, H4, H5, and H12 where the coactivators LXXLL receptor interaction domain operates (L is leucine and X is any amino acid) (Figure 1.4) (164, 165). The ligand binding domains (LDBs) of the ERα and ERβ have high homology, especially the amino acid residues which directly contact with the ligand or consist of the ligand binding cavity (166). Therefore E_2 and several other ligands bind to ERα and ERβ with similar

affinity. However, due to the relatively small ligand binding cavity in ERα, a number of ligands exhibit receptor-selective affinity and exert different biological response (161, 167).

The tissue distribution profiles of the two types of ER indicate that they may have similar but also unique roles in E_2 action *in vivo*. ERα is primarily located in the pituitary, uterus, mammary gland, testis, liver, kidney, heart, and skeletal muscles. ERβ is present in the ovary and prostate. The gonad, thyroid, adrenals, and various regions of the brain have relatively similar levels of *ERα* and *ERβ* mRNA. However, the cellular distribution of *ERα* and *ERβ* mRNA differs within the tissues that coexpress both subtypes of ER (168). Characterization of mice lacking either *Erα*, *Erβ*, or both have shed light on the function of each ER subtype (168). The physiological importance of ERα after binding with E_2 involves maintenance of the hypothalamic-pituitary axis, bone mineral density, glucose metabolism, cardiovascular function, mating behavior, and the development of mammary gland. By contrast, ERβ is responsible for the regulating ovulation, several aspects of mating behavior, and the immune system. The two subtypes also have different intrinsic mechanistic properties such that ERβ can function as an inhibitor of ERα and reduces the strength of E_2 acting *via* ERα (169). Both sexes of *Erα* knockout mice are infertile, and while male *Erβ* knockout mice are fertile, the females are subfertile. Female mice that lack ERα have a hypoplastic uterus, a condition that is insensitive to E_2, donor embryos do not implant, immature follicles are present in the ovary and they do not ovulate. On the other hand, female *Erβ* knockout mice have a normal response to E_2 in the uterus and can support pregnancy. The appearance of the *Erβ* knockout mouse ovary is normal but it exhibits reduced ovulation (168).

In the anterior pituitary, E_2 binding can be localized within the gonadotroph, lactotrope, somatotrope, and thyrotrope cells. The rodent pituitary contains three forms of ER, including the predominant ERα, the truncated pituitary-specific form of ERα called TERP, and ERβ. In

both normal rodent primary cells and several cell lines including pituitary αT3-1 cells, only ERα and TERP proteins are expressed at noteworthy levels, whereas the expression of ERβ in the pituitary is extremely low (5, 170-173). Furthermore, mice with a disrupted *Erα* gene have compromised pituitary function, whereas *Erβ* knockout mice appear to have normal pituitary function (174, 175). Thus, the response of gonadotroph cells to E_2 is primarily mediated by ERα.

1.3.2 ERE-dependent genomic actions

The main genomic effect of E_2 occurs *via* the ERE-dependent E_2-ER signaling pathway. Binding of E_2 to the ER permits the receptor to separate from heat shock proteins (hsps) and promotes ER dimerization to form homodimers or heterodimers (159, 176, 177). Subsequently the ER accumulates at various permutations of a palindromic DNA sequence, 5′-GGTCAnnnTGACC, which is the so-called consensus ERE with three central nonspecific nucleotides. Natural EREs lose affinity for ER with an increase in the number of nucleotides deviating from the consensus sequence, especially when the changes occur in both bisects of the ERE palindrome (178). In the human genome, however, most E_2 target genes possess non-palindromic EREs (179). The requisite sequences for imperfect EREs have been identified *in vitro* (180). The two ER subtypes are assumed to bind to EREs in an identical approach because both ERα and ERβ communicate with the same nucleotides in the consensus ERE (181). The ER-ERE complex recruits coregulatory proteins, thus affecting chromatin remodeling and/or providing a bridge to other transcription factors that mediate assembly of the transcriptional machinery (182).

1.3.3 ERE-independent genomic actions

It is evident that ER not only regulates genes that contain EREs but also influences the expression of those that do not, and this enables ER to regulate a broader range of genes. These genes normally possess binding sites for a variety of heterogeneous transcription factors, including AP-1, Sp-1, and nuclear factor κB (NFκB) which interact with ER through protein-protein binding.

The ERα-AP-1 protein (Fos and Jun) interaction increases the transcription of genes such as ovalbumin (183), insulin-like growth factor I (*IGF-I*) (184), collagenase (185) and cyclin D1 (186, 187). Activation of the ERα-AP-1 complexes induced by E_2 requires the AF-2 domain of ERα, which binds p160 to form a multiprotein (176, 188). A direct connection between ERα and c-Jun is necessary for ERα/AP-1 action. Moreover, the coactivator glucocorticoid receptor interacting protein 1 (GRIP-1) forms a triple complex with c-Jun and ERα which stabilizes the ERα/c-Jun complex (188).

The interaction between ERα and Sp-1 regulates several genes that contain GC-rich promoter sequences, including E2F1 (189), low-density lipoprotein receptor (190), c-fos (191), and cyclin D1 (192). The actions of ER at Sp-1 binding sites rely on the ligand, the cell type, and the receptor subtype. Although both ERα and ERβ form complexes with Sp-1, only ERα induces consensus Sp-1 element-linked reporter gene activity; ERβ by contrast exhibits minimal or decreased basal reporter gene activity. Additionally, it has been shown that the AF-1 domain of ERα is critical for ERα/Sp-1-mediated transactivation (193).

Other than AP-1 and Sp-1, the interactions of ERα with two other transcription factors, NFκB and CCAAT/enhancer binding protein β (C/EBPβ), decrease interleukin-6 gene expression (194). In terms of ERE-independent genomic actions, ERs do not interact directly

with DNA but act by tethering other transcription factors. However, in this context, the DBD of the ERs is also crucial for proper protein-protein interactions and for the recruitment of coregulator proteins to the promoter region (130, 183-186, 194, 195).

1.3.4 ERα phosphorylation

Activation of ERα by its ligand is associated with increases in overall receptor phosphorylation and this regulates certain receptor functions. Eight phosphorylation sites have been identified within the human ERα. Four of these sites (Ser^{104}, Ser^{106}, Ser^{118}, and Ser^{167}) are located in the A/B domain, one is in C domain (Ser^{236}), and the other three sites (Ser^{305}, $threonine^{311}$ and $tyrosine^{537}$) are found within the LBD (Figure 1.5) (196). Among them, Ser^{118} and Ser^{167} are the major E_2-inducible phosphorylation sites in ERα isolated from different cell lines (197-203). Phosphorylation of the serine residues modulates the down-regulation of ERα by the ubiquitin-proteasome pathway (204); its nuclear localization (205), and transcriptional activity (198, 199). ERα phosphorylation at Ser^{118} is detected after transfection of human ERα into COS-1 cells. Mutation of this serine to alanine causes a significant reduction in transcriptional activation by ERα of reporter genes containing an ERE (199). In contrast, another study has determined that the S118A-ERα mutation alone can not significantly abolish E_2 stimulated transcriptional activity when compared to the wild-type ERα. Only the combined mutation of three of the amino-terminal phosphorylation sites (Ser^{104}, Ser^{106} and Ser^{118}) to alanine residues significantly reduces the ERα transcriptional activity, highlighting the importance of multiple phosphorylation of these amino acids for full receptor function (198). Tyrosine 537 is located immediately amino-terminal to the AF-2 activation helix and is conserved in the ERα sequence of every species (206). It is regarded as a basal phosphorylation site (197).

1.3.5 Nuclear coactivators

The recruitment of coactivators to ERα allows the resulting complex to bridge the receptor to the general transcription machinery, leading to chromatin structure remodeling, and thereby facilitating gene expression (207). Several ERα co-activators have been identified including members of the P160 or steroid receptor coactivator (SRC) family, P300, cAMP response element-binding protein-binding protein (CBP), and the P300/CBP-associated factor (PCAF) (178, 182).

The SRC family members of proteins are composed of ligand-dependent coactivators that enhance the transcriptional activation of several nuclear receptors such as ER and PR (182). This family is divided into three classes based on their sequence homology: SRC-1/NcoA-1 belongs to class I; TIF2/glucocorticoid receptor-interacting protein /NCoA-2 fits in class II, and pCIP/activator of thyroid and retinoic acid receptor/amplified in breast cancer 1/SRC-3 is a class III coactivator (208). Recruitment of SRC to the ER depends on the integrity of helix 12 with the carboxy-terminal region of the ER. Upon ligand binding, repositioning of helix 12 of the ER forms a hydrophobic cleft acting as "a charged clamp" to interact with LXXLL motifs in the coactivators (209-211). It has also been reported that SRC-1 enhance transcriptional activation of ERα through both AF-1 and AF-2, acting synergistically to achieve receptor full activity (212).

CBP, and its homologue P300, are another class of coactivators that exhibit histone acetylase activity. They were first identified as nuclear proteins that functionally interact with the cAMP response element binding protein (CREB) and the viral adenovirus oncoprotein E1A, respectively. Subsequently they were suggested to potentiate activation of the thyroid hormone receptor, the glucocorticoid receptor (GR) and the ER (213, 214). Purified P300 is

found to significantly enhance ligand-dependent ERα actions on a chromatin template, suggesting a role for the histone acetyltransferase activity of P300 in chromatin remodeling (215). Although CBP/P300 directly interacts with nuclear receptors through the LXXLL motifs in CBP/P300 and the LBD of the nuclear receptors (214), this somehow appears to be dispensable for transcriptional activation *in vitro* (216). Instead, P300/CBP is indirectly recruited to nuclear receptor target genes by the SRC coactivators, which serve as an assembly point for multimeric activation complexes (217). Both CBP and P300 have also been associated with the regulation of a large numbers of transcription factors (218). Competition for limiting levels of these proteins within a cell results in cross-talk between different signaling pathways, suggesting that CBP/P300 proteins are key mediators of signal integration (219).

PCAF is the first mammalian histone acetyltransferase found original from sequence homology to the yeast Gcn5p protein (220). Chromatin is composed of nucleosomal core particles in which DNA is wrapped around histone octamers containing histones H3 and H4 heterotetramer and two heterodimers of H2A and H2B. In each of the core histones, there is a lysine rich N-terminal tail, the majority of which can be acetylated in transcriptionally active chromatin. Recombinant PCAF generally acetylates lysine 14 of H3 and lysine 8 of H4 (220, 221). PCAF also functions as a transcriptional coactivator, thus contributing to transcriptional activation by chromatin structure remodeling in GR, ER and the retinoic acid receptor/retinoid X receptor (RAR/RXR). PCAF contains an extended amino terminus that interacts with other coactivators such as CBP/P300 and members of SRC family to form a multiprotein (213, 222, 223). It is interesting that though CBP is compulsory for the roles of several transcription factors, the function of PCAF and SRC appears to be more specific. The DNA-bound transcription factors can determine recruitment of CBP, PCAF and SRC. In

addition, signal cascades probably participate in the regulation of the complex assembly (223, 224).

Many studies have reported that steroid hormone receptors are activated and bind to specific regions in the promoters of target genes, and this occurs with the recruitment of certain coactivators to increase the target gene expression. However, how this occurs in the context of gonadotropin subunit gene expression in the pituitary function remains unclear.

1.3.6 Ligand-independent activation of ER

It was originally thought that steroid hormone receptors were only activated by their own ligands. However, ligand-independent activation of a steroid hormone receptor has been found to occur in response to signaling pathways from membrane regulatory molecules including growth factors, cAMP, dopamine, cytokines, and other regulators. The activation of steroid hormone receptors by other factors instead of their own ligands represents a prime function by which membrane receptors and steroid hormone receptors cross-talk at the level of the gene transcription, and is a mechanism by which the cellular environment modulates the functions of steroid hormone receptors as transcriptional regulators (225).

Numerous studies have reported that epidermal growth factor (EGF), transforming growth factor alpha (TGFα), IGF-1, heregulin, insulin, act through several signaling pathways to transcriptionally activate genes whose promoters contain a consensus ERE in an ER-dependent manner (201, 226-231). For instance, EGF mimics estrogenic effects in ovariectomized mice, resulting in increased uterine and vaginal cell proliferation (232). The inhibitory effects of ICI 164,384 on EGF-stimulated cell proliferation are observed in wild-type mice but not in *Erα* knockout mice, suggesting an association of EGF signaling with the ligand-independent activation of the ERα (233). In addition, growth factor-activated

ERα transcriptional activity is dependent on the phosphorylation state of ERα. EGF or IGF activates ERK1/2, which mediates phosphorylation of ERα at Ser^{118}, leading to the ligand-independent transactivation of ERα (226, 234). A 90 kDa ribosomal S6 kinase (RSK) is an ERK substrate and a mediator of the ERK signaling pathway. EGF or phorbol myristate activates RSK, causing specific phosphorylation of ERα at Ser^{167} (235). PKA overexpression has been linked to improved proliferation in normal breast samples, breast malignant transformation, poor prognosis in breast tumor, as well as antiestrogen resistance (236). Up-regulation of PKA activity induces the ligand-independent activation of ERα (201, 226, 237) and increases receptor phosphorylation (198, 238-240). The ligand-independent and ERE-dependent activation of the other subtype of ER, ERβ, is also induced by forskolin and 3-isobutyl-1-methylxanthine, resulting in increases in intracellular cAMP in transient transfections of Hela cells (237).

AKT is a serine/threonine protein kinase that is a downstream target of PI3K. PI3K and AKT activate human ERα in the absence of E_2 (241, 242). Cyclins are subunits of cyclin-dependent kinase (CDK) complexes, which regulate cell cycle progression. Cyclin D1 is overexpressed at a significant level in human breast cancers together with expression of ERα. Overexpression of cyclin D1 stimulates ERα transcriptional functions in the absence of E_2 (243, 244). The cyclin A/CDK2 complex phosphorylates ERα at Ser^{104} and Ser^{106}, and these modifications potentiate the transcriptional activity of ERα in a ligand-independent manner (245). Furthermore, cyclin D1 also interactes with PCAF to facilitate the association between PCAF and the ERα. Overexpression of PCAF potentiates cyclin D1-stimulated ERα activity in a dose-dependent manner (246), suggesting the importance of this coactivator.

In the pituitary, the ligand-independent activation of ERα contributes to gene expression. In αT3-1 gonadotroph cells, cAMP stimulates ERα in a ligand-independent manner *via*

PKA-dependent pathways. Because several physiological factors stimulate cAMP levels in the pituitary, cAMP can influence ERα activity in the pituitary *in vivo* (5). GnRH-I also stimulates an ERE-promoter activity in the same cell line (20). However, the molecular mechanisms responsible for this, and whether the ligand-independent activation of ERα by GnRH-I might modulate the expression of relevant endogenous genes in the pituitary remain unclear.

1.4 Progesterone receptor

1.4.1 Progesterone receptor

Pogesterone (P4) is critical in the regulation of mammary gland development, ovulation, blastocyst implantation, epithelial cell proliferation, uterus contractility, and reproductive behavior (247, 248). In the neuroendocrine system, P4 exerts negative feedback effects on both hypothalamic GnRH release and pituitary gonadotropin production (249, 250), which include homeostatic suppression of pulsatile GnRH secretion (251), as well as the pre-ovulatory GnRH and gonadotropin surges (252). The main regulation of female reproduction by P4 depends on the binding and activation of PR, which is another member of the nuclear receptor superfamily. The two main isoforms of PR are a full length PR-B and an amino-terminally truncated PR-A, lacking the first 164 amino acids of PR-B. The two isoforms are attained from the transcription of a same gene from two specific promoters and a translation start at two substitute AUG initiation codons (253). Both PR isoforms have three major functional domains (Figure 1.6) (254). The amino-terminal transactivation domain is poorly conserved and contains a functionally important AF-1 region. Apart from AF-1, PR-B

also possesses an AF-3 region that is responsible for the recruitment of coactivators to the receptor, and which modulates target gene activation and promoter specificity (255, 256). An inhibitory domain, which recruits transcriptional corepressors, is located in the amino-terminal transactivation domain (257). The DBD is composed of about 66-68 amino acids. The LBD contains a ligand-dependent AF-2, which is requisite for hormone-dependent enrollment of coactivators.

The occurrence of PR-A and PR-B is conserved in several vertebrate species comprising humans and rodents (258). The two PR isoforms are usually coexpressed in normal cells *in vivo*; however, the ratios of the isoforms differ in reproductive tissues as a result of development (259) and hormonal levels (260), as well as during carcinogenesis (261). In the brain of E_2-treated rhesus macaques, the hypothalamus expresses a high level of PR-B, but the pituitary contains an excess of PR-A (262). PR-A is found to be the predominant isoform in the LβT2 cell line, or pituitary cells from ovariectomized rats or mice. There is a relatively lower level of PR in the LβT2 cell line compared to the primary pituitary cells, and it is unaffected by E_2 alone or with P4 treatment (263). Regarding other tissues, the PR-A and PR-B levels and their ratio vary extensively during the menstrual cycle in the human endometrium (264, 265). Overexpression of PR-B is associated with advanced endometrial, cervical, and ovarian malignancy (266, 267). The PR-A and PR-B isoforms have dissimilar transactivational properties which are particular to the cell type and the target gene promoters. Generally in a diversity of cell types, the PR-B isoform is a strong transcriptional activator of some PR-dependent promoters, whereas PR-A is inactive. Furthermore, the PR-A isoform represses the transcription of PR-B, ER, the androgen receptor, GR, and the mineralocorticoid receptor when the receptors are co-expressed in cultured cells (255, 268, 269). In addition, the PR-B and PR-A isoforms have effect on different target genes

expression; for instance, of 94 P4-regulated genes in breast cancer cell lines, approximately 70% are uniquely regulated by PR-B, 4% are regulated by PR-A and not by PR-B, and 26% are regulated by both PR isoforms (270).

The *Pr* knockout mice model has provided extensive substantiation for the essential roles of PR subtypes in female reproduction. More specifically, both *Pr-a* and *Pr-b* knockout female mice display impaired gonadotropin regulation and pregnancy-associated mammary gland morphogenesis, anovulation, and uterine dysfunction (13, 271-273). Specifically, *Pr-a* knockout mice have irregular uterine and ovarian function, whereas the defeat of *Pr-b* result in flawed mammary gland development during pregnancy (274, 275).

1.4.2 Ligand-dependent activation of PR

The function of intracellular PR as a ligand-activated transcription factor is well characterized and is similar to that of ER (276). In the absence of P4, PRs form complexes with a number of chaperone molecules containing hsp90, hsp70, hsp40, Hop, and p23. The associations are required for the accurate protein folding and creation of stable complexes for competent binding ligand (277). Following P4 binding, the PRs exhibit conformational changes leading to hsp dissociation, receptor phosphorylation, dimerization, nuclear translocation, binding to progesterone response elements (PREs) with a specific sequence as 5′-AGAACAnnnTGTTCT, and subsequent gene transcription (278). If expressed in equal ratios, the PR-A and PR-B proteins dimerize to form the three distinct dimer types, A:A or B:B homodimers or A:B heterodimers, that bind DNA. The presence of the specific AF-3 domain of PR-B in these complexes contains differential transactivation properties, which may influence the entire repertoire of physiological responses to P4 (114, 279, 280).

Similar to ER, PR may alternatively control genes expression by tethering to other

transcription factors including AP-1, SP-1, or the signal transducer and activator of transcription (STAT) (270, 281-283). All of these regulation routes contribute to the biological response to P4.

1.4.3 PR phosphorylation

The role of PR phosphorylation is still elusive; it may be responsible for the modulation of ligand-dependent (284) and ligand-independent (285, 286) hormone sensitivity, nuclear localization, receptor turnover, interaction with co-regulators, and transcriptional activities. Fourteen PR phosphorylation sites have been identified (Figure 1.7) (287, 288). Of these sites, serines 102, 294, 345 and 400 can be phosphorylated by the ligand (289, 290). The phosphorylation of serines at positions 81, 162, 190, 294 and 400 seem to occur in a ligand-independent manner (289).

1.4.4 Ligand-independent activation of PR

O'Malley's group published the pioneering study on the ligand-independent activation of the nuclear receptor family, where cellular phosphorylation pathways were first discovered to activate PR in the absence of a ligand (291). Next, they reported that dopamine activated PR in both cultured cells and living animals by acting on its own D1 membrane receptor (292, 293). Later studies have demonstrated that several growth factors and kinases phosphorylate specific serine sites and activate the PR, a process with physiological significance. For instance, in neuroendocrine cells, the ligand-independent activation of PR in response to dopamine is found to mediate sexual behavior in rodents (272, 293). In breast cancer cells EGF strongly activates MAPK, thus inducing PR phosphorylation at Ser^{294} and its rapid nuclear accumulation, and mutation of Ser^{294} to Ala (S294A) abolishes EGF-mediated

translocation (294). In addition, heregulin, an EGF family member, also stimulates the MAPK signaling pathway and induces PR phosphorylation at Ser^{294}, nuclear translocation, DNA binding, and transcriptional activity in a ligand-independent manner in T47D breast cancer cells (285, 294). Casein kinase II and MAPK phosphorylate PR at Ser^{81} (295) and Ser^{294} (284, 296), respectively. Cyclin A/CDK2 complexes phosphorylate eight of the 14 sites *in vitro*; these include serines 25, 162, 190, 213, 400, 554, 676, and Thr^{430} (286, 289, 297), although only 5 of them including serines 162, 190, 213, 400 and 676, have been confirmed to be phosphorylated *in vivo* (286, 289, 295, 297). CDK2 is also shown to enhance the translocation of phosphorylated PR at Ser^{400} to the nucleus. Overexpression of CDK2 increases PR transcriptional activity with or without treatment with progestin. The ligand-independent transactivation of PR is specifically blocked by the mutation of Ser^{400} to alanine (S400A) (286), suggesting that CDK2 regulates unliganded PR by stimulating Ser^{400} phosphorylation.

In addition to the ligand-independent transcriptional activity of PR by growth factors, the cross-talk between GnRH-I and PR, which is believed to play an imperative role in the GnRH-I self-priming effect, has been reported in rodent pituitary cells. The self-priming effect of GnRH-I is one of the fundamental pathways involved in the GnRH-I-induced release of gonadotropin; this effect is defined as increased gonadotropin secretion from gonadotrophs in response to a second stimulation by GnRH-I (298, 299). This pathway markedly potentiates the pituitary responsiveness to GnRH-I. Numerous animal studies have suggested that such amplifying effects of serial GnRH-I pulses are crucial to the genesis of the preovulatory LH surge at the mid-point of the menstrual cycle. Interestingly, the administration of a pulse of GnRH-I to primary rat pituitary cells cultured with E_2 potentiates the LH secretion in response to subsequent GnRH-I pulses, and this is blocked by a PR

antagonist, RU488. Similarly, forskolin increases the response to a pulse of GnRH-I in rat pituitary cells; this is also reduced by RU488 in the absence of P4 (250). Furthermore, P4, GnRH-I, or 8-bromo-CAMP induce CAT activity in the pituitary cells transfected with the PRE-Elb-CAT plasmid. CAT activity is blocked by RU488, suggesting that a GnRH-I-triggered signaling cascade transactivates PR in a ligand-independent manner (300). The GnRH-I self-potentiation is shown to depend on PR in experiments using *Pr* knockout mice that lack either isoform *a* or *b* of the *Pr*. Wild-type mice exhibit a robust GnRH-I self-priming effect. In contrast, *Pr* knockout mice receiving two GnRH-I pulses present no additional increase in plasma LH levels, suggesting that the activation of PR is essential for the existence of the GnRH-I self-priming effect (13). Pituitary cells from ovariectomized wild-type or *Pr* knockout mice challenged with hourly pulses of GnRH-I achieve similar results. The cells from *Pr* knockout mice exhibit a blunted GnRH-I self-priming response (301).

Data from our laboratory have provided further evidence of the cross-talk between GnRH and PR in mouse pituitary cells. More specifically, GnRH-I and GnRH-II activate a PRE-luciferase reporter gene in a ligand-independent manner through the PKC and PKA signaling pathways; the effect of GnRH-I is more profound than that of GnRH-II. GnRH-I and GnRH-II also phosphorylate PR at Ser^{294} and induce PR translocation to the nucleus. Furthermore, interactions between PR and SRC-3 increase after GnRH treatment. Most importantly, GnRH-I and GnRH-II induce the assembly of PR and SRC-3 to the PREs of the luciferase reporter gene, as well as the *Gsuα* subunit gene promoter. These effects rely on GnRHR since knockdown of GnRHR using siRNA reduces activation of PR by GnRH (21). More recently, our laboratory has shown that GnRH-I increases *Fshb* through the ligand-independent activation of PR in LβT2 cells. GnRH-I stimulates PRE-luciferase

reporter gene activity and *Fshb* mRNA levels; GnRH-I is more effective than GnRH-II. While this is attenuated by PKA and PKC inhibitors, PR phosphorylation at Ser^{249} is only blocked by inhibition of PKC. In addition, treatment with GnRH-I increases the interaction between PR and SRC-3; this interaction is believed to be imperative for the induction of *Fshb* by GnRH-I because transfection of SRC-3 siRNA markedly reduces the GnRH-I effect. Importantly, knocking down PR by using siRNA significantly reduces the GnRH-I activation of a PRE-luciferase reporter gene and *Fshb* mRNA levels. ChIP assays also demonstrate that GnRH-I induces binding of PR to the PRE within the promoter of *Fshb* (302). From these studies it is concluded that the effects of GnRH on *Gsuα* and *Fshb* gene expression depend, at least in part, on the transactivation of PR in a ligand-independent manner. This is mediated by PR phosphorylation, nuclear translocation and loading of PR and SRC-3 at the PRE within the promoters of *Gsuα* and *Fshb*.

Throughout the human menstrual cycle and mouse estrous cycle, the gonadotropins are coordinately and differentially regulated in essentially the same way. The physiological importance of induction of *Gsuα* by GnRH through the cross-talk with PR is proposed as the normal mechanism of the preovulatory LH surge (21, 299). The routes for GnRH-I self priming require the PR and serve as functional pathway to ensure the LH surge. Apart from this, GnRH-I is suggested to activate the PR in the absence of P4 and to promote the accumulation of *Fshb* gene expression in LβT2 cells. This contributes to the differential regulation of gondotropin gene expression during the luteal-follicular transition and leads to the selection of dominant follicles. There are still several issues that remain uncertain in relation to the ligand-independent activation of PR by GnRH, and its effects on *Gsuα* gene expression. Although GnRH-I treatment increases the assembly of PR and SRC-3 to the PRE in the promoter of *Gsuα*, and promotes GSU protein levels (21), direct evidence for the

GnRH-I dependent induction of the *Gsuα* gene through PR is lacking. Furthermore, the induction of *Gsuα* gene expression by GnRH-I involving the transactivation of PR was only previously studied in αT3-1 cells. Further research is needed to demonstrate that the induction of *Gsuα* gene expression is mediated by the cross-talk between GnRH and PR also occurs in other pituitary cell lines, such as LβT2 cells, which are more representative of mature pituitary gonadotrophs.

1.5 AP-1

The AP-1 proteins are basic leucine zipper transcription factors which comprise c-Jun, JunB, JunD, c-Fos, Fosb, Fra-1 and Fra-2; the members of this family are early response genes which can be stimulated by several extracellular subjects compassing UV irradiation, oxidative stress, steroid hormones, as well as growth factors (118, 303). AP-1 proteins attach to particular promoter areas, otherwise known as 12-O-tetradecanoylphorbol 13-acetate response elements, to regulate several genes expression which participate in cell growth, differentiation and metastasis (304). Increased AP-1 levels often result in the amplification of target gene expression. AP-1 activity can be regulated at several levels, for instance, AP-1 dimer formation, transcription and posttranslation issues, as well as communication with accessory proteins (305). In terms of the structure, AP-1 members are similar to other transcription factors which possess transactivation domains, DNA binding domains, and leucine zippers which form dimerization areas to allow the creation of effective transcriptional items (303). The Jun family members generate dimers among themselves to be active in transcription. However, the Fos family members do not form stable dimers but bind to the Jun members to form heterodimers.

Although all types of Fos-Jun and Jun-Jun dimers bind the consensus AP-1 binding site in the promoters of target genes, functional assays have revealed some discrepancies in their binding ability and stability to unique AP-1 sites, as well as transcriptional activation. Typically, the binding of heterodimers of Fos-Jun to DNA is more stable than homodimers (306). In addition, c-Jun dimers are the strongest with respect to target gene expression as compared to the JunB or JunD homodimers (307). Heterodimers consisting of Fosb are more stable than those composed of Fra-1 or c-Fos binding to DNA (308). Due to differences in c-Jun to JunB ratios, AP-1 dimers stimulate either cellular growth or differentiation in keratinocytes (309) and serve as activators or inhibitors of cell death in erythroid cells (310). The essential function of AP-1 in malignancy has been broadly studied. It has been found that c-Jun and c-Fos are involved in oncogenic transformation (311, 312). Increase of Fra-1 at least in part involves in the proliferation of estrogen-independent breast cancer cells (313). The regulation of AP-1 activity by retinoic acid partially mediates anti-carcinogenesis in AP-1-luciferase transgenic mouse (314).

Experimentally manipulated deficiencies in individual AP-1 proteins in mice or cultured cells provide a more precise technique to ascertain the physiological roles. Fibroblasts obtained from lacking both *c-fos* and *Fosb* mice display diminished cell growth. Mice with both *c-fos* and *Fosb* deletions are approximately one third smaller than their wild-type siblings or the corresponding single mutants (315). Unlike a deficiency in one of the Fos members, deletion of one individual Jun protein in fibroblasts results in considerable alteration of cell growth. The c-jun mutation induces a dramatic increase in cell cycle transit time, the most rigorous flaws which cause fibroblasts only have one or two cell division rounds before they display a growth arrest phenotype in culture (120, 316, 317).

The importance of AP-1 in the pituitary has been confirmed by several studies.

Administration of GnRH-I stimulates transcription of the early response genes, including Fosb, c-Fos, and c-Jun either in cultured cells or in hypogonadotropic animals (318-321). Furthermore, the induction of the *Fshb* gene by GnRH-I involves the transcription factor AP-1. In αT3-1 and HeLa cells transfected with mouse *Gnrhr*, GnRH-I increases the AP-1 binding activity. Mutation of putative AP-1 sites in the *Fshb* promoter reduces GnRH-I induction of *Fshb* in heterologous HeLa cells (322). In the ovine FSHβ promoter, purified c-Jun protein binds to putative AP-1 sites (323). In mouse LβT2 gonadotroph cells, regulation of the mouse *Fshb* promoter by GnRH-I is mediated, at least in part, by the induction of multiple AP-1 subtypes. These subtypes integrate to a site consisting of a half-site of the AP-1 consensus binding sequence that overlaps the element binding to the basal transcription factor NF-Y. GnRH-I stimulates the interaction of NF-Y and AP-1, as well as the co-occupation to this site *in vivo* (324). In LβT2 cells transfected with a human *FSHB* promoter reporter construct, GnRH-I stimulates *FSHB* promoter activity in a concentration- and time-dependent manner, *via* the ERK1/2 and p38 signaling pathways. GnRH-I also induces the synthesis of AP-1 proteins, including Fosb, c-Fos, JunB, and c-Jun, as well as AP-1 complex formation. AP-1 binds to a conserved *cis*-element in the transcriptional initiation site of the *FSHB* promoter. There is also the other site that localizes more proximally with lower affinity. Mutations of these *cis*-elements reduce the GnRH-I-stimulated *FSHB* promoter activity; undoubtedly, the interruption of the site with higher affinity is more effective. A dominant-negative Fos protein restrains the GnRH-I-stimulated *FSHB* transcriptional activity in a concentration-dependent manner, confirming the central role of endogenous AP-1 proteins (325).

The involvement of AP-1 in the induction of *Gnrhr* by GnRH-I and glucocorticoids has been recognized. GnRH-I induces *Gnrhr* mRNA levels in primary rat pituitary cells (139). In

the αT3-1 and LβT2 cell lines, GnRH-I stimulates endogenous *Gnrhr* mRNA levels and a mouse *Gnrhr*-luciferase promoter activity (123, 326-328). An AP-1-binding site is the critical promoter element in the regulation of the mouse *Gnrhr* by GnRH-I in αT3-1 cells (326). In addition to GnRH-I, glucocorticoids have also been shown to induce *Gnrhr* gene expression in GnRH-deficient rodents pituitary cells (329). In LβT2 cells, dexamethasone alone upregulates both the expression of an endogenous *Gnrhr* gene and that of a transfected mouse *Gnrhr* promoter-reporter construct (327, 330). These results are further confirmed in the somatolactotrope GGH3 cell line, where glucocorticoids strengthens the mouse *Gnrhr* promoter activity (143). Importantly, an AP-1 site in the mouse *Gnrhr* promoter is required for this up-regulation by glucocorticoids, indicating that the GR combines with AP-1 proteins to increase transcription of the mouse *Gnrhr* gene (331, 332). A more recent study has indicated a rapid nongenomic and genomic cross-talk machinery between the GnRNR and GR signaling pathways in LβT2 cells. GnRH-I and dexamethasone increase both the mouse *Gnrhr* promoter activity and endogenous *Gnrhr* gene expression, which requires GR. ChIP and immunofluorescence analyses indicate that both GnRH-I and dexamethasone enhance the mouse *Gnrhr* gene through nuclear localization and connection of the GR with the AP-1 binding site on the mouse *Gnrhr* promoter. Furthermore, GnRH and dexamethasone synergistically activate the endogenous *Gnrhr* promoter *via* a mechanism involving the recruitment of SRC-1 to the AP-1 region in the promoter of *Gnrhr* (333). These studies provide evidence that AP-1 proteins are necessary for *Gnrhr* gene expression and that this involves protein-protein interactions between AP-1 and GR.

Taken together, the available data suggest that AP-1 plays important roles in the pituitary. GnRH-I rapidly induces the expression of several AP-1 family genes, including mouse *Fosb* (318), which contains an ERE in its promoter (334). As indicated previously, GnRH-I also

increases the activity of a ERE-luciferase reporter gene in mouse pituitary αT3-1 cells (20). It is unclear whether GnRH-I stimulates *Fosb via* the ligand-independent activation of ERα, and contributes to *Fshb* expression. Intriguingly, other than the GR (333), GnRH-I and GnRH-II activate PR in a ligand-independent manner (21, 302). GnRH-I also increases *Gnrhr* gene expression and a mouse *Gnrhr*-luciferase reporter gene activity (333). As confirmed by previous studies and using the bioinformatic analysis tool, Genomatix (http://www.genomatix.de/en/index.html), the mouse *Gnrhr* is composed of a PRE and an AP-1 binding site in the promoter (326, 333). But whether induction of *Gnrhr* by GnRH is mediated by ligand-independent activation of PR and ER in the pituitary remains unclear.

1.6 Hypothesis and objectives

This project sets out to address the hypothesis that ligand-independent activation of ERα and PR by GnRH involves in target genes expression in mouse gonadotrophs. In this project, we have re-examined the cross-talk between ERα/PR and the GnRH-mediated signaling pathways that regulate the activation and expression of specific genes in mouse LβT2 and/or αT3-1 pituitary cells.

Objective 1. In Chapter 2, I tested the hypothesis that GnRH-mediated phosphorylation of the ERα contributes to *Fosb* expression in mouse gonadotrophs in 5 sets of experiments:

1) The activation of ERα by GnRH-I and GnRH-II in LβT2 cells was investigated.

2) The requirement of GnRHR for GnRH-mediated ERE-luciferase was examined.

3) The phosphorylation of ERα and promotion of ERα interactions with its coactivators by GnRH-I and GnRH-II were evaluated.

4) The co-recruitment of ERα and PCAF to the *Fosb* promoter ERE after GnRH treatments was verified.

5) Whether GnRH treatments increased *Fshb* expression was measured.

Objective 2. In Chapter 3, I investigated whether the GnRH-I-mediated activation of PR contributes to *Gsuα* expression in mouse gonadotrophs in 4 sets of experiments:

1) The activation of PR by GnRH-I in αT3-1 and LβT2 cells was tested.

2) The stimulation of *Gsuα* gene expression by GnRH-I in αT3-1 and LβT2 cells was evaluated.

3) The involvement of PKC during the activation of PR and its effects on *Gsuα* expression was elucidated.

4) The induction of *Gsuα* gene expression by GnRH-I requires PR was examined.

Objective 3. In Chapter 4, I inspected the cross-talk between GnRH and PR in the induction of *Gnrhr* in mouse gonadotrophs in 4 sets of experiments:

1) The ability of GnRH-I and GnRH-II to activate a mouse *Gnrhr*-luciferase promoter in LβT2 and αT3-1 cells was measured.

2) The ability of GnRH-I and GnRH-II to activate AP-1-luciferase gene in LβT2 and αT3-1 cells was evaluated.

3) The requirement for the PR in GnRH-I-induced mouse *Gnrhr* promoter activity was tested.

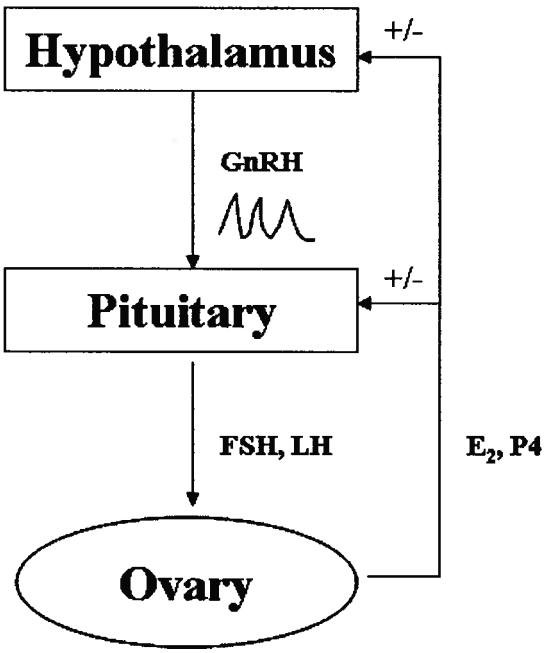


Figure 1. 1 Hypothalamic-pituitary-gonadal axis.

E_2, estradiol; FSH, follicle stumulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; P4, progesterone; +, positive feedback; -, negative feedback; ∿∿, pulsatile secretion.

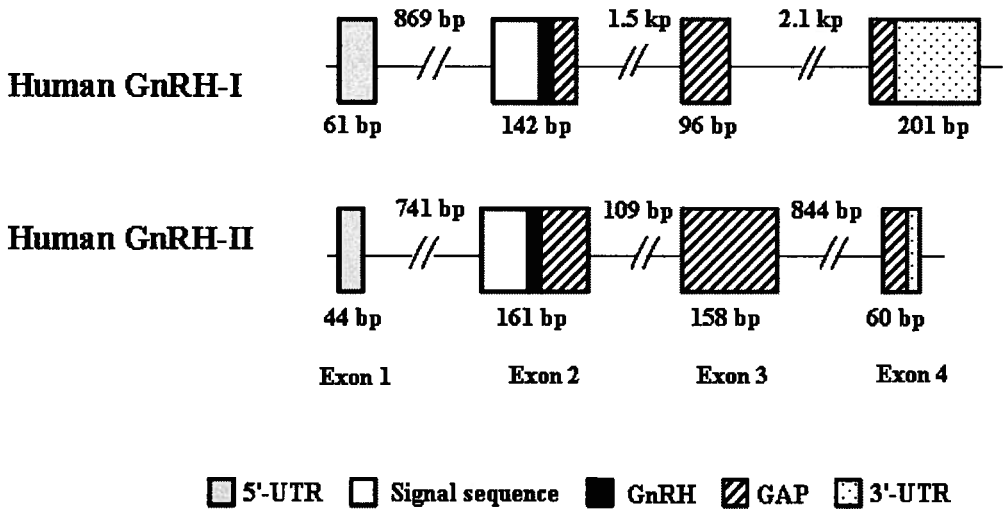


Figure 1. 2 Genomic structures of human GnRH-I and GnRH-II genes.

Two forms of GnRH, termed GnRH-I and GnRH-II, have been identified in humans. Their genes consist of 4 exons (boxes) interrupted by 3 introns (thin lines). The encoded preprohormones contain a signal sequence, a GnRH decapeptide, a conserved GKR cleavage site, and subsequently a GAP. bp, base pairs; GAP, gonadotropin-releasing hormone-associated peptide; GnRH, gonadotropin-releasing hormone; kp, kilo base pairs; UTR, untranslated regions. Modified from Cheng CK and Leung PC, 2005 (34).

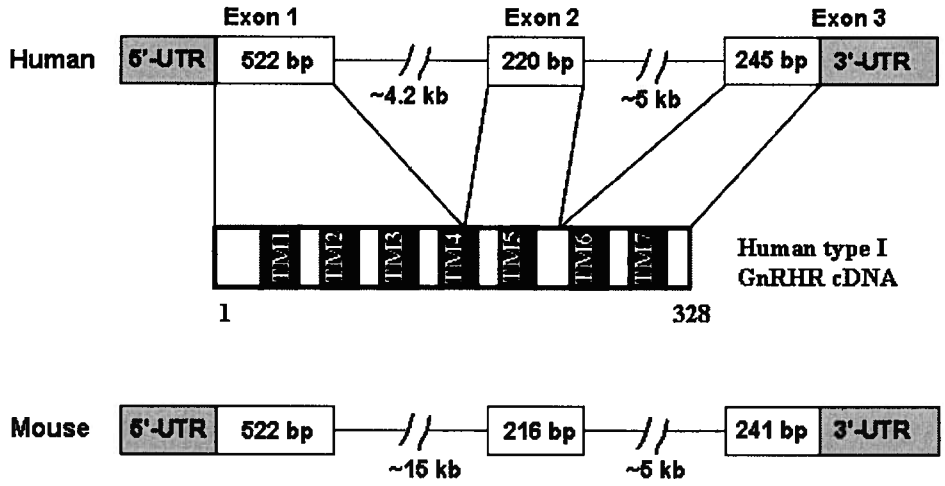


Figure 1. 3 Genomic structures of human and mouse GnRHR type I genes.

In human and mouse the genes coding for the type I GnRH receptor are composed of three exons separated by two introns. In human, exon 1 consists of the 5′-UTR and encodes the first 3 TM domains and a part of the fourth TM domain. Exon 2 codes the remainder of the fourth TM domain, the fifth TM domain, and part of the third intracellular loop. Exon 3 consists of the rest of the open reading frame and contains the 3′-UTR. bp, base pairs; GnRHR, GnRH receptor; kb, kilo base pairs; TM, transmembrane helices; UTR, untranslated regions. Modified from Cheng CK and Leung PC, 2005; and Hapgood JP et al, 2005 (34, 78).

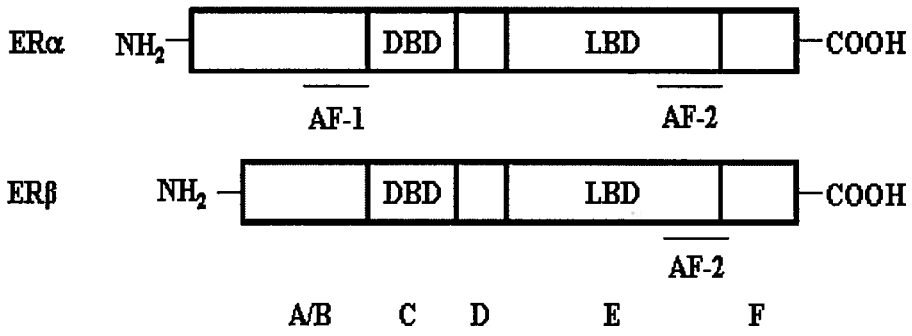


Figure 1. 4 Structural organization of human ERα and ERβ.

Both ER subtypes share a highly conserved DBD and moderately conserved LDB. The ligand-dependent transcriptional activities are mediated through AF-2 in both subtypes. ERα contains a constitutive AF-1 in the N terminus. AF, transactivation function; DBD, DNA binding domain; ER, estrogen receptor; LBD, ligand binding domain. Modified from Hall JM and McDonnell DP, 2005 (165).

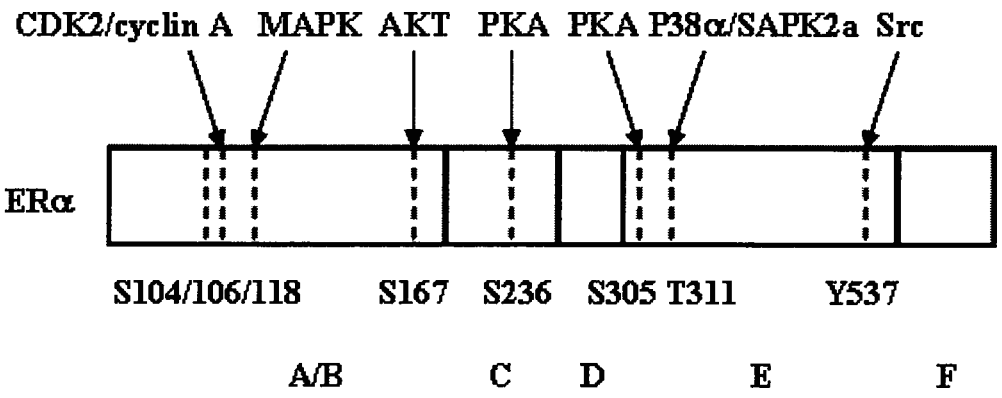


Figure 1. 5 Schematic representation of ER phosphorylation sites.

Eight phosphorylation sites have been identified within the human ERα. Four of these sites (Ser^{104}, Ser^{106}, Ser^{118}, and Ser^{167}) are located in the A/B domain, one is in C domain (Ser^{236}), and the other three sites (Ser^{305}, $threonine^{311}$ and $tyrosine^{537}$) are found within the E domain. CDK, cyclin-dependent kinase; ER, estrogen receptor; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; S104/106/118, Ser^{104}, Ser^{106} and Ser^{118}; S167, Ser^{167}; S236, Ser^{236}; S305, Ser^{305}; SAPK, stress-activated protein kinase; T311, $threonine^{311}$; T537, $tyrosine^{537}$. Modified from Al-Dhaheri MH and Rowan BG, 2006 (196).

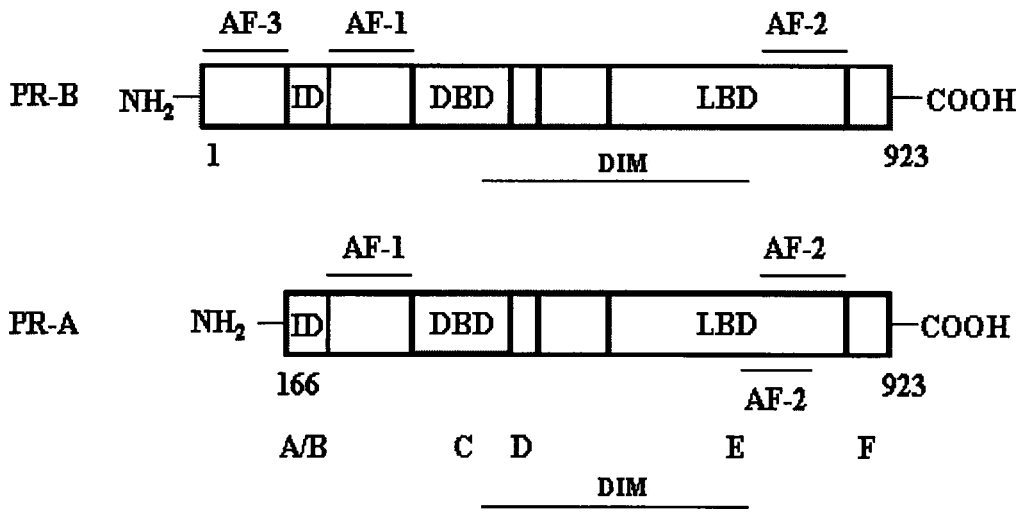
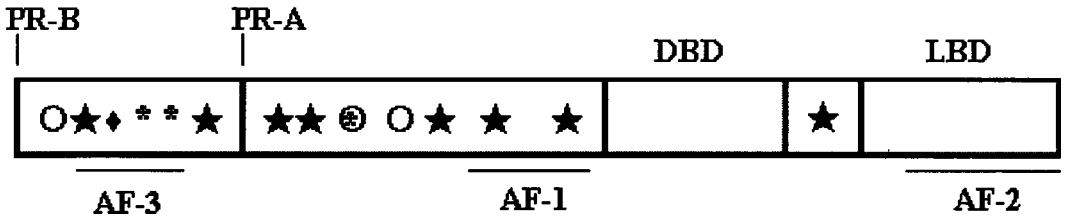


Figure 1. 6 Structural organization of the human PR-A and PR-B isoforms.

Numbers stand for the amino acid position in each protein. AF, transactivation function; DBD, DNA binding domain; DIM, sequences important for receptor dimerization; ID, inhibitory domain; LBD, ligand binding domain; PR, progesterone receptor. Modified from Mulac-Jericevic B and Conneely OM, 2004 (254).



O MAPK consensus sites (20, 294, 345)
♦ Casein Kinase II site (81)
★ CDK2 sites (25, 162, 190, 213, 400, 430, 554, 676)
* Unknown kinases (102, 130, 294)
Hormone-dependent sites: 102, 294, 345, 400
Basal sites: 81, 162, 190, 400

Figure 1. 7 Schematic representation of human PR phosphorylation sites.

There are totally 13 serine residues and 1 threonine residue in human PR which represent basal and ligand-induced phosphorylation sites. MAPK, casein kinase II, and CDK2 can also phosphorylate PR. AF, transactivation function; CDK, cyclin-dependent kinase; DBD, DNA binding domain; LBD, ligand binding domain; MAPK, mitogen-activated protein kinase; PR, progesterone receptor. Modified from Lange CA, 2004 and Lange CA, 2008 (287, 288)

Chapter 2 GnRH-mediated phosphorylation of estrogen receptor α contributes to *Fosb* expression in mouse gonadotrophs[1]

2.1 Introduction

In the pituitary, numerous hormones and signaling cascades intersect to control the reproductive system. Critically important in this regard is GnRH-I which is released into the hypophyseal portal system in a pulsatile manner to stimulate the biosynthesis and secretion of FSH and LH (30, 54, 335, 336). A second GnRH subtype, GnRH-II, displays a different spatial pattern of expression and has specific functions in other reproductive tissues, such as the placenta and ovary (34, 337). The mammalian genome also contains two distinct GnRH receptor genes (type I and type II GnRHR), but type II GnRHR has never been found to be expressed in the mouse or human, and GnRH-I and GnRH-II function through the type I GnRHR in these species (81). After binding to its receptor, GnRH-I activates the PKA, PKC, PI3K and MAPK signaling pathways, which are all indirectly involved in regulating gonadotropin subunit genes (110-114, 116, 338).

The pulsatile binding of GnRH-I to the type I GnRHR on pituitary gonadotrophs also induces the expression of immediate early response genes, including AP-1 which comprises either Jun/Jun homodimers or Jun/Fos heterodimers. In mouse LβT2 pituitary cells, GnRH-I stimulates the production of AP-1 components via the MAPK signaling pathway (324, 325, 339), and subsequently up-regulates *Fshb* promoter activity (322, 325).

Feedback regulation of pituitary gonadotropin production by estrogen is also essential for controlling reproductive cycles. E_2 is the most important physiological ligand of the ERα,

[1] A version of this chapter has been published. **Chen J, An BS, Cheng L, Hammond GL, Leung PC** 2009 Gonadotropin-releasing hormone-mediated phosphorylation of estrogen receptor-alpha contributes to fosB expression in mouse gonadotrophs. Endocrinology 150:4583-4593

and it promotes ERα dimerization and binding to EREs that are often located in the promoters of estrogen sensitive genes (176, 340). When ERα binds to an ERE (178) it recruits co-regulatory proteins that influence chromatin remodeling and/or provide a bridge to other transcription factors that mediate assembly of the transcriptional machinery (182). Several ERα co-activators have been identified, including members of the P160 or SRC family, P300, CBP proteins, and PCAF (178, 182). In addition to this classical genomic mechanism of ligand-induced activation of ERα, its transcriptional activity can be influenced by signaling pathways that alter its phosphorylation status (20, 197, 198, 201, 226). These signaling pathways are normally triggered by growth factors and peptide hormones, such as GnRH-I, and they work in concert with E_2 to influence the timing and/or magnitude of the ERα-mediated effects on gene expression, as observed in mouse αT3-1 pituitary cells (20). However, since the latter immortalized mouse gonadotrophs are considered developmentally immature and do not express the gondotropin β subunit genes (341), we have re-examined the cross-talk between E_2 and GnRH-mediated signaling pathways that regulate ERα activation of gene expression in mouse LβT2 pituitary cells, because they express both *Fshb* and *Lhb* subunit genes (342, 343). In particular, our experiments set out to define the molecular mechanisms that mediate the rapid ERα-dependent responses to GnRH in these pituitary cells.

2.2 Materials and methods

2.2.1 Cells and cell culture

The mouse gonadotroph-derived LβT2 (342) cell line was generously provided by Dr.

P.L. Melon (Department of Reproductive Medicine, University of California, San Diego, CA) and maintained in monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 0.1 mg/ml streptomycin (Invitrogen, Burlington, ON) in humidified 5% CO_2, 95% air at 37 C. The cells were passaged when they reached about 90% confluence using a trypsin/EDTA solution (0.05% trypsin, 0.5 mM EDTA), and kept in phenol-red free medium and charcoal-treated FBS for 4 days before experiments.

2.2.2 Plasmid and ERE-luciferase reporter gene assays

The pERE-tk-Luc reporter plasmid containing two copies of a consensus ERE was kindly provided by Dr. J. Larry Jameson (Northwestern University Medical Schol, Chicago, IL, USA), and prepared for transfections using Qiagen Plasmid Maxi Kits (Qiagen, Mississauga, ON). Transient transfections of the pERE-tk-Luc were performed using FuGENE 6.0 (Roche Diagnostics, Quebec, QC) together with a Rous sarcoma virus (RSV)-lacZ plasmid to correct for transfection efficiencies. One day before transfection, 12×10^5 LβT2 cells were seeded in six-well plates. One microgram of pERE-tk-Luc and 0.5μg of RSV-lacZ were dissolved in 100μl culture medium containing 3μl of FuGENE 6.0 without serum. The DNA mixture was incubated for 25 min at room temperature and then applied to the cells. After set times of culture (1 - 48 h), cellular lysates were obtained after addition of 150μl reporter lysis buffer (Promega Corp., San Luis Obispo, CA), and assayed for luciferase activity using a Lumat LB 9507 luminometer (EG&G, Berthold, Germany). β-Galactosidase activity was also measured using the β-Galactosidase Enzyme Assay System (Promega Corp.) to normalize for transfection efficiencies. Promoter activity was calculated as luciferase/β-galactosidase activity. To knock-down the cell levels of specific proteins,

indicated amounts of siRNAs (ERα, GnRHR or PCAF from Qiagen) were co-transfected together with pERE-tk-Luc and RSV-lacZ using FuGENE 6.0.

2.2.3 Nuclear extraction, immunoblotting and immunoprecipitation

Cells seeded in 10cm dishes were washed with cold PBS and harvested with 1 ml 10 mM Hepes, pH 7.9, 10 mM KCl, 10 mM EDTA, 1 mM dithiothreitol, 40 µl/ml 10% IGEPAL (Nonidet P40 Substitute), and 10 µl/ml protein inhibitor cocktail. Cell lysates were placed in an orbital rocker for 15 min at 4 C. After centrifugation (14,000 g at 4 C for 5 min), the supernatant cytoplasmic protein was collected. Nuclear pellets were obtained and re-suspended in 20 mM Hepes, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 50% glycerol, 1 mM dithiothreitol, and 10 µl/ml protein inhibitor cocktail (Sigma, St. Louis, MO); mixed in an orbital rocker for 2 h at 4 C, and then centrifuged (14,000 g at 4 C for 5 min) to obtain nuclear protein extracts.

The protein content was determined using a Bradford assay (Bio-Rad Laboratories, Mississauga, ON), and 40 µl aliquots were resolved by 10% SDS-PAGE and electrotransferred to a Hybond-C membrane (Amersham Biosciences, Morgan, ON). After blocking, the membranes were incubated (overnight 4 C) with specific antibodies against: phospho-ERα (Ser^{118}), phosphor-ERα (Ser^{167}) (Cell Signaling Technology, Inc., Pickering, ON), ERα, Fosb, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), or type I GnRHR (Lab Vision Corporation, Montreal, QC). Horseradish peroxidase-conjugated secondary antibodies were then incubated with the membranes. After washing, immunoblots were examined using the ECL chemiluminescent system (Amersham Pharmacia Biotech, Piscataway, NJ) followed by exposure to Kodak X-Omat film.

Nuclear protein extracts were incubated with ERα antibody (10 ug/ml), followed by

addition of the antibody capture affinity ligand included in an immunoprecipitation kit (Upstate, Lake Placid, NY) for 1 h at room temperature. The immunoprecipitated proteins were then subjected to 8% SDS-PAGE and western blotting using appropriate antibodies (SRC-1, catalogue number 05-522, glucocorticoid receptor-interacting protein-1 (SRC-2), catalogue number 06-986, activator of thyroid and retinoic acid receptor/ amplified in breast cancer 1 (SRC-3), catalogue number 05-490: Upstate; P300, catalogue number sc-585, CBP, catalogue number sc-1211, PCAF, catalogue number sc-8999: Santa Cruz Biotechnology). After incubation with secondary antibodies, the immunoreactive proteins on western blots were detected, as described above.

2.2.4 Real-time RT-PCR

Total RNA was extracted from cell cultures using Trizol (Invitrogen, Burlington, Canada). The RNA concentration was measured based on absorbance at 260 nm. The isolated RNA was reverse transcribed into first-strand cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega BioSciences, San Luis Obispo, CA, USA). The primers used for SYBR Green real-time RT-PCR were designed using Primer Express Software v2.0 (PerkinElmer Applied Biosystems, Foster City, CA), and were as follows: *Egr-1* mRNA (sense, 5′-GAGCGAACAACCCTATGAGC and antisense, 5′-AGGCCACTGACTAGGCTGAA); *Fosb* mRNA (sense, 5′-GAGGGAGCTGACAGATCGAC and antisense, 5′-TTCCTTAGCGGATGTTGACC); Annexin A5 mRNA (sense, 5′-GAAGCCCTCACGACTCTACG and antisense, 5′-TATCCCCCACCACATCATCT); *Lhb* mRNA (sense, 5′-GGCCGCAGAGAATGAGTTCT and antisense, 5′-CTCGGACCATGCTAGGACAGTAG); *Fshb* mRNA (sense,

5′-CCCAGCTCGGCCCAATA and antisense, 5′-GCAATCTTACGGTCTCGTATACCA); and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA (sense, 5′-CATGGCCTTCCGTGTTCCTA and antisense, 5′-GCGGCACGTCAGATCCA). Real-time PCR was performed using the ABI prism 7000 Sequence Detection System (PerkinElmer Applied Biosystems) equipped with a 96-well optical reaction plate. The reactions were set up in a 20 μl reaction mixture containing 10 μl SYBR Green PCR Master Mix (PerkinElmer Applied Biosystems), 4 μl of cDNA template and 6 μl of primer mixture (2 μM). Real time PCR conditions were 50 C for 2 min, 95 C for 10 min, followed by 40 cycles of 95 C for 15 sec and 60 C for 1 min. All experiments were run in duplicate, and mRNA levels were normalized against the amount of *Gapdh* mRNA.

2.2.5 Chromatin immunoprecipitation (ChIP)

All reagents, buffers and supplies were provided in a ChIP-IT™ kit (Active Motif, Inc., Carlsbad, CA) and described previously (344). Briefly, the LβT2 cells were cross-linked with 1% formaldehyde for 10 min at room temperature. The cells were treated with glycine Stop-Fix solution, re-suspended in lysis buffer, and incubated on ice for 30 min. Then the cells were homogenized and nuclei were re-suspended in shearing buffer, and applied to ultrasonic disruption situations optimized before the experiments to generate 100-400 bp DNA fragments. The chromatin was pre-cleared with Protein G beads and 1 μg of the following antibodies were added: negative control IgG, anti-ERα and anti-PCAF. Protein G beads were then supplied to the antibody/chromatin incubation mixtures. After several times of washing, elution buffer was used to remove antibody bound protein/DNA complexes from the beads. The samples then were incubated with NaCl and RNase at 65 C for 4 h to reverse cross-links and remove RNA. The samples were treated with proteinase K for 2 h at 42 C and

the DNA was purified using gel exclusion columns. The purified DNA was subjected to PCR amplification (1 cycle of 94 C for 3 min; 40 cycles of 94 C for 20 sec; 64 C for 30 sec and 72 C for 30 sec) of the *Fosb* promoter region that contains a known ERE (334) using forward (5′-AGGAGGCCCCTGGATTACATC) and reverse (5′-GTACCACCTTTGGCCTGGAA) primers. As an input control, 10% of each chromatin preparation was used in a parallel PCR reaction. The PCR products were resolved by 2% agarose gel electrophoresis and visualized after ethidium bromide staining.

2.2.6 Data analysis

For transfection assays, data are shown as the mean ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA and the GraphPad Prism 4 statistical software (GraphPad Software, Inc., San Diego, CA), and $p<0.05$ was considered statistically significant.

2.3 Results

2.3.1 GnRH-I and GnRH-II rapidly and transiently activate ERα in LβT2 cells

When LβT2 cells containing an ERE-luciferase reporter plasmid were treated with 100 nM GnRH-I or GnRH-II, we observed rapid but transient increases in luciferase activity which peaked at 12 h, with GnRH-I being more potent than GnRH-II (Figure 2.1A). In contrast to the robust 12 and 7 fold responses obtained after 12 h treatment with GnRH-I and GnRH-II, respectively, we observed only a 2-3 fold induction of the ERE-luciferase reporter activity after cells were treated with 100 nM E_2, which occurred within 6 h and was

maintained at this level for 48 h (Figure 2.1B). In dose response assays, in which cells were treated with 0.1 nM to 1 μM GnRH-I or GnRH-II, maximum increases in ERE-luciferase reporter activity were seen with 10nM GnRH-I and 100 nM GnRH-II (Figure 2.1C), while the 2 fold response in cells treated with 10 pM E_2 was not increased at higher E_2 concentrations up to 100 nM (data not shown).

Since ERα is the predominant ER subtype in LβT2 cells, we used an siRNA to knock down the amounts of ERα in these cells, and observed about a 50% reduction in ERα levels by western blotting (see upper panel of Figure 2.1D). When these ERα siRNA-treated cells were treated for 6 h with 100 nM GnRH-I or GnRH-II in the absence of E_2, the response of an ERE-luciferase reporter gene was also about half that observed in control cells (Figure 2.1D). Moreover, we observed a significant ($p<0.05$) reduction in the modest increase in ERE-luciferase reporter gene activity in ERα siRNA-treated cells exposed to 100 nM E_2 for 6 h (Figure 2.1D).

Although activation of the ERE-luciferase reporter gene by both GnRH subtypes in LβT2 cells is very much more robust than the response obtained after E_2 treatment, we wished to determine whether co-treatments with E_2 and the GnRH subtypes might act synergistically. The results of this experiment, however, indicate that E_2 does not further enhance the transient responses obtained after treatment with either GnRH subtype over and above that expected after E_2 treatment alone (Figure 2.1E and 2.1F).

2.3.2 GnRHR is required for GnRH-mediated ERE-luciferase activation

To verify that the activation of the ERE-luciferase reporter gene by GnRH-I and GnRH-II is mediated by the GnRHR, LβT2 cells were co-treated with its antagonist, antide. This almost completely blocked the induction of luciferase activity by both GnRH subtypes,

while co-treatment with an ER antagonist, ICI 182,780, did not (Figure 2.2A). By contrast, activation of the ERE-luciferase reporter gene by E_2 was attenuated by ICI 182,780 under the same conditions (Figure 2.2A). To further confirm that the GnRHR is required for GnRH-induced activation of the ERE-luciferase reporter gene, we used an siRNA to knock-down GnRHR levels in LβT2 cells, and checked this by western blotting (upper panel of Figure 2.2B). This siRNA treatment resulted in a > 50% reduction in the ERE-reporter gene activation by both GnRH subtypes, but did not influence the E_2-dependent activation of the reporter gene (Figure 2.2B), thus confirming that the GnRHR specifically mediates the ligand-independent activation of the ERE-luciferase reporter gene by both forms of GnRH in LβT2 cells.

2.3.3 GnRH treatments affect ERα phosphorylation and promote ERα interactions with PCAF

In several different cell lines, treatments with E_2 and agents that stimulate various signal transduction pathways result in the phosphorylation of ERα at Ser^{118} and/or Ser^{167} (197-199, 201, 226). To determine which of these phosphorylation sites are regulated by GnRH in the nucleus and cytoplasm of LβT2 cells, antibodies that recognize ERα at Ser^{118} and Ser^{167} were used in western blotting experiments. This demonstrated that GnRH-I or GnRH-II treatments result in rapid and transient increases in ERα phosphorylation at Ser^{118} in the nucleus (Figure 2.3A and 2.3B), while phosphorylation of ERα at Ser^{118} was not observed after GnRH or E_2 treatments in the cytoplasm (Figure 2.3D and 2.3E). On the other hand, both GnRH subtypes induce phosphorylation of ERα at Ser^{167} in the nucleus and cytoplasm, and were more effective than E_2 (Figure 2.3A, 2.3C, 2.3D and 2.3F). To study the signaling pathways induced by GnRH-I or GnRH-II in terms of the phosphorylation of ERα, cells were

pre-treated with inhibitors of PKA (H89), PKC (GF109203X), PI3K (LY 294002) or MAPK (PD 98059) prior to treatment with the GnRH subtypes. This experiment shows that after treatments with either GnRH subtypes, phosphorylation of ERα at Ser^{118} is reduced by PKC, PI3K or MAPK inhibitors but not by the PKA inhibitor, while all four inhibitors attenuate the phosphorylation of ERα at Ser^{167} (Figure 2.3G).

We then examined whether GnRH treatments promote the association of ERα with specific coactivators in LβT2 cells by immunoprecipitation of ERα complexes with an ERα antibody, and identification of interacting co-activators by western blotting. While we were unable to identify any increase in the co-immunopreciptation of ERα with SRC-1, SRC-2, SRC-3, CBP or P300 after the cells were treated with GnRH subtypes, both GnRH-I and GnRH-II increased interaction of the ERα with PCAF by almost 2 fold (Figure 2.4A).

Since our data showed that both GnRH subtypes stimulate recruitment of PCAF by ERα, a PCAF siRNA was used to further explore whether PCAF is an essential component of the ERα-dependent activation of the ERE-luciferase reporter gene by GnRH subtypes. A western blot demonstrated that the siRNA treatment decreased cellular PCAF levels (upper panel of Figure 2.4B), and resulted in significant reductions in reporter gene activation by GnRH-I or GnRH-II (Figure 2.4B).

2.3.4 GnRH treatments promote the co-recruitment of ERα and PCAF to the *Fosb* promoter ERE

It is known that GnRH treatments induce the expression of several genes that contain EREs in their promoters, including the early growth response gene 1 (*Egr-1*), *Fosb,* annexin A5, and *Lhb* genes (318, 334, 345). When we examined the expression of these genes by real-time RT-PCR after GnRH treatments, we observed rapid (within 30 min) and transient

(lost by 6 h) increases in the expression of *Fosb* (Figure 2.5A upper panel) and *egr-1* (not shown). By contrast, the annexin A5 mRNA level increased progressively until 12 h, after which it decreased rapidly, and there was no increase in *Lhb* mRNA at all (not shown). In addition, GnRH treatments increased Fosb protein levels within 3-6 h (Figure 2.5A). When the cells were co-treated with E_2 and the GnRH subtypes, E_2 does not further influence the transient responses of *Fosb* mRNA levels obtained after treatment with either GnRH subtype (Figure 2.5B). When LβT2 cells were pre-treated as above with an ERα siRNA, this again reduced ERα levels (as seen in Figure 2.1D upper panel) and attenuated the stimulation of *Fosb* expression after GnRH treatments (Figure 2.5C), while there was no effect on Egr-1 and annexin A5 mRNA levels (not shown). When the cells were pre-treated with an PCAF siRNA which reduced PCAF levels (as seen in Figure 2.4B upper panel), this also attenuated the stimulation of *Fosb* expression after GnRH treatments (Figure 2.5C). To further define the signaling pathways involved in the induction of *Fosb* by GnRH-I or GnRH-II, cells were pre-treated with inhibitors of PKA, PKC, PI3K or MAPK prior to treatment with the GnRH subtypes, and we then compared the fold inductions of *Fosb* mRNA and Fosb protein levels. The results show that only PKA and PKC inhibitors significantly attenuate the increases in *Fosb* mRNA and Fosb protein levels in response to GnRH-I or GnRH-II (Figure 2.5D).

We next used a ChIP assay to determine whether the GnRH-mediated induction of endogenous *Fosb* gene expression requires the assembly of ERα and PCAF at the ERE within its promoter region (334). This indicated that both GnRH-I and GnRH-II treatments cause rapid (within 1 h) recruitment of ERα to the *Fosb* promoter ERE, and this occurred in concert with the recruitment of PCAF at the same site within 1 h after GnRH-I treatment and by 3 h after GnRH-II treatment (Figure 2.5E).

2.3.5 GnRH treatments increase *Fshb* expression

The most important physiological function of gonadotrophs is to synthesize gonadotropin under the regulation of GnRH. An AP-1 half-site within the *Fshb* promoter binds AP-1 after GnRH-I treatment and is required for the maximal induction of *Fshb* mRNA levels (324). We therefore examined the changes in *Fshb* mRNA levels in LβT2 cells by real-time RT-PCR after GnRH treatments, and this revealed transient (lost by 24 h) increases in the expression of *Fshb* after both GnRH-I and GnRH-II treatments (Figure 2.6A). To assess which signaling pathways might be involved in the induction of *Fshb* expression by GnRH-I or GnRH-II, cells were pre-treated with inhibitors of PKA, PKC, PI3K or MAPK prior to treatment with the GnRH subtypes, and we then compared the fold inductions of *Fshb* mRNA levels. The results indicate that only PKA and PKC inhibitors significantly attenuate the increases in *Fshb* mRNA levels in response to GnRH-I or GnRH-II (Figure 2.6B).

2.4 Discussion

In gonadotrophs, signaling pathways that mediate the actions of GnRH and steroid hormones converge to regulate the expression of gonadotropin genes. It has been shown in αT3-1 cells that cAMP (5) and GnRH-I (20) both stimulate ERE-containing promoters in an estrogen-independent manner, but these cells do not express the gonadotropin β subunit genes. We have therefore used the mouse pituitary LβT2 cell line, because it expresses both gonadotropin β subunit genes and is considered to be a developmentally mature pituitary cell line (342, 343). Our studies show that ERα is activated by both GnRH subtypes in these cells,

and that GnRH-I is consistently more potent than GnRH-II in this regard. Most importantly, when compared to other previous studies using αT3-1 cells, we have now explored the molecular signaling pathways involved in the apparent ligand-independent activation of ERα in LβT2 cells.

As observed in αT3-1 cells (5), we found that GnRH treatments of LβT2 cells are much more effective in increasing the expression of an ERE reporter gene, when compared to equimolar amounts of E_2. In this context, a maximum response was obtained with 10 nM GnRH-I, which approximates the concentration of GnRH-I in the pituitary. However, GnRH-II was also maximally effective at 100 nM, and this difference in the effectiveness of these two GnRH subtypes has also been observed previously in monkey and rat pituitary cells (346), and attributed to the fact that the type 1 GnRH receptor binds GnRH-1 better than GnRH-II (65).

It is widely appreciated that phosphorylation of ERα regulates its ligand binding activity, nuclear translocation, dimerization, and ability to regulate transcription (196, 325, 347). It is also known that ligand binding increases the phosphorylation of ERα at Ser^{118} which increases the ERE-binding affinity of the receptor (197), and that substitution of Ser^{118} with alanine reduces its transcriptional activity (198, 199). Other studies have shown that the ligand-independent activation of nuclear hormone receptors, including ERα, involves a change in their phosphorylation status. For instance, activation of PKA or PKC signal transduction pathways leads to an increase in phosphorylation of ERα when it is transiently expressed in COS-1 cells (198), while cAMP treatments of HeLa and COS-1 cells results in phosphorylation of ERα via PKA or MAPK pathways (240, 348). In addition, treatments of cancer cells with growth factors, such as EGF, result in the phosphorylation of specific serine residues in the AF-1 domains of both ERα and ERβ, which are crucial to their transcriptional

responses (234, 348-350). Subsequent studies have indicated that growth factors activate the MAPK pathway which in turn phosphorylates the Ser^{118} residue of ERα (226, 234). It has also been shown that Ser^{167} of ERα can be phosphorylated in vitro by MAPK and AKT (197, 241, 242, 351). Our treatments of LβT2 cells with either GnRH subtype resulted in significant increases in the amounts of phosphorylated ERα at Ser^{167} in both nucleus and cytoplasm, as well as the amount of phosphorylated ERα at Ser^{118} in the nucleus. Interestingly, within the same 1-12 h time-frame, E_2 treatments did not enhance phosphorylation of ERα at Ser^{167}, and this may explain why the E_2-dependent activation of ERα was much less effect in these pituitary cells than observed after treatments with the GnRH subtypes.

It is also known that phosphorylation of ERα influences the recruitment of its co-activators, resulting in enhanced transcriptional activation (234, 350, 352) through histone modifications and recruitment of the basal transcriptional machinery (353). The binding of ligands by ERα is known to alter its affinity for co-activators, but co-activators are also recruited to nuclear receptors in a ligand-independent manner, in response to other stimuli. For instance, we have previously reported that SRC-3 is required for PR transactivation of the gonadotropin α-subunit in αT3-1 cells after treatment with GnRH subtypes (21). We therefore examined whether ERα interacts with various co-activator proteins in LβT2 cells after 1-6 h stimulation with GnRH, and found a specific increase in ERα interaction with PCAF within this time-frame.

It is known that PCAF interacts with multiple receptors including RXR-RAR heterodimers, ERα, AR and GR, and that its recruitment by these nuclear hormone receptors plays a key role in their transcriptional properties (222, 354). To demonstrate that PCAF plays a role in GnRH-induced, ERα-mediated transcription in LβT2 cells, we knocked down

PCAF levels by using an siRNA approach, and found that a reduction in cellular PCAF levels by about 50% reduces the ability of both GnRH subtypes to trigger ERE-luciferase reporter gene expression, as well as *Fosb* mRNA levels.

Although the physiological importance of ligand-independent activation of ERα remains unclear, the magnitude of the responses we observe are remarkable and suggest that they must have some impact on the gonadotrophs which are exposed to large pulsatile fluctuations in the GnRH-I levels in the hypophyseal portal system. It is therefore of interest that GnRH-1 treatments of LβT2 cells increase *Fshb* gene expression (330), because others have shown that the *Fshb* gene in LβT2 cells is regulated by the AP-1 transcription factor complex: a heterodimer of fos and jun family members, the relative levels of which influence the activity AP-1 as a transcription factor (355, 356). It has been shown that an AP-1 half-site within the *Fshb* promoter is occupied by AP-1 following GnRH-I treatment, and that this is essential for the maximal induction of *FshB* mRNA levels by GnRH-I in these cells (324). Moreover, GnRH-I has been found to stimulate AP-1 complex formation and Fosb synthesis, and that a dominant-negative FOS protein dose-dependently inhibited GnRH-I stimulated human FSHB transcription (325). Although little is known about the regulation of Fosb by estrogens, an ERE has been located within the *Fosb* promoter (334), and we therefore explored the possibility that the GnRH effects on increasing *Fosb* expression in LβT2 cells are mediated via the ligand-independent activation of ERα. As reported previously (318, 324, 325, 330), we observed a very robust and rapid (within 30 min) increase in *Fosb* mRNA levels after GnRH treatments, and found that this effect could be attenuated by an siRNA-mediated reduction in ERα levels. Unlike the effect of GnRH treatment on ERE-reporter gene expression, which appears to be mediated by multiple signaling pathways (PKA, PKC, PI3K and MAPK, data not shown), the rapid induction of *Fosb* gene expression by both GnRH

subtypes seems to involve only PKA and PKC signaling pathways, and this may due to the differences in the ERE/chromatin context of the endogenous *Fosb* gene promoter versus the ERE-driven luciferase reporter gene construct. We have also found that the induction of *Fshb* gene expression by both GnRH subtypes is only attenuated by PKA and PKC inhibitors. More importantly, although E_2 and GnRH treatments both result in the co-recruitment of ERα and PCAF to the *Fosb* promoter ERE, the binding of ERα and PCAF at this site is much more prolonged after treatment with the GnRH-I when compared to E_2. Moreover, the recruitment of ERα and PCAF to the *Fosb* promoter ERE occurs more rapidly and is much more prolonged after GnRH-I treatment than after GnRH-II treatment.

In conclusion, our results demonstrate that GnRH-mediated phosphorylation of ERα in mouse LβT2 pituitary cells results in its rapid association with PCAF, and that co-recruitment of ERα and PCAF to an ERE within the *Fosb* promoter likely enhances its transcriptional activation, which in turn is known to activate other genes in pituitary cells including the *Fshb* subunit gene (Figure 2.7). In a physiological context, the ligand-independent activation of ERα by GnRH in pituitary cells may be most important under conditions when estrogen levels are low and GnRH pulse amplitude is high, such as during the luteal/follicular transition phase of the menstrual cycle.

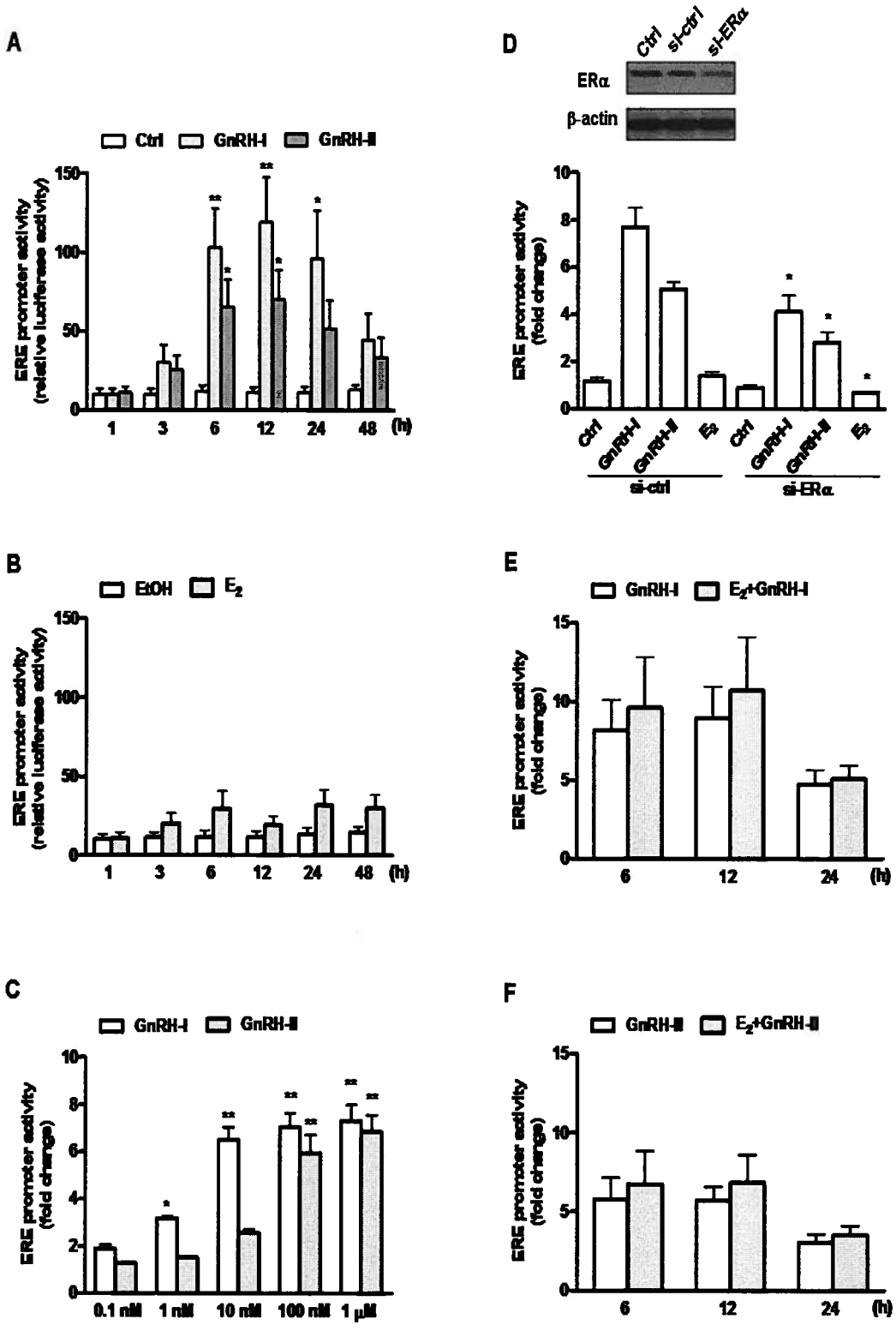
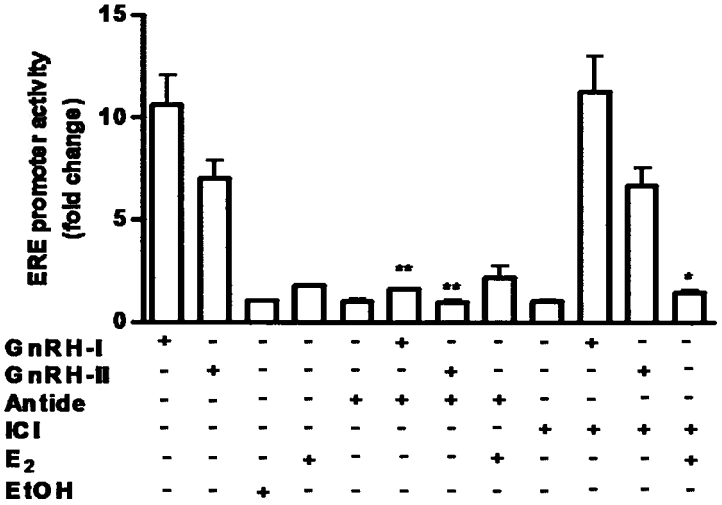

A
Ctrl
GnRH-I
GnRH-II
ERE promoter activity (relative luciferase activity)
150
100
50
0
1
3
6
12
24
48
(h)
B
EtOH
E2
ERE promoter activity (relative luciferase activity)
150
100
50
0
1
3
6
12
24
48
(h)
C
GnRH-I
GnRH-II
ERE promoter activity (fold change)
10
8
6
4
2
0
0.1 nM
1 nM
10 nM
100 nM
1 μM
D
Ctrl
si-ctrl
si-ERα
ERα
β-actin
ERE promoter activity (fold change)
10
8
6
4
2
0
Ctrl
GnRH-I
GnRH-II
E2
Ctrl
GnRH-I
GnRH-II
E2
si-ctrl
si-ERα
E
GnRH-I
E2+GnRH-I
ERE promoter activity (fold change)
15
10
5
0
6
12
24
(h)
F
GnRH-II
E2+GnRH-II
ERE promoter activity (fold change)
15
10
5
0
6
12
24
(h)

Figure 2.1 Effects of GnRH-I or GnRH-II on the trans-activation of an ERE-reporter gene in LβT2 cells.

A-C, the ERE-luciferase reporter gene together with a (RSV)-lacZ plasmid were transiently transfected into LβT2 cells by FuGENE 6.0. The cells were either treated with 100 nM GnRH-I, GnRH-II (A) or 100 nM E_2 (B) over a 48 h time course, or with 0.1 nM to 1 μM GnRH-I or GnRH-II for 6 h (C). The cell lysates were assayed for luciferase activity and measurements of β-galactosidase activity as a control for transfection efficiency. D, LβT2 cells were co-transfected with the ERE-luciferase reporter gene and a (RSV)-lacZ plasmid, with control siRNA (si-ctrl) or an siRNA for ERα (si-ERα). The efficiency of the siRNA was tested by immunoblotting for ERα (upper panel). After treatment with 100 nM GnRH-I, GnRH-II, or E_2, the cell lysates were assayed for luciferase activity and measurements of β-galactosidase activity as a control for transfection efficiency. E and F, the ERE-luciferase reporter gene together with a (RSV)-lacZ plasmid were transiently transfected into LβT2 cells by FuGENE 6.0. The cells were treated with 100 nM GnRH-I (E) or GnRH-II (F) with or without E2 for 6, 12 and 24 h. The cell lysates were assayed for luciferase activity and measurements of β-galactosidase activity as a control for transfection efficiency. Results of at least three independent experiments are expressed as mean ± SEM luciferase activity/β-galactosidase activity (i.e., relative luciferase activity) in A and B or fold change in C, D, E and F. *: $p<0.05$ or **: $p<0.01$ compared to untreated control (Ctrl) in A and C, and the untreated control value set at 1 in C, D, E and F. D, *: $p<0.05$ compared to the respective treatment after transfection with control siRNA (si-ctrl).

A


ERE promoter activity
(fold change)
15
10
5
0
**
**
*
GnRH-I + - - - - + - - - + - -
GnRH-II - + - - - - + - - - + -
Antide - - - - + + + + - - - -
ICI - - - - - - - - + + + +
E2 - - - + - - - + - - - +
EtOH - - + - - - - - - - - -

B

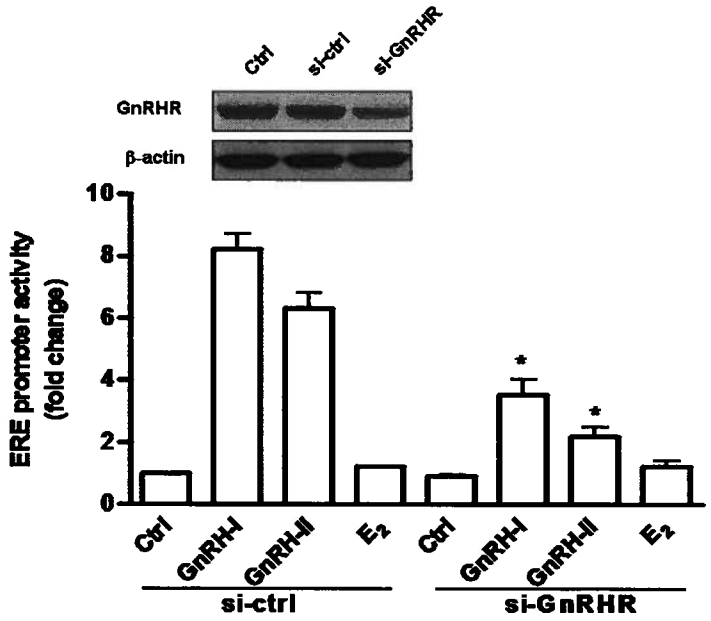

Ctrl
si-ctrl
si-GnRHR
GnRHR
β-actin
ERE promoter activity
(fold change)
10
8
6
4
2
0
*
*
Ctrl
GnRH-I
GnRH-II
E2
Ctrl
GnRH-I
GnRH-II
E2
si-ctrl
si-GnRHR

Figure 2.2 GnRHR mediates the activation of ERE by GnRH-I and GnRH-II.

A, the ERE-luciferase reporter gene and a (RSV)-lacZ plasmid were transiently transfected into LβT2 cells. The cells were treated with 100 nM GnRH-I, or GnRH-II alone, or together with antide (type I GnRHR antagonist), or ICI 182,780 (ICI, ER antagonist) for 6 h. B, LβT2 cells were cotransfected with the ERE luciferase reporter gene and a (RSV)-lacZ plasmid, with control siRNA (si-ctrl) or an siRNA for GnRHR (si-GnRHR). The efficiency of the siRNA was tested by immunoblotting for GnRHR (upper panel). After treatment with 100 nM GnRH-I, GnRH-II, or E_2 for 6 h, the cell lysates were collected for luciferase assay and measurements of β-galactosidase activity as a control for transfection efficiency. Results are expressed as mean ± SEM fold change in ERE promoter activity expressed as luciferase activity/β-galactosidase activity of at least three independent experiments. A, **: $p<0.01$ *versus* treatment without antide. *: $P<0.05$ *versus* E_2. B, *: $p<0.05$ *versus* the respective treatment after transfection with control siRNA.

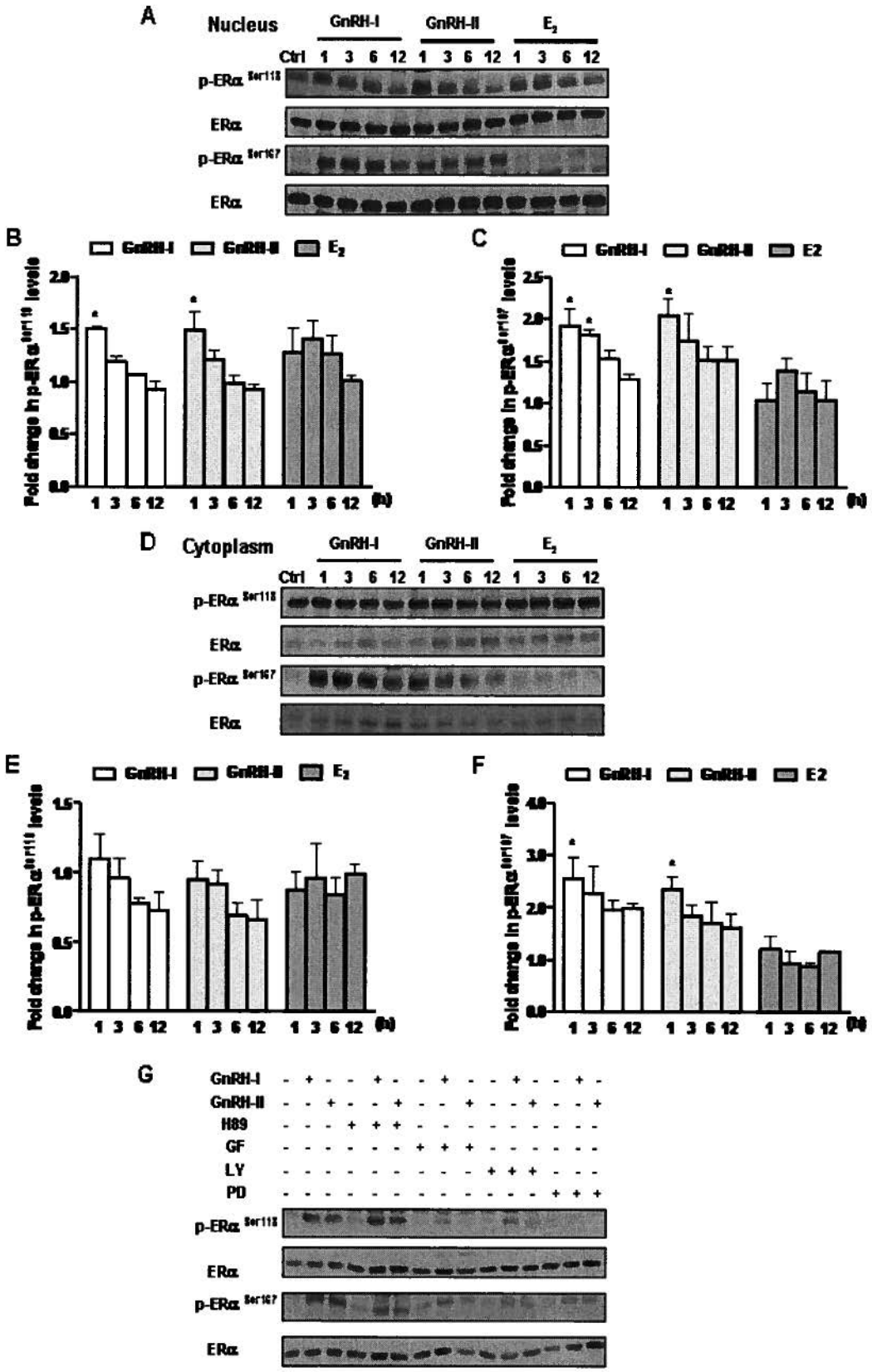

A
Nucleus
GnRH-I
GnRH-II
E2
Ctrl 1 3 6 12 1 3 6 12 1 3 6 12
p-ERα Ser118
ERα
p-ERα Ser167
ERα
B
GnRH-I GnRH-II E2
Fold change in p-ERα Ser118 levels
1 3 6 12 1 3 6 12 1 3 6 12 (h)
C
GnRH-I GnRH-II E2
Fold change in p-ERα Ser167 levels
1 3 6 12 1 3 6 12 1 3 6 12 (h)
D
Cytoplasm
GnRH-I
GnRH-II
E2
Ctrl 1 3 6 12 1 3 6 12 1 3 6 12
p-ERα Ser118
ERα
p-ERα Ser167
ERα
E
GnRH-I GnRH-II E2
Fold change in p-ERα Ser118 levels
1 3 6 12 1 3 6 12 1 3 6 12 (h)
F
GnRH-I GnRH-II E2
Fold change in p-ERα Ser167 levels
1 3 6 12 1 3 6 12 1 3 6 12 (h)
G
GnRH-I
GnRH-II
H89
GF
LY
PD
p-ERα Ser118
ERα
p-ERα Ser167
ERα

Figure 2.3 Regulation of ERα phosphorylation at Ser^{118} and Ser^{167} by GnRH-I or GnRH-II. The LβT2 cells were treated with 100 nM GnRH-I, GnRH-II, or E_2 for 1, 3, 6 and 12 h. Equal amounts of nuclear (A, B, C) or cytoplasmic lysates (D, E, F) were electrophoresed on SDS-10% gels, and western blotted to nitrocellulose for detection with antibodies specific for phosphor-$ERα^{Ser118}$, phosphor-$ERα^{Ser167}$, or ERα. Control (Ctrl) represents untreated cells. A, D, western blots are representative data from three independent experiments. B, C, E, F, relative pixel intensity of protein bands from western blots for ERα phosphorylation at Ser^{118} or Ser^{167}. Data are presented as the mean ± SEM of three independent experiments. *: $p<0.05$ *versus* untreated control value set at 1. G, the cells were also treated with 100 n M GnRH-I, GnRH-II for 1 h alone, or after pre-treatment with 10μM H89 (PKA inhibitor), 10μM GF109203X (GF, PKC inhibitor), 50μM LY294002 (LY, PI3K inhibitor) or 50μM PD98059 (PD, MAPK inhibitor). Equal amounts of cell lysates were electrophoresed on SDS-10% gels, and western blotted to nitrocellulose for detection with antibodies specific for phosphor-$ERα^{Ser118}$, phosphor-$ERα^{Ser167}$, or ERα. Immunoblots shown are representative of three independent experiments.

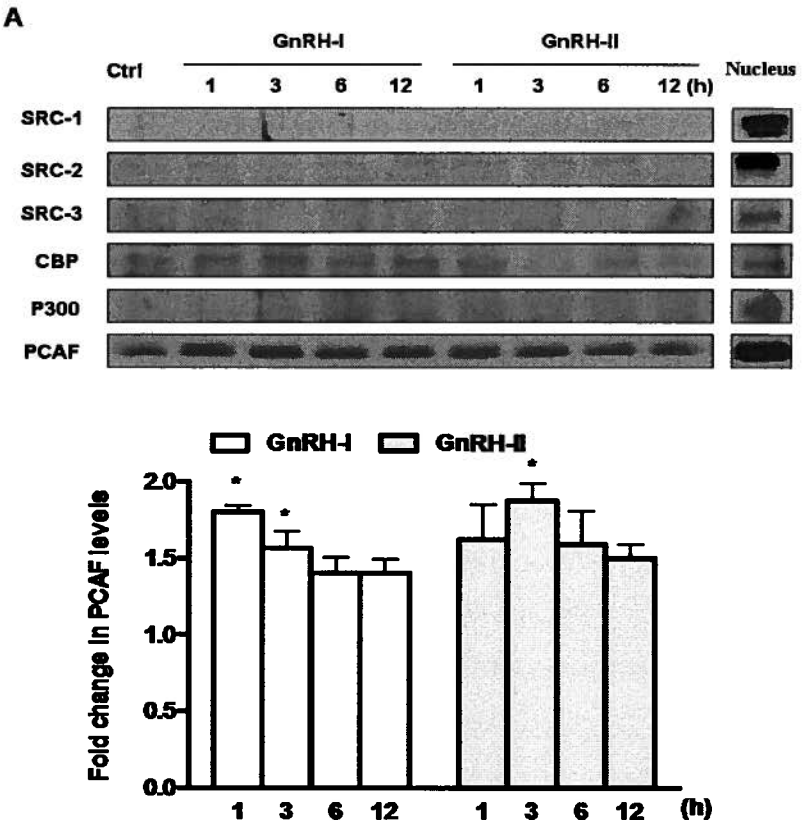
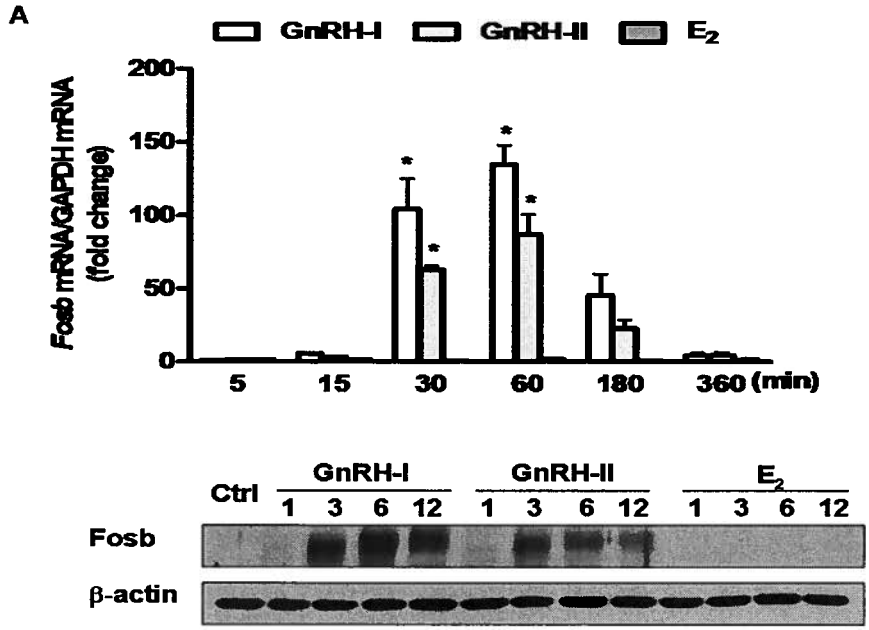
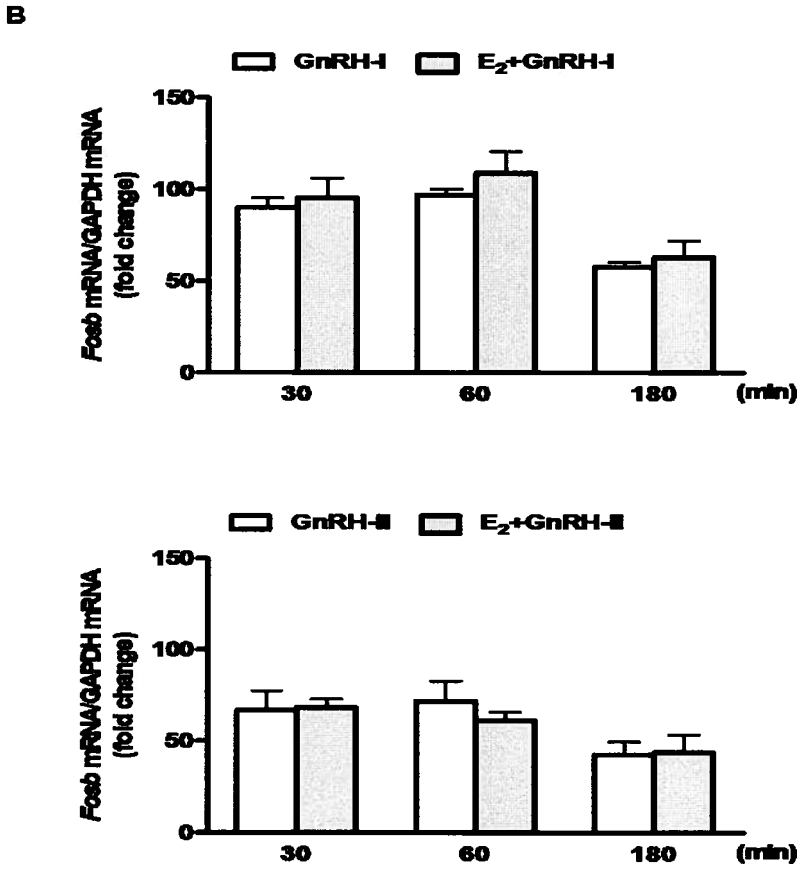

A
GnRH-I
GnRH-II
Ctrl
1 3 6 12 1 3 6 12 (h)
Nucleus
SRC-1
SRC-2
SRC-3
CBP
P300
PCAF
GnRH-I
GnRH-II
Fold change in PCAF levels
2.0
1.5
1.0
0.5
0.0
1 3 6 12 1 3 6 12 (h)
B
Ctrl
si-ctrl
si-PCAF
PCAF
β-actin
ERE promoter activity (fold change)
10
8
6
4
2
0
Ctrl
GnRH-I
GnRH-II
E2
Ctrl
GnRH-I
GnRH-II
E2
si-ctrl
si-PCAF

Figure 2.4 Interactions between ERα and PCAF after GnRH-I or GnRH-II treatments and effects on the transcription activity of ERα.

A, LβT2 cells were treated with 100 nM GnRH-I or GnRH-II for increasing lengths of time, and nuclear lysates were immunoprecipitated using an anti-ERα antibody. The immunoprecipitates were then probed with anti-SRC-1, SRC-2, SRC-3, CBP, P300 or PCAF antibodies. The nuclear lysates (Nucleus) were also probed with the individual antibodies as an input control for the immunoprecipitations. The western blot shown is representative of three independent experiments (upper panel). Relative pixel intensity of protein bands from western blots of PCAF from ERα immunoprecipitation experiments are presented as the mean ± SEM of three independent experiments (lower panel). B, LβT2 cells were cotransfected with the ERE-luciferase reporter gene and a (RSV)-lacZ plasmid, with control siRNA (si-ctrl) or an siRNA for PCAF (si-PCAF). The efficiency of the siRNA was tested by immunoblotting for PCAF (upper panel). After treatment with 100 nM GnRH-I, GnRH-II, or E_2, the cell lysates were assayed for luciferase activity and measurements of β-galactosidase activity as a control for transfection efficiency. Results are expressed as mean ± SEM fold change in ERE promoter activity expressed as luciferase activity/β-galactosidase activity of at least three independent experiments. A, *: $p<0.05$ *versus* untreated control value set at 1. B, *: $p<0.05$ *versus* the respective treatment after transfection with control siRNA.


A
GnRH-I
GnRH-II
E2
Fosb mRNA/GAPDH mRNA (fold change)
200
150
100
50
0
5
15
30
60
180
360 (min)
Ctrl
GnRH-I
GnRH-II
E2
1 3 6 12 1 3 6 12 1 3 6 12
Fosb
β-actin


B
GnRH-I
E2+GnRH-I
Fosb mRNA/GAPDH mRNA (fold change)
150
100
50
0
30
60
180
(min)
GnRH-II
E2+GnRH-II
Fosb mRNA/GAPDH mRNA (fold change)
150
100
50
0
30
60
180
(min)

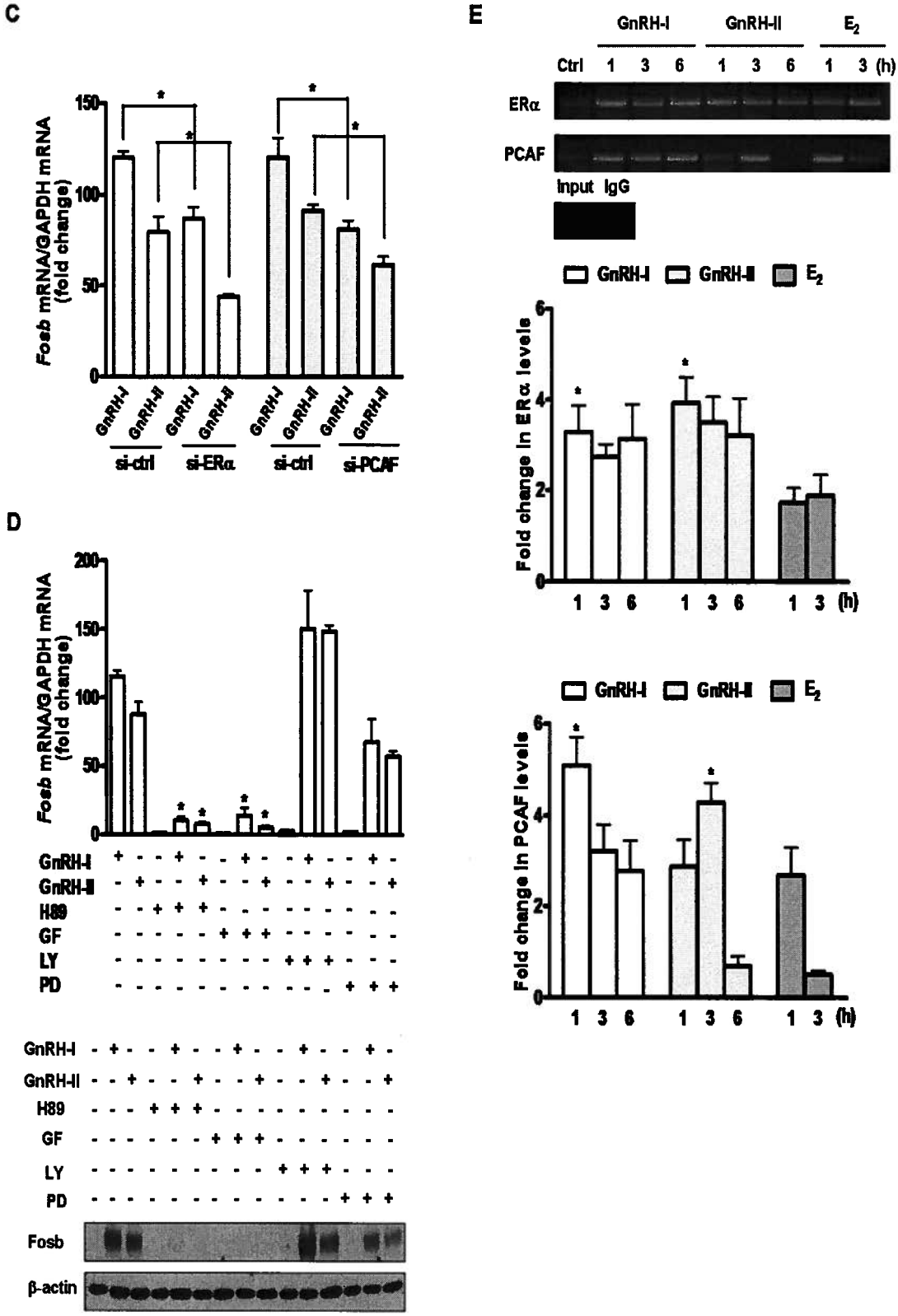
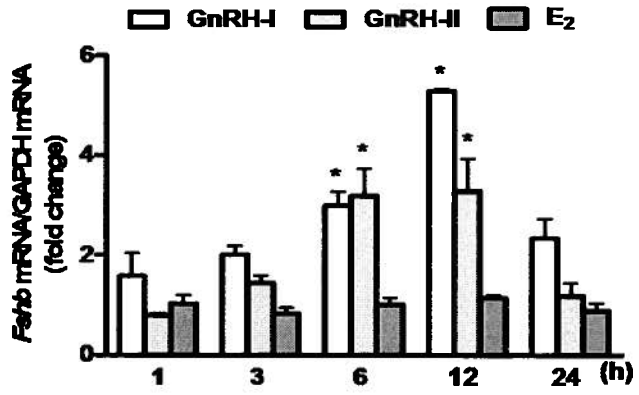

C
Fosb mRNA/GAPDH mRNA (fold change)
150
100
50
0
GnRH-I
GnRH-II
si-ctrl
si-ERα
si-PCAF
D
Fosb mRNA/GAPDH mRNA (fold change)
200
150
100
50
0
GnRH-I
GnRH-II
H89
GF
LY
PD
Fosb
β-actin
E
GnRH-I
GnRH-II
E2
Ctrl
1 3 6 1 3 6 1 3 (h)
ERα
PCAF
Input IgG
Fold change in ERα levels
Fold change in PCAF levels
1 3 6 1 3 6 1 3 (h)

Figure 2.5 The induction of *Fosb* gene expression and co-recruitment of ERα and PCAF to an ERE within the *Fosb* promoter after GnRH treatments.

A, LβT2 cells were either untreated or treated with 100 nM GnRH-I, GnRH-II or E_2 for 5, 15, 30, 60, 180 and 360 min. Total RNA was extracted and reverse transcribed into first-strand cDNA. The levels of *Fosb* mRNA were measured by real-time RT-PCR (upper panel). The LβT2 cells were either untreated or treated with 100 nM GnRH-I, GnRH-II or E_2 for 1, 3, 6, or 12 h. Equal amounts of cell lysates were electrophoresed on SDS-10% gels, and western blotted to nitrocellulose for detection with antibodies specific for Fosb (lower panel). Control (Ctrl) represents untreated cells. Data of real-time RT-PCR are presented as the mean ± SEM of three independent experiments. Immunoblots shown are representative of three independent experiments. *: $p<0.05$ *versus* untreated control value set at 1. B, LβT2 cells were treated with 100 nM GnRH-I (upper panel) or GnRH-II (lower panel) with or without E2 for 30, 60 and 180 min. Total RNA was extracted and reverse transcribed into first-strand cDNA. The levels of *Fosb* mRNA were measured by real-time RT-PCR. Data of real-time RT-PCR are presented as the mean ± SEM of three independent experiments. C, LβT2 cells were transfected with control siRNA (si-ctrl), siRNA for ERα (si-ERα) or PCAF (si-PCAF). After treated with 100 nM GnRH-I or GnRH-II for 1 h, total RNA was extracted and reverse transcribed into first-strand cDNA. The levels of *Fosb* mRNA were measured by real-time RT-PCR. Data of real-time RT-PCR are presented as the mean ± SEM of three independent experiments. *: $p<0.05$ *versus* the respective treatment after transfection with control siRNA (si-ctrl). D, LβT2 cells were treated with 100 n M GnRH-I, GnRH-II for 1 h alone, or after pre-treatment with 10μM H89, 10μM GF109203X (GF), 50μM LY294002 (LY) or 50μM PD98059 (PD). Total RNA was extracted and reverse transcribed into first-strand cDNA. The levels of *Fosb* mRNA were measured by real-time RT-PCR. The cells were also treated with 100 n M GnRH-I, GnRH-II for 3 h alone, or after pre-treatment with H89, GF109203X, LY294002 or PD98059. Equal amounts of cell lysates were electrophoresed on SDS-10% gels, and western blotted to nitrocellulose for detection with antibodies specific for Fosb. Data of real-time RT-PCR are presented as the mean ± SEM of three independent experiments. Immunoblots shown are representative of three independent experiments. *: $P<0.05$ *versus* treatment without inhibitor. E, LβT2 cells were treated with 100 nM GnRH-I, GnRH-II, or E_2 for 1, 3, or 6 h and the nuclear proteins were then cross-linked. Sheared

chromatin was immuno-precipitated (IP) with ERα and PCAF antibodies, and recovered chromatin was subjected to PCR analysis using primers spanning an ERE within the *Fosb* promoter. Non-specific IgG was used in all ChIP reactions as a control. An ethidium bromide-stained gel of PCR products showed a representative of ChIP analysis (upper panel). Relative pixel intensity of PCR products bands for ERα and PCAF are presented as the mean ± SEM fold change of three independent experiments (lower panel). *: $p<0.05$ *versus* untreated control set as 1.

A


GnRH-I
GnRH-II
E_2
Fshb mRNA/GAPDH mRNA (fold change)
0
2
4
6
1
3
6
12
24
(h)

B

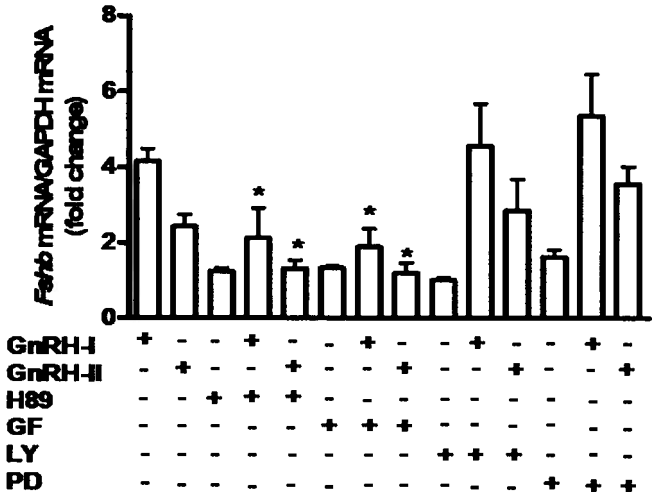

Fshb mRNA/GAPDH mRNA (fold change)
0
2
4
6
8
GnRH-I
GnRH-II
H89
GF
LY
PD

Figure 2.6 Increased expression of *Fshb* in LβT2 cells after treatments with GnRH subtypes. A, LβT2 cells were either untreated or treated with 100 nM GnRH-I, GnRH-II or E_2 for 1, 3, 6, 12 and 24 h. Total RNA was extracted and reverse transcribed into first-strand cDNA. The levels of *Fshb* mRNA were measured by real-time RT-PCR. Data of real-time RT-PCR are presented as the mean ± SEM of three independent experiments. *: $p<0.05$ *versus* untreated control value set at 1. B, LβT2 cells were treated with 100 n M GnRH-I, GnRH-II for 12 h alone, or after pre-treatment with 10μM H89, 10μM GF109203X (GF), 50μM LY294002 (LY) or 50μM PD98059 (PD). Total RNA was extracted and reverse transcribed into first-strand cDNA. The levels of *Fshb* mRNA were measured by real-time RT-PCR. Data of real-time RT-PCR are presented as the mean ± SEM of three independent experiments. *: $P<0.05$ *versus* treatment without inhibitor.

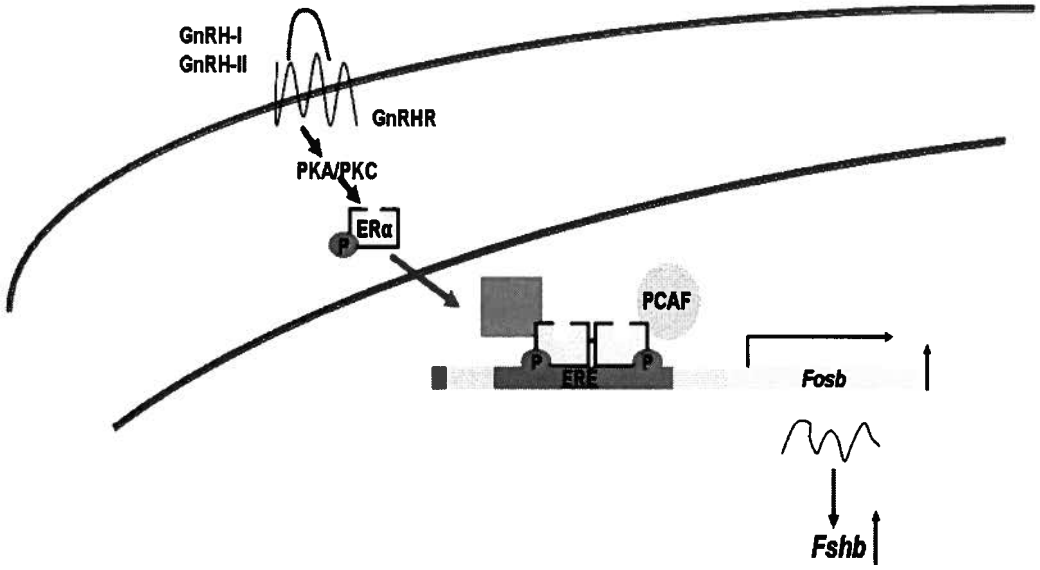


Figure 2.7 GnRH-mediated phosphorylation of ERα contributes to *Fosb* expression in mouse gonadotrophs

After binding with GnRHR, GnRH-I and GnRH-II active PKA and PKC signaling pathways and mediate phosphorylation of ERα in mouse LβT2 pituitary cells. These result in ERα rapid association with PCAF, and that co-recruitment of ERα and PCAF to an ERE within the *Fosb* promoter likely enhances its transcriptional activation, which in turn is known to activate other genes in pituitary cells including the *Fshb* subunit gene. ER, estrogen receptor; ERE, estrogen-response element; FSH, follicle stumulating hormone; GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor; PCAF, P300/CBP-associated factor; PKA, protein kinase A; PKC, protein kinase C.

Chapter 3 GnRH-I-mediated activation of progesterone receptor contributes to *Gsuα* expression in mouse gonadotrophs[2]

3.1 Introduction

The hypothalamic-pituitary-gonadal axis is important for maintaining normal reproductive function. GnRH is secreted in a pulsatile manner from neurons in the hypothalamus into the hypophyseal portal system (13). After binding to the GnRH receptor on the surface of gonadotrophs in the anterior pituitary, GnRH controls synthesis and secretion of FSH and LH, which consist of a common GSUα and a unique β subunit (1), and which play a systematic role in gametogenesis, folliculogenesis and steroidogenesis in the gonads (2, 4, 357). Subsequently, steroid hormones, including E_2 and P4, feed back to regulate expression and secretion of GnRH and gonadotropin at the level of the hypothalamus and pituitary (5).

The function of intracellular PRs as ligand-activated transcription factors is well characterized (278). There are two main isoforms of PR, a full length PR-B and N-terminally-truncated PR-A; the two isoforms are derived by the transcription of a single gene from two different promoters (253). Following P4 binding, PRs exhibit conformational changes leading to phosphorylation, dimerization, nuclear translocation, binding to PREs, and subsequent gene transcription (278). Apart from being activated by their ligands, steroid hormone receptors, including PRs, are also activated after phosphorylation in a ligand-independent manner by peptide growth factors including EGF and heregulin (285, 286).

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[2] A version of this chapter has been published. **Chen J, An BS, So WK, Cheng L, Hammond GL, Leung PC** 2010 Gonadotropin-releasing hormone-I-mediated activation of progesterone receptor contributes to gonadotropin alpha-subunit expression in mouse gonadotrophs. Endocrinology 151:1204-1211

Cross-talk between GnRH and PR occurs in rodent pituitary cells and is believed to play an important role in the GnRH self-priming effect (21, 250, 358, 359), which is defined as increased gonadotropin secretion from gonadotrophs in response to a second stimulation by GnRH (299). As a powerful servo-mechanism, this potentiates the pituitary responsiveness to GnRH-I severalfold. In rat primary anterior pituitary cells cultured in the absence of P4, the PR antagonist RU486 inhibits GnRH self-priming and prevents cAMP augmentation of GnRH-stimulated LH secretion. This is consistent with the fact that a GnRH-stimulated PKA cascade acts, in part, through transcriptional activation of PR (250). This GnRH self-potentiation appears to depend on PR, as it is completely absent in PR knock-out mice (13). Therefore, it is suggested that after binding to its receptor on gonadotrophs, GnRH prompts signaling pathways and activates PR in a ligand-independent manner (300), which mediates the GnRH self-priming effect. However, the mechanisms of PR involvement in GnRH self-priming, which results in the abrupt and exponential increase in pituitary responsiveness at the time of the LH surge, remain unclear.

We have previously demonstrated that GnRH-I activates a PRE-luciferase reporter gene in mouse αT3-1 pituitary cells through PKA and PKC transduction pathways. Furthermore, the binding of PR and its coactivator SRC-3 to the PRE in the murine *Gsuα* promoter is enhanced by GnRH-I, and is coincident with an increase in GSUα protein levels, indicating that the influence of GnRH-I on the common *Gsuα* gene expression depends on the PR (21). In this study, we have utilized LβT2 mouse pituitary cells that express both gonadotropin β genes and the *Gsuα* gene, and are considered more mature gonadotrophs, as well as αT3-1 cells to provide further direct evidence that induction of *Gsuα* by GnRH-I depends on the ligand-independent activation of PR.

3.2 Materials and methods

3.2.1 Cells and cell culture

The mouse gonadotroph-derived αT3-1 (341) and LβT2 (342) cell lines were generously provided by Dr. P.L. Melon (Department of Reproductive Medicine, University of California, San Diego, CA) and maintained in monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin (Invitrogen, Burlington, ON) in humidified 5% CO_2, 95% air at 37 C. The cells were passaged when they reached about 90% confluence, using a trypsin/EDTA solution (0.05% trypsin, 0.5 mM EDTA). Cells were kept in phenol-red free medium containing charcoal-treated FBS for four days prior to experiments.

3.2.2 Plasmids, siRNA and transient transfection assay

A PRE-luciferase reporter plasmid containing two copies of a consensus progesterone responsive element (PRE) upstream of the thymidine kinase promoter was provided by Dr. D. P. McDonnell (Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC). The siRNAs for PR were obtained from Qiagen Inc. (Mississauga, ON, Canada), together with a nonspecific siRNA (si-ctrl) that was used as a negative control.

Transient transfections of the PRE-luciferase reporter gene or siRNA were performed using FuGENE 6.0 (Roche Diagnostics, Quebec, QC) together with a Rous sarcoma virus (RSV)-lacZ plasmids to correct for transfection efficiencies. One day before transfection, αT3-1 and LβT2 cells were seeded in six-well plates. One microgram of PRE-luciferase reporter gene and 0.5 μg of RSV-lacZ were applied to the cells. After set times of culture (0-24 h), cellular lysates were obtained after addition of 150 μl reporter lysis buffer (Promega

Corp., San Luis Obispo, CA) and were assayed for luciferase activity using a Lumat LB 9507 luminometer (EG&G, Berthold, Germany). β-Galactosidase activity was also measured using a β-galactosidase enzyme assay system (Promega Corp.) to normalize for transfection efficiencies. Promoter activity was calculated as luciferase/β-galactosidase activity.

3.2.3 Immunoblotting

The protein content of cell lysates was determined using a Bradford assay (Bio-Rad Laboratories, Mississauga, ON); 20 µl aliquots were resolved by 10% SDS-PAGE and electrotransferred to a Hybond-C membrane (Amersham Biosciences, Morgan, ON). After blocking, the membranes were incubated (overnight 4 C) with specific antibodies against PR A/B and actin (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies were then incubated with the membranes. After washing, immunoblots were examined using the ECL chemiluminescent system (Amersham Pharmacia Biotech, Piscataway, NJ) followed by exposure to Kodak X-Omat film.

3.2.4 Real-time RT-PCR

Total RNA was extracted from cell cultures using Trizol (Invitrogen, Burlington, Canada). The RNA concentration was measured based on absorbance at 260 nm. Isolated RNA was reverse transcribed into first-strand cDNA using M-MLV reverse transcriptase (Promega Corp.). The primers used for SYBR Green real-time RT-PCR were designed using Primer Express Software v2.0 (PerkinElmer Applied Biosystems, Foster City, CA). The primers for *Gsua* mRNA were: sense, 5′- TGTTGCTTCTCCAGGGCATAT and antisense, 5′- TGGAACCAGCATTGTCTTCTTG. The primers for *Gapdh* mRNA were: sense, 5′-CATGGCCTTCCGTGTTCCTA and antisense, 5′-GCGGCACGTCAGATCCA. Real-time

PCR was performed using an ABI prism 7000 Sequence Detection System (PerkinElmer Applied Biosystems). Reactions were set up using SYBR Green PCR Master Mix (PerkinElmer Applied Biosystems). Relative quantification of *Gsuα* mRNA levels was performed using the comparative cycle threshold method with GAPDH as an endogenous control and with the formula $2^{-\Delta\Delta Ct}$.

3.2.5 Data analysis

Data are presented as the mean ± SEM of at least three independent experiments. Data were analyzed by one-way ANOVA followed by Tukey's test by using the GraphPad Prism 4 statistical software (GraphPad Software, Inc., San Diego, CA); $p<0.05$ was considered statistically significant.

3.3 Results

3.3.1 GnRH-I rapidly and transiently activates PR in αT3-1 and LβT2 cells

The basal levels of PR-B are higher in LβT2 cells than in αT3-1 cells, whereas the latter cells contain much higher levels of ERα (Figure 3.1A). Induction of PR expression by E_2, which has synergistic effects with P4 on PR-mediated transcription, has been documented in many cell lines including those of pituitary origin (21, 263). In our experiments, we cultured αT3-1 and LβT2 cells in the presence of 0.2 nM E_2 for 2 days before treatment with GnRH-I or GnRH-II, and induction of PR was only observed in αT3-1 cells (Figure 3.1B).

After transfection of a PRE-luciferase reporter gene, 10 nM GnRH-I alone increased luciferase levels only at 8h in αT3-1 cells. With the inclusion of E_2, luciferase activity

increased with a peak at 8h, and then declined (Figure 3.1C). In LβT2 cells, GnRH-I transiently increased luciferase levels, which peaked at 8 h. However, there was no apparent difference in PRE-luciferase promoter activity in these cells after pretreatment with E_2 (Figure 3.1D). This finding may be due to the induction of PR by E_2 in αT3-1 cells but not in LβT2 cells (Figure 3.1B). In dose-response assays in which cells were treated with 0.1 nM to 1 μM GnRH-I, maximum increases in PRE-luciferase reporter activity were seen at 10 nM - 1 μM GnRH-I in αT3-1 cells (Figure 3.1E) and at 1 nM - 1 μM GnRH-I in LβT2 cells (Figure 3.1F). Moreover, there was a >3-fold change in PRE-luciferase reporter activity when the LβT2 cells were treated with 0.1 nM GnRH-I. Based on the results of this experiment, we used 10 nM in the following studies.

Since our data and a previous study (21) showed that both GnRH subtypes and P4 activate a PRE-luciferase reporter gene, we wished to determine whether co-treatment with P4 and the GnRH subtypes might result in a synergistic effect. The results of this experiment indicate that in αT3-1 cells, P4 further increased the response after GnRH-I, but not GnRH-II, treatment (Figure 3.1G). In LβT2 cells, in contrast, there was no synergistic effect between P4 and either GnRH subtype (Figure 3.1H).

3.3.2 GnRH-I enhances *Gsuα* gene expression in αT3-1 and LβT2 cells

Employing real-time RT-PCR to interrogate the effect of GnRH-I on *Gsuα* gene expression, we found that GnRH-I increased *Gsuα* mRNA accumulation, which reached its highest level at 24 h in both cell lines, while E_2 did not further enhance the GnRH-I effect (Figure 3.2A and 3.2B). The influence of GnRH-I on *Gsuα* mRNA levels in both cell lines at 24 h was greater than that observed after treatment with the same concentration (10 nM) of GnRH-II (Figure 3.2C and 3.2D). It should also be noted that, under these conditions, the

co-administration of P4 with either GnRH-I or GnRH-II did not result in any additive effects on *Gsuα* mRNA levels in either cell line (Figure 3.2C and 3.2D).

3.3.3 Activation of PR and its effects on *Gsuα* expression involves PKC

Since PR can be phosphorylated at Ser249 upon activation of PKA and PKC signaling pathways by GnRH-I in both αT3-1 (21) and LβT2 cells (360), we pretreated cells with inhibitors of PKA (H89) or PKC (GF109203X) prior to treatment with GnRH-I, and compared the subsequent induction of PRE-luciferase reporter gene activities. The results show that both H89 and GF109203X significantly attenuated the activation of the PRE-luciferase reporter gene in response to GnRH-I in both cell lines (Figure 3.3A and 3.3B).

To explore whether the activation of PR by GnRH-I is mediated by the GnRHR, both cell lines were pretreated with its antagonist, antide. Antide significantly blocked the induction of PRE-luciferase reporter activity by GnRH-I, while pre-treatment with a PR antagonist, RU486, did not (Figure 3.3A and 3.3B).

In order to study the signaling transduction pathways involved in the GnRH-I stimulated accumulation of *Gsuα* transcripts in these cell lines, αT3-1 and LβT2 cells were pretreated with PKA or PKC inhibitors and then challenged with GnRH-I. The results show that only GF109203X had inhibitory effects on GnRH-I-induced *Gsuα* expression, while H89 did not (Figure 3.4A and 3.4B). As in the PRE-luciferase reporter gene study, antide abolished induction of *Gsuα* by GnRH-I, while RU486 had no influence (Figure 3.4A and 3.4B).

3.3.4 GnRH-I-induced *Gsuα* gene expression requires PR

In the next set of experiments, we used an siRNA approach to assess the role of PR in

GnRH-I-modulated *Gsuα* gene expression in αT3-1 and LβT2 cells. When the PR levels in αT3-1 cells were knocked-down using two PR siRNAs separately (Figure 3.5A upper panel), approximate 40% reductions in GnRH-I stimulated *Gsuα* mRNA levels were observed (Figure 3.5A). Similar results were obtained when PR levels were knocked-down using a PR siRNA in LβT2 cells (Figure 3.5B). These data suggest that downstream signaling events involving PR are required for the GnRH-I induction of *Gsuα* gene expression.

3.4 Discussion

The development of immortalized mouse pituitary gonadotrophs, including αT3-1 and LβT2 cells, has provided the opportunity to analyze mechanisms of regulation of the gonadotropin genes by GnRH-I and steroid hormones (341, 342). In particular, the immortalized LβT2 cell line expresses many markers of a mature gonadotroph, rendering it more physiologically relevant to *in vivo* systems (5, 23, 343, 361, 362). Our previous study using αT3-1 cells demonstrated that, coincident with an increase in GSUα protein levels, GnRH-I increases assembly of SRC-3 and PR at the PRE in the *Gsuα* promoter, leading to the conclusion that the induction of *Gsuα* gene transcription by GnRH-I occurs at least partially through the ligand-independent activation of PR (21). In this study, we have found that induction of *Gsuα* gene expression by GnRH-I can be significantly attenuated by PR siRNA, thus providing direct evidence that transactivation of PR is important for GnRH-I effects on the common gonadotropin α subunit gene, which involves PKC signaling (Figrue. 3.6).

The GnRH self-priming effect involves the PR (300). Although E_2 treatment leads to increases in PR levels in primary rat and mouse pituitary cells, and thereby contributes to the

robust GnRH self-priming effect, E_2 pre-incubation does not influence PR levels in LβT2 cells (263). In the present study, we confirmed this but found that E_2 increases PR levels in αT3-1cells. Since ERα levels are much higher in αT3-1cells compared with LβT2 cells, we also conducted a dose response experiment of E_2 on PR expresssion in LβT2 cells. However, 0.2 nM - 1μM E_2 did not increase PR levels in LβT2 cells (data now shown). Thus, the more mature LβT2 gonadotroph cells differ in this regard as compared to αT3-1cells. The failure of E_2 treatment to induce a significant increase in PR expression in LβT2 cells may be due to differences in basal PR levels or their state of differentiation. Moreover, the different induction of PR by E_2 in the two cell lines may explain why GnRH-I and P4 synergize to activate a PRE-luciferase reporter gene in αT3-1 cells, but not in LβT2 cells. Consistent with the activation of the PRE-luciferase gene, we found that GnRH-I treatment of both cell lines leads to an increase in *Gsuα* mRNA levels at 24 h. In contrast to the additive effect of P4 on GnRH-I-induced PRE promoter activity in αT3-1 cells, there is no further enhancement of *Gsuα* mRNA levels when the cells are co-treated with GnRH-I and P4. These data indicate that while GnRH-I induces *Gsuα* expression, P4 cannot influence *Gsuα* expression in a ligand-dependent manner despite the fact that it increases the expression of a PRE-luciferase reporter gene.

Pulsatile secretion of GnRH-I regulates pituitary gonadotropin production. In the current study, we used a relatively low dose of GnRH-I which more closely resembles its physiological concentration in the pituitary (363, 364). Although the distinguishing feature of GnRH-II is its wide distribution in extrahypothalamic regions of the brain and outside the nervous system, it is also present in preoptic and medio-basal hypothalamic areas (65). In agreement with previous data obtained in gonadotrophs (21), GnRH-II exerts somewhat milder effects on a PRE-luciferase reporter gene and *Gsuα* mRNA levels compared with the

same dose of GnRH-I.

In pituitary gonadotrophs, binding of GnRH-I to its G protein-coupled receptor (GnRHR) initiates the PKC signaling pathway to stimulate transcription of the *Gsuα* subunit gene (110). Our results show that PKC and possibly PKA signaling pathways are important for GnRH-I activation of the PRE-luciferase reporter gene. However, only PKC signaling mediates the increase in levels of *Gsuα* mRNA by GnRH-I treatment in these two cell lines. Our previous studies have shown that phosphorylation of PR at Ser^{249} occurs in response to GnRH-I in both αT3-1 and LβT2 cells (21, 302), and that only PKC influences the phosphorylation of PR at the same site in LβT2 cells (302). Thus, in terms of downstream signals triggered by the GnRHR, PKC induces PR phosphorylation and a subsequent increase in endogenous *Gsuα* expression.

Cross-talk between PR and GnRH-I is associated with a GnRH-I self-priming system in transfected anterior pituitary cells in the presence of E_2 (250, 299, 300), and this depends on the presence of PR (13). Our previous study also suggested that activation of GnRHR by GnRH-I and GnRH-II in αT3-1 cells prompts PKA and PKC signaling pathways, which activate PR in a ligand-independent manner and ultimately induce the loading of PR and SRC-3 onto PRE in the *Gsuα* promoter. To provide further direct evidence that PR activation is involved in the induction of *Gsuα* expression by GnRH-I, we knocked down PR protein levels using siRNAs. This reduction in PR levels resulted in an approximately 40% reduction of GnRH-I-induced *Gsuα* mRNA levels. Supporting the concept that PR is required for GnRH-I-induced *Gsuα* expression, there is a PRE sequence within the mouse *Gsuα* promoter, which sequesters PR in association with SRC-3 (21). In terms of *Gsuα* gene expression in the pituitary cells, our results therefore indicate that GnRH-I induces *Gsuα* by transactivation of PR in the absence of its own ligand, P4.

The gonadotropins are coordinately and differentially regulated throughout the menstrual cycle. During the follicular phase, circulating E_2 levels increase gradually. At midcycle, there is an LH surge that induces resumption of meiosis, ovulation and luteinization. Although the LH surge has been intensively studied, the basis for the triggering of LH remains unclear. Apart from positive feedback of E_2, the self-priming effect of GnRH-I plays a fundamental role in triggering the ovulatory gonadotrophin surge in mammals, including humans (299). Together with our previous results (21, 302), the present results show that ligand-independent activation of PR by GnRH-I is involved in *Gsuα* and *Fshb* gene expression and suggest that this is important for the mid-cycle increase of gonadotropin release. Moreover, our results using αT3-1 and LβT2 cells indicate that GnRH-I activates PR in a ligand-independent manner and promotes the accumulation of *Gsuα* mRNA, and this likely contributes to the self-priming effect of GnRH-I during the follicular phase of the menstrual cycle.

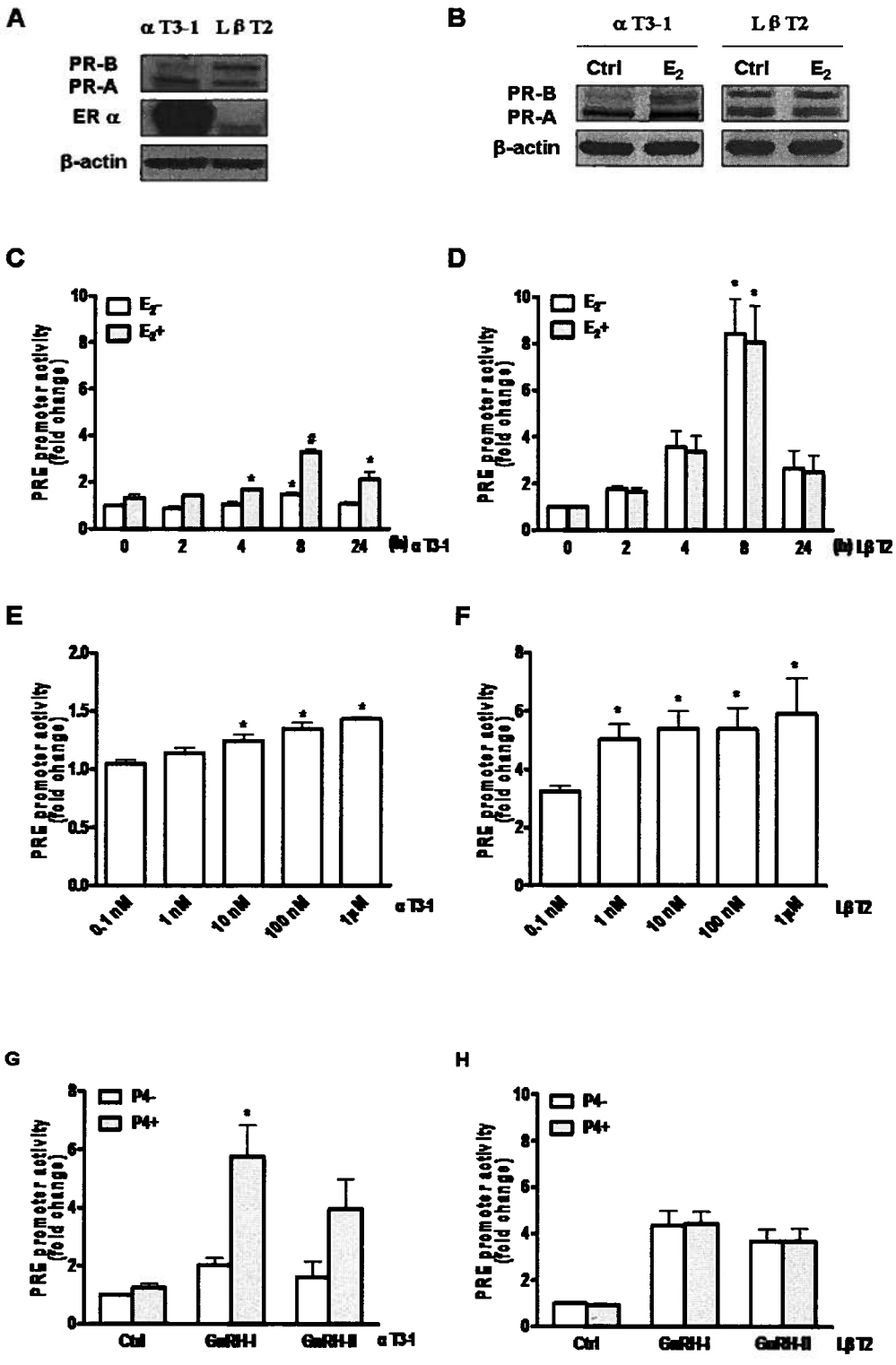

A
αT3-1
LβT2
PR-B
PR-A
ER α
β-actin
B
αT3-1
LβT2
Ctrl
E2
Ctrl
E2
PR-B
PR-A
β-actin
C
E2-
E2+
PRE promoter activity (fold change)
0
2
4
8
24
(h) αT3-1
D
E2-
E2+
PRE promoter activity (fold change)
0
2
4
8
24
(h) LβT2
E
PRE promoter activity (fold change)
0.1 nM
1 nM
10 nM
100 nM
1μM
αT3-1
F
PRE promoter activity (fold change)
0.1 nM
1 nM
10 nM
100 nM
1μM
LβT2
G
P4-
P4+
PRE promoter activity (fold change)
Ctrl
GnRH-I
GnRH-II
αT3-1
H
P4-
P4+
PRE promoter activity (fold change)
Ctrl
GnRH-I
GnRH-II
LβT2

Figure 3.1 GnRH-I activates the PR in αT3-1 and LβT2 cells.

A, basal levels of PR (PR-B and PR-A) and ERα in αT3-1 and LβT2 cells were determined by western blotting. B, after 2 days incubation in the absence or presence of 0.2 nM E_2, the levels of PR (PR-B and PR-A) and actin were determined by western blotting in αT3-1 and LβT2 cells. C-H, the PRE-luciferase reporter gene, together with a (RSV)-lacZ plasmids, was transiently transfected into αT3-1 (C, E and G) and LβT2 cells (D, F and H) by FuGENE 6.0. The cells were either pretreated with or without 0.2 nM E_2 for 48 h and then challenged with 10 nM GnRH-I over a 24 h time course (C and D), or treated with 0.1 nM to 1 μM GnRH-I for 8 h (E and F), or were incubated with 0.2 nM E_2 and then treated with 10 nM GnRH-I or GnRH-II with or without 100 nM P4 for 8 h (G and H). Cell lysates were assayed for luciferase activity and measurements of β-galactosidase activity were made as a control for transfection efficiency. Results of at least three independent experiments are expressed as mean ± SEM fold change of PRE promoter activity. C-F, *: $p<0.05$ compared to GnRH-I treatment in the absence of E_2 at 0 h in C and D, or compared to untreated control value set at 1 in E and F. C, #: $p<0.05$ compared to GnRH-I treatment in the absence of E_2 at 8 h. G, *: $p<0.05$ compared to the respective treatment without P4.

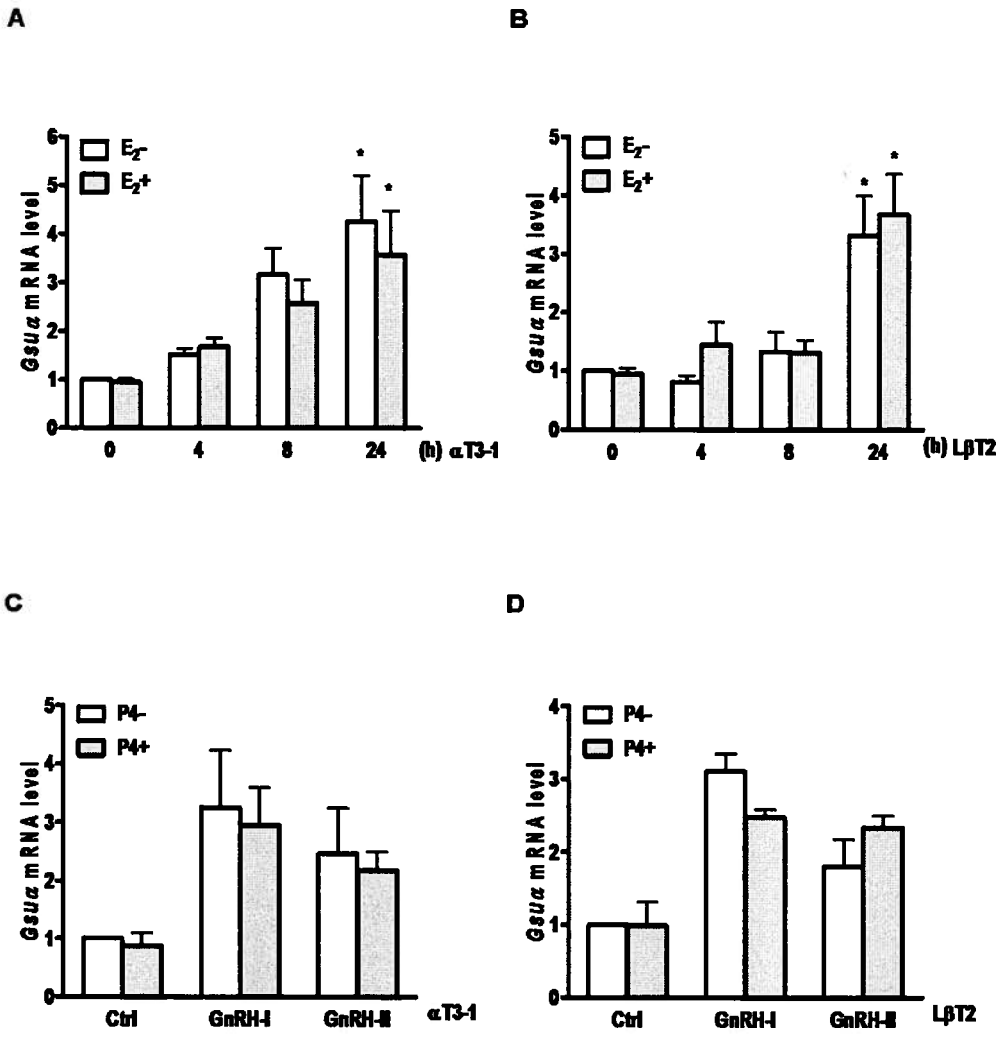
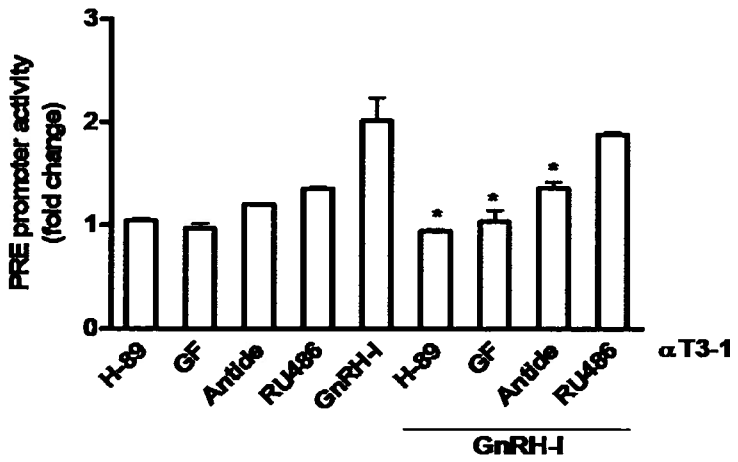


Figure 3.2 GnRH-I enhances *Gsua* gene expression in αT3-1 and LβT2 cells.

A and B, after 2 days incubation in the absence or presence of 0.2 nM E_2, αT3-1 and LβT2 cells were treated with 10 nM GnRH-I over a 24 h time course and the chronological changes of *Gsua* mRNA levels were monitored by real-time PCR. C and D, after 2 days incubation with 0.2 nM E_2, 100 nM P4 was co-treated with 10 nM GnRH-I or GnRH-II for 24 h, followed by real-time PCR to monitor the expression levels of *Gsua*. Results are expressed as mean ± SEM (i.e., *Gsua* mRNA level) of three independent experiments. *: $P<0.05$ compared to GnRH-I treatment in the absence of E_2 at 0 h.

A


PRE promoter activity
(fold change)
3
2
1
0
H-89
GF
Antide
RU486
GnRH-I
H-89
GF
Antide
RU486
αT3-1
GnRH-I

B

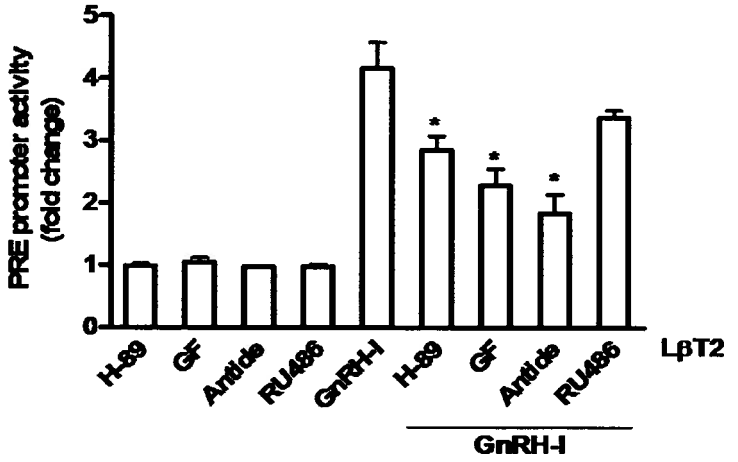

PRE promoter activity
(fold change)
5
4
3
2
1
0
H-89
GF
Antide
RU486
GnRH-I
H-89
GF
Antide
RU486
LβT2
GnRH-I

Figure 3.3 PKA and PKC inhibitors reduce GnRH-I induced PRE-luciferase reporter gene activity in αT3-1 and LβT2 cells.

A and B, PRE-luciferase reporter genes, together with a (RSV)-lacZ plasmids, were transiently transfected into αT3-1 (A) and LβT2 (B) cells. After 2 days incubation in the presence of 0.2 nM E_2, the cells were either treated with 10 nM GnRH-I alone or pre-treated with 10 μM H89 (PKA inhibitor), 10 μM GF (GF109203X, PKC inhibitor), 10 nM antide, or 1 μM RU486 over a 8 h time course. Cell lysates were assayed for luciferase activity and measurements of β-galactosidase activity were taken as a control for transfection efficiency. Results are expressed as mean ± SEM fold change of PRE promoter activity of three independent experiments. *: $P<0.05$ compared to GnRH-I treatment alone.

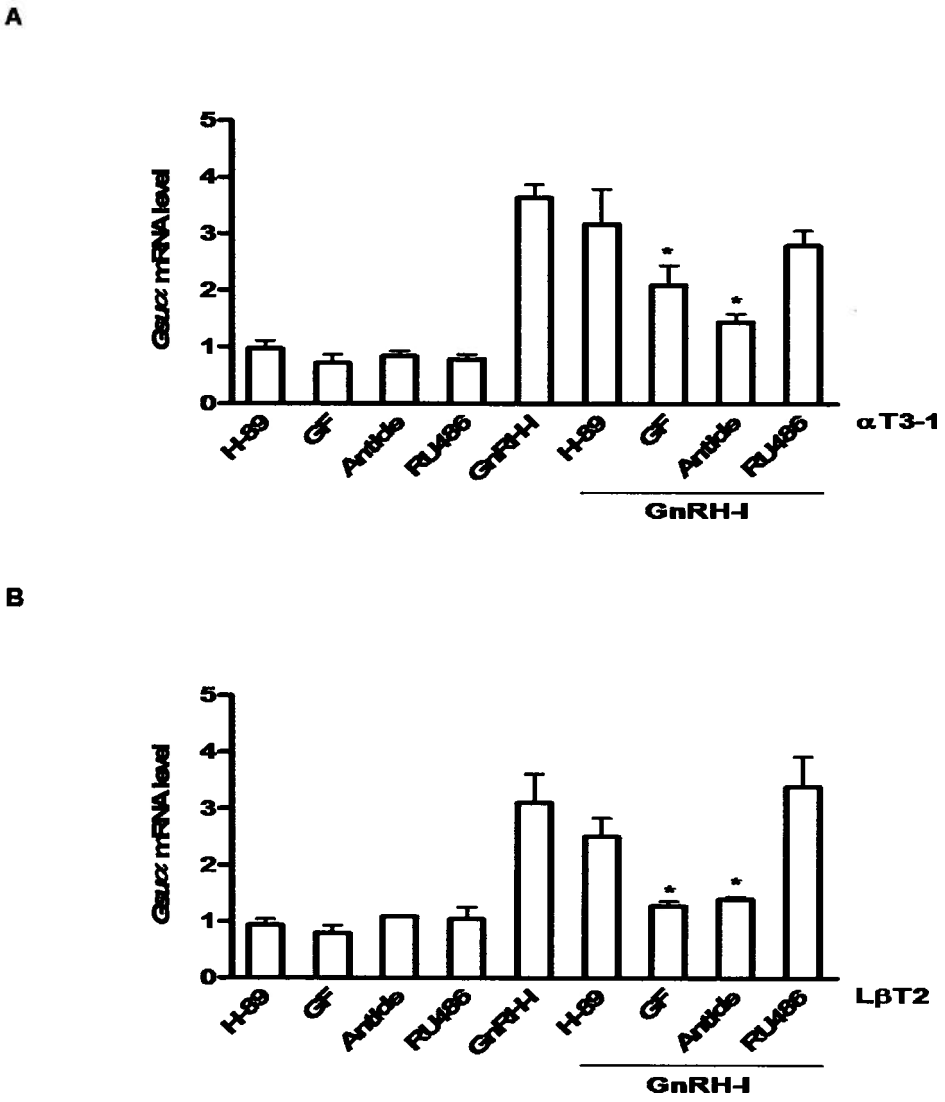
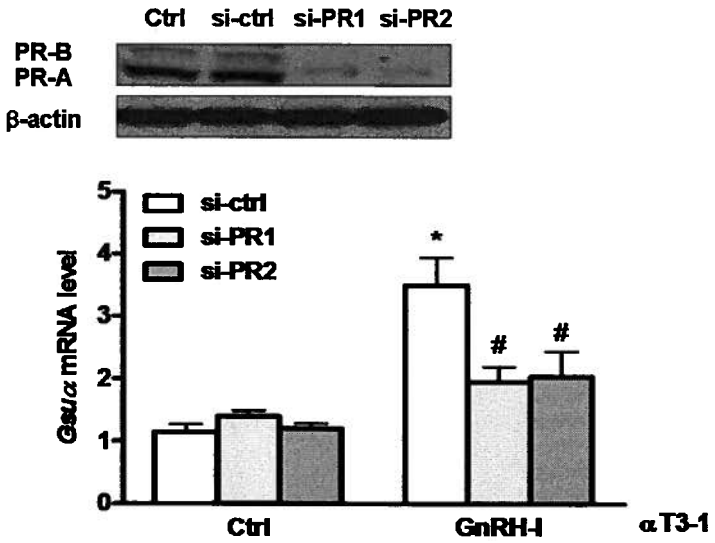


Figure 3.4 PKC inhibitors reduce GnRH-I induced increases in *Gsuα* mRNA levels in αT3-1 and LβT2 cells.

A and B, after 2 days incubation in the presence of 0.2 nM E_2, the cells were treated with 10 nM GnRH-I alone, or pre-treated with 10 μM H89 (PKA inhibitor), 10 μM GF (GF109203X, PKC inhibitor), 10 nM antide, or 1 μM RU486 over a 24 h time course. Total RNA was isolated and subjected to real-time PCR to determine the *Gsuα* mRNA level. Results are expressed as mean ± SEM (i.e., *Gsuα* mRNA level) of three independent experiments. *: $P<0.05$ compared to GnRH-I treatment alone.

A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si-ctrl
si-PR1
si-PR2
PR-B
PR-A
β-actin
si-ctrl
si-PR1
si-PR2
Gsuα mRNA level
0
1
2
3
4
5
*
#
#
Ctrl
GnRH-I
αT3-1

B

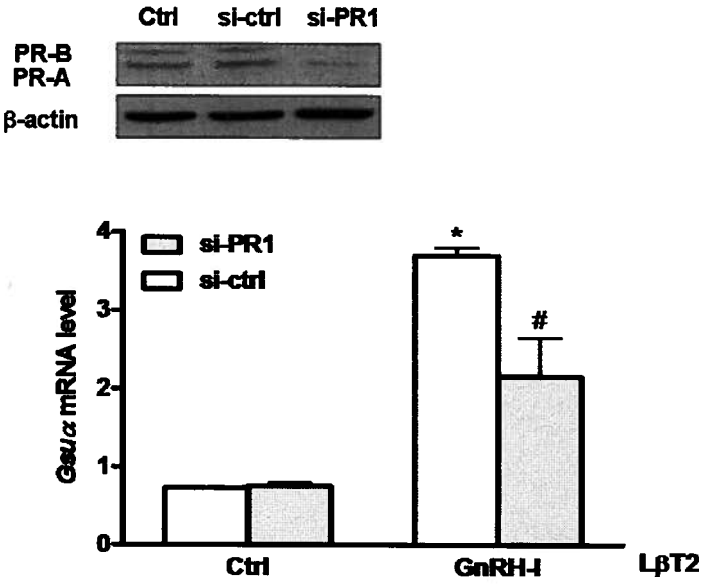

Ctrl
si-ctrl
si-PR1
PR-B
PR-A
β-actin
si-PR1
si-ctrl
Gsuα mRNA level
0
1
2
3
4
*
#
Ctrl
GnRH-I
LβT2

Figure 3.5 GnRH-I-modulated *Gsuα* gene expression requires the PR.

A and B, αT3-1 (A) and LβT2 (B) cells were transfected with 150 nM control siRNA (si-ctrl) or siRNAs for PR (si-PR1 and si-PR2 in αT3-1 cells, si-PR1 in LβT2 cells) for 48 h. The efficiency of the siRNAs was tested by immunoblotting (upper panel of A and B). To determine the role of PR in GnRH-I regulated *Gsuα* mRNA levels, transient transfection was performed using the above conditions and total RNA after GnRH-I treatment was isolated and subjected to real-time PCR. Results are expressed as mean ± SEM (i.e., *Gsuα* mRNA level) of three independent experiments. *: $P<0.05$ compared to cells transfected with an siRNA control (si-ctrl). #: $P<0.05$ compared to cells transfected with an siRNA control and followed by GnRH-I treatment.

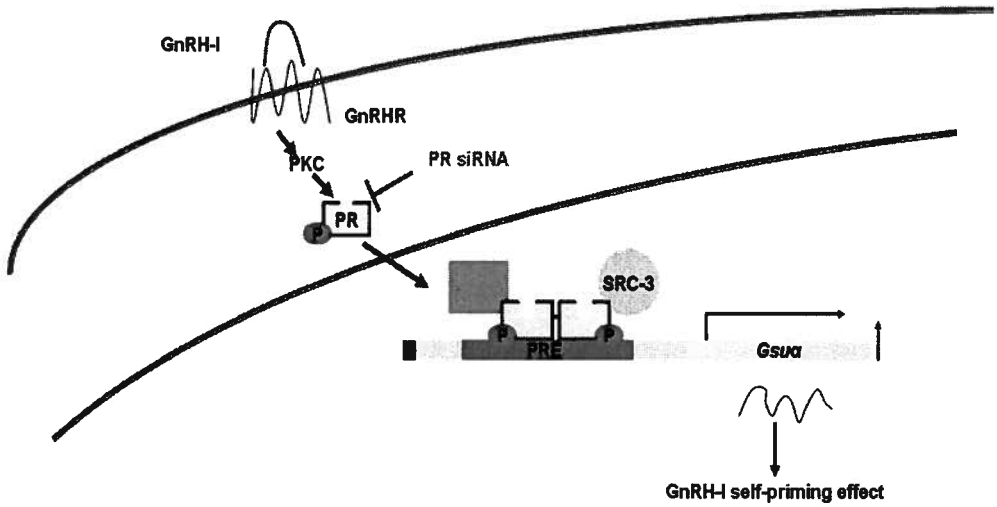


Figure 3.6 GnRH-I-mediated activation of PR contributes to *Gsuα* expression in mouse gonadotrophs

After binding with GnRHR, GnRH-I actives PKC signaling pathway and mediates phosphorylation of PR in mouse pituitary cells. These result in PR association with SRC-3, and that co-recruitment of PR and SRC-3 to a PRE within the *Gsuα* promoter likely enhances its transcriptional activation, which is known to contribute to GnRH-I self priming effect. The induction of *Gsuα* by GnRH-I is significantly reduced by knockdown of PR using an siRNA approach. GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor; GSUα, gonadotropin α subunit; PR, progesterone receptor; PRE, progesterone-response element; PKC, protein kinase C; SRC-3, steroid receptor coactivator.

Chapter 4 Cross-talk between GnRH and PR in the induction of *Gnrhr* in mouse gonadotrophs

4.1 Introduction

GnRH-I plays a vital role in the regulation of mammalian reproductive function. The responsiveness of the pituitary to GnRH-I relies on the amounts of GnRHR on the cell surface. Pituitary *Gnrhr* gene expression is dynamically modulated by GnRH-I pulses and concentration in rat: the highest expression level of *Gnrhr* in the pituitary has been reported to be associated with a 30 min GnRH-I pulse frequency, which leads the optimum synthesis and release of LH, while lower expression of *Gnrhr* is observed with a 2 h GnRH-I pulse frequency, which is related to optimum synthesis and release of FSH (138, 365). Continuous exposure to high concentrations of GnRH-I results in down-regulation of *Gnrhr* mRNA levels (49). The difference in the *Gnrhr* mRNA levels influenced by high and low frequency GnRH-I pulses is approximately 2-3 fold (139). In mouse gonadotroph αT3-1 cells, the response of *Gnrhr* to GnRH-I seems controversial. One study has observed that GnRH-I increases mouse *Gnrhr* promoter activity in a reporter gene assay (326). However, other studies find no changes in *Gnrhr* mRNA levels in response to continuous exposure to GnRH-I (366), or time- and concentration-dependent decreases in the levels of *Gnrhr* mRNA in response to GnRH-I (367). The disparity among these studies may be due to the difference of cell culture conditions and the duration of GnRH stimulation.

In terms of the mechanisms involved in the regulation of *Gnrhr* by GnRH-I treatments, deletion, mutation and functional transfection studies in the murine αT3-1 cell line have shown that the responsiveness of the mouse *Gnrhr* gene to GnRH-I is localized to

two distinct DNA elements including an AP-1 binding site, which appears to be necessary and sufficient to mediate a full GnRH-I response. In this regard, GnRH-I rapidly induces a member of the Fos/Jun heterodimer to form complexes with AP-1 binding site, and this is found to involve the PKC signal transduction pathway (326).

The PR is a member of the steroid hormone receptor superfamily. It has two main isoforms obtained from the transcription of an individual gene from two separate promoters, a full length PR-B, and N-terminally-truncated PR-A lacking the first 164 amino acids of PR-B. Ligand activation of PR is well characterized (276). In general, PRs are complexed with hsp in the absence of the ligand. After binding with P4, PRs exhibit conformational changes leading to hsp dissociation, receptor phosphorylation, dimerization, loading to PRE, and induce subsequent gene transcription (278). On the other hand, PR may alternatively change gene expression by tethering to other transcription factors such as AP-1, SP-1, or STAT (270, 281-283).

Ligand-independent activation of PR and glucocorticoid receptor (GR) by GnRH-I has been identified in gonadotrophs. In rodent pituitary cells, cross-talk between GnRH-I and PR is believed to play a role in the GnRH-I self-priming effect (21, 250, 358, 359), which serves to induce the preovulatory LH surge. The GnRH-I self-priming effect depends on PR, as it is completely absent in *Pr* knock-out mice (13). Previous data in our laboratory have indicated that in αT3-1 and LβT2 cells, GnRH-I activates PR in a ligand-independent manner and promotes the accumulation of *Gsuα* mRNA (21, 368). This likely contributes to the self-priming effect of GnRH-I during the follicular phase of the menstrual cycle and also the proestrus phase of mouse estrous cycle. Furthermore, GnRH-I also activates PR and promotes the accumulation of *Fshb* mRNA in LβT2 cells. This is suggested to contribute to the enhancement of FSH levels during the luteal to follicular phase transition, which is

important for the selection and maturation of dominant follicles (302). In LβT2 cells, transactivation of GR by GnRH-I contributes to *Gnrhr* expression. GnRH-I and dexamethasone increase mouse *Gnrhr* promoter activity *via* an AP-1 site, and also induce endogenous mouse *Gnrhr* mRNA expression. GR is required because knocking down endogenous GR levels by siRNA reduces both the dexamethasone and GnRH-I effects. In addition, GnRH-I and dexamethasone up-regulate GR nuclear translocation, and ChIP assays demonstrate that GnRH-I and dexamethasone enhance the interaction of the GR with the AP-1 binding site on the mouse *Gnrhr* promoter (333).

The murine *Gnrhr* promoter contains an AP-1 binding site (326, 333). We confirmed this by using the bioinformatic analysis tool, Genomatix, and also found a potential PRE in the promoter. Since GnRH-I activates luciferase reporter genes under the control of PRE and AP-1 binding sites in mouse gonadotrophs (21, 302, 333, 368), and there is cross-talk between GnRH-I and PR (21, 302, 368), we set out to determine whether the ligand-independent activation of PR by GnRH-I contributes to the induction of mouse *Gnrhr* gene using mouse pituitary cell lines.

4.2 Materials and methods

4.2.1 Cells and cell culture

The mouse gonadotroph-derived αT3-1 (341) and LβT2 (342) cell lines were kindly provided by Dr. P.L. Melon (Department of Reproductive Medicine, University of California, San Diego, CA) and maintained in monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin

(Invitrogen, Burlington, ON) in humidified 5% CO_2, 95% air at 37 C. The cells were passaged when they grew approximately 90% confluence, utilizing a trypsin/EDTA solution (0.05% trypsin, 0.5 mM EDTA). Cells were maintained in phenol-red free medium containing charcoal-treated FBS for four days prior to experiments.

4.2.2 Plasmids, siRNA and transient transfection assay

A fusion construct prepared by ligation of the 1.2-kb 5'-flanking region of the mouse *Gnrhr* gene (designated -1164/+62 bp) into the luciferase reporter plasmid, pXP2, was generously provided by Dr. U. B. Kaiser (Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts) (326, 369). The pAP1-luciferase plasmid which contains seven copies of a consensus AP-1 site was obtained from Stratagene (La Jolla, CA). The siRNAs for PR and a nonspecific siRNA (negative control, si-ctrl) were obtained from Qiagen Inc. (Mississauga, ON, Canada).

Transient transfections of the mouse *Gnrhr*-luciferase, pAP1-luciferase reporter genes, or siRNA were performed using Lipofectamine 2000 (Invitrogen, Burlington, ON) together with a RSV-lacZ plasmids to correct for transfection efficiencies. After seeding in six-well plates for 24 h, the cells were transfected with 1 μg of mouse *Gnrhr*-luciferase, or pAP1-luciferase reporter genes, together with 0.5 μg of RSV-lacZ. After set times of culture, cellular lysates were obtained by adding 150 μl reporter lysis buffer (Promega Corp., San Luis Obispo, CA) which were assayed for luciferase activity using a Lumat LB 9507 luminometer (EG&G, Berthold, Germany). β-Galactosidase activity was also examined using a β-galactosidase enzyme assay system (Promega Corp.) to normalize for transfection efficiencies. Promoter activity was calculated as luciferase/β-galactosidase activity.

4.2.3 Immunoblotting

Equal amounts of protein were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking, the membranes were incubated overnight at 4 C with specific antibodies against PR A/B and actin (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies were incubated with the membranes. After washing, immunoblots were examined using the ECL chemiluminescent system (Amersham Pharmacia Biotech, Piscataway, NJ) followed by exposure to Kodak X-Omat film.

4.2.4 Real-time RT-PCR

Total RNA was extracted from cell cultures using Trizol (Invitrogen, Burlington, Canada). The RNA concentration was calculated based on absorbance at 260 nm. Isolated RNA was reverse transcribed into first-strand cDNA using M-MLV reverse transcriptase (Promega Corp.). The primers for mouse *Gnrhr* mRNA were: sense, 5′-TCTTCTCGCAATGTGTGACC and antisense, 5′-TAGCGAATGCGACTGTCATC. The primers for *Gapdh* mRNA were: sense, 5′-CATGGCCTTCCGTGTTCCTA and antisense, 5′-GCGGCACGTCAGATCCA. Real-time PCR was performed using an ABI prism 7000 Sequence Detection System (PerkinElmer Applied Biosystems). Reactions were set up using SYBR Green PCR Master Mix (PerkinElmer Applied Biosystems). Relative quantification of mouse *Gnrhr* mRNA levels was performed using the comparative cycle threshold method with GAPDH as an endogenous control and with the formula $2^{-\Delta\Delta Ct}$.

4.2.5 Data analysis

Data are presented as the mean ± SEM of at least three independent experiments. Data

were analyzed by one-way ANOVA followed by Tukey's test by using the GraphPad Prism 4 statistical software (GraphPad Software, Inc., San Diego, CA). Statistical significance was defined as $p<0.05$.

4.3 Results

4.3.1 GnRH-I and GnRH-II transiently activate mouse *Gnrhr* in LβT2 cells

After transfection of a mouse *Gnrhr* promoter-luciferase reporter gene, 100 nM GnRH-I and GnRH-II transiently increase luciferase levels by approximately 4 and 2.5 fold, respectively, during 6-12 h in LβT2 cells. There is no apparent difference in the luciferase reporter gene activity after treatment with 100 nM P4 in LβT2 cells (Figure 4.1). In the mouse gonadotroph αT3-1 cell line, 100 nM GnRH-1, GnRH-II or P4 has no effects on mouse *Gnrhr* promoter-luciferase reporter gene (Figure 4.2).

4.3.2 GnRH-I rapidly and transiently activates the AP-1 luciferase gene in LβT2 and αT3-1 cells

We first checked the mouse *Gnrhr* promoter sequence by using the Genomatix program. In accordance with a previous study (326, 333), there is an AP-1 binding site at -338 to -327 bp. We also found a potential PRE in the promoter of *Gnrhr* at -232 to -213 bp. Our previous data have indicated that GnRH-I and GnRH-II enhance PRE-luciferase reporter gene activity in both the LβT2 and αT3-1 cell lines (21, 302, 368).

To examine whether GnRH treatments influence the transcriptional activity of AP-1, LβT2 cells were transfected with a synthetic AP-1-luciferase reporter gene. When cells are treated with 100 nM GnRH-I or GnRH-II, approximately 300 and 150 folds increases,

respectively, in luciferase activity are observed. The much greater response of the AP-1-luciferase reporter gene after treatments with GnRH-I and GnRH-II, compared with that of untreated cells, reflects the presence of seven AP-1 sites in the AP-1-luciferase construct, when compared to only one in the *Gnrhr* promoter. In contrast to the results observed with GnRH-I and GnRH-II, treatment with P4 does not increase the transcriptional activity of the AP-1-luciferase reporter gene, compared with vehicle control in LβT2 cells (Figure 4.3). Similarly, when αT3-1 cells are transfected with the AP-1-luciferase reporter gene, and then treated with GnRH-I, luciferase activity is significantly increased during 6-24 h and reaches its highest level at 12 h, when it is approximately 3.5 fold higher than the control. However, treatments with GnRH-II and P4 do not enhance the AP-1-luciferase reporter gene activity in αT3-1 cells (Figure 4.4).

4.3.3 GnRH-I-induced mouse *Gnrhr* promoter activity requires PR

In the next set of experiments, we used a siRNA approach to assess the role of PR in GnRH-I-modulated mouse *Gnrhr* promoter activity in LβT2 cells. When the PR levels in LβT2 cells are knocked down by a PR siRNA (Figure 4.5 upper panel), significant reductions in both GnRH-I and GnRH-II stimulated mouse *Gnrhr* promoter-luciferase gene activity are observed (Figure 4.5). These suggest that downstream signaling events involving PR are required for the GnRH-I and GnRH-II induction of mouse *Gnrhr* promoter activity.

4.4 Discussion

During the human menstrual cycle and mouse estrous cycle, the biosynthesis and secretion of gonadotropins from the pituitary gonadotrophs are tightly regulated as evidenced

by predictable and reproducible alterations in circulating levels. This relies primarily on GnRH pulse amplitude and frequency, which fluctuates during the rodent estrous cycle and the human menstrual cycle. The responsiveness of pituitary gonadotrophs to GnRH-I correlates directly with variations in GnRHR concentrations on the cell surface which is partially mediated at the level of gene expression (136, 137). In this study, we have found that both GnRH-I and GnRH-II increase mouse *Gnrhr* promoter activity and this may be due to the activation of PR (Figure 4. 6). In the future, we will further verify whether GnRH-I and GnRH-II increase endogenous mouse *Gnrhr* mRNA, and whether these effects depend on the transactivation of PR by using a siRNA approach. Since our previous data indicate that GnRH-I treatment causes phosphorylation of the PR at Ser^{249} through activating PKC signaling pathways, we hypothesize that PKC signaling involves in the induction of mouse *Gnrhr* gene expression. Because SRC-3 has been found to assemble to the PRE in the promoter of *Gsuα* in αT3-1 cells (21), it will be important to determine whether this or other coactivators bind to PR by co-immunoprecipitation. Assembly of the PR to the PRE on a transfected mouse *Gnrhr* promoter, as well as the endogenous mouse *Gnrhr* promoter will be assessed by ChIP assays. Our previous data have indicated that transactivation of ERα by GnRH-I and GnRH-II increases *Fosb* expression (370). Further studies are required to determine whether Fosb and Jun family proteins are recruited to AP-1 binding site in the promoter of *Gnrhr*.

The concentration of GnRHR in the pituitary is regulated by several hormonal factors, most notably by its own ligand. The effects of GnRHR by GnRH-I are cell and species specific. Homologous activation of the mouse *Gnrhr* promoter by GnRH-I involves an AP-1 binding site (123, 326), but previous data from our laboratory have indicated that treatment with GnRH-I results in a concentration- and time-dependent repression of human *type I*

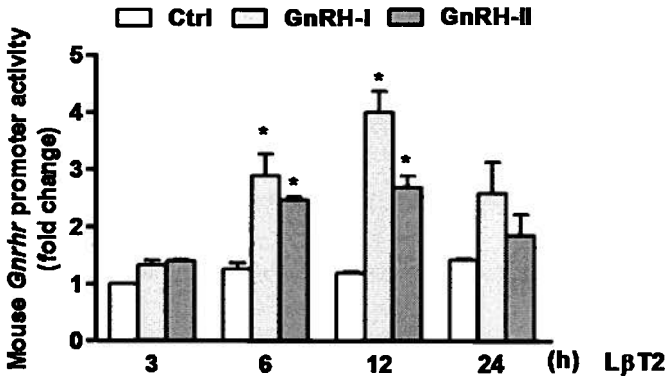
GnRHR promoter activity in αT3-1 cells, which is mediated by the PKC signaling cascade. Subsequent studies have indicated that mutation of an AP-1-like motif abolishes the sensitivity of the promoter to GnRH-I, and electrophoretic mobility shift assays reveal that GnRH-I stimulated c-Fos DNA binding activity in the AP-1-like binding motif is responsible for the downregulation of the human *type I GnRHR* gene transcription (371). In the current study, we have found that GnRH-I and GnRH-II transiently enhance mouse *Gnrhr* promoter activity in LβT2 cells, but not in αT3-1 cells. It is not known if the different experimental paradigms or mechanisms account for differences in the gene responsiveness to GnRH-I in the human and rodent gonadotrophs. However, disparities in *Gnrhr* promoter activities in the two mouse gonadotroph cell lines may be due to differences in their developmental differentiation.

The dynamic changes in GnRHR levels in pituitary gonadotrophs during the estrous cycle (372-374) or after gonadectomy (139, 375) strongly implicate steroid hormones in the regulation of *Gnrhr* gene expression. In αT3-1 cells, P4 represses human *type I GnRHR* promoter activity in concentration- and time-dependent manners, while P4 stimulates the human *type I GnRHR* promoter in JEG-3 cells. Moreover, mutation of an imperfect PRE in the human *type I GnRHR* promoter attenuates the P4 effect on its transcriptional activity (157). Overexpression of two human PR isoforms also indicates that PR-B is a fundamental modulator in mediating the down-regulatory effect of P4 in this context. However, in JEG-3 cells the two PR isoforms have differential roles such that PR-B stimulates while PR-A inhibits the activity of the human *type I GnRHR* promoter (157). We have found that P4 has no effect on mouse *Gnrhr* promoter activity in the mouse gonadotroph cell lines, indicating that ligand-dependent activation of PR in mouse gonadotrophs may not be involved in mouse *Gnrhr* expression.

A few studies suggest that ligand-independent activation of the PR occurs in mouse pituitary cells (21, 302, 368). Steroid receptors other than PR can also be activated in a ligand-independent manner. For instance, growth factors and GnRH subtypes transactivate the ERα *via* specific signaling pathways and phosphorylation of ERα at Ser^{118} and Ser^{167} (226, 370). A recent study has found that after GnRH-I binding with GnRHR, cross talk occurs with the GR, inducing GR phosphorylation at Ser^{234} *via* PKC and MAPK signaling, resulting nuclear translocation and transactivation of a glucocorticoid response element in mouse LβT2 cells (333). By using siRNA technology in the current study, we have first indicated that the PR is required for transcriptional regulation of mouse *Gnrhr* promoter activity. We also searched the human *GnRHR* promoter using a bioinformatic approach, and in accordance with a previous study (157), we have found that it contains AP-1 and PR binding sites. Whether activation the PR by GnRH and subsequent loading of the PR at PRE and/or AP-1 binding site occur in the human pituitary which regulates *GnRHR* gene expression require further study.

In conclusion, we demonstrate for the first time that GnRH-I and GnRH-II increase mouse *Gnrhr* promoter activity *via* ligand independent activation of the PR in LβT2 cells (Figure 4. 6). Because GnRH-I regulates many genes in gonadotrophs, this represents a mechanism by which the activation of PR by GnRH-I may modulate the expression of several GnRH-I and PR target genes. The induction of *Gnrhr* expression by the GnRH-mediated activation of the PR may be involved in the self-priming effect of GnRH-I before the LH surge. It may also have broader physiological implications because GnRHR and PR are widely expressed in many extrapituitary tissues.

A



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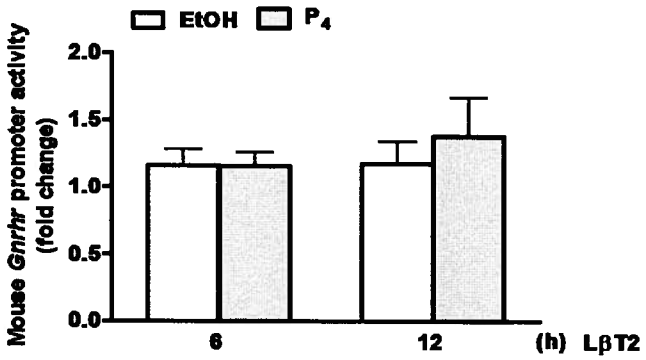
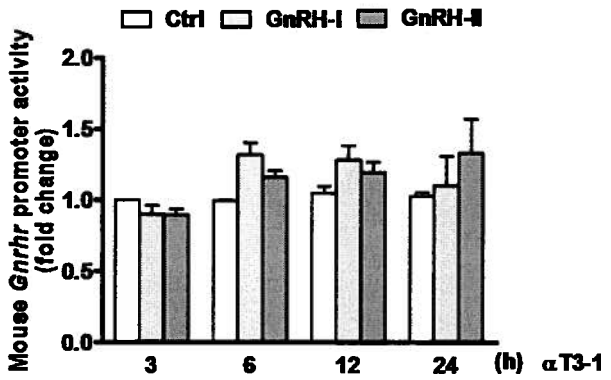


Figure 4.1 GnRH-I and GnRH-II activate mouse *Gnrhr* in LβT2 cells.

The mouse *Gnrhr* promoter-luciferase reporter gene together with a (RSV)-lacZ plasmid were transiently transfected into LβT2 cells using Lipofectamine 2000. The cells were either treated with 100 nM GnRH-I, GnRH-II (A) or 100 nM P4 (B) over a 24 h time course. The cell lysates were assayed for luciferase activity and measurements of β-galactosidase activity as a control. Results are shown as mean ± SEM fold change in mouse *Gnrhr* promoter activity expressed as luciferase activity/β-galactosidase activity of three independent experiments. *: $p<0.05$ compared to untreated control value set at 1 (Ctrl).

A



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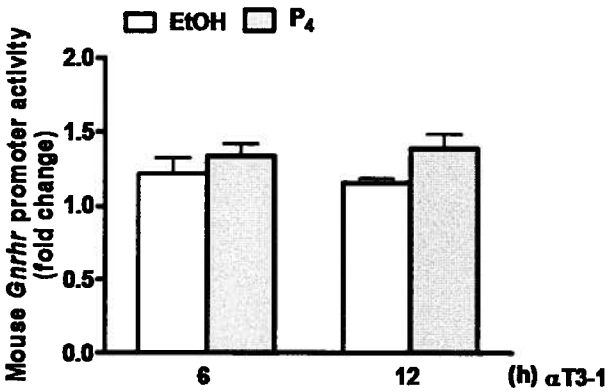


Figure 4.2 GnRH-I and GnRH-II have no effects on mouse *Gnrhr*-luciferase promoter activity in the αT3-1 cell line.

The mouse *Gnrhr* promoter-luciferase reporter gene together with a (RSV)-lacZ plasmid were transiently transfected into αT3-1cells using Lipofectamine 2000. The cells were either treated with 100 nM GnRH-I, GnRH-II (A) or 100 nM P4 (B) over a 24 h time course. The cell lysates were assayed for luciferase activity and measurements of β-galactosidase activity as a control. Results are shown as mean ± SEM fold change in mouse *Gnrhr* promoter activity expressed as luciferase activity/β-galactosidase activity of three independent experiments.

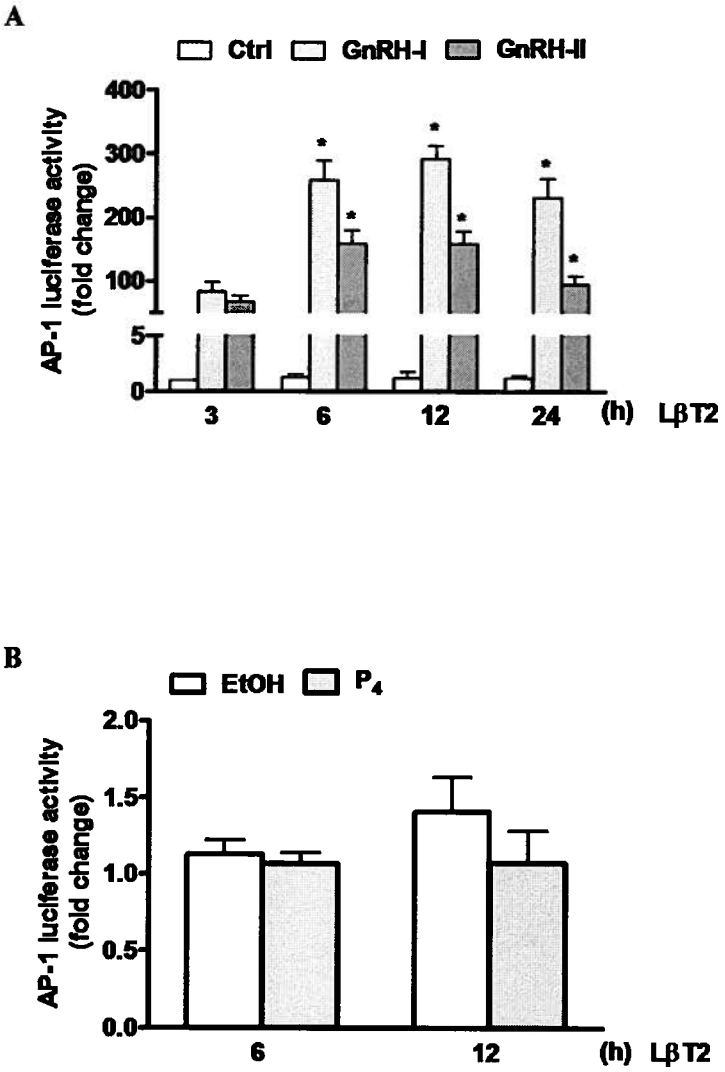
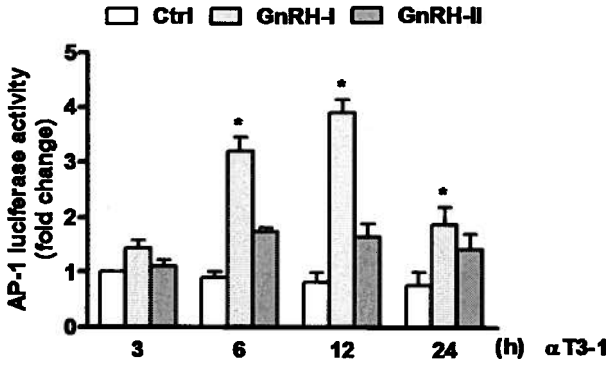


Figure 4.3 GnRH-I and GnRH-II activate AP-1 luciferase activity in LβT2 cells.

The luciferase reporter gene under the control of a promoter containing seven AP-1 binding sites together with a (RSV)-lacZ plasmid was transiently transfected into LβT2 cells by Lipofectamine 2000. The cells were either treated with 100 nM GnRH-I, GnRH-II (A) or 100 nM P4 (B) over a 24 h time course. The cell lysates were assayed for luciferase activity and measurements of β-galactosidase activity as a control. Results are shown as mean ± SEM fold change in AP-1 promoter activity expressed as luciferase activity/β-galactosidase activity of three independent experiments. *: $p<0.05$ compared to untreated control value set at 1 (Ctrl).

A



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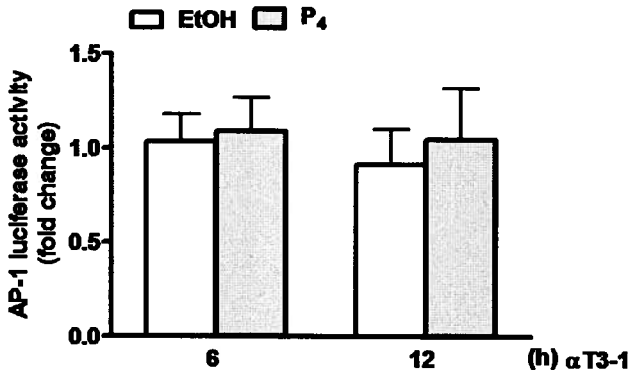


Figure 4.4 GnRH-I rapidly and transiently activates AP-1 luciferase activity in αT3-1 cells. The luciferase reporter gene under the control of a promoter containing seven AP-1 binding sites together with a (RSV)-lacZ plasmid was transiently transfected into αT3-1 cells by Lipofectamine 2000. The cells were either treated with 100 nM GnRH-I, GnRH-II (A) or 100 nM P4 (B) over a 24 h time course. The cell lysates were assayed for luciferase activity and measurements of β-galactosidase activity as a control. Results are shown as mean ± SEM fold change in AP-1 promoter activity expressed as luciferase activity/β-galactosidase activity of three independent experiments. *: $p<0.05$ compared to untreated control value set at 1 (Ctrl).

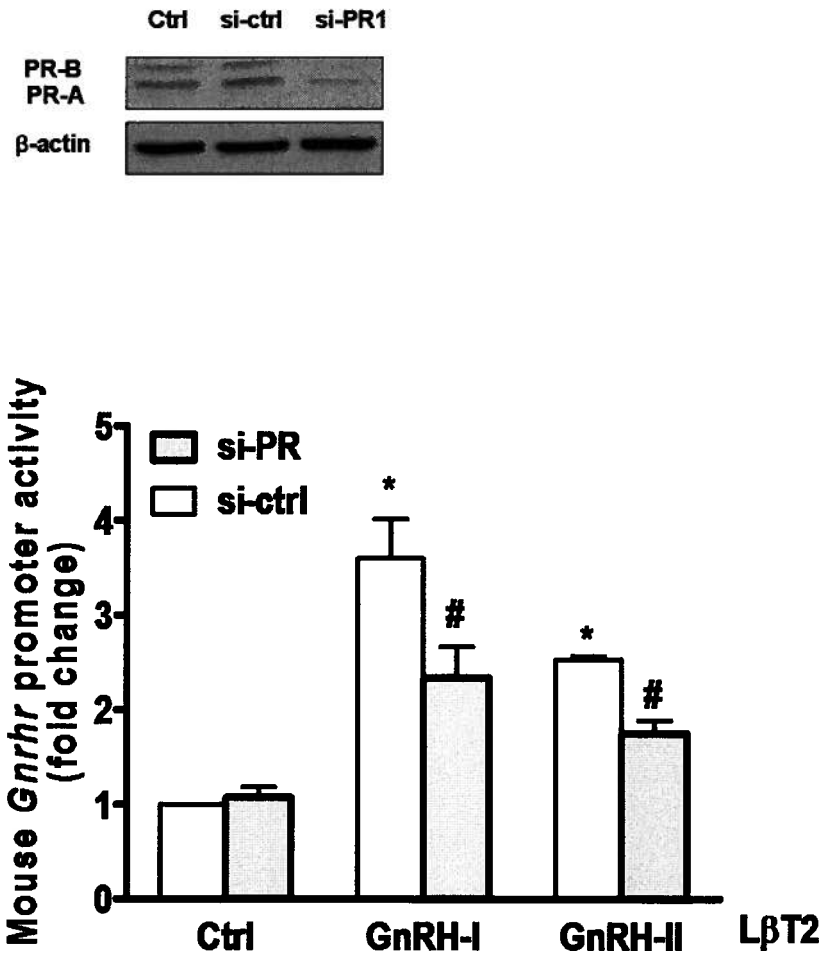


Figure 4.5 GnRH-I-induced mouse *Gnrhr* promoter activity requires PR.

LβT2 cells were cotransfected with the mouse *Gnrhr* promoter-luciferase reporter gene and a (RSV)-lacZ plasmid, with control siRNA (si-ctrl) or an siRNA for PR (si-PR). The efficiency of the siRNA was tested by immunoblotting for PR (upper panel). After treatment with 100 nM GnRH-I or GnRH-II for 6 h, the cell lysates were collected for luciferase assay and measurements of β-galactosidase activity as a control. Results are shown as mean ± SEM fold change in mouse *Gnrhr* promoter activity expressed as luciferase activity/β-galactosidase activity of three independent experiments. *: $P<0.05$ compared to cells transfected with an siRNA control (si-ctrl). #: $P<0.05$ compared to cells transfected with an siRNA control and followed by the respective treatment.

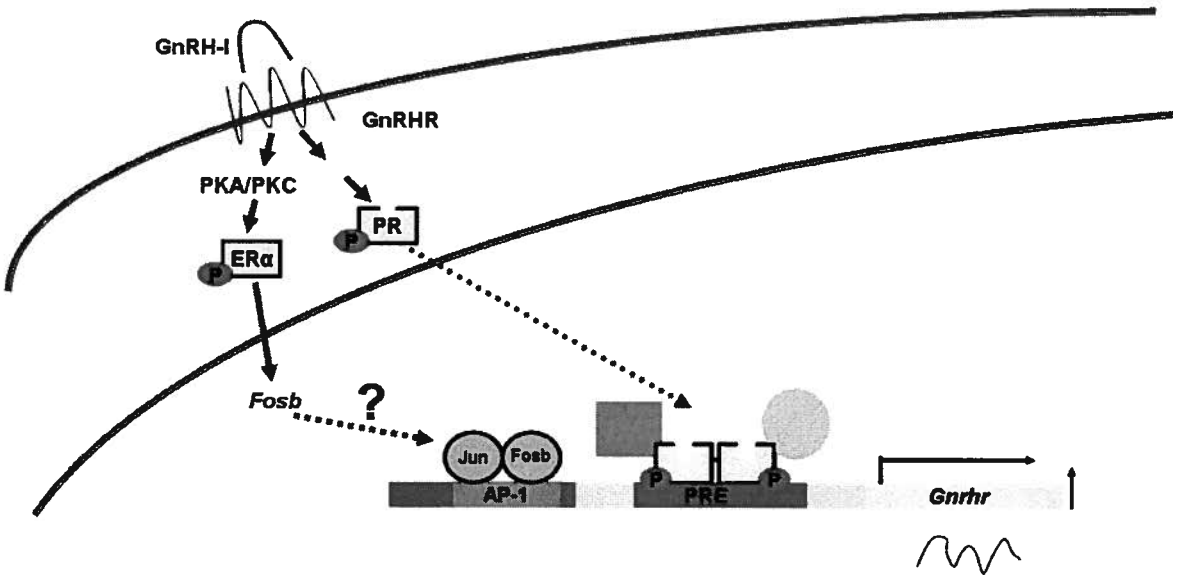


Figure 4.6 Cross-talk between GnRH-I and PR in the induction of *Gnrhr* promoter activity in mouse gonadotrophs.

After binding with GnRHR, GnRH-I stimulates PR phosphorylation, loading to the PRE, and induces *Gnrhr* expression. On the other hand, GnRH-I also activates ERα after stimulating PKA and PKC signaling pathways and increases *Fosb* expression. Whether Fosb and Jun family protein form heterodimer and load to AP-1 binding site to increase *Gnrhr* expression need further study. AP-1, activator protein-1; ER, estrogen receptor; GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor; PKA, protein kinase A; PKC, protein kinase C; PR, progesterone receptor; PRE, Progesterone-response element.

Chapter 5 Conclusion and future work

The molecular events and mechanisms that control the dynamic flux of the hypothalamic-pituitary-gonadal axis are of particular physiological significance. Some of these mechanisms involve GnRH-I, a decapeptide that mediates reproductive competence through the stimulation of gonadotropin synthesis and secretion from pituitary gonadotrophs. Many GnRH-I agonists and antagonists have been developed for a variety of remedies in assisted reproductive technologies, gynecological diseases, and cancer treatment. Cross-talk between GnRH and steroid hormone receptors has been recognized from previous studies (20, 21, 302, 333).

In this project, we have studied specific mechanisms and provide further evidence that GnRH activates both ERα and PR in a ligand-independent manner in mouse gonadotrophs. In the LβT2 cell line, we have found that both GnRH-I and GnRH-II activate the ERE-luciferase reporter gene, and that GnRH-I is consistently more potent in this regard. In addition, GnRH-I and GnRH-II phosphorylate ERα at Ser^{118} in the nucleus and at Ser^{167} in both nucleus and cytoplasm, and enhance ERα binding to its coactivator PCAF. Most importantly, this transactivation of ERα by GnRH-I and GnRH-II are involved in the rapid and transient induction of the immediate early response gene, *Fosb*, since co-treatment with ERα siRNA or PCAF siRNA attenuates this induction, and GnRH-I and GnRH-II induce the assembly of ERα and PCAF to an ERE within the *Fosb* promoter.

We have also found that GnRH-I activates a PRE-luciferase reporter gene and endogenous *Gsuα* gene expression in both the αT3-1 and LβT2 pituitary cell lines. PKA and PKC inhibitors attenuate the GnRH-I induced up-regulation of the PRE-luciferase reporter gene in both cell lines, while only the PKC inhibitor significantly reduces the GnRH-I

stimulated increase in *Gsuα* mRNA levels. Knockdowning endogenous PR levels using an siRNA approach also significantly reduces the GnRH-I activation of *Gsuα* mRNA levels, and this provides direct evidence that the activation of PR by GnRH-I contributes to *Gsuα* expression. Furthermore, both GnRH-I and GnRH-II promote the mouse *Gnrhr*-luciferase promoter activity in LβT2 cells. This is due to the activation of the PR, because a PR siRNA significantly reduces both the GnRH-I and GnRH-II effects on mouse *Gnrhr* promoter activity. We therefore conclude that the induction of *Fosb* and *Gsuα* expression, as well as *Gnrhr* promoter activity by GnRH-I in mouse gonadotrophs is mediated by the ligand-independent activation of ERα and PR, respectively.

5.1 Physiological relevance of the experimental conditions design

Primary pituitary cell cultures contain only about 5% gonadotrophs and are difficult to manipulate *in vitro*. Currently there are no human gonadotroph cell lines available. The development of immortalized mouse pituitary gonadotroph cell lines, including αT3-1 and LβT2 cells, has allowed analyses of the mechanisms regulating gonadotropin genes by GnRH and nuclear receptors within a single population of gonadotroph cells. Both αT3-1 and LβT2 cell lines were derived from transgenic mice pituitary tumors (341, 342). The αT3-1 cell line only expresses *Gsuα* gene but not the gonadotropin β subunit gene, and they represent gonadotrophs present at mouse embryonic day 13.5, and are therefore regarded as relatively immature. The gonadotroph-derived LβT2 cell line expresses both the common *Gsuα* subunit gene and the unique β subunit gene , as observed in mouse gonadotrophs by embryonic day 17.5, rendering it more physiologically relevant to *in vivo* systems in mature animals (5, 23, 343, 361, 362, 376).

We assessed the relative transfection efficiencies by using siGLO transfection indicator or an EGFP expression vector in αT3-1 and LβT2 cells before the transfection experiments. The transfection efficiencies after 48 hours for siRNA were evaluated by the fluorescence signal and were approximately 70% in αT3-1, and approximately 60% in LβT2 cells. The transfection efficiencies for DNA were similar to siRNA in both cell lines (data not shown). From these pilot studies we have modified the transfection conditions such as cell confluence and transfection duration in these two cell lines, which we believe represent ideal models for the study of cross talk between GnRH and steroid hormone receptors.

5.2 GnRH activates specific signaling pathways in the transactivation of ERα and PR

The type I GnRHR is a special type of G protein-coupled receptor that primarily uses the Gq protein for its downstream cascades, and this activates multiple signaling pathways that in turn regulate the transcription of several genes including the gonadotropin subunit genes. Elucidating the coordination of different GnRH-induced signaling pathways is of considerable interest in understanding the mechanism of action of GnRH in the pituitary. In our studies, we have found that inhibitors of the PKA, PKC, PI3K and MAPK signaling cascades significantly reduce the induction of an ERE-reporter gene by GnRH-I and GnRH-II (data not shown). Only inhibitors of PKA and PKC signaling reduce the rapid and transient activation of *Fosb* gene expression by both GnRH subtypes in LβT2 cells. The GnRH-I induction of a PRE-luciferase reporter gene also involves the PKC and PKA signaling pathways. Interestingly, only the PKC inhibitor significantly impairs the increase of *Gsuα* mRNA levels by GnRH-I treatment in both the αT3-1 and LβT2 cells. Previous studies in our laboratory have indicated that the PKC inhibitor, GF109203X, decreases the GnRH-I

mediated phosphorylation of the PR at Ser^{249} in both the αT3-1 and LβT2 cells (302). This may explain why only the PKC inhibitor attenuates enhancement of *Gsuα* mRNA levels by GnRH-I.

Normally inhibitor studies are used to define the specificity of signaling cascades, and for this purpose a number of inhibitors of specific pathways are frequently examined to provide confidence that particular signaling pathways are involved. Only single inhibitors of PKC, PKA, as well as single antagonists of type I GnRHR and PR signaling were used in my studies of the cross talk between GnRH-I and PR. This is because PKC signaling in GnRH-I induced *Gsuα* expression has been well-documented previously, and PKA signaling did not mediate the effects of GnRH-I on *Gsuα* mRNA levels in my studies. Thus, the use of multiple inhibitors was considered unnecessary. In addition to the use of RU486 and antide, which are well-know antagonists of the PR and type I GnRHR, respectively, we also used siRNA to knock down PR levels and this resulted in a significant reduction of *Gsuα* levels after GnRH-I treatment. In an earlier study, we also used an siRNA to knock down endogenous GnRHR levels in αT3-1 cells, and this significantly reduced GnRH-I enhanced PRE-luciferase reporter gene activity compared to control cells with normal levels of *Gnrhr* (302).

5.3 GnRH-I has more robust effects than GnRH-II in mouse gonadotrophs

Both GnRH-I and GnRH-II perform their functions by binding the same receptor in mouse, i.e., GnRHR. The physiological importance of GnRH-II remains largely unknown in the pituitary. Although our results have shown that the effects of GnRH-I and GnRH-II are similar in most experiments, we routinely tested both GnRH subtypes in terms of the

ligand-independent activation of ERα to increase our level of confidence that GnRH-I consistently has more robust effects on all the GnRHR-mediated responses we have studied. The induction of an ERE-luciferase reporter gene by GnRH-I is greater compared to that mediated by GnRH-II, as shown in Figure 2.1A. However, in Figure 2.1C, the magnitude of the induction at 100 nM of each ligand is comparable. This difference is due to the relatively large error bars in Figure 2.1A, but in both experiments the induction of the ERE-luciferase reporter gene by 100 nM GnRH-I or GnRH-II is significant. In a dose-dependent experiment, a maximum response is obtained with 10 nM GnRH-I and 100 nM GnRH-II. In studies of the ligand-independent activation of ERα by GnRH-I and GnRH-II, we utilized the same concentration of GnRH-I and GnRH-II, i.e., 100 nM. We have confirmed that GnRH-I has stronger effects than GnRH-II on the ligand-independent activation of ERα, as well as of PR in the previous study (21). Therefore, in a subsequent study of how the cross-talk between GnRH and PR might influence *Gsuα* mRNA levels, we only tested GnRH-I. Based on the dose-dependent study, we utilized a low dose of GnRH-I, i.e., 10 nM, which more closely represents its physiological concentration in the pituitary (377).

5.4 Single mutations (S118A or S167A) of ERα phosphorylation sites are not sufficient to affect Fosb protein levels

The phosphorylation of ERα is induced by its ligand and by other factors that influence its nuclear translocation, dimerization, and transcriptional activity. In this project, we have ascertained that treatments of LβT2 cells with either GnRH-I or GnRH-II stimulate the phosphorylation of ERα at Ser^{167} in both the nucleus and cytoplasm, as well as the phosphorylation of ERα at Ser^{118} in the nucleus. However, E_2 does not significantly increase

the phosphorylation of ERα at Ser^{167}. We next tried to study whether phosphorylation at these two sides was important for the induction of the endogenous *Fosb* gene by substituting alanine for the serine residues by site-directed mutagenesis. After 24-72 h transfection of the wild-type ERα, or the ERα S118A or S167A mutant into LβT2 cells, western blot analysis shows a dramatic increase in ERα levels (Figure 5.1). Surprisingly, when treated with either GnRH-I or GnRH-II, the S118A or S167A ERα mutants do not significantly affect Fosb levels compared to the cells transfected with the wild-type ERα (Figure 5.1).

We then determined the lowest concentrations of GnRH-I and GnRH-II that induced *Fosb* mRNA levels by real-time PCR. The results show that 1 nM of GnRH-I and 10 nM of GnRH-II significantly increase the *Fosb* mRNA levels (Figure 5.2). However, when the cells are treated with lower concentrations of GnRH-I or GnRH-II (1 nM and 10 nM, respectively), the S118A or S167A ERα mutants still have no effect on *Fosb* mRNA levels compared to the wild-type ERα (data not shown). This lack of effect may be masked by the basal levels of ERα in LβT2 cells which are sufficient to induce endogenous *Fosb* gene expression by GnRH. Furthermore, although Ser^{118} and Ser^{167} are the most common phosphorylation sites of ERα, other sites may still be required for GnRH-induced *Fosb* expression in mouse gonadotrophs. However, we have not studied other phosphorylation sites due to the unavailability of antibodies. Further investigations to assess the importance of other phosphorylation sites using site-directed mutagenesis therefore need to be conducted. It can also be assumed that the phosphorylation of only one site within ERα will not influence *Fosb* gene expression by GnRH, and to confirm this both phosphorylation sites may need to be mutated.

5.5 PCAF regulates gene expression in the pituitary after interactions with ERα.

Nuclear receptor coactivators are necessary for the proficient transcriptional modulation by nuclear receptors (378, 379). The importance of these coactivators has been noted in several diseases, such as cancer and some neurological disorders (380). The enrollment of coactivators is a rate-limiting step in the steroid receptor-induced gene transcription in *in vitro* studies (378, 381), and coactivator acetylation, methylation, phosphorylation and chromatin remodeling influence nuclear receptor mediated transcriptional activities (378). PCAF belongs to the Gcn5 related *N*-acetyl transferase superfamily of acetyltransferases and has high similarity with GCN5 over the whole sequence (220). The mechanisms underlying the ligand-dependent and -independent activation of ERα together with the loading of coactivators at specific promoter sequences to induce target gene expression at the pituitary level remain largely unknown. In this project, GnRH-I and GnRH-II rapidly increase the ligand-independent activation of ERα in LβT2 cells, as well as binding of ERα and PCAF to the ERE in the promoter of the *Fosb* gene. To our knowledge, this is the first time that PCAF has been shown to participate in gene expression in the pituitary after assembly with ERα. Additionally, we have obtained evidence that both GnRH-I and GnRH-II stimulate the assembly of ERα and PCAF to the ERE in a transfected luciferase reporter gene construct (data not shown). The full transcriptional activity of nuclear receptors depends, at least in part, on the recruitment of coactivators. Whether PCAF action involves the activation of other genes after binding with ERα to the ERE in the gene promoter, and the overall importance of this nuclear receptor coactivator in the pituitary require further studies.

5.6 GnRH-I activates PR in a ligand-independent manner to induce *Gsuα* expression

The PR can be activated by both ligand-dependent or ligand-independent mechanisms, and these mechanisms are not mutually exclusive, and mostly likely act in synergy in most cases. In this study we focus on the ligand-independent activation of PR by GnRH-I and its role in the regulation of *Gsuα* expression and mouse *Gnrhr* promoter activity in αT3-1 and LβT2 cells. In both cell lines, the PR can be activated in a ligand-independent manner by GnRH-I.

The self-priming effect of GnRH-I is a powerful servo-mechanism that amplifies the pituitary responsiveness to GnRH-I by several fold. This coordinates the increased pulses of GnRH-I into the hypophysial portal system with increased pituitary LH secretion so that both GnRH-I and LH simultaneously reach a peak just before ovulation. Animal studies have indicated that such potentiating effects of serial GnRH-I stimulation are critical to generating the preovulatory LH surge during folliculogenesis (250). A human study among healthy postmenopausal women examined the kinetic characteristics of gonadotropin release in response to pulses of exogenous GnRH-I and has found that the self-priming effects of GnRH-I exist in the human (382). PR plays a central role in the preovulatory LH surge at the midcycle, and *Pr* knockout mice provide a model for analyzing cellular pathways participating in this function (13). Together with previous data (21), we have provide direct evidence that after binding with type I GnRHR, GnRH-I at a physiological concentration prompts the PKC signaling cascade and then activates PR in the absence of the ligand; this activation induces *Gsuα* mRNA levels after loading to the promoter PRE together with SRC-3. This mechanism likely enhances the self-priming effect of GnRH-I during the follicular phase of the human menstrual cycle and the proestrus phase of the mouse estrous

cycle.

In experiments designed to knockdown the levels of specific endogenous gene products by using siRNA, it is better to utilize two siRNAs directed against the sequence of interest in addition to the control siRNA, in order to authenticate the response. We used a second siRNA for PR in αT3-1 cells. Both siRNAs significantly reduce PR levels and are equally effective in reducing GnRH-I stimulated *Gsuα* mRNA levels. However, we were not able to successfully knock down PR levels with the second siRNA in LβT2 cells, and we were therefore not able to evaluate its effects in LβT2 cells. Furthermore, there are two isoforms of PR, PR-A and PR-B. So far it is still not known whether the ligand-independent activation of PR by GnRH-I relies on PR-A, PR-B, or both. This may require further study by applying specific siRNA for either PRA or PRB.

In addition to the PR knockdown experiments, our findings could be strengthened if PR overexpression is also performed in the presence or absence of GnRH-I. We have since tried to overexpress PR-A and PR-B in both cell lines using an hPR B plasmid provided by Dr. P. Chambon (Institut National de la Santé et de la Recherche Médicale, University Louis Pasteur, Paris, France), and a pOP13-hPR A plasmid from Dr. Graham (University of Sydney Westmead Hospital, Sydney, Australia). However, we have been unable to attain significant over-expression of human PR-A and PR-B in these cell lines. However, since the siRNA-mediated knockdown of PR significantly reduces *Gsuα* mRNA levels stimulated by GnRH-I in both αT3-1 and LβT2 cells, and our previous data have shown that GnRH-I increases assembly of SRC-3 and PR at the PRE in the *Gsuα* promoter (21), we believe our current results provide sufficient evidence that PR is crucial for GnRH-I effects on the gonadotropin α subunit gene.

We have not attempted to mutate the PRE in the *Gsuα* promoter, which would provide

important insight into the role of PR in the transactivation of *Gsuα* by GnRH-I. Nonetheless, our previous ChIP assay data have indicated that GnRH-I increases loading of SRC-3 and PR at the PRE in the *Gsuα* promoter. We have since examined the *Gsuα* promoter sequence amplified in the ChIP assay using the Genomatix program, and found no other non-PRE elements that are known to interact with PR, such as AP-1 sites and Sp1 sites (data not shown). In addition, we show in this study that the stimulation of *Gsuα* gene expression by GnRH-I can be significantly attenuated by PR siRNA. Together, these data provide direct evidence that activation of PR is critical for GnRH-I effects on the *Gsuα* gene.

5.7 The physiological importance of transactivation of ERα and PR by GnRH-I

The significance of growth factors and peptide hormones in the induction of ERα and PR transcriptional activity is still far from being fully understood. One possible explanation is that peptide hormone receptors are maintained at relatively high levels where steroid hormone receptor levels are low, such as in the pituitary. Furthermore, signaling cascades initiated by growth factors and peptide hormones may modulate the transcriptional activity of ligand-occupied nuclear receptors and increase the magnitude of target gene expression. It remains unclear whether the target genes activated by the steroid hormone receptors in response to growth factors and peptide hormones or steroid hormone are identical; it is also unclear whether these genes are preferentially or selectively regulated by a specific pathway (383). The GnRH agonists and antagonists have been widely utilized in *in vitro* fertilization and treatment of carcinoma, including endometrial and breast cancer. A better understanding of the molecular mechanisms underlying the ligand-independent activation of ERα and PR by GnRH will improve our assessment of the physiological function of GnRH and the nuclear

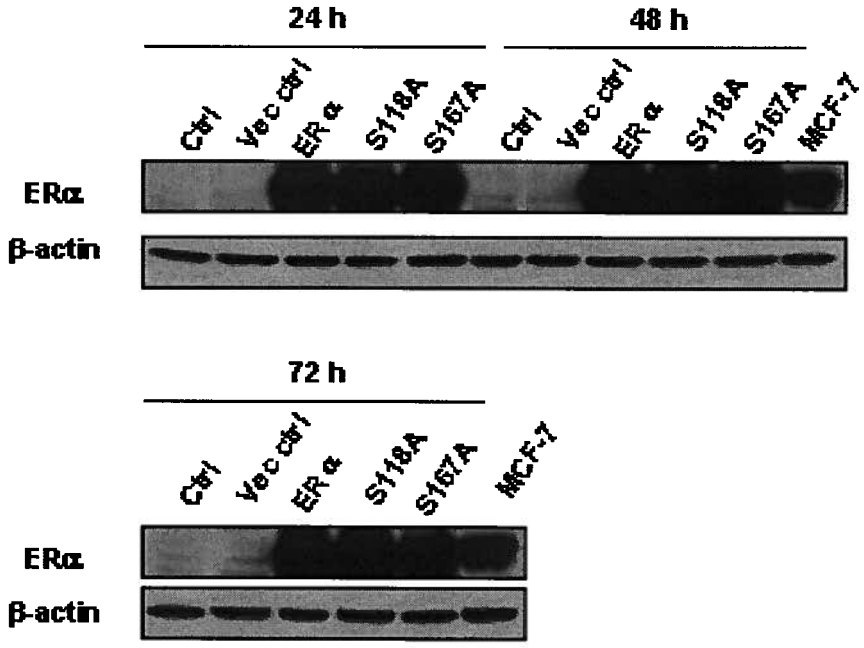
receptors, as well as the therapeutic side effects of GnRH agonists.

The gonadotropins are regulated in a concert with GnRH, steroid hormones and other factors throughout the mouse estrous cycle and human menstrual cycle. During the luteal/follicular transition phase, circulating steroid hormone levels are relatively low. However, during the late follicular phase, increased E_2 levels reach a threshold for a period of time before inducing the LH surge. Our results are the first to demonstrate that in mouse LβT2 pituitary cells, GnRH mediates the phosphorylation of ERα and the co-recruitment of ERα and PCAF to an ERE within the *Fosb* promoter, which enhanced the transcriptional activation. This mechanism likely activates the *Fshb* subunit gene in pituitary cells.

The ligand-independent activation of ERα by GnRH in pituitary cells may be most important under conditions when the GnRH pulse amplitude is high, such as during the transition from luteal to follicular phase of the human menstrual cycle and the proestrus phase of the mouse estrous cycle. Together with previous data from our laboratory (21), we have provided direct evidence that in αT3-1 and LβT2 cells, GnRH-I activates PR in a ligand-independent manner and promotes the accumulation of *Gsuα* mRNA, and this likely contributes to the self-priming effect of GnRH-I during the follicular phase of the human menstrual cycle and the proestrus phase of the mouse estrous cycle. Furthermore, the cross-talk between GnRH and PR has been studied in the context of a mouse *Gnrhr* reporter-luciferase reporter gene in LβT2 cells, indicating that the mechanism of transactivation of PR by GnRH also influences other genes containing PRE. It is also possible that this may be a mechanism of action in the self-priming effect of GnRH-I. Further studies are required to determine whether the activation of PR by GnRH can induce endogenous mouse *Gnrhr* expression, and if the stimulation of Fosb influences *Gnrhr* expression (Figure 5.3 and 5.4). GnRH-I induces the ligand-independent activation of both

ERα and PR to mediate the expression of specific genes in mouse gonadotrophs; the genes are important in a certain period during the human menstrual cycle and mouse estrous cycle. It needs further evaluation that the activation of ERα and PR by GnRH is involved in other gene alterations in the pituitary. Apart from this, physiologically GnRH is released in a pulsatile manner from the hypothalamus. In the current project I have only tested the effects of continuous concentration of GnRH. More detailed studies of GnRH pulse frequency and/or amplitude, or the effects of other intercellular stimuli on the selective activation of ERα or PR are required.

A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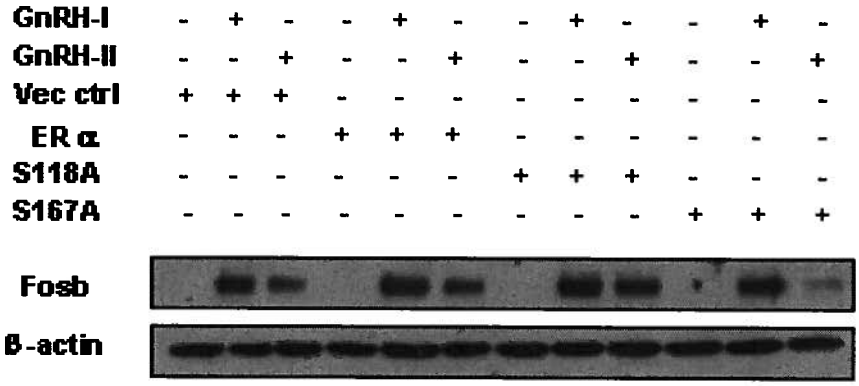
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Fosb
β-actin

Figure 5. 1 Effects of mutation of Ser^{118} and Ser^{167} on GnRH-I and GnRH-II induced Fosb protein levels.

A, LβT2 cells were transfected with a vector control (Vec ctrl), wild-type ERα (ERα), mutant S118A or S167A ERα for 24, 48 and 72 h. Equal amounts of cell lysates were electrophoresed on SDS-10% gels, and western blotted to nitrocellulose for detection with antibodies specific for ERα. Control (Ctrl) represents untransfected cells. MCF-7 cells were utilized as positive control. B, LβT2 cells were transfected with a vector control (Vec ctrl), wild-type ERα (ERα), mutant S118A or S167A ERα for 24 h and then treated with 100 n M GnRH-I or GnRH-II for 3 h. Equal amounts of cell lysates were electrophoresed on SDS-10% gels, and western blotted to nitrocellulose for detection with antibodies specific for Fosb.

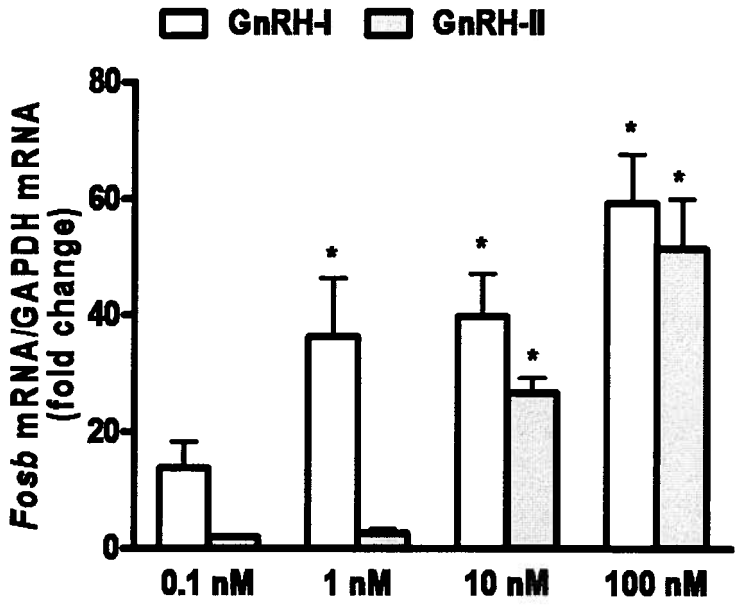

Figure 5. 2 Effects on *Fosb* mRNA levels by different concentration of GnRH-I and GnRH-II.

LβT2 cells were either untreated or treated with 0.1-100 nM GnRH-I or GnRH-II for 1 h. Total RNA was extracted and reverse transcribed into first-strand cDNA. The levels of *Fosb* mRNA were measured by real-time RT-PCR. Data of real-time RT-PCR are presented as the mean ± SEM of three independent experiments. *: $p<0.05$ *versus* untreated control value set at 1.

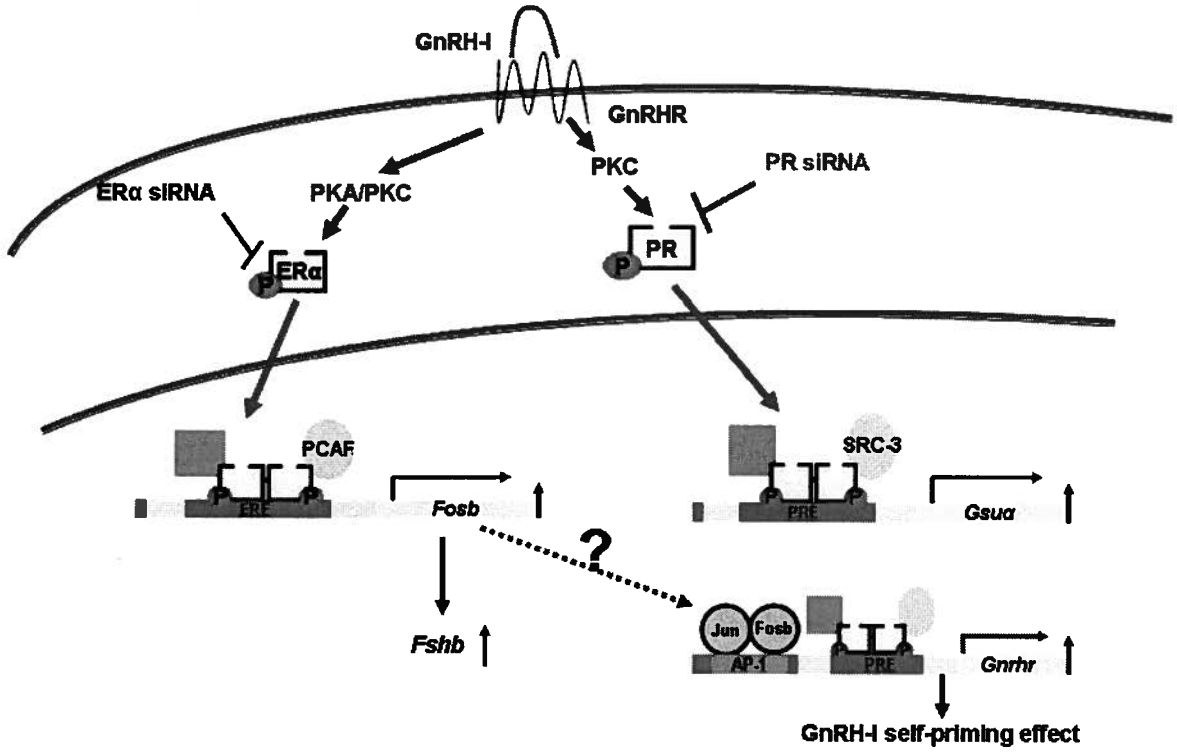

Figure 5. 3 Transactivation of ERα and PR by GnRH-I in mouse gonadotrophs.

In mouse LβT2 pituitary cells, GnRH-I actives PKA and PKC signaling pathways which mediate the phosphorylation of ERα. Then ERα and PCAF are recruited to an ERE within the *Fosb* promoter to enhance its transcriptional activation. This mechanism likely activates the *Fshb* subunit gene in pituitary cells. In addition, GnRH-I activates PR in a ligand-independent manner through the PKC signaling pathway. It promotes the binding of PR and coactivators to PREs in the *Gsuα* and *Gnrhr* promoters, resulting in increased *Gsuα* mRNA levels and *Gnrhr* promoter activity. These likely contribute to the self-priming effect of GnRH-I. Whether Fosb and Jun form a heterodimer and load at an AP-1 binding site to increase *Gnrhr* expression needs to be confirmed.

AP-1, activator protein-1; ERα, estrogen receptor; ERE, estrogen-response element; FSH, follicle stumulating hormone; GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor; GSUα, gonadotropin α subunit; PCAF, P300/CBP-associated factor; PKA, protein kinase A; PKC, protein kinase C. PR, progesterone receptor; PRE, progesterone-response element; SRC-3, steroid receptor coactivator.

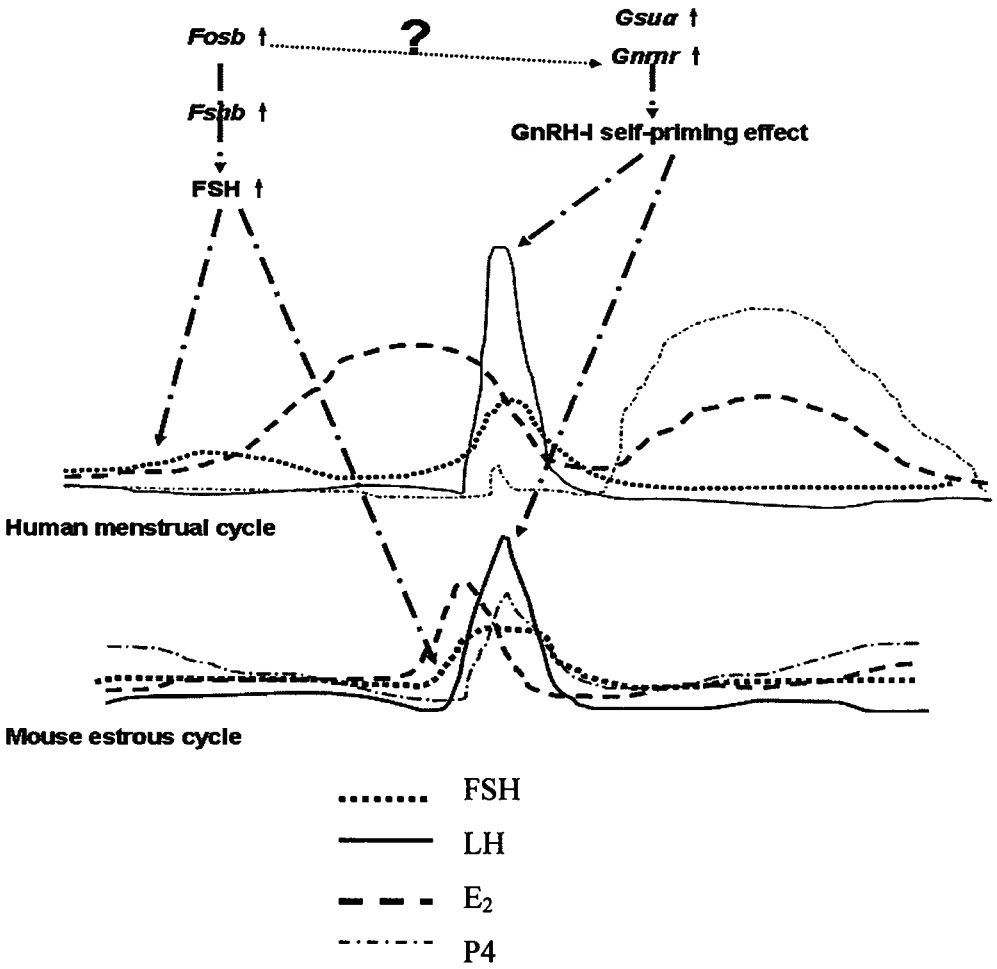

Figure 5. 4 The physiological importance of *Fosb, Gsuα* and *Gnrhr* in human menstrual cycle and mouse estrous cycle.

Fosb, Gsuα and *Gnrhr* are important for the cyclic hormonal changes during the human menstrual cycle and mouse estrous cycle. The induction of *Fosb* is crucial for *Fshb* gene expression which enhances FSH levels. This may be most important under conditions when the GnRH pulse amplitude is high, such as during the luteal/follicular transition phase of the human menstrual cycle, and proestrus phase in mouse estrous cycle. In addition, the accumulation of *Gsuα* and *Gnrhr* mRNA levels likely contributes to the self-priming effect of GnRH-I, which is important to the LH surge in human menstrual cycle and mouse estrous cycle. Whether stimulation of *Fosb* involves in *Gnrhr* expression needs further study.

E_2, estradiol; FSH, follicle stumulating hormone; GnRH, gonadotropin-releasing hormone; *Gnrhr*, GnRH receptor; GSUα, gonadotropin α subunit; LH, luteinizing hormone; P4, progesterone.

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