THE DYNAMIC INTERPLAY OF CHROMATIN MODIFYING FACTORS AND DNA-BOUND TRANSCRIPTION FACTORS IN THE REGULATION OF MOUSE GONADOTROPIN GENES VIA MULTIPLE SIGNALING PATHWAYS

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Summary

The acetylation state of the histones, regulated by histone acetyltransferases (HATs) and deacetylases (HDACs), within the chromatin is correlated with transcriptional competence. We investigated how the regulation of the pituitary gonadotropin genes, follicle stimulating hormone β-subunit (FSHβ) and luteinizing hormone β-subunit (LHβ), involves recruitment of HATs and HDACs via the various signaling pathways. Trichostatin A (TSA), a HDAC inhibitor, and gonadotropin-releasing hormone (GnRH) were discovered to stimulate expression of FSHβ and LHβ genes in immature mouse gonadotropes and of FSHβ also in mature gonadotropes. In these mature gonadotropes which express LH freely but FSH only at very low levels, we show using chromatin immunoprecipitation (ChIP), that HDAC1 and HDAC7 occupy the FSHβ gene promoter, but that HDAC1 is removed following GnRH treatment, and HDAC7 following activin treatment.

Transfection of siRNA constructs into these cells has revealed that the HATs CBP, Tip60, GCN5 and pCAF are recruited for the expression of FSHβ gene at basal, GnRH- and estradiol-mediated levels, but they are not required for the basal expression of the LHβ gene. ChIP analysis revealed that GCN5, but not Tip60 is present on the FSHβ gene promoter in unstimulated conditions, while estrogen treatment stimulates recruitment of Tip60 but not GCN5. However, both GCN5 and Tip60 are recruited to this promoter following GnRH treatment. ChIP analysis combined with transfection of siRNA contructs in these cells, showed that the recruitment of both GCN5 and Tip60 to the GnRH-stimulated promoter requires both ERα and *c*-Fos. Promoter studies on the FSHβ gene showing that Tip60 synergises with *c*-Fos and *c*-Jun, suggest that Tip60 recruitment may be via AP-1. Protein-protein interaction assays revealed that the

recruitment Tip60 to the estradiol-stimulated promoter is likely to be dependent on the physical interaction with the liganded DNA-bound ERα.

In addition, we provide evidence for the occurrence of crosstalking between the GnRH, estradiol and activin signaling pathways via Smad4 which acts as a transcriptional corepressor by possibly interacting with ERα and subsequently regulating the HAT-HDAC exchanges on the promoter.

Overall, our data affirms the relationship between the regulation of transcription factors by hormonally induced signaling cascades and the recruitment of HATs and HDACs in gonadotropin gene regulation.

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AP-1	Activating protein 1
CREB	cAMP response element binding protein
CBP	CREB-binding protein
ChIP	Chromatin immunoprecipitation
DAG	Diacylglycerol
Egr-1	Immediate growth factor 1
ER	Estrogen receptor
ERα	Estrogen receptor α
ERE	Estrogen response element
Ets	E box
FSH	Follicle stimulating hormone
FSHβ	Follicle stimulating hormone β-subunit
GnRH	Gonadotropin-releasing hormone
GnRHr	Gonadotropin-releasing hormone receptor
GTF	General transcription factor
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
IP3	Inositol 1,4,5 triphosphate
LH	Luteinizing hormone
LH-2	LIM homeobox gene
LHR	Luteinizing hormone receptor
LHβ	Luteinizing hormone β-subunit
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MYST	MOZ, Ybf2/Sas3, Sas2, Tip60
PCAF	p300/CBP-associated factor
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
p-LIM	LIM homeobox gene
Ptx1 or Pitx1	Pituitary homeobox 1
RLU	Relative light unit
RT-PCR	Reverse transcriptase PCR
SRC	Steroid receptor cofactor

SRC-1	Steroid receptor cofactor 1
Sf-1	Steroidogenic factor 1
Sp-1	Selective promoter factor 1
TAF	TBP-associated factor
TBP	TATA-binding protein
TGF-β	Transforming growth factor β
TFC	Transcription factor complex
Tip60	Tat-interacting Protein
TSA	Trichostatin A
USF	Upstream transcription factor

Chapter 1 Introduction

1.1 The Gonadotropins

As a control center for integration of hormonal signals in the reproductive axis, the mature vertebrate anterior pituitary gonadotrope exclusively synthesizes and secretes both pituitary gonadotropins: the follicle stimulating hormone (FSH) and the luteinizing hormone (LH).

FSH and LH are members of the family of pituitary glycoprotein hormones that also includes the thyroid-stimulating hormone (TSH) and chorionic gonadotropin (CG; Pierce and Parsons, 1981). LH and FSH are synthesized and secreted from the gonadotropes located in the anterior pituitary gland (Figure 1), and are controlled by the binding of the hypothalamic neurohormone, gonadotropin-releasing hormone (GnRH), to the GnRH receptors (GnRHr) on the gonadotrope cell surface (Pierce and Parsons, 1981). Each of these heterodimeric hormones contains a common α-subunit and a hormone-specific β-subunit that defines its biological activity and physiological specificity. The two subunits are linked by noncovalent interactions stabilized by a β cysteine loop that forms a "seatbelt" encircling loop 2 of the α-subunit (Lapthorn *et al.*, 1994; Wu *et al.*, 1994).

FSH and LH both play crucial roles in mammalian reproduction (simplified in Figure 2). Upon their synthesis and release, FSH and LH act on the ovary and testes

Figure 1. Functional connections between the hypothalamus and pituitary gland. Whilst the anterior pituitary gland is not anatomically connected with the hypothalamus, it is functionally connected with this part of the brain. Nerve cells in the hypothalamus secrete neurohormones that act on the endocrine cells of the anterior lobe to stimulate or inhibit their synthesis and secretion. Abbreviations: AL, anterior lobe; PL, posterior lobe; MB, mammillary body (adapted from Nussey and Whitehead, 2001).

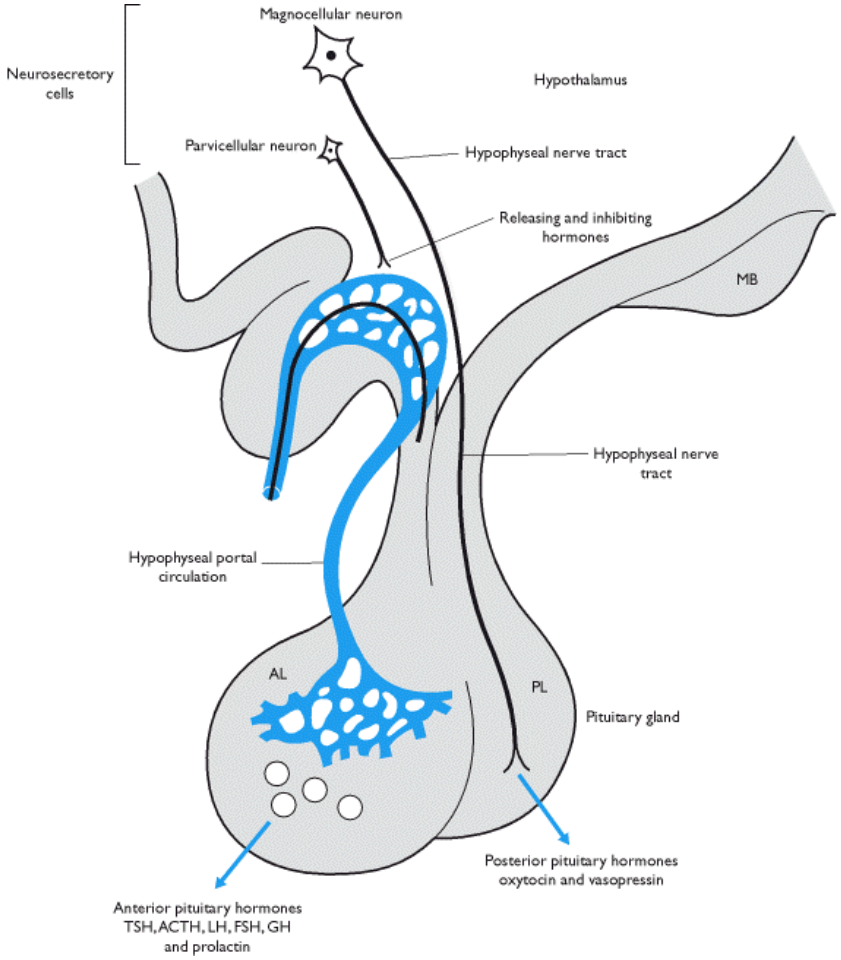
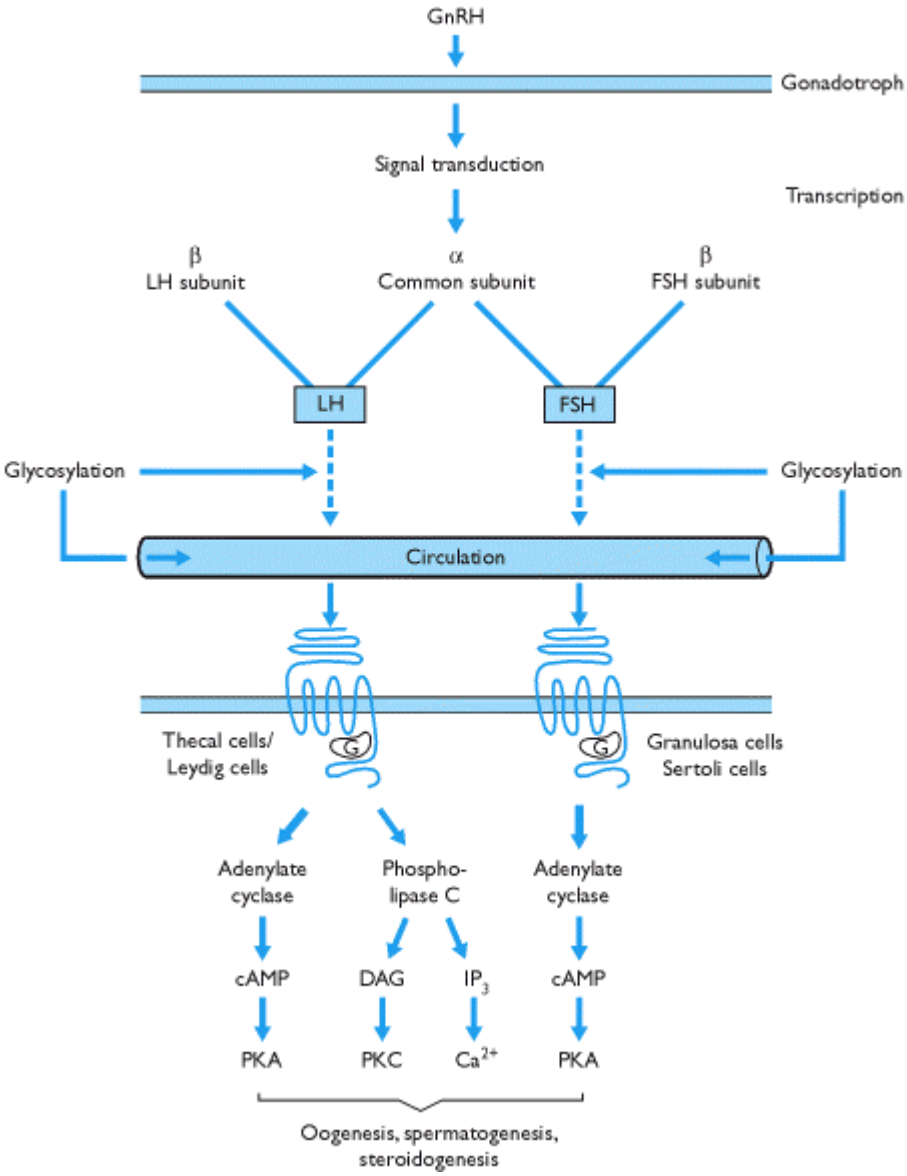


Figure 2. The actions of gonadotropins. GnRH stimulates transcription of the genes coding for the common α and specific β subunits of LH and FSH. Glycosylation of the proteins occurs in the pituitary and may be modified in the circulation. Glycosylation sites and the charge of different LH and FSH isoforms alter their biological potency. They act on gonadal cells via typical G-protein linked receptors. Abbreviations: PKA/C, protein kinase A/C; DAG, diacylglycerol; IP_3, inositol 1, 4, 5-trisphosphate (adapted from Nussey and Whitehead, 2001).

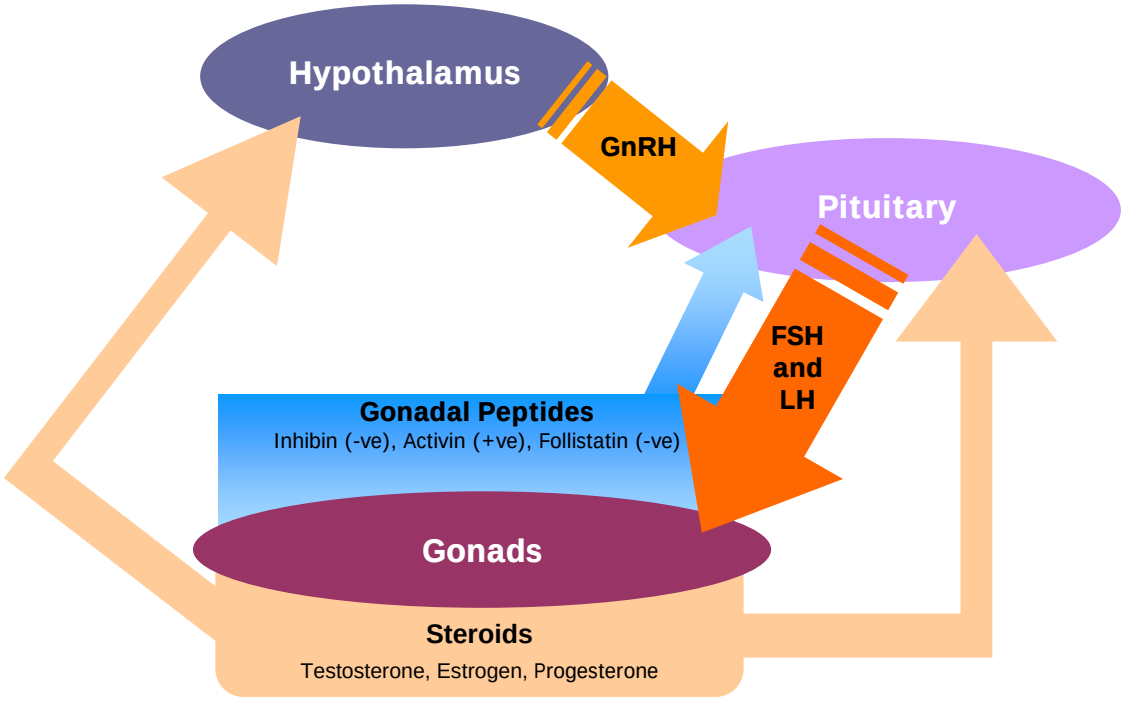


leading to puberty and subsequent fertility. FSH is primarily involved in spermatogenesis in the male (Kumar *et al.*, 1997; Tapanainen *et al.*, 1997) and folliculogenesis in the female (Aittomaki *et al.*, 1996). In the female, it is also involved in the conversion of androgens to estrogens. In the testis, LH acts through plasma membrane receptors on the Leydig cells to maintain general metabolic processes and regulate steroidogenic enzymes, especially for the production of androgens (Dufau, 1988). In the ovary, LH promotes the maturation of follicular cells. After the initial inductive effect of FSH on LH receptor (LHR) expression in the small and medium follicles, LH enhances the subsequent stages of follicular development and steroidogenesis in granulosa and luteal cells (Richards and Hedin, 1988). The LH surge triggers ovulation by promoting the rupture of the preovulatory follicle and the release of the ovum. In addition, LH causes the formation of the corpus luteum from the ruptured ovarian follicles remaining after ovulation. In some species, it may also be responsible in the synthesis of progesterone and its subsequent release by the corpus luteum (Norris, 1997).

Three genes, encoding the α-, FSH β- and LH β-subunits, are expressed in the same type of pituitary cell in mammals (Liu *et al.*, 1988). They are temporally regulated and are subject to the complex control of many factors (Figure 3) including GnRH (Papavasiliou *et al.*, 1986; Kato *et al.*, 1989), steroid hormones (testosterone, estrogen and progesterone) and gonadal peptides (activin, inhibin and follistatin: Gharib *et al.*, 1990; Miller, 1993).

The gonadotrope cell lineage originates in the ventral part of the embryonic pituitary and is characterized initially by the expression of the α-subunit gene at

Figure 3. Regulation of gonadotropin gene expression. GnRH, synthesized in and released from the hypothalamus, binds to GnRH receptors on the surface of the gonadotrope. This leads to the synthesis and secretion of LH and FSH, which stimulate the production of steroid hormones. Testosterone, estrogen and progesterone negatively or positively regulate the synthesis of the gonadotropins directly at the pituitary or indirectly by modulating GnRH secretion from the hypothalamus. The gonadal peptides, inhibin, activin and follistatin, also have roles in the regulation of gonadotropin gene expression by exerting positive or negative feedback (adapted from Brown and McNeilly, 1999).



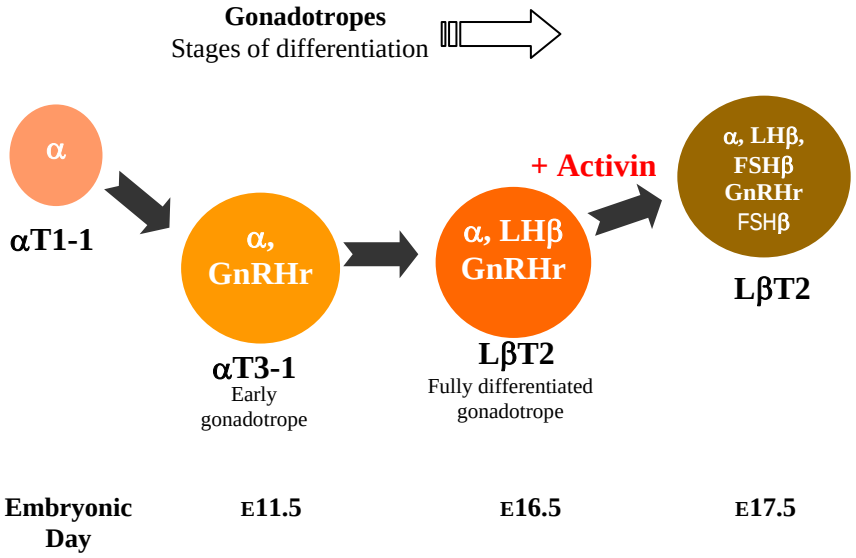
approximately embryonic day 11.5 (e11.5) of gestation in the mouse. This is followed by a clear progression of cell differentiation marked by the expression of the individual β-subunits of LH on e16.5 and FSH on e17.5 (Japon *et al.*, 1994; Figure 4).

The Mouse Gonadotrope: αT3-1 and LβT2 Clonal Cell Lines Two murine pituitary clonal cell lines, the αT3-1 and LβT2 cell lines were previously generated by targeted oncogenesis in transgenic mice (Alarid et al., 1996).

The αT3-1 cell line represents an immature gonadotrope that is not fully differentiated, expressing α-subunit, GnRH receptor (GnRHr) and Sf-1, but neither the LHβ gene nor the FSHβ gene (Windle *et al.*, 1990). The LβT2 cell line represents a fully differentiated gonadotrope, expressing GnRH receptor, both α- and LH β-subunits; it can also be induced to express the FSHβ gene with exposure to activin (Graham *et al.*, 1999; Figure 4).

Both cell lines express all of the known transcription factors (Alarid et al., 1996), which have been shown to be essential for LHβ expression, and yet the αT3-1 cells do not appear to synthesize LHβ. In contrast, the LβT2 cells synthesize and secrete intact and biologically active LH, and this response was found to be strongly augmented by GnRH (Turgeon *et al.*, 1996). These cells also exhibit intracellular calcium fluxes in response to GnRH, indicating fidelity of gonadotrope physiological behavior (Thomas *et al.*, 1996). Notably, the identities and functions of the factors absent in αT3-1 that are responsible for this differential expression are currently unknown.

Figure 4. The gonadotrope cell lines along the developmental cell lineages of the anterior pituitary. The distinct stages of differentiation are represented by the immortalized pituitary cell lines created by target oncogenesis in transgenic mice. In this study, the αT3-1 (early gonadotrope) and LβT2 (fully differentiated gonadotrope) cell lines were used as comparative model systems to investigate FSHβ and LHβ gene-specific transcription mechanisms at both basal and GnRH-regulated levels. Abbreviations: GnRHr, GnRH receptor.



1.2 Transcriptional Regulation of FSHβ and LHβ Genes

Reproductive development and function in mammals depend on the exquisite regulation of gonadotropin release by the anterior pituitary gonadotropes.

The expression of the gonadotropin genes is transcriptionally regulated by two fundamental overlaying mechanisms: the basal gene expression targets expression of these genes specifically to the gonadotropes, after which GnRH, gonadal steroids and peptides regulate gene expression at puberty (Brown and McNeilly, 1999).

Basal Gene Expression During embryogenesis, the basal FSHβ and LHβ gene expression is activated and maintained by a controlled cascade of temporally and spatially regulated transcription factors (Figures 5 and 6A) via multiple signaling pathways that control the expression of these transcription factors; giving rise to gonadotrope differentiation (Treier *et al.*, 1998). The basal gene expression is activated in and targeted to the gonadotrope cells, and is maintained throughout development. However, when the GnRH secretion is increased, expression of these genes is upregulated, reaching maximum values at puberty, while blocking the GnRH input causes the gonadotropin expression to return to basal levels (McNeilly *et al.*, 1991).

Figure 5. The relationship between the organogenesis of the anterior pituitary and the sequential activation of genes encoding transcription factors known to regulate gonadotropin gene expression in mice. Shaded bars indicate the initiation and duration of gene expression. Rathke's pouch is formed from the hypophyseal placode of oral ectoderm that invaginates at approximately embryonic day 9.5 (e9.5). Cell proliferation then occurs in the anterior pituitary before pituitary cell-specific genes can be identified. The earliest identifiable marker of pituitary gene expression is the common α-subunit, which is activated at e10.5, a full 6 days before LHβ (e16.5) and FSH (e17.5) gene expression is detected. NE: neuroectoderm (adapted from Brown and McNeilly, 1999).

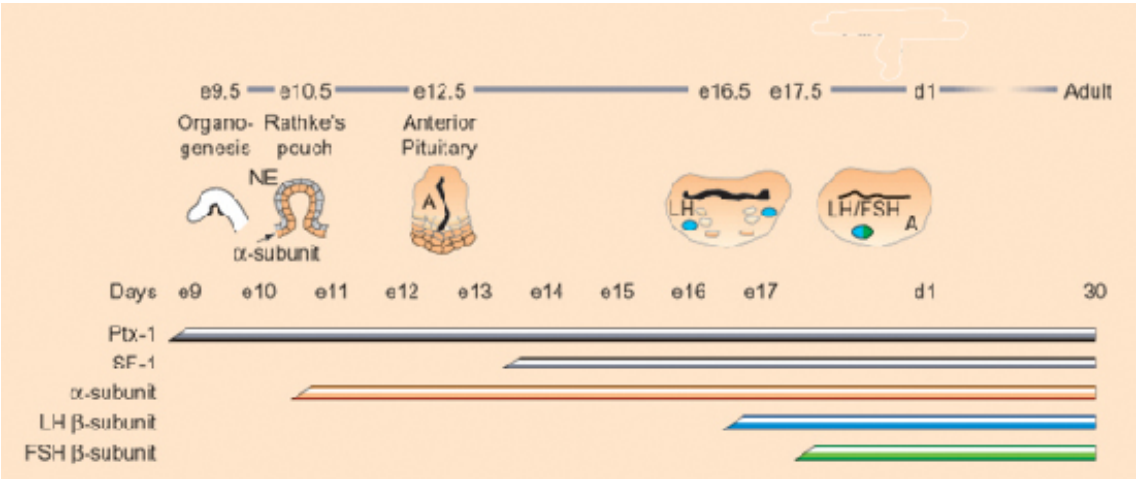
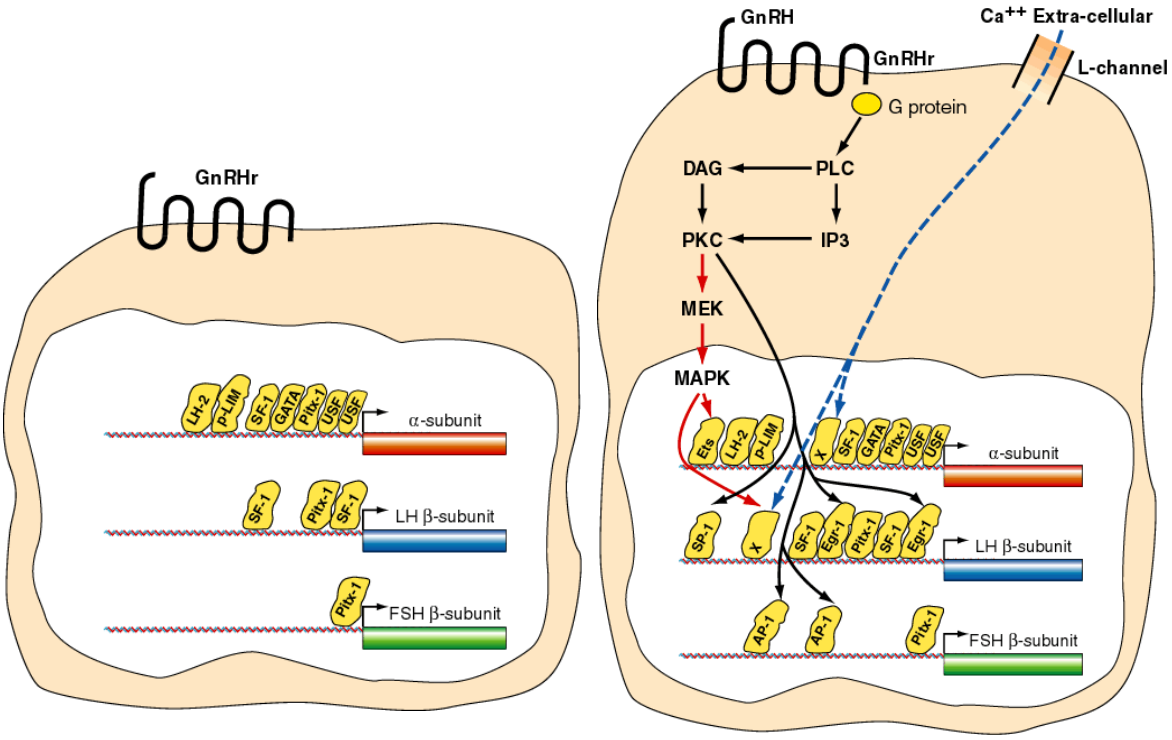


Figure 6. A model of basal and GnRH-modulated gonadotropin subunit gene expression. (A) Transcription factors involved in basal gonadotropin subunit gene expression are activated during anterior pituitary development and are shown bound to their cognate DNA elements in the gonadotropin subunit gene promoters. **(B)** The GnRH-regulated transcription factors, shown together with the putative second messenger signaling pathways. Interactions between basal and GnRH-regulated transcription factors are as follows: Sf-1 synergises with Egr-1 (Lee *et al.,* 1996; Halvorson *et al.,* 1998); LH-2 synergises with Ets (Roberson *et al.,* 1994); GnRH regulates SP-1 (Kaiser *et al.,* 1998b), Egr-1 (Halvorson *et al.,* 1999), Ets (Roberson *et al.,* 1995) and AP-1 (Strahl *et al.,* 1998). Extracellular Ca^{2+} influx upregulates unknown factor x, which transactivates α-subunit and LHβ promoters (Holdstock *et al.,* 1996; Weck *et al.,* 1998). Abbreviations: PLC, phopholipase C; MEK: mitogen-activated protein kinase kinase or MAPKK; MAPK, mitogen-activated protein kinase. Other abbreviations are as in Figure 2 (adapted from Brown and McNeilly, 1999).



(A) Basal Gene Expression **(B) GnRH-modulated Gene Expression**

As previously mentioned the gonadotrope cell lineage is characterized initially by the expression of α-subunit transcripts and, after a further 6 days, by the expression of LHβ and FSHβ transcripts (Japon *et al.,* 1994). Transcription factors, including Sf-1, Ptx1, Hesx1-Rpx, pLim-Lhx3 and Otx1, that activate gonadotropin gene transcription are expressed during this developmental window.

Steroidogenic factor 1 (Sf-1or Ad4BP) is an orphan nuclear receptor that is necessary for the expression of all components of the steroidogenic pathway as well as the development of the steroidogenic tissues (Luo *et al.*, 1994; Luo *et al.*, 1995; Morohashi and Omura, 1996; Parker and Schimmer, 1997). It is specifically found in the gonadotrope cell lineage of anterior pituitary cells, but is also expressed in other non-pituitary steroidogenic tissues including the adrenal glands, gonads, and placenta, and also in the ventromedial nucleus of the hypothalamus (Ikeda *et al.*, 1993; Ikeda *et al.*, 1995). It possesses an N-terminal DNA binding domain and a C-terminal ligand-binding domain (Jacob and Lund, 1998). Sf-1 stimulates the LHβ gene expression (Halvorson *et al.*, 1996; Keri and Nilson, 1996), and also that of FSHβ (Jacobs *et al.*, 2003). In addition, Sf-1 is also involved in the transcriptional regulation of the gonadotropin α-subunit gene (Horn *et al.*, 1992), the LH receptor (Cammas *et al.*, 1997), the steroid hydroxylase genes (Hu *et al.*, 1997), the andrenocorticotropin receptor (Waterman, 1994), the prolactin receptor (Halvorson *et al.*, 1996) and the steroidogenic acute regulatory protein (Sugawara *et al.*, 1997). Although Sf-1 stimulates α-subunit and LHβ gene expression, Sf-1 gene expression is activated at embryonic day 13.5 (e13.5), after the activation of α- subunit gene expression on e10.5, while the earliest LHβ transcripts appear at e16.5 (Figure 5). It appears that,

after activation of α-subunit gene expression; undiscovered transcription factors suppress Sf-1 and LHβ gene activation in precursor gonadotropes.

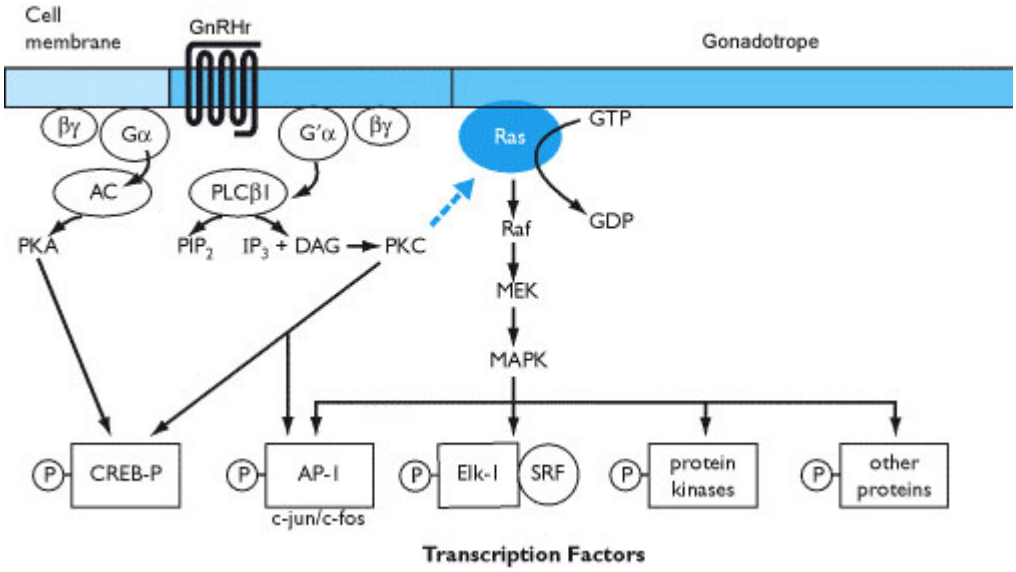
Pituitary homeobox (Ptx1 or Pitx1) is expressed in several anterior pituitary cell lineages and transactivates most of the anterior pituitary cell-specific genes, including the gonadotropin α-subunit, FSHβ and LHβ genes (Tremblay *et al.*, 1998). More recently, it was observed that Ptx1 activates the rat FSHβ gene through both direct and indirect interactions with the FSHβ gene promoter: both in unstimulated and GnRH-exposed cells (Zakaria *et al.*, 2002). In addition, our lab has shown that its action involves a synergistic effect with Sf-1 alone or in combination with the estrogen receptor on the proximal promoter of the Chinook salmon LHβ (Melamed *et al.*, 2002).

GnRH-modulated Gene Expression The decapeptide GnRH plays a critical role in reproductive development and function in vertebrates by stimulating the biosynthesis and secretion of the pituitary gonadotropins. The GnRH responsiveness is through the activation of intracellular signaling transduction pathways stimulated by the binding of GnRH to the GnRH receptor (GnRHr; Figure 6B). The GnRHr is a G-protein-coupled seven transmembrane receptor that signals predominantly via the protein kinase C (PKC) pathway. This stimulatory signal is relayed to the gene via transcription factors that are activated and/or modified allowing them to bind to discrete DNA sequences on the gonadotropin gene promoters and thus transactivate gene expression. Activation of the GnRH pulse generator occurs prior to the onset of puberty and results in increased GnRH release which, in turn, stimulates gonadotropin gene expression (Cattanach *et al.*, 1977).

The GnRH Signaling Pathway The GnRH receptor activates $_{L}$-type calcium channels, causing an influx of calcium into the cell. Phospholipase C (PLC) is also activated, leading to the cleavage of phosphatidylinositol-diphosphate (PIP_2), located in the cell membrane, into inositol 1, 4, 5-triphosphate (IP3) which mediates calcium release from intracellular stores, and diacylglycerol (DAG). Increased concentrations of intracellular calcium together with DAG production lead to activation of protein kinase C (PKC), which in turn leads to activation of other protein kinases, such as the ubiquitous mitogen-activated protein kinases (MAPK) which are involved in various cellular functions, including cell growth, differentiation, transformation, cell cycle and apoptosis (Naor; 1990; Roberson *et al.*, 1995; Haisenleder *et al.*, 1998). The activated MAPKs translocate to the nucleus and stimulate sequence-specific transcription factors via phosphorylation. Once the transcription factors are activated, they bind to promoter DNA sequences and trigger the expression of gonadotropin subunit genes (Marshall and Kelch, 1986; Gharib *et al.*, 1990; Naor *et al.*, 2000; Shacham *et al.*, 2001; Kraus *et al.*, 2001; Figure 7).

In the mammalian gonadotrope, GnRH differentially regulates LH and FSH biosynthesis through preferential sensitivity of the gonadotropin subunit gene promoters to either calcium influx or PKC/MAPK cascade (Roberson *et al.*, 1993; Schoderbek *et al.*, 1993; Sundaresan *et al.*, 1996; Weck *et al.*, 1998; Saunders *et al.*, 1998). The frequency and amplitude of the GnRH pulses delivered to the anterior pituitary, which vary during different phases of the estrous cycle, directs differential regulation of the gonadotropin gene expression (Levine and Ramirez, 1982). In general, higher GnRH amplitude stimulates LH synthesis while FSH synthesis is increased at lower GnRH pulses (Papavasiliou *et al.*, 1986). The effect of the variation

Figure 7. Signal transduction pathways activated by GnRH. GnRH can activate a number of transcriptional factors by phosphorylation. This may involve the MEK-MAP pathway (serine-threonine kinases) or direct activation of transcription factors by protein kinase (PK) A, PKC or the Ca^{2+}-calmodulin activated CaM protein kinase (not shown). Abbreviations: AC, adenylate cyclase; PIP_2, phosphatidylinositol 4,5-bisphosphate; Ras, monomeric G-protein; Raf/MEK, kinases; CREB-P, cyclic AMP response element binding protein; SRF, serum response factor. Other abbreviations are as in Figures 2 and 6 (adapted from Nussey and Whitehead, 2001).



in GnRH pulse pattern is also associated with differential LH and FSH release, with higher frequency resulting in greater LH secretion while lower frequency stimulates FSH release (Dalkin *et al.*, 1989).

Regulation of GnRH-modulated Gene Expression by Gonadal Steroids and Peptides FSHβ gene transcription is highly sensitive to the negative feedback effects of steroids. Experiments using nuclear run-on assays demonstrate that estradiol and progesterone downregulate FSHβ gene transcription (Phillips *et al.,* 1988). Studies *in vitro* of the FSHβ promoter localize the effect of estrogen to Activating Protein 1 (AP-1) sites, which also confer GnRH responsiveness (Miller and Miller, 1996; Strahl *et al.,* 1998).

Evidence for a direct inhibitory effect of steroids on LHβ gene expression is weak or contradictory. It appears that there is no direct interaction of estradiol receptor with the LHβ promoter (Keri *et al.,* 1994), indicating that any inhibitory effects of steroids on LHβ gene expression are not mediated via a classical estrogen response element (ERE) on the LHβ gene promoter. Studies *in vitro*, in which LβT2 cells were cultured with and without estradiol, showed that estradiol did not affect LHβ mRNA levels, but did increase GnRHr mRNA levels (Turgeon *et al.,* 1996). This observation implies that steroids affect LHβ synthesis by increasing GnRHr gene expression. In contrast, studies *in vivo* suggest that estradiol negatively regulates LHβ transcription by downregulating expression of Sf-1 (Brown and McNeilly, 1997).

Inhibin and activin are members of the transforming growth factor-β (TGF-β) family of cytokines (Heldin *et al.,* 1997). While activin upregulates FSHβ gene

expression (Weiss *et al.,* 1995), inhibin antagonizes the stimulatory effect of activin on FSHβ gene expression. Inhibin action downregulates transcription of the FSHβ but has no effect on the transcription of the LHβ (Clarke *et al.,* 1993). Follistatin, a potent activin-binding protein, inhibits binding of activin to its receptors and hence specifically inhibits the synthesis and secretion of FSH by the pituitary (Shimasaki *et al.*, 1988).

Molecular Mechanisms of Steroid Actions The transcriptional regulation of gene expression by steroid hormones is based on a two-step model that involves high affinity binding of the hormone to the specific steroid hormone receptors, usually within the cytoplasm of the target cells, followed by activation of the hormone-receptor complex which translocates into the nucleus. The activated receptors bind to the hormone responsive elements of target promoters as monomers (Johnston *et al*., 1997), homodimers (Vanacker *et al*., 1999) or heterodimers (Beato, 1989) to activate transcription.

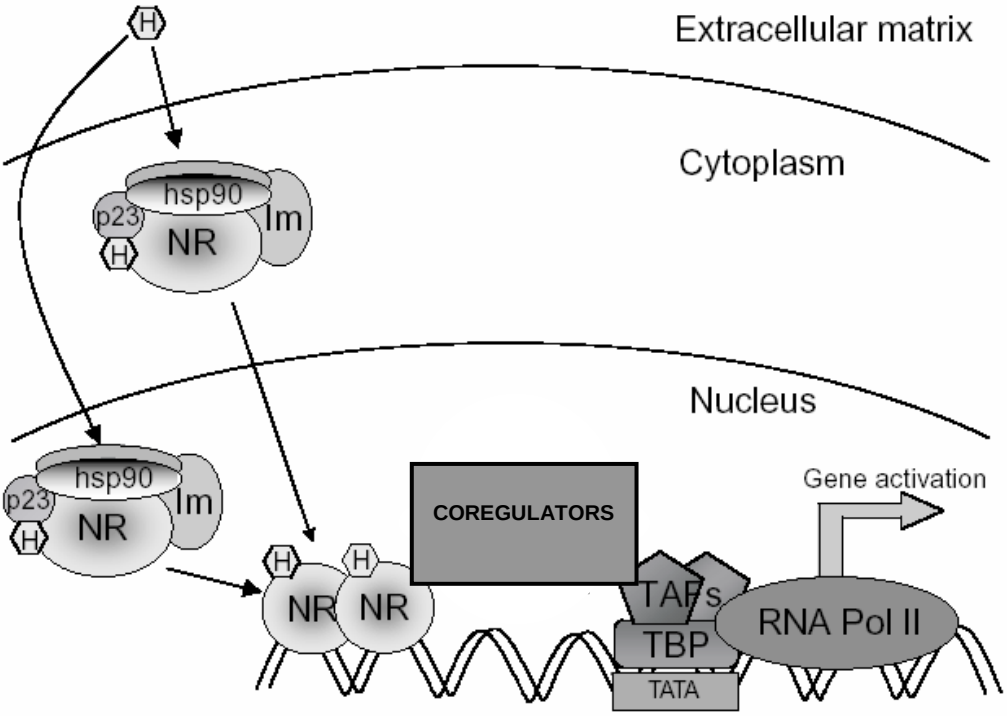
In estrogen target tissues, estrogen signaling is mediated by estrogen receptors α and β (ERα and ERβ), both of which belong to the nuclear receptor superfamily of hormone activatable transcription factors (Muramatsu and Inoue, 2000). The mechanism of estrogen action is best understood for ERα, which is maintained in the nucleus as a hormone-responsive heteromeric complex associated with the 90-kDa heat shock protein (hsp90) chaperone machinery (Pratt and Toft, 1997). Hormone binding triggers a dissociation of receptor from the chaperone proteins, allowing it to homodimerize and bind with high affinity to the specific palindromic DNA

sequences, ERE of target gene promoters leading to increased transcriptional activity of the target genes (Aranda and Pascual, 2001; Figure 8).

In the simplest sense, steroid nuclear receptors, once established on their response elements, initiate the recruitment of a variety of multi-protein complexes to promoter sequences (Hager *et al.*, 2004). These complexes stabilize and enhance the assembly of basal transcriptional complexes that contain RNA polymerase II in addition to other general transcription factors, which leads to initiation of transcription (Ratajczak, 2001). Traditional models suggest that these factors remain statically bound to each other and to the promoter until other signals are received to reduce transcription. Recent findings demonstrate that the processes and actions involved are much more complex than traditional models convey, and that the movement of receptors and coactivators is remarkably dynamic (Metivier *et al.*, 2003).

Some nuclear receptors can also modulate gene expression by mechanisms independent of binding to a hormone responsive element (HRE). Thus they can alter the expression of genes that do not contain an HRE through positive or negative interference with the activity of other transcription factors, a mechanism generally referred to as "transcriptional cross-talk" (Gottlicher *et al*., 1998). The ERs utilize protein-protein interactions to enhance transcription of genes that contain the AP-1 sites (Gaub *et al*., 1990). ERα and ERβ have been shown to signal in opposite ways at AP-1 sites: ERα activates transcription, whereas the liganded ERβ inhibits AP-1-dependent transcription (Maruyama *et al.*, 2001). Moreover, transient transfection studies in αT3 cells revealed that ER can also be activated by the PKA or PKC/MAPK signaling pathways, leading to the transactivation of an ER-responsive

Figure 8. Hormonal signaling pathway mediated via nuclear receptors resulting in activation or repression of specific target genes. Abbreviations: H, hormone; NR, nuclear receptor; hsp90, heat-shock protein; Im, immunophilin; p23, 23-kDa chaperone protein; TAFs, transcription-associated factors; TBP, TATA-binding protein; RNA Pol II, RNA polymerase II (adapted from Ratajczak, 2001).



promoter in a ligand-independent manner (Demay *et al*., 2001; Schreihofer *et al*., 2001). This ligand-independent ER activity likely depends on its phosphorylation in the AF-1 domain, allowing interaction with specific coactivators (Hall *et al*., 2001).

Mechanisms of activin-stimulated gene expression involving Smads

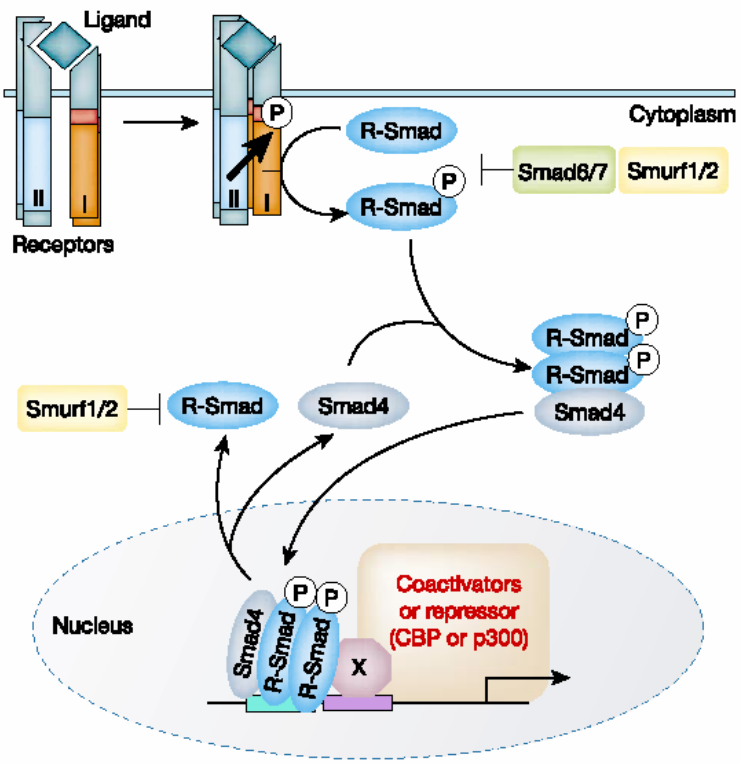
Activin regulates FSH transcription through interaction with the cell-surface receptors that are members of the TGF-β family of transmembrane receptor kinases, resulting in both the elevation of FSHß mRNA levels and FSH secretion (Matthews and Vale, 1991; Attisano *et al*., 1992). Activin signal transduction (Figure 9) is mediated by a heterodimeric receptor complex consisting of two-ligand binding type II receptors (ActRII) and two signaling transducing type I receptors (ActRI; Massagué, 1998). Upon ligand binding, ActRII phosphorylates the ActRI on multiple serine/threonine residues located in a conserved GS-rich motif located just N-terminal to the kinase domain. The phosphorylated receptor is then activated to signal to its downstream targets, the Smad family of proteins which transmit TGF-β signals from the cell surface into the nucleus (Attisano and Wrana, 2000; Zimmerman and Padgett, 2000).

The Smad family of proteins comprises three major classes: the first class is the receptor-regulated Smads (R-Smads) that includes the activin receptor-targeted Smad 2 and 3. These Smad proteins are phosphorylated by the type I receptor on the last two serines at the extreme carboxyl terminus (Abdollah *et al*., 1997). The second class of Smad proteins is the common mediator Smad (Co-Smad) comprising of the Smad4 found in mammals and the highly related Smad4β/Smad10 in *Xenopus*. These Co-Smads associate with phosphorylated R-Smads and the resulting R-Smad/Co-Smad complex then translocates to the nucleus to induce specific gene responses

(Lagna *et al*., 1996; Howell, *et al*., 1999; Masuyama, *et al*., 1999). Activin signaling is prevented by the third class of Smad proteins known as the inhibitory Smads (I-Smads). These proteins antagonize signaling by interacting directly with the receptor to prevent access to and phosphorylation of R-Smads, or by interfering with R-Smad/Smad4 complex, or by recruiting E3 ubiquitin ligases, Smurf1 (Smad-ubiquitination-regulatory factor 1) and Smurf2 (Arora and Warrior, 2001), that mediate ubiquitination and the consequent degradation of the R-Smads (Welt *et al*., 2002).

Once in the nucleus, the R-Smad/Co-Smad complex may bind specific gene promoters through the consensus motif GCTC or AGAC (Massagué and Wotton, 2000). However, this binding is of low affinity and specificity, and might not be required on all promoters. For example, Pardali *et al*. (2000) showed that Smad3 and Smad4 functionally cooperate with Sp-1 to activate the human p21 promoter in hepatoma HepG2 cells. This activation requires the ubiquitous Sp-1 to bind to the proximal promoter. Using Smad3 and Smad4 DNA-binding site mutants, the Smad proteins could still transactivate the p21 promoter as efficiently as wild type Smads, indicating that the Smad3 and Smad4 binding sites are not crucial. The same Smad proteins synergize with *c*-Jun/*c*-Fos at the AP-1 binding site of the collagenase I promoter to induce transcriptional activation in response to TGF-β. Mutational analyses of the *c*-Jun protein and the AP-1 binding site in the promoter revealed that the interaction of *c*-Jun with DNA is necessary for the transcriptional activation, whereas similar analysis of Smad3 revealed that its binding to the DNA, is required although there appears to be little importance for the actual DNA binding sequence (Qing *et al*., 2000).

Figure 9. Mechanism of TGF-β receptor and Smad activation. At the cell surface, the ligand binds a complex of transmembrane receptor serine/threonine kinases (type I and II) and induces transphosphorylation of the type I receptor by type II receptor kinases. The consequently activated type I receptors phosphorylate selected Smads, and these receptor-activated Smads (R-Smads) then form a complex with a common Smad4**.** Activated Smad complexes translocate to the nucleus, where they regulate transcription of target genes, through physical interaction and functional cooperation with DNA binding transcription factors (X) and coactivators (CBP or p300) or corepressors. Activation of R-Smads by type I receptor kinases is inhibited by Smad6 or Smad7. R-Smads and Smad4 shuttle between the nucleus and cytoplasm. The E3 ubiquitin ligases Smurf1 and Smurf2 mediate ubiquitination and consequent degradation of R-Smads, yet can also interact with Smad6/7 and thereby ubiquitinate the type I receptors (adapted from Derynck and Zhang, 2003).



These studies, together with the short consensus Smad recognition sequence, suggest that Smad-dependent gene expression only involves weak binding of Smad to its recognition sequence, and requires interactions with other transcription factors (Welt *et al*., 2002). The activin-mediated regulation of gene expression therefore likely involves a series of interconnecting signal transduction pathways, culminating with the concerted actions of the DNA-binding Smad proteins and other transcription factors and associated co-factors.

It was reported that the Smad3 and Smad4 transcription factors are responsible for mediating the stimulation of the rat FSHβ promoter by activin in LβT2 cells. Functional analysis identified the target site of the promoter through which the transcription factors act. Moreover, the pituitary-specific Ptx-2 transcription factor and its corresponding binding site on the promoter were also shown to be involved in regulating the FSHβ subunit gene in a tissue-specific manner. This was the first paper to identify Smad transcription factors acting in concert with a pituitary-specific nuclear transcription factor to mediate the activin stimulation of the FSHβ subunit gene (Suszko, *et al*., 2003). In another recent study in primary ovine pituitary and LβT2 cells, activin activated several signaling pathways with different time course but only the Smad2 pathway appeared to be directly implicated in the expression and release of FSH in LβT2 cells (Dupont *et al*., 2003). Taken together, these two studies suggest that Smad3/Smad4 complex is at least partly responsible for the activin-induced expression of the FSHβ gene via the Smad2 pathway.

Possible Crosstalk between Signaling Pathways Crosstalk between different signaling pathways is common in signal transduction and a number of

studies have shown how the different components of the TGF-β signaling pathway crosstalk with the GnRH-signaling pathway.

In one study, Atfi and colleagues (1997) reported that TGF-β initiates a signaling cascade leading to stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) activation. The expression of dominant-interfering forms of various components of the SAPK/JNK signaling pathways including Rho-like GTPases, MAPK kinase kinase 1 (MEKK1), MAPK kinase 4 (MKK4), SAPK/JNK, or c-Jun abolishes TGF-β-mediated signaling. This study demonstrates that the SAPK/JNK activation, which is a part of the GnRH-mediated pathway, contributes to TGF-β signaling. Similarly, Frey and Mulder (1997) had the same observation in breast cancer cells.

More recently, Smads have also been linked to the MAPK pathway. It was shown that anti-Bone Morphogenic Protein (BMP; Chordin), Fibroblast Growth Factor 8 (FGF8) and Insulin-like Growth Factor 2 (IGF2) signals are integrated in the embryo via the regulation of Smad1 phosphorylation. The neural induction results from the combined inhibition of BMP receptor serine/threonine kinases and activation of receptor tyrosine kinases that signal through MAPK and phosphorylate Smad1 in the linker region, further inhibiting Smad1 transcriptional activity (Pera *et al*., 2003).

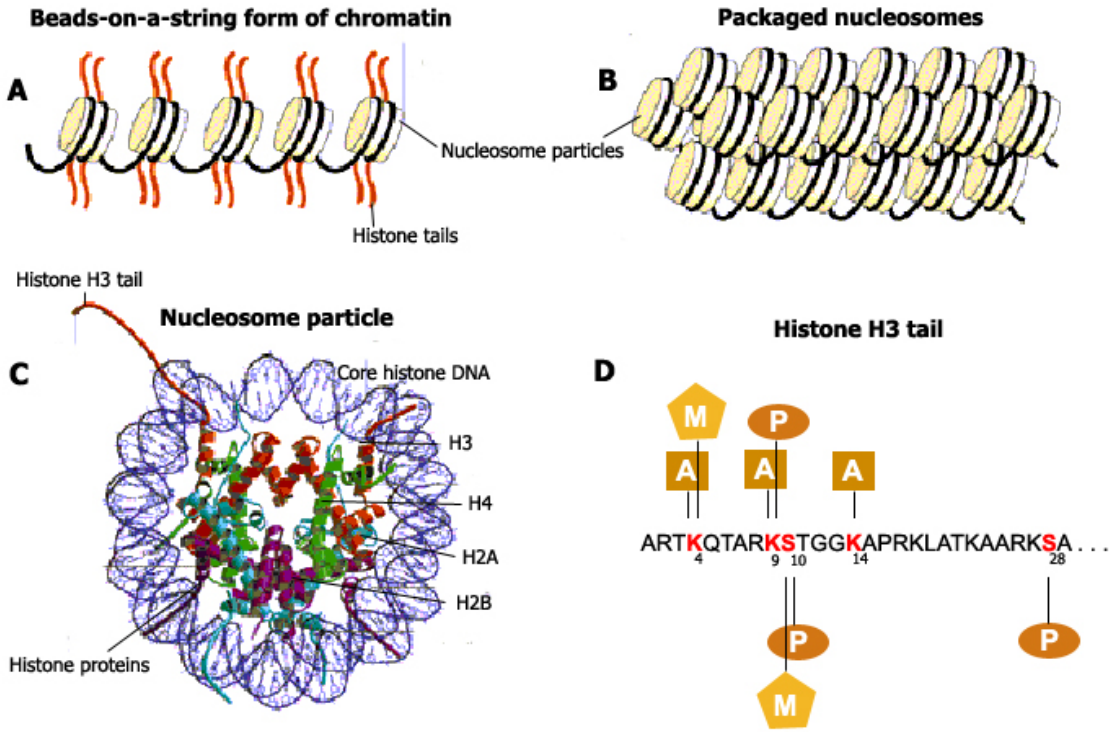
1.3 Chromatin Structure and Function

The eukaryotic cell makes use of the nucleosome in the organization of the genome, so enabling it to fit the minute dimensions of a nucleus, but at the same time being able to provide sufficient accessibility of the DNA substrate to regulatory factors (Kornberg and Lorch, 1999a; Wu and Grunstein, 2000).

The nucleosome, the fundamental repeating unit of chromatin, is comprised of 145-147 base pairs of DNA irregularly wrapped 1.65 turns around an octameric core of histone proteins via multiple interactions between histones and the phosphate backbone on deoxyribose moieties (Wolffe, 1992). This irregular DNA wrapping provides a degree of flexibility in the structure that may play a role during localized perturbation of histone-DNA interactions critical for gene expression (Kornberg and Lorch, 1999a; Luger *et al.*, 1997; Luger and Richmond, 1998).

The histone octamer-DNA complex consists of histones forming a rough cylinder comprised of two heterodimers of histones H3 and H4, flanked by two heterodimers of histones H2A and H2B (Luger *et al.*, 1997; Figure 11). The linker histone, H1, affords stability to the assembly of the octameric core into higher-order structures characteristic of chromatin (Figure 10).

Figure 10. Chromatin organization and the histone H3 amino-terminal tail. **(A)** The nucleosome particles that make up chromatin are depicted as yellow cylinders. The DNA is depicted as black strands and the amino-terminal histone tails are shown as red wavy lines. **(B)** Higher-order chromatin characteristic of condensed chromatin or heterochromatin is shown as packaged nucleosomes. **(C)** The high-resolution structure of the nucleosome core particle (Luger *et al.*, 1997) shows the DNA double helix (blue) and the histone H3 dimer (red), H4 (green), H2A (aqua) and H2B (purple). **(D)** Histone H3 tail region from yeast is shown with the modifications that are known to regulate gene activity. Acetylation is represented by (A), phosphorylation is represented by (P) and methylation is represented by (M). Modifications that promote transcriptional activation are shown above the sequence and modifications that induce transcriptional silencing or chromosome condensation are shown below the sequence (adapted from Marmostein, 2001).

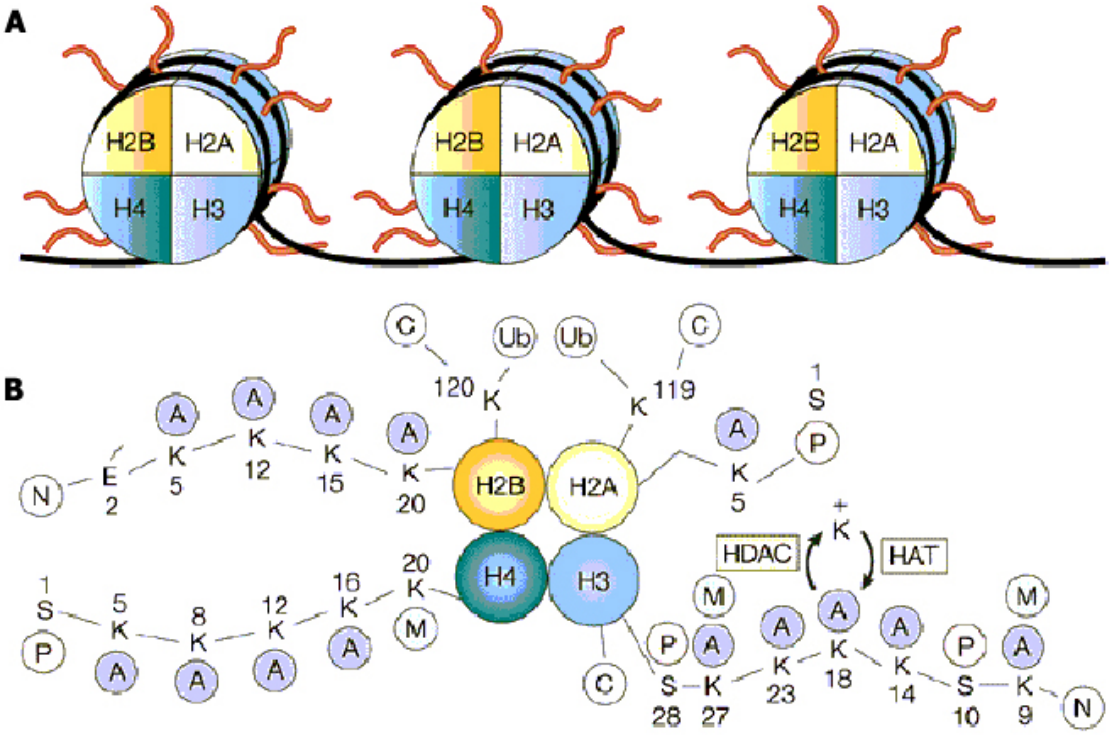


The four histone subunits that comprise the octamer are very basic. Each of them possesses a carboxy-terminal: a highly globular domain that comprises about 75 % of the amino acid content and forms the interior of the core of the nucleosome particle. The remaining amino-terminal portion of the core histone proteins has a flexible lysine rich tail region, which contains about half of the positively charged residues. These amino-terminal tails pass through and around the enveloping DNA double helix such that the DNA is arranged around the core, allowing the interactions between the amino-terminal tails of the histones and the adjacent nucleosome particles.

The amino-terminal tails contain most of the post-translational modification sites of the core histones (Figure 11). However, the functions of these post-translational modifications, which include methylation, ADP-ribosylation, phosphorylation, ubiquitylation and acetylation (Bradbury, 1992), are largely unclear (Kornberg and Lorch, 1999b; Gregory *et al.*, 2001). Nonetheless, these modifications, in particular acetylation, are crucial in modulating gene expression, as they affect the interaction of DNA with transcription-regulatory non-nucleosomal protein complexes (Kouzarides, 2000; Strahl and Allis, 2000; Roth *et al.*, 2001). Apart from their involvement in regulating transcription, these modifications are also correlated with other nuclear activities such as replication and chromatin assembly (Durrin *et al.*, 1991; Thompson *et al.*, 1994; Grunstein, 1997).

In the light of the importance of these amino-terminal lysine rich histone tails with regard to DNA-regulatory protein interactions, Strahl and Allis proposed that a regulatory ‘code’ resides in the pattern of post-translational modifications, of which

Figure 11. Schematic model of the structure of histones in nucleosomes. (A) Each histone is present in two copies, so the DNA (black) wraps around an octamer of histones — the core nucleosome. **(B)** The amino-terminal tails of core histones showing that the lysines (K) in the amino-terminal tails of histones H2A, H2B, H3 and H4 are potential acetylation/deacetylation sites for histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation neutralizes the charge on lysines. A, acetyl; C, carboxyl terminus; E, glutamic acid; M, methyl; N, amino terminus; P, phosphate; S, serine; Ub, ubiquitin (adapted from Davie, 1998).



these tails are the target (Strahl and Allis, 2000; Figure 11). They proposed that the non-histone proteins and multiprotein complexes, which form the transcription-activating and transcription-repressing molecular machinery, are able to read this 'code'. Most recently, it was revealed that the cascade of the human Interferon-β (IFNβ) gene activation was via a point-by-point interpretation of the histone 'code' through the ordered recruitment of bromodomain-containing transcription complexes (Agalioti *et al.*, 2002). Of these modifications, histone acetylation has received the most analysis, and forms the basis of an evolving model for how histone modifications may modulate transcription. Two classes of chromatin-modifying enzymes affect the acetylation status of histones: histone acetyltransferases (HATs) and histone deacetylases (HDACs; see Section 1.4).

The association of histones with DNA is modulated, at least in part, by changing the charge interactions between the amino-terminal histone tails and the DNA (Sivolob *et al.*, 2000). In the absence of modification, the basic histone tails are tightly associated with the acidic DNA backbone, which helps prevent DNA access by transcriptional activators. Once lysine acetylation occurs, the overall charge of the amino-terminal tail is mitigated, thus relieving association of the histone tails with DNA, and, possibly, also changing the conformation of the core nucleosome particle itself, allowing easier access for transcription factors to associate with DNA for gene activation. Conversely, histone deacetylation enhances the association between the histone tails and the DNA, thereby augmenting transcriptional repression. It was observed that histone acetylation increases the α-helical content of the histone tails of the nucleosome; giving indications that histone tail acetylation might be important in the regulation of the chromatin conformation (Wang *et al.*, 2000).

It was further suggested that the combination of tight histone-DNA contacts, and the organization of nucleosomes into higher-order structures, can restrict access to DNA by proteins involved in transcription (Lee and Young, 2000). The flexible amino-terminal histone tails that protrude from nucleosomes may also promote fiber formation, either through direct contact with nucleosomes or by interactions with linker DNA (Fletcher and Hansen, 1995).

Several mechanisms have been identified for nucleosome-potentiated gene expression (Figure 12). Activation of the mouse mammary tumor virus promoter involves synergistic interactions between multiple transcription factors that occur when their binding sites are appropriately positioned by nucleosomes (Chavez and Beato, 1997). Estrogen-regulated transcription of the *Xenopus* vitellogenin B1 promoter is potentiated by generation of a nucleosome-dependent loop (Schild *et al.*, 1993). The mouse transcriptional activator Hnf3 can stably bind its target sequence only when the DNA is packaged into a nucleosome (Cirillo and Zaret, 1999). Hence, DNA packaging into chromatin probably provides a distinct physical context for each promoter, henceforth increasing the options available for regulation of specific genes.

Transcriptional activators recruit both chromatin-remodeling factors and the general transcriptional machinery after binding to gene promoters of protein-coding genes (Ptashne and Gann, 1997; Sauer and Tjian, 1997; Struhl, 1999; Figure 13). The discovery that chromatin-remodeling (or –modifying) enzymes are components of multiple complexes involved in transcription initiation and elongation, reaffirms the importance of chromatin remodeling in gene regulation. These factors are able to regulate gene expression through covalent modification of the nucleosomes, as is the

Figure 12. Effects of nucleosomes on binding of activators and the transcriptional machinery. (A) Nucleosomes interfere with binding of activators (*green*) or elements of the transcription machinery (*blue*) and **(B)** serve a positive role in transcription by positioning two distinct DNA segments to create a complete binding site. **(C)** The nucleosomes position independent activator binding sites to permit synergistic binding of activators, and also **(D)** alter the orientation or distance between factors, thereby stimulating interactions required for transcription (adapted from Lee and Young, 2000).

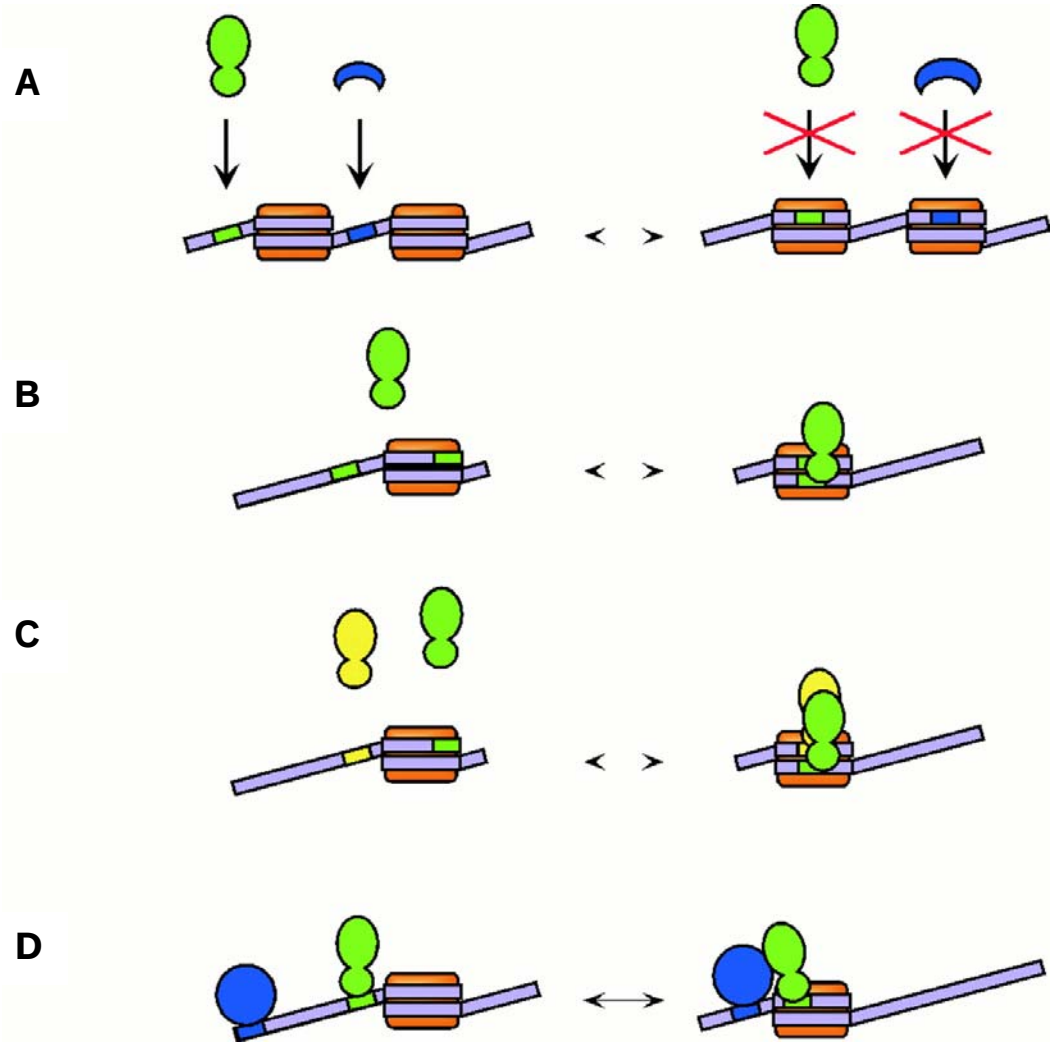
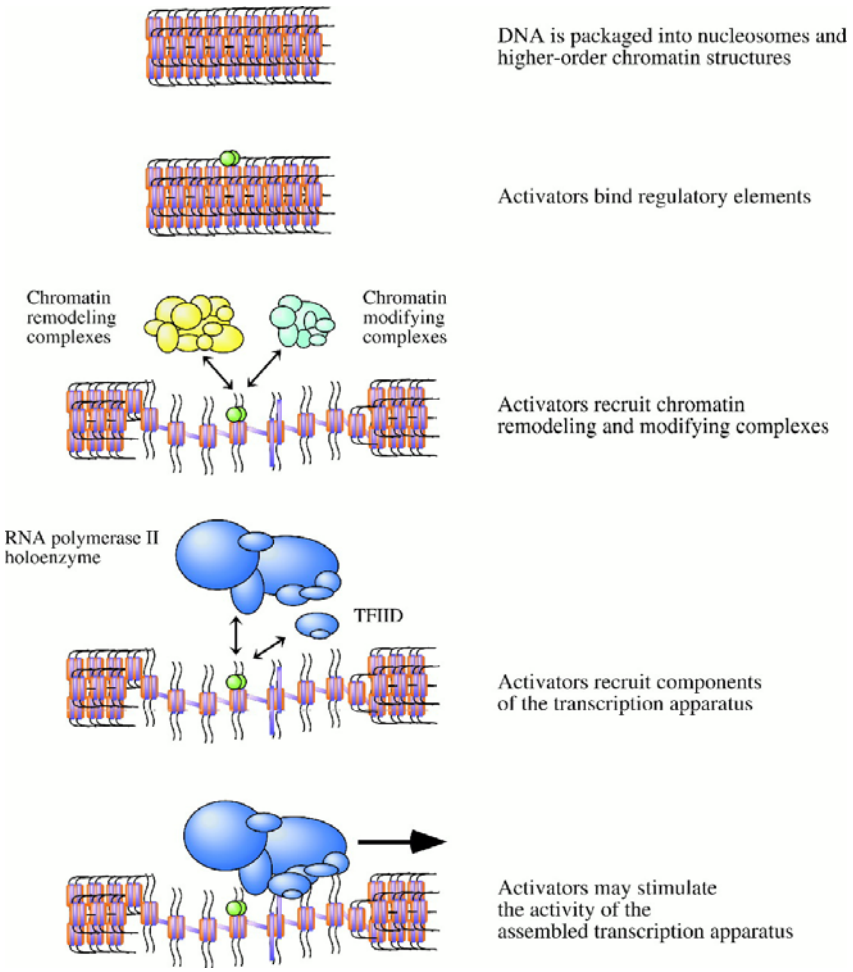


Figure 13. A model for the role of activators in transcription initiation. Activators can bind and recruit chromatin remodeling and modifying complexes that influence local chromatin structure thereby possibly increasing the stability of the activator-DNA complex and affecting access of promoter sequences for binding of the transcription apparatus. Activators also bind and recruit the transcription initiation complex, probably through the concerted interactions of a few large complexes. The diagram suggests an order to the process of gene activation, although it is not clear that this occurs at all promoters in vivo. The diagram shows a single activator bound at the promoter, but promoters typically contain multiple activator binding sites (based on Lee and Young, 2000).



case with Gcn5 acetyltransferase of the SAGA complex, or through noncovalent modifications as in the case with the Swi/Snf complex (Kingston and Narlikar, 1999; Brown *et al.*, 2000; Vignali *et al.*, 2000).

1.4 Roles of Histone Acetyltransferases and Deacetylases in Gene Regulation

For nearly four decades, acetylation of internal lysine residues of core histone amino-terminal domains has been known to correlatively associate with transcriptional activation in eukaryotes (Allfrey, 1964 and 1966). Recent discoveries (Strahl and Allis, 2000; Gregory et al., 2001; Roth et al., 2001) showing that several transcriptional regulators possess intrinsic HAT and HDAC activities strongly suggest that histone acetylation and deacetylation each plays a causative role in regulating transcription.

Transcriptional activation is linked to the active acetylation of core histones (Lee *et al.*, 1993; Brownell and Allis, 1996; Vettese-Dadey *et al*., 1996; Wolffe and Pruss, 1996; Grunstein, 1997; Ura *et al*., 1997). Histone acetylation occurs post-translationally, and reversibly, on the lysine residues embedded in the amino-terminal tails of core histones (Figure 14). HATs transfer the acetyl moiety from acetyl coenzyme A to the ε-NH_3 groups of these internal lysine residues so neutralizing the positive charge and increasing the hydrophobicity. In the opposing deacetylation reaction, HDACs remove the acetyl groups re-establishing the positive charge in the histones (Figure 15).

Figure 14. Histone acetylation-deacetylation cycle. Equilibrium of steady-state histone acetylation is maintained by opposing activities of histone acetyltransferases and deacetylases. Acetyl coenzyme A is the high-energy acetyl moiety donor for histone acetylation. Histone acetyltransferases (HATs) transfer the acetyl moiety to the ε-NH_3 group of internal lysine residues of histone N-terminal domains. Reversal of this reaction is catalyzed by histone deacetylases (HDACs; based on Kuo and Allis, 1998).

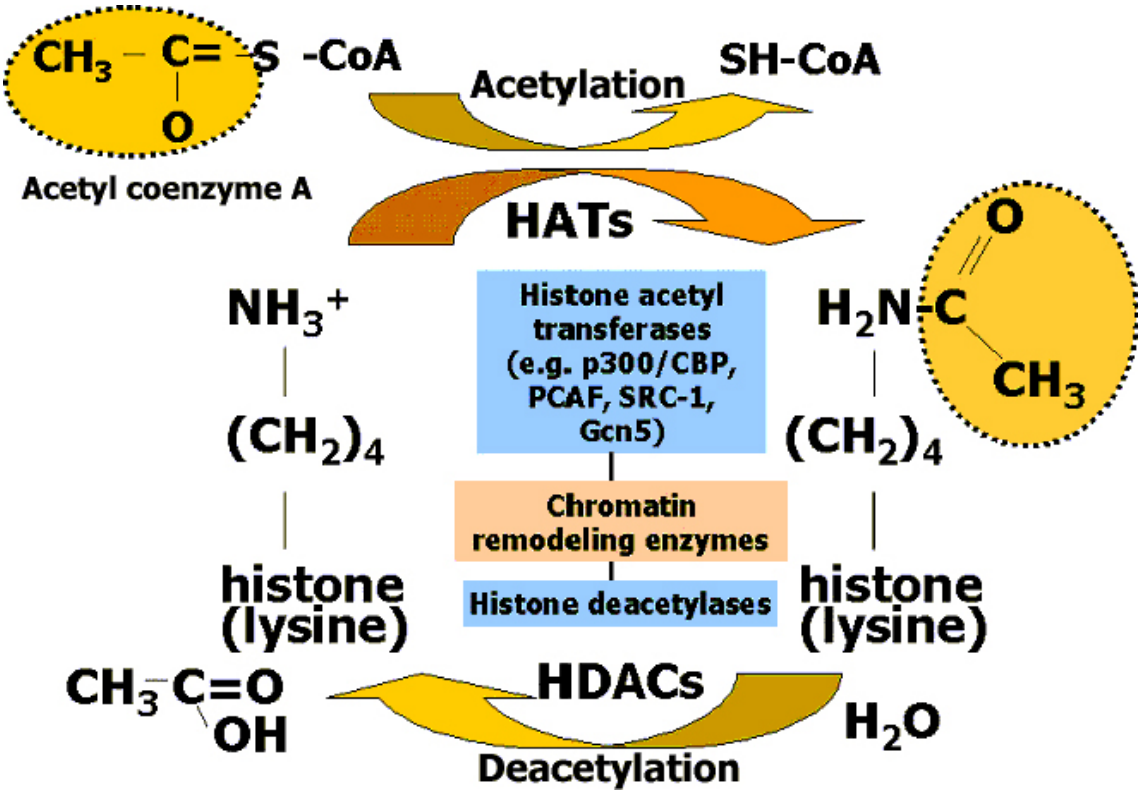
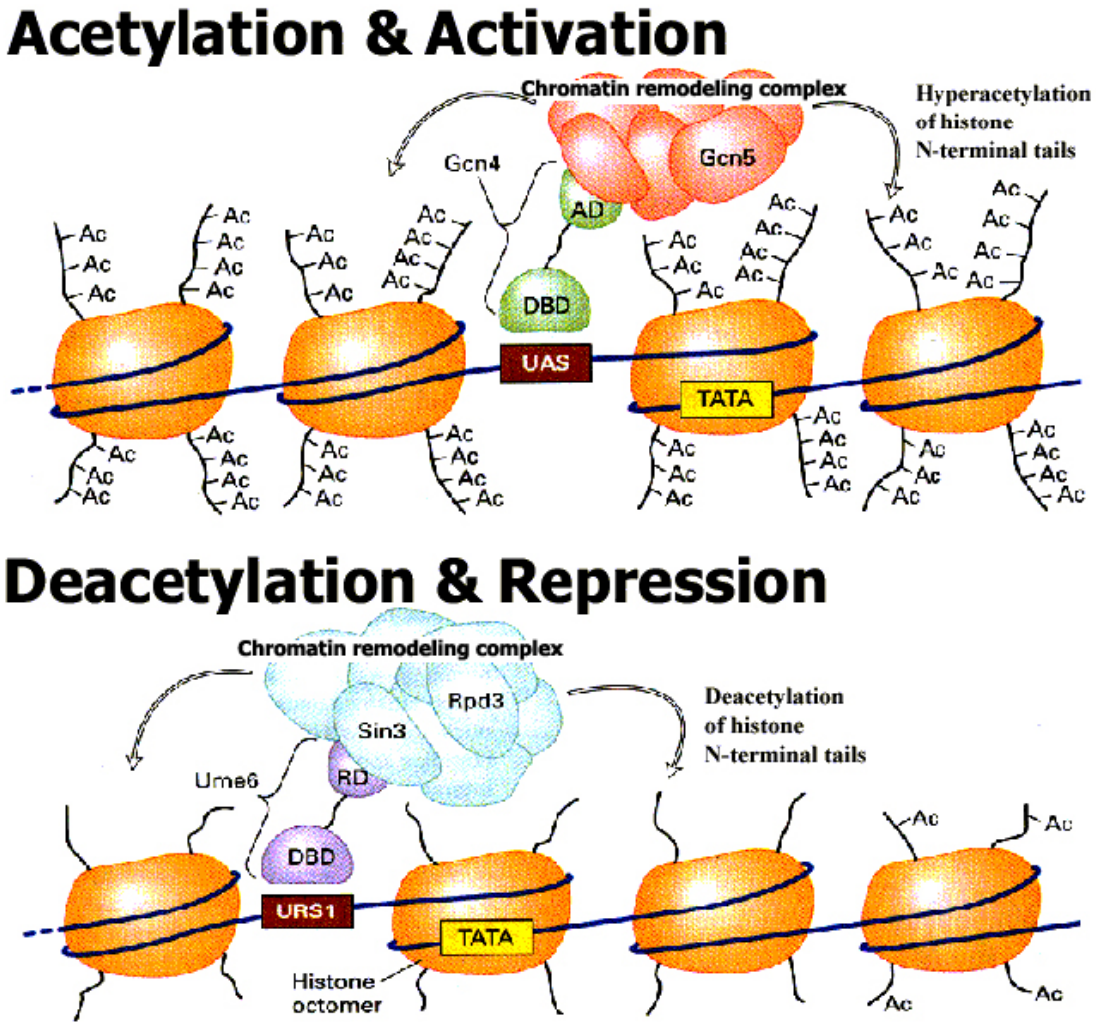


Figure 15. Acetylation and Activation, Deacetylation and Repression. Acetylation of histones releases the wrapping of DNA around histones, and reduces the charge, which results in less interaction with DNA. Is also reduces the stability of interaction of histone tails with DNA. Furthermore, it disrupts the secondary structures within H3 and H4 N-terminals, resulting in further destabilization of the interactions with DNA. All these, facilitate the binding of transcription factors to their binding sites in isolated nucleosomes. Deacetylation reverses these effects. Abbreviations: DBD, DNA binding domain; URS1, upstream control element; RD, repression domain; UAS, upstream activating sequences; AD, activation domain; Gcn5, a histone acetylase; Rpd3, a histone deacetylase (adapted from Lodish *et al.*, 2000).



The HATs are divided into five families (Table 1). These include the Gcn5-related acetyltransferases (GNATs); the MYST (for 'MOZ, Ybf2/Sas3, Sas2 and Tip60')-related HATs; p300/CBP HATs; the general transcription factor HATs, which include the TFIID subunit TAF250; and the nuclear hormone-related HATs SRC1 and ACTR (SRC3).

HATs do not bind directly to DNA but are recruited to promoters by means of DNA-bound transcription factors (Roth *et al.*, 2001). The acetylation of histone tail lysines is not random; indeed, HATs preferentially acetylate specific histone lysine substrates (Strahl and Allis, 2000; Roth *et al.*, 2001). HATs function in association with protein complexes that can include an array of other HATs, transcription co-activators and corepressors.

The mammalian HDACs are ordered into three classes (Table 2). Class I deacetylases include HDACs 1, 2, 3 and 8; class II deacetylases include HDACs, 4, 5, 6, 7, 9 and 10 (Grozinger *et al.*, 2001; De Ruijter *et al.*, 2003). The third class of HDACs is the conserved nicotinamide adenine dinucleotide-dependent Sir2 family of deacetylases (Grozinger *et al.*, 2001).

HDACs are involved primarily in the repression of the gene transcription by virtue of the compaction of chromatin structure that accompanies the removal of charge-neutralizing acetyl groups from the histone lysine tails (Gregory *et al.*, 2001; Kochbin *et al.*, 2001; Figure 15). As with HATs, HDACs are found in multiprotein complexes that regulate gene transcription. HDACs do not bind directly to DNA but are recruited

Table 1. Characteristics of HAT families (based on Kuo and Allis, 1998).

HAT family	*HAT (and complexes associated with it)*	*Histones acetylated by recombinant HAT*	*Histones acetylated by HAT complex*	*Interactions with other HATs*
GNAT	GCN5	H3 >>H4	H3, H2B	p300; CBP
	pCAF	H3 >> H4	H3, H4	p300; CBP
	Hat1 (HatB)	H4 >> H2A	H4, H2A	
	Elp3 (elongator)	H2A, H2B, H3, H4		
	Hpa2	H3 > H4		
MYST	Esa1	H4 >> H3, H2A	H2A, H4	
	Mof	H4 >> H3, H2A	H4	
	Sas2	Unknown		
	Sas3	Unknown	H3	
	MORF	H4 > H3		
	Tip60	H4 >> H3, H2A		
p300/CBP	p300	H2A, H2B, H3, H4		pCAF; GCN5
	CBP	H2A, H2B, H3, H4		pCAF; GCN5
General transcription factors	TAF250 (TFIID)	H3 >> H2A		
	TFIIIC		H3, H4 > H2A	
	Nut1 (mediator)		H3 >> H4	
Nuclear receptor cofactors	SRC1	H3 > H4		p300; CBP; pCAF
	ACTR (SRC3)	H3 > H4		p300; CBP; pCAF

Table 2. HDAC classes and their sizes (adapted from Marks *et al.*, 2003).

Class	*Enzyme*	*Size (amino acids)*
I (Rpd3-like)	HDAC1	482
	HDAC2	488
	HDAC3	428
	HDAC8	377
II (Hda1-like)	HDAC4	1084
	HDAC5	1122
	HDAC6	1215
	HDAC7	855
	HDAC9	1011
	HDAC10	>700
III (Sir2-like)	SIRT1	747
	SIRT2	373
	SIRT3	399
	SIRT4	314
	SIRT5	310
	SIRT6	355
	SIRT7	400

to DNA by protein complexes that vary in their subunit composition (Khochbin *et al*., 2001).

Several aspects of the nucleosome function are affected by acetylation of internal lysines. For example, the affinity of histone N-terminal domains for DNA is significantly reduced (Hong *et al.,* 1993); indeed, histone acetylation alters the linking number of close circular DNAs *in vivo* (Norton *et al.*, 1989). Besides the subtle changes in nucleosome structure (Garcia-Ramirez *et al.*, 1995; Bauer *et al.*, 1994), modulation of the histone tail domain seems to influence interactions between nucleosomes and other nonhistone chromosomal proteins. For example, certain proteins, such as yeast silencing information regulators Sir3p and Sir4p, (Hecht *et al.*, 1995) and the general repressor Tup1p (Edmondson *et al.*, 1996), interact preferentially with hypoacetylated histone N-terminal domains; histone acetylation may interfere with such interactions. The crystal structure of defined nucleosome core particles has been solved (Luger *et al.*, 1997). Based on the current 2.8-Å structure, it appears that, at least under the condition of crystallization, histone tails may play some role in internucleosomal interaction affecting higher-order structure of chromatin. Acetylation seems likely to impose some effects on this aspect of chromatin structure (Luger *et al.*, 1997).

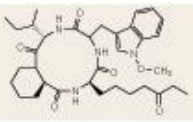
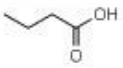
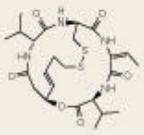
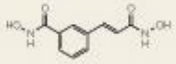
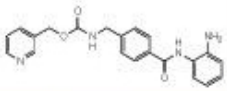
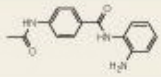
Both HATs and HDACs also target proteins other than histones, including transcription factors. Deacetylation of these substrates; which include p53, E2F, GATA1, TFII and TFIIF, might explain the ability of HDACs to regulate gene expression by a mechanism that is distinct from their effects on chromatin (Deckert and Struhl, 2001; Gray and Ekstrom, 2001; Kochbin *et al.*, 2001). HDACs have been

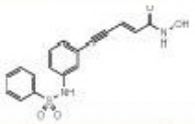
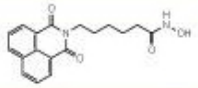
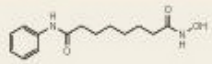
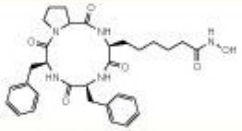
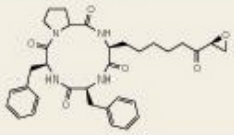
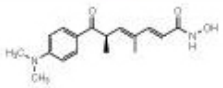
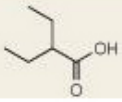
found in complexes with proteins that are involved in the regulation of cell-cycle progression and apoptosis. These include glucocorticoid receptors (Ito *et al.*, 2000), DNA methytransferase 1 (Robertson *et al.*, 2000) and chicken ovalbumin upstream promoter transcription factor (Smirnov *et al.*, 2000). These proteins are involved in pathways that have diverse effects ranging from altered gene transcription (for example, DNA methytransferase) to effects on cell-cell interactions.

Inhibition of HDACs The fact that different HDACs have diverse functions (Grozinger *et al*., 2001; De Ruijter *et al*., 2003; Turner, 2002; Khochbin *et al*., 2001; McKinsey *et al*., 2001), even though these are not well understood, makes it desirable to develop selective inhibitors. With inhibition of HDACs by HDAC inhibitors, such as trichostatin A (TSA; Yoshida et al., 1990), histones are acetylated, and the DNA that is tightly wrapped around a deacetylated histone core relaxes. The classes of compounds that are identified as HDAC inhibitors (Table 3) include: short chain fatty acids (such as 4-phenylbutyrate and valproic acid); hydroxamic acids (such as suberoylanilide hydroxamic acid, pyroxamide, TSA and oxamflatin); cyclic tetrapeptides (such as trapoxin, apicidin and depsipeptide); and benzamides (such as MS-275). Hydroxamic-acid containing HDAC inhibitors have been suggested to interact with the catalytic site of HDACs, thereby blocking substrate access to the active zinc ion at its base, as shown by X-ray crystallographic studies (Finnin *et al.*, 1999).

TSA, which was developed as an antifungal agent, has relatively high reactivity (active at nanomolar concentrations) and is unstable, has been useful in studying HDAC function (Yoshida *et al.*, 1990; Jung *et al.*, 1999; Marks *et al.*, 2000).

Table 3. Histone deacetylase inhibitors. The structures of the HDAC inhibitors are shown (adapted from Grozinger and Schreiber, 2002).

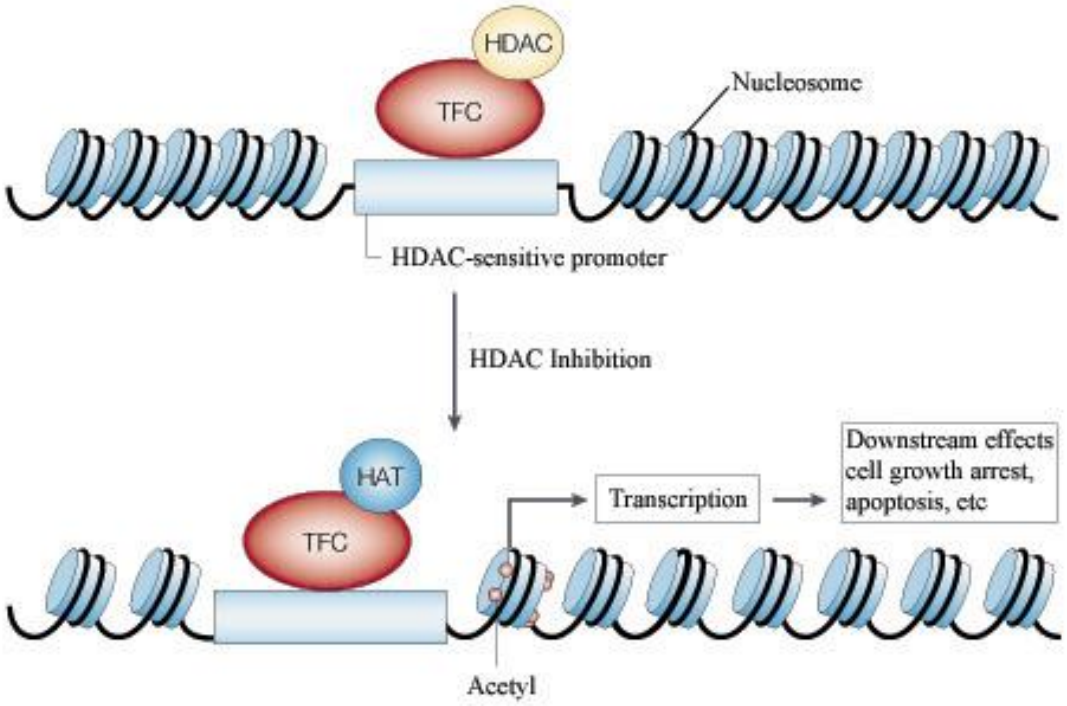
Name	Structure
Apicidin	
Butyrates	
Depsipeptide (FR901228, FK-228)	
Depudecin	
m-Carboxy cinnamic acid bishydroxamic acid (CBHA)	
MS-275	
CI-994 (*N*-acetyl dinaline)	

Name	Structure
Oxamflatin	
Pyroxamide	
Scriptaid	
Suberoylanilide hydroxamic acid (SAHA)	
TPX-HA analogue (CHAP)	
Trapoxin	
Trichostatin A (TSA)	
Valproic acid	

In one such study, it was revealed that an active site consisting of a tubular pocket, a zinc-binding site and two Asp–His charge-relay systems were present in the HDAC catalytic core structure, and it also established the mechanism of HDAC inhibition (Finnin *et al.*, 1999).

Marks and colleagues (Marks *et al.*, 2001) have proposed a mechanism for the HDAC inhibition by inhibitors such as TSA (Figure 16). They suggested that, in the promoter region of a subset of genes, there exist specific sites that recruit the transcription factor complex (TFC) with HDAC and that the exposure to HDAC inhibitors leads to an accumulation of acetylated histones in the nucleosomes bringing about increased transcription of this subset of genes, which in turn, leads to downstream effects.

Figure 16. Mechanism of action of histone deacetylase inhibitors. With inhibition of HDACs by HDAC inhibitors such as trichostatin A (TSA), histones are acetylated, and the DNA that is tightly wrapped around a deacetylated histone core relaxes. There are specific sites in the promoter region of a subset of genes that recruit the transcription factor complex (TFC) with HDAC, and that accumulation of acetylated histones in the nucleosomes leads to increased transcription of this subset of genes, which, in turn, leads to downstream effects that result in cell growth arrest, differentiation and/or apoptotic cell death (adapted from Marks *et al.*, 2001).



1.5 Hypotheses and Aims

Mammalian reproduction and development depend on the exquisite and precise regulation of the gonadotropin genes (Section 1.2). Chromatin remodeling as a result of post-translational modifications of the nucleosomal histone tails is pivotal in gene regulation (Kouzarides et al, 2000; Strahl and Allis, 2000; Roth et al, 2001). In particular, the acetylation state of histones within chromatin is correlated with transcriptional competence (Allfrey et al, 1964; Pogo et al, 1966).

The objective of this study was to investigate the correlation between gonadotropin gene expression and nucleosomal histone acetylation and deacetylation catalyzed by HATs and HDACs respectively. We wanted to know whether hyperacetylation of the histone and/or non-histone proteins promotes gonadotropin gene expression. The murine gonadotrope was used because it provides an excellent model system as the gonadotropin genes, LHβ and FSHβ, are not expressed in the immature αT3-1 clonal cell line, while only the LHβ gene is expressed in the mature LβT2 clonal cell line.

We hypothesized that the repression of these genes is due to histone deacetylation by HDACs. The effects of the HDAC inhibitor, TSA, which on the gonadotrope cell model were assessed in order to provide an insight into the

mechanisms involved in the repression of the LHβ and FSHβ genes in immature and mature gonadotropes at both basal and GnRH-modulated gene expression levels.

We hypothesized further that the effects of acetylation due to HDAC inhibition are far ranging, affecting not only the histone tails but also non-chromatin substrates. In investigating the effects of TSA on non-chromatin targets at both the basal and GnRH-modulated levels of gene expression, transient transfection studies, which exclude the gonadotropin chromatin environment as the causative effect of any gene transcriptional activity, were carried out using the LHβ and FSHβ promoter constructs.

In the reversible process of acetylation/deacetylation, HDACs and HATs play opposite roles. We postulated that, if HDACs are negatively regulating the gene expression of the gonadotropins, the HATs may also be involved. We investigated which HATs are involved and the possible mechanisms of their recruitment to the promoter by various signaling pathways while considering the possibility that different combination of HATs, HDACs, other cofactors and transcription factors come into play for various signaling cascades.

To study the molecular mechanisms of possible crosstalk between these signals, we then focused on how Smad proteins may affect the GnRH signaling pathway and the steroidal actions in gonadotropes. We also investigate how such crosstalk may relate to the regulatory functions of HAT/HDAC complexes and their exchanges at the promoter.

Chapter 2 Materials and Methods

2.1 Plasmids

2.1.1 Promoter Constructs

FSHβ and LHβ promoter constructs used in the promoter studies are: (1) the Chinook salmon (CS) FSHβ-chloramphenicol acetyl transferase (CAT); (2) the CS LHβ-CAT constructs (Xiong *et al*, 1994; Chong *et al*, 2001); and (3) the FSHβ-firefly luciferase (LUC) (Feng *et al*, unpublished).

2.1.2 Short Interfering RNA (siRNA) Constructs

The following siRNA constructs targeting sequences were already available: ERα (Luo *et al*, unpublished), *c*-Fos (Zhu *et al*, unpublished), HDAC1 and HDAC2 (Koh *et al*, unpublished). For others, the following procedures describe how they were made.

2.1.2.1 Vectors

The pSuper Basic (OligoEngine) vector was used in concert with a pair of custom oligonucleotides that contain a unique 19-nt sequence derived from the mRNA transcript of the gene targeted for suppression. Prior to ligation with the annealed oligonucleotides, the pSuper Basic vector was linearized with BglII and HindIII.

2.1.2.2 siRNA Design

The oligonucleotides (Table 4) for the siRNA constructs were designed using OligoEngine RNAiDesign Tools (http://www.oligoengine.com).

Table 4. Oligonucleotides used for making siRNA constructs

Gene	Oligonucleotides	Accession No.
GCN5	Forward: 5'-GATCCCCAGGCCAATGAAACCTGCAATTCAAGAGATTGCAGGTTTCATTGGCCTTTTTTA-3' Reverse: 5'-AGCTTAAAAAAGGCCAATGAAACCTGCAATCTCTTGAATTGCAGGTTTCATTGGCCTGGG-3'	NM_020004
pCAF	Forward: 5'-GATCCCCAGTAAAGAGCCCAAAGACCTTCAAGAGAGGTCTTTGGGCTCTTTACTTTTTTA-3' Reverse: 5'-AGCTTAAAAAAGTAAAGAGCCCAAAGACCTCTCTTGAAGGTCTTTGGGCTCTTTACTGGG-3'	NM_020005
Tip60	Forward: 5'-GATCCCCGGCCAAGACACCTACCAAGTTCAAGAGACTTGGTAGGTGTCTTGGCCTTTTTA-3' Reverse: 5'-AGCTTAAAAAGGCCAAGACACCTACCAAGTCTCTTGAACTTGGTAGGTGTCTTGGCCGGG-3'	NM_178637
CBP	Forward: 5'-GATCCCCCACAGCCTCACAATCAACATTCAAGAGATGTTGATTGTGAGGCTGTGTTTTTA-3' Reverse: 5'-AGCTTAAAAACACAGCCTCACAATCAACATCTCTTGAATGTTGATTGTGAGGCTGTGGGG-3'	XM_148699
Smad4	Forward: 5'-GATCCCCCCCCAAGCGCGTATATAAAGTTCAAGAGACTTTATATACGCGCTTGGGTTTTTA-3' Reverse: 5'-AGCTTAAAAACCCAAGCGCGTATATAAAGTCTCTTGAACTTTATATACGCGCTTGGGGGG-3'	NM_008540

The oligos were dissolved in sterile nuclease-free water to a concentration of 3 mg/mL. The annealing reaction was assembled by mixing 1 μL of each oligo (forward and reverse) with 48 μL annealing buffer (100 mM NaCl and 50 mM HEPES pH 7.4). The mixture was incubated at 90 ^{o}C for 4 min, and then at 70 ^{o}C for 10 min. The annealed oligos were then slowly cooled to 4 ^{o}C in a stepwise fashion (reducing the temperature by 6 ^{o}C each time and incubating at each temperature for 4 min). The annealed oligos were ligated separately into the linearized vectors as described in Section 2.1.5. Other procedures involving molecular cloning are also described in Section 2.1.5.

2.1.2.3 Verification of Suppresion of Target Genes by siRNA Constructs

Western blot analysis (Section 2.12) or RT-PCR (primers used are listed in Table 19; as described in Section 2.9) was employed to verify the suppresion of the target genes by the siRNA constructs.

2.1.3 Expression Vectors

The following expression vectors were already available (all from mouse unless otherwise stated): human ERα (F. Pakdec, France), Egr-1 (J. Milbrandt, St Louis), Smad1, Smad2, Smad3, Smad4, Smad5 (Wang *et al*, unpublished), Sf-1 (K. Parker, Oregon), Ptx-1 (J. Drouin, Montreal), *c*-Jun and *c*-Fos (Chong *et al*, unpublished). For others, the following procedures describe how they were made.

pCMV-Script® vector (Strategene) was linearized with NotI and EcoRI according to the procedures in Section 2.1.5.2. The primers (Table 5) for the expression vectors were designed to include two RE sites, one at each end, and according to the cDNA sequences of the genes. These primers all have a Tm that falls in the range of 60-63 ^{o}C. The primers were used to generate inserts by PCR, which were then cut appropriately and ligated separately into the linearized vectors as described in Section 2.1.5. Other procedures involving molecular cloning are also described in Section 2.1.5.

Table 5. Primers used for making expression vectors

Gene	Primer	Expected size	RE Sites
GCN5	Forward: 5'-ATAAGAATGCGGCCGCATGGCGGAACCTTCCCA-3' Reverse: 5'-GGAATTCCCTACTTGTCGATGAGCCCTCC-3'	~2500bp	NotI and EcoRI
Tip60	Forward: 5'-ATAAGAATGCGGCCGCATGGCGGAGGTGGGG-3' Reverse: 5'-GGAATTCCTCACCACTTTCCTCTCTTGATC-3'	~1500bp	NotI and EcoRI

2.1.4 Mammalian Two Hybrid Constructs

The following mammalian two hybrid constructs were already available: pVP16A, pM, pVP16T, pM53 (all from Promega), human ERα pVP/pM, Sf-1 pVP, Ptx-1 pVP/pM, *c*-jun pVP/pM, *c*-fos pVP/pM (Zhu *et al*, unpublished), pRL-CMV (Promega), pCMV-Luc (kindly donated by Dr. Chan Woon Khiong). For others, the following procedures describe how they were made.

2.1.4.1 Vectors

The cloning vector pG5CAT (Clontech) was used as a template to PCR the DNA sequences of GAL4 binding sites with the following pair of primers:

5'-GGGGTACCTCGAGGACAGTACTCCGCT-3'; and

5'-GAAGATCTTTTAGCTTCCTTAGCTCCTG-3'

This produced an insert (~100 bp) with KpnI and BglII RE sites. The insert was cut, and subsequently ligated into a linearized pGL3-basic vector (Promega) to make the pGL3-GAL4 construct, which contains the firefly luciferase reporter gene for assaying protein-protein interactions.

2.1.4.2 Primers

The primers (Table 6) for the mammalian two hybrid constructs were designed to include two RE sites, one at each end. These primers all have a Tm that falls in the range of 60-63 ^{o}C. The primers were used to generate inserts by PCR, which were then cut appropriately and ligated separately into the two linearized pVP and pM vectors as described in Section 2.1.5. Other procedures involving molecular cloning are also described in Section 2.1.5.

Table 6. Primers used for making mammalian two hybrid constructs

Gene	Primer	Expected size	RE Sites
GCN5	Forward: 5'-GGAATTCATGGCGGAACCTTCCCA-3' Reverse: 5'-GCTCTAGACTACTTGTCGATGAGCCCTCC-3'	~2500bp	EcoRI and XbaI
Tip60	Forward: 5'-GGAATTCCATGGCGGAGGTGGGG-3' Reverse: 5'-GCTCTAGATCACCACTTTCCTCTCTTGCTC-3'	~1500bp	EcoRI and XbaI
CBP1	Forward: 5'-GGAATTCATGAAACTGATACAGCAGCAGCTGG-3' Reverse: 5'-GCTCTAGACTATCCACTAGCTGGAGATCCCA-3'	~250bp	EcoRI and XbaI
CBP2	Forward: 5'-GGAATTCATGGTTCGAAAAGGCATGGCATG-3' Reverse: 5'-GCTCTAGACTATTCTTCTAGTTCTTTTTGTATTTTATAGATTTTCTC-3'	~200bp	EcoRI and XbaI
CBP3	Forward: 5'-GGAATTCATGGACCCCTTTGTTTATACCTGCA-3' Reverse: 5'-GCTCTAGACTAGTTGAGGCAGAAGGGCACA-3'	~350bp	EcoRI and XbaI
Smad3	Forward: 5'-GGAATTCATGTCGTCCATCCTGCCC-3' Reverse: 5'-GCTCTAGACTAAGACACACTGGAACAGCGG-3'	~1280	EcoRI and XbaI
Smad4	Forward: 5'-GGAATTCATGGACAATATGTCTATAACAAATACACCA-3' Reverse: 5'-GCTCTAGATCAGTCTAAAGGCTGTGGGTCC-3'	~1400	EcoRI and XbaI
Smad4 MH1	Forward: 5'-GGAATTCATGGAAAGTGAAACCTTTGCAAAAAGA-3' Reverse: 5'-GCTCTAGATCCAGGTGAGACAACCCG-3'	~280	EcoRI and XbaI
Smad4 MH2	Forward: 5'-GGAATTCATGGAGTACTGGTGCTCCATTGCTT-3' Reverse: 5'-GCTCTAGAGTGAAGGTGAATCTCAATCCAGC-3'	~550	EcoRI and XbaI
Smad4 SAD	Forward: 5'-GGAATTCATGCCATACACACCTAATTTGCCTCA-3' Reverse: 5'-GCTCTAGAAGGAGCAGGATGATTGGAAATG-3'	~120	EcoRI and XbaI

2.1.5 Molecular Cloning

2.1.5.1 Preparation of Inserts

For making expression vectors, the inserts were generated by PCR using cDNA synthesized from total RNA extracted from LβT2 cells (as described in Sections 2.5 and 2.8) as template. For making mammalian two hybrid constructs, the inserts were generated by PCR using either available protein expression vectors containing the gene of interest, or cDNA as template. The components of each PCR reaction is given in Table 7, and the cycling parameters in Table 8.

Table 7. Components in each PCR reaction. A negative control without the DNA template was also included to ensure non-contamination. *For cDNA template, 5 μL out of the total 20 μL synthesized was used. For expression vectors as template, an amount of about 100 ng to 500 ng was used.

Components	Volume (μL)
10X buffer	10
dNTP (10 mM)	2
Forward Primer (10 μM)	2
Reverse Primer (10 μM)	2
Template*	-
DyNAzyme II DNA Polymerase (Finnzymes)	2
Sterile distilled water	Made up to 100 μL

Table 8. Cycling parameters for PCR. * Extension times are dependent on the expected sizes of the inserts (60 s = 1kb).

Cycle	Steps
1 (x1)	**Step 1**: 94 °C for 5 min
2 (x35)	**Step 1:** 94 °C for 30 s
	Step 2: 55 °C for 30 s
	Step 3*: 72 °C for 30 to 180 s
3(x1)	**Step 1:** 72 °C for 5 min

The PCR products were loaded on a 1.0 % agarose/EtBr gel after mixing with 6X loading dye. The electrophoresis was carried out at 80-100 V until the dye front reached two thirds of the gel. 1X TAE buffer was used as the running buffer for electrophoresis. A 100 bp or 1 kb DNA ladder (GeneRuler^{TM}, MBI Fermentas) was used for size estimation of the DNA fragments. The EtBr-stained linear DNA fragments were observed under UV illumination and the bands corresponding to the expected sizes of the inserts were cut out and used in DNA gel extraction procedure.

The DNA fragments were extracted from the agarose gel using the QIAEX II Agarose Gel Extraction kit (Qiagen) according to the

manufacturer's instructions. The DNA concentration was measured as described in Section 2.1.6.3.

2.1.5.2 Restriction Enzyme (RE) Digestion of Inserts/Vectors

The inserts and vectors (200 ng and 1 μg respectively) were used for RE digestion with the appropriate pair of restriction enzymes (Tables 5 and 6; Promega) according to the manufacturer's instructions. RE digestion was performed in sequential reaction steps. After each reaction step, it was followed immediately by DNA purification using QIAquick® PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

2.1.5.3 Ligation of Inserts into Vectors

Ligation of the inserts to the vectors was carried out in a 1:6 molar ratio of vector : insert using T4 DNA ligase (Promega) according to the manufacturer's instructions. Transformation of the overnight ligation mixtures into DH5-α competent cells was carried out (Section 2.1.5.5) and subsequent small- and large- scale DNA preparations (Section 2.1.6) were performed. The correct constructs were selected by colony PCR (Section 2.1.5.6) and verified by sequencing (Section 2.1.5.7).

2.1.5.4 Preparation of Frozen Competent *E. coli* DH5-α Cells

A tube of glycerol stock of DH5-α *E. coli* cells was thawed and 50 μl was used to innoculate 2 mL of Lauria Bertiani (LB) medium. This was shaken in a 37 °C incubator set at 200 rpm for 18 h. Of this culture, 100 μl was transferred to 250 mL of LB medium and left to shake at 250 rpm at 37

°C till the optical density at 600 nm (OD_{600}) reached a value of 0.3 to 0.6. The bacterial culture was centrifuged at 4000 x g for 15 min at 4 °C. The pellets were resuspended on ice in 1/20 volume TSS [LB broth (pH 6.1), 10 % PEG 3350 or 8000, 5 % DMSO (dimethylsulfoxide, Sigma) 10 mM $MgCl_2$, 10 mM $MgSO_4$ and final pH 6.5]. The competent cells were kept on ice for 10 min before being aliquoted in 200 μL in sterile centrifuge tubes and snap-frozen in liquid nitrogen and stored at -80 °C.

2.1.5.5 Transformation of *E. coli* DH5-α Cells

DH5-α competent cells (from Section 2.1.5.4; 50 μL per transformation reaction) were thawed on ice and the ligation mixture (10 μL) was added to the cells before gentle mixing and incubation on ice for 30 min. The cells/DNA mixture was then incubated at 42 °C for 90 s, and immediately after that, placed on ice. LB medium (500 μL) was added to the mixture, and shaken in a 37 °C incubator set at 250 rpm for 60 min. The mixture was then centrifuged at high speed for 10 s, and the LB medium was discarded leaving about 50 μL in the microfuge tube. The cell pellet was resuspended, and the cells were then plated onto a LB-ampicillin agar plate pretreated with 40 μL of 10% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 4 μL of 100 mm isopropyl-β-D-thiogalactopyranoside (IPTG). The plate was incubated overnight at 37 °C.

2.1.5.6 Selection of Colonies by Colony PCR

The ligation was examined using colony PCR. A single colony was picked and swirled inside 10 µl distilled water of which 5 µl were used as template for PCR reaction. The PCR amplification was carried out using two oligonucleotide primers flanking the multiple cloning sites of the vectors (pGL3-basic, pCMV-script, pVP16 or pM). The sequences of these primers are shown in Table 9. The PCR reaction components and cycling parameters are given in Tables 10 and 11 respectively. The amplicon was analyzed in 1 % agarose/EtBr gel (Section 2.1.5.2). The size of the DNA fragment band determines the status of insertion of target DNA into the vectors.

Table 9. Primers used for colony PCR reaction

Primer	Sequence	Tm	Vector
RVprimer3	5'-CTAGCAAATAGGCTGTCCCCCAG-3'	55	pGL3-basic
pGL3REV	5'-CTGCCAACCGAACGGACATTTCGAAGTACTCAGC-3'	55	pGL3-basic
pVP-FWD	5'-GCCGACTTCGAGTTTGAG-3'	45.2	pVP16
pM-FWD	5'-TCATCGGAAGAGAGTAG-3'	39.5	pM
pVP/pM-REV	5'-GGTTCAGGGGGAGGTGTGGG-3'	54.9	pVP16 and pM
T3	5'-ATTAACCCTCACTAAAGGGA-3'	55	pCMV-Script
T7	5'-GTAATACGACTCACTATAGGGCGA-3'	55	pCMV-Script

Table 10. Components in each PCR reaction. A negative control without the DNA template was also included to ensure non-contamination.

Components	Volume (µL)
10X buffer	2.5
dNTP (10 mM)	0.5
Forward Primer (10 µM)	0.5
Reverse Primer (10 µM)	0.5
Bacterial mix	5
DyNAzyme II DNA Polymerase (Finnzymes)	0.5
Sterile distilled water	Made up to 25 µL

Table 11. Cycling parameters for PCR. *Annealing temperature depends on the Tm of the primers used. **Extension times are dependent on the expected size of the amplicon (60 s = 1kb).

Cycle	Steps
1 (x1)	**Step 1**: 94 °C for 5 min
2 (x35)	**Step 1:** 94 °C for 30 s
	Step 2*: 55 °C for 30 s
	Step 3:** 72 °C for 30 to 180 s
3(x1)	**Step 1:** 72 °C for 5 min

2.1.5.7 DNA Sequencing to Verify Constructs

DNA sequencing was carried out for verification of the constructs. For siRNA and protein expression constructs, T3 or T7 (Table 9) was used. For the pGL3-Gal4 construct, RVprimer3 or pGL3REV (Table 9) was used. For mammalian two hybrid constructs, pVP-FWD, pM-FWD or pVP/pM-REV (Table 9) was used.

The ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Perkin Elmer) was used for the sequencing. The sequencing reaction mix was prepared by combining the components in a separate tube as in Table 12. The precipitation of the PCR fragment was carried out according to the manufacturer's protocol. Automated sequencing loading buffer (2 μL of 5X deionized formamide, 1X 20 mM EDTA, pH 8.0 with blue dextran [50 mg/mL]) was added to the PCR pellet. Automated sequencing was then performed using the ABI Prism 377 DNA Automated Sequencer (Perkin Elmer). The sequencing reactions were performed in a thermal temperature cycler (iCycler™, BIO-RAD) as in Table 13.

Table 12. Preparation of the sequencing reaction mixtures.

Components	Quantity (µl)
Terminator Ready Reaction Mix	4
DsDNA template (200 ng)	1
Primer (1.6 pmol)	1
Deionised water	4
Total	**10**

Table 13. Cycling parameters for sequencing reaction.

Step	Temperature (°C)	Time	No. of Cycles
Denaturation	95	30 s	25
Annealing	50	15 s	
Extension	60	4 min	
Soak	4	Indefinite	1

2.1.6 Plasmid Isolation and Preparation

2.1.6.1 Small-scale (Mini-prep) Preparation of Plasmids

Selected colonies were grown in 5 mL LB medium supplemented with 100 µg/µL ampicillin. The culture was shaken in a 37 °C incubator set at 250 rpm for 18 h. This was followed by centrifuging 2 mL of the cultures at 10,000 x g for 10 min. The plasmid DNA was extracted using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega).

2.1.6.2 Large-scale (Maxi-prep) Preparation of Plasmids

After verifying the result from the digestion reaction, the selected colony was grown in 5 mL LB-ampicillin medium. After about 8 h, the culture was poured into a 500 mL conical flask containing 250 mL Terrific Broth (3 g bacto-tryptone, bacto-yeast extract, 1 mL glycerol, 225 mL water and 25 mL of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4) containing ampicillin (100 µg/mL). The culture was shaken in a 37 °C incubator set at 250 rpm for about 16 h. The Quantum Prep plasmid maxiprep kit (BIO-RAD) was used to isolate plasmid

DNA. The large-scale plasmid preparation was air-dried and the DNA was resuspended in 300 μL deionized water.

2.1.6.3 Analysis of DNA Purity and Concentration

To analyze the amount and purity of DNA, spectrophotometric readings at $OD_{260\ nm}$ and $OD_{280\ nm}$ were taken after DNA purification. The ratios of $OD_{260\ nm}/OD_{280\ nm}$ were taken as the basis of DNA purity.

2.2 Cell Culture

2.2.1 Mouse Gonadotrope Clonal Cell Lines

Mouse gonadotrope αT3-1 and LβT2 cells (a gift from Dr P. Mellon, San Diego) were used.

2.2.2 Cell Media

The αT3-1 cells were cultured in Minimum Essential Medium (MEM) containing MEM Sodium Pyruvate and MEM Non-essential amino acids with 10 % qualified FBS, 10 mM HEPES and 100 μg/mL penicillin and 100 μg/mL streptomycin (Gibco-BRL). The LβT2 cells were cultured in Dulbeco's modified Eagle's Medium (DMEM) containing 4.5 g/L glucose with 10 % certified FBS, 10 mM HEPES and 100 μg/mL penicillin and 100 μg/mL streptomycin (Gibco-BRL).

2.2.3 Splitting of Cells

The medium was removed, and the cells rinsed with HBSS (1X HBSS, 10 mM HEPES) once. Appropriate amount of trypsin (Sigma) was added to the culture dishes, swirled around and incubated for 2 min. The reaction was stopped by addition of cell-specific media (2X volume of trypsin used). The cells were detached from the dishes by pipetting and cells were collected in a 50 mL falcon tube.

2.2.4 Plating and Incubation

The cells were counted and subsequently plated on appropriate culture dishes. For 6-well culture dishes, $6x10^5$ cells in 2 mL per well were plated. For 60 mm culture dishes, $5x10^6$ cells in 5 mL were plated. For 96-well microplates, $2x10^4$ cells in 100 μL per well were plated. The cells were then incubated at 37 °C under 5 % CO_2, and were grown according to the required confluency before proceeding with experiments.

2.2.5 Storing of Cells

The cells were rinsed once with HBSS. Trypsin was added together with the specific cell media to collect the cells in a 1.5 mL cryogenic microfuge (NUNC). The tube was centrifuged at 2000 rpm for 2 min. The supernatant was discarded. The pellet was resuspended with freezing solution (90 % specific cell media and 10 % DMSO). The tubes of cells were then incubated at -80^{o}C overnight before storing them in a liquid nitrogen storage tank.

2.2.6 Recovery of Cells

The tube of frozen cells was placed in ice to thaw. The outside of the tube was sterilized with 70% ethanol solution. The contents of the tube were then directly poured into a 100 mm culture dish containing 10 mL of cell-specific medium. The dish was then swirled gently and incubated at 37 °C under 5 % CO_2 for 24 h before the cells were rinsed once with 1X HBSS and new media replaced. The cells were then grown according to the required confluency before splitting the cells to be used for experiments.

2.3 Transient Transfections

2.3.1 Preparation of Cells for Transfection

αT3-1 and LβT2 cells were plated in appropriate culture dishes one to two days before transfection. They were incubated at 37 °C under 5 % CO_2, and were 50-80 % confluent by the day of the transfections. The cells were rinsed and new media added just before transfection.

2.3.2 Transfection of αT3-1 Cells

Transfection of αT3-1 cells was carried out using the FuGENE6 reagent (Roche, USA), at a ratio of FuGENE6 reagent (μL) to plasmid DNA (μg) of 4:2. FuGENE6 reagent was added directly into 96 μl of serum-free media. The contents were mixed and the diluted FuGENE6 transfection reagent was incubated for 5 min at room temperature. Plasmid DNA (2 μg) was added and the tube was gently tapped to mix the contents. It was then incubated for 15 min at room temperature. The 100 μl of mix were added drop wise to each well of the 6-well plates. The plates were swirled to ensure even dispersal before incubation for a further 48 h.

2.3.3 Transfection of LβT2 Cells

Transfection of LβT2 cells was carried out using the GenePorter2 reagent (Gene Therapy Systems, USA) according to the manufacturer's instructions. Briefly,

the hydrated GenePorter2 reagent was diluted with serum-free medium according to Table 14. An appropriate amount of plasmid DNA was mixed with a volume of the DNA diluent B according to Table 15, by pipetting several times, and then incubated for 5 min at room temperature. The DNA solution was then added to the diluted GenePorter2 reagent, and incubated for 5 to 30 min to form GenePorter2/DNA lipoplexes. The lipoplexes were added drop-wise directly to the cells. The plates were swirled to ensure even dispersal before incubation for a further 24 h. Before proceeding with other procedures, the cells were rinsed and new media added.

Table 14. Volumes of transfection reagent

DNA (μg)	**DNA diluent B** (μL)	**GenePorter2** (μL)	**Serum-free medium** (μL)
0.5	12.5	1.75	10.75
6.0	150	21	129
8.0	200	28	172
10.0	250	35	215

Table 15. Transfection volume and DNA amount for various culture dishes.

Culture dish	**DNA** (μg)	**Transfection volume** (mL)
96-well	0.5	0.1
6-well	6.0	1.0
60 mm	8.0	2.5
100 mm	10.0	5.0

2.4 Hormonal and TSA Exposure

αT3-1 and LβT2 cells were exposed to 100 nM GnRH ([D-Ala6]-LHRH; Sigma; dissolved in the culture medium) and/or 10 nM β-Estradiol (Sigma; dissolved in 100 % ethanol and diluted with the culture medium) and/or 100 ng/mL Activin (human recombinant Activin A; Sigma; dissolved in 0.05 M Tris, 0.15 M NaCl, pH 7.4) for 24 h before harvest. They were treated with TSA (Sigma; dissolved in the culture medium) at concentrations 20 ng/mL, 100 ng/mL and 200 ng/mL for 24 h before harvest. The hormones, peptide and TSA were added at 0.1 % of the total culture media volume to give the final concentration as stated.

2.5 Total RNA Isolation

TRIzol® Reagent (Gibco BRL) was used to isolate total RNA from αT3-1 and LβT2 cell cultures (grown in monolayer) in accordance with the manufacturer's instructions. Briefly, the media from the culture dishes were discarded by pipetting. TRIzol® reagent (1 mL) was added to each 35 mm dish, and incubated at room temperature (RT) for 1 min. The lysate was then passed through a pipette several times, before further incubating for another 3 min at RT. The lysate was then transferred to a new 1.5 mL Eppendorf tube and 0.2 mL chloroform was added and the tube was shaken vigorously by hand for 15 s before incubation at RT for 2 min. The sample was centrifuged at 12,000 rpm at 4 ^{o}C for 15 min. After centrifugation, the upper aqueous phase was transferred to a new 1.5 mL Eppendorf tube and the RNA was precipitated from the aqueous phase by mixing with 0.5 mL isopropyl alcohol.

The sample was then incubated at RT for 10 min and centrifuged at 12,000 rpm at 4 ^{o}C for 10 min. After centrifugation, the supernatant was removed and the RNA pellet was washed with 1 mL 75 % ethanol (with DEPC-treated water: 0.1 % DEPC). The sample was vortexed and centrifuged at 5, 000 rpm at 4 ^{o}C for 5 min. At the end of the procedure, the RNA pellet was briefly air-dried for 5 min and dissolved

in 200 μL of DEPC-treated water, and stored at –70 ^{o}C in aliquots of 20 μL, until further use.

The isolated total RNA was run on a on a 1 % agarose/ethidium bromide (EtBr) gel to check for the integrity of the nucleic acid. To analyze the amount and purity of total RNA, spectrophotometric readings at OD_{260nm} and OD_{280nm} were taken.

2.6 Genomic DNA Isolation

Normal LβT2 cells grown in 100 mm culture dishes (as previously) were used for genomic DNA isolation. The medium was discarded and DNAzol® Reagent (4 mL; Life Technologies) was added to each culture dish. After 1 min the lysate was passed through the pipette several times. It was further incubated at RT for another 3 min. The lysate was centrifuged for 10 min at 14, 000 rpm at 4 ^{o}C. Following centrifugation, the resulting supernatant was transferred to a fresh 1.5 mL Eppendorf tube. DNA was precipitated from the lysate/homogenate by adding 1 mL of 100 % ethanol.

The sample was mixed by inversion and stored at RT for 2 min. The DNA precipitate was spooled with a pipette tip and transferred to a clean 1.5 mL Eppendorf tube. Following this, the DNA precipitate was washed twice with 1 mL 75 % ethanol. At each wash, the DNA was suspended in ethanol by inverting the tube three times. The DNA was allowed to settle to the bottom of the tube before removing the ethanol by pipetting. After the DNA was air dried for 15 s, it was dissolved in 400 μL of 8 mM NaOH.

The size and purity of the genomic DNA were analyzed: 1 μg of genomic DNA and 1 μg of control human genomic DNA were loaded on a 1.0 % agarose/EtBr

gel to check for the size the genomic DNA. The quality of the genomic DNA was also analyzed by *Dra* I digestion. The mixtures were loaded into the gel and electrophoresis was carried out at 100 V for 45 min. 1X TAE buffer was used as the running buffer for electrophoresis. A 1 kb DNA ladder (GeneRuler^{TM}, MBI Fermentas) was used as a molecular weight marker for size estimation of the DNA fragments. The EtBr-stained linear DNA fragments were observed under UV illumination and the gel was photographed using a digital camera.

2.7 Northern Blot Analysis

2.7.1 Construction of cDNA Probes

FSHβ and LHβ double-stranded cDNA probes were prepared for detection of the FSHβ and LHβ mRNAs on the Northern blot membrane. As a control, the double-stranded cDNA probe for murine 60S ribosomal protein mRNA, a housekeeping mRNA, was also used.

2.7.1.1 FSHβ and LHβ cDNA Probes

Gene-specific primers to amplify the exon 2 of the FSHβ gene, and exon 3 of the LHβ gene. The four oligonucleotides used for PCR are shown in Table 16. Each reaction (Table 17) was cycled in the PE Biosystems DNA thermal Cycler 480 using the cycling parameters outlined in Table 18. QIAquick® PCR Purification Kit (Qiagen) was used to purify the PCR fragments according to the manufacturer's instructions. The purified PCR product (10 μL) was analyzed with 1 % agarose/EtBr gel electrophoresis to ensure the correct size of the PCR fragments (FSHβ and LHβ cDNA probes). To analyze the amount and purity of the cDNA, spectrophotometric readings at $OD_{260\ nm}$ and $OD_{280\ nm}$ were taken after purification. The ratios of $OD_{260\ nm}$

/$OD_{280\ nm}$ were taken as the basis of cDNA purity. The purified cDNA probes were stored at –20 °C, in aliquots of 10 μL

Table 16. Oligonucleotides used for PCR-based cDNA probe construction.

Primers	Sequence	Tm	Amplicon size
mFSHβ-PCR1-F	5'-GACGTAGCTGTTTACTTCCCAGACC-3'	65.2	~178bp
mFSHβ-PCR1-F	5'-GCAGTAGCCCGCACACCAAGAGG-3'	63.4	
mLHβ-PCR1-F	5'-TGCTTTGCCTCCTGTGCCCAGCC-3'	56.5	~226bp
mLHβ-PCR1-F	5'-TCAGAGGAGGAGGAGGCCG-3'	63.8	

Table 17. The components in each PCR reaction for cDNA probe amplification

Components	Volume (μL)
10X buffer	5
dNTP (10 mM each)	1
Forward Primer	1
Reverse Primer	1
Genomic DNA (template)	1
Taq polymerase	1
Sterile distilled water	Made up to 50 μL

Table 18. Cycling parameters for PCR in cDNA probe amplification

Cycle	Steps
1 (x1)	**Step 1**: 94 °C for 5 min
2 (x30)	**Step 1:** 94 °C for 45 s
	Step 2: 57 °C for 45 s
	Step 3: 72 °C for 1 min
3 (x1)	**Step 1:** 72 °C for 5 min

2.7.1.2 60s Ribosomal Protein cDNA Probe

The plasmid pGEM-T® Easy vector (Promega) containing the murine 60S ribosomal protein cDNA sequence was kindly donated by A/P Lim Tit Meng from Department of Biological Sciences, NUS. Sufficient plasmid was

prepared and purified as previously. The purified plasmid was then digested in 20 μL reaction volume with 1 μL EcoRI restriction enzyme (New England, BioLabs; 20,000 U/mL) at 37 °C for 90 min before analysis on a 1 % agarose/EtBr gel to verify for the correct size of the insert. The band (365 bp) corresponding to the insert on the agarose gel was excised. The cDNA was purified using GENECLEAN III Kit (BIO 101) according to the manufacturer's instructions. Analysis and measurement of the amount and purity of the cDNA was carried out as in section 2.7.1.1. The purified cDNA probe was stored at –20 °C, in aliquots of 10 μL.

2.7.1.3 Verification of cDNA Probes

DNA sequencing was carried out for verification of the correct DNA sequences (as in section 2.1.5.7). For DNA sequencing of the FSHβ and LHβ cDNA probes, the same primers used in the PCR (Table 16) were used. For the 60S ribosomal protein cDNA probe, a primer was designed on the pGEM-T® Easy vector (Promega):

5'-ATGGTCCAGCGTTTGACATACCGCCG-3'.

2.7.2 RNA Gel Electrophoresis and Transfer

Total RNA (10 μg) extracted from αT3-1 and LβT2 cells was denatured for 15 min at 55 ^{o}C in a solution consisting of 12.5 μL formamide, 4.5 μL formaldehyde and 2.5 μL 10X MOPS buffer (0.01M EDTA, 0.05 M sodium acetate [pH 7.0], 0.2 M 3-[N-morpholino]-propane-sulphonic acid [MOPS]). The denatured RNA was incubated on ice for 10 min, and then separated by electrophoresis in a denaturing formaldehyde gel in 1X MOPS buffer. RNA in the gel was transferred to a nylon membrane (Roche)

according to the manufacturer's instruction. The membrane was baked for 2 h at 80 ^{o}C.

2.7.3 Radioactive cDNA Probe Labeling

All the three cDNA probes were labeled with radioactive [α-^{32}P] dATP (3000 Ci/mmol; Amersham Biosciences) using the Megaprime^{TM} DNA Labeling Systems (Amersham Life Science), following the manufacturer's instructions. MicroSpin^{TM} G-50 Columns (Amersham Pharmacia Biotech) were used to remove excess dNTP (including the [α-^{32}P] dATP) from the reaction, following the manufacturer's protocol.

2.7.4 Hybridization and Detection

A prehybridization solution consisting of 1.5 mL 10 % SDS (sodium dodecyl sulfate), 6 mL 25 % dextran sulfate and 7.5 mL DEPC-treated deionized water was heated for 10 min at 65 ^{o}C. NaCl (0.87 g) was then added and the mixture was heated for another 10 min before adding to the membrane, which was arranged between nylon sheets and rolled up into the hybridization tube. Incubation was then carried for 4 h at 65 ^{o}C in a rotating hybridization oven (Hybaid). After prehybridization, the labeled cDNA probe was boiled for 10 min and added to the hybridization tube. The membrane was hybridized for another 18 h at 65 ^{o}C. After hybridization, the membranes were washed twice in 50 mL of 2X SSC for 5 min at room temperature followed by twice in 50 mL 2X SSC and 1 % SDS for 30 min at 60 ^{o}C and finally, twice in 50 mL of 0.5X SSC and 0.1 % SDS for 15 min at room temperature. The membrane was allowed to dry slightly and sealed within a ziplock bag before exposure to an X-ray film (Kodak X-Omat™ AR) in an autoradiography cassette at –

70 °C for 24-72 h. Autoradiograms were developed using a Kodak X-Omat developer machine.

2.8 cDNA Synthesis

2.8.1 First-Strand cDNA Synthesis

In a nuclease-free microcentrifuge tube, 1 μL of oligo-$(dT)_{18}$ (500 μg/mL; New England BioLabs), 5 μg of total RNA (extracted as previously), 1 μL of 10 mM dNTP mix (10 mM each dATP, dCTP, dGTP and dTTP at neutral pH) and sterile distilled water were added, making the final total volume to 20 μL. The contents of the tube were collected by brief centrifugation after mixing. The mixture was heated to 65 ^{o}C for 5 min and chilled quickly on ice before adding 4 μL of 5X First-strand Buffer (SuperScript®, Promega), 2 μL 0.1 M DTT and 1μL of DEPC-treated sterile distilled water. The contents of the tube were mixed gently and incubated at 42 ^{o}C for 2 min. SuperScript® II RT-enzyme (1 μL; Promega) was added to the mixture, and mixed by pipetting gently up and down. Finally, it was incubated for 80 min at 42 ^{o}C before inactivation at 70 ^{o}C for 15 min. The cDNA was immediately used as a template or stored at –20 ^{o}C for later use.

2.9 Reverse Transcriptase PCR Analysis

2.9.1 Primers

The primers used for amplification of DNA fragments of mouse FSHβ, LHβ, CBP, Tip60, pCAF, GCN5 and Smad4 coding regions are given in Table 19. Primers for the control, which amplified the DNA fragment of murine 60s ribosomal protein coding region, are also given in Table 19.

Table 19. Primers used for RT-PCR Analysis

Primers	Sequence	Tm (^{o}C)	Amplicon size
mFSHβ-PCR1-F	5’-CAATCTGGGGGTTCAGCGAG-3’	65.2	~178bp
mFSHβ-PCR1-F	5’-CCTTGGGCACCTGGCTTTAT-3’	63.4	
mLHβ-PCR1-F	5'-ATTGGTGACAGAGAGGACATC-3'	56.5	~226bp
mLHβ-PCR1-F	5-CCAATACCAACATAAAGCCTGCTG-3'	63.8	
mFSHβ-PCR2-F	5’-AGCACTGACTGCACCGTGAG-3’	62.7	~600bp
mFSHβ-PCR2-F	5’-CCTCAGCCAGCTTCATCAGC-3’	63.5	
mLHβ-PCR2-F	5'-GCCTGTCAACGCAACTCTGG-3'	64.3	~300bp
mLHβ-PCR2-F	5-CAGGCCATTGGTTGAGTCCT-3'	62.4	
mCBP-PCR-F	5’-GGAATTCATGGGCACACCGCTTTCTCAG-3’	61.2	~600bp
mCBP-PCR-R	5’-CCCAAGCTTCTACTGCATGACAGGGTCAATTTC-3’	61.0	
mTip60-PCR-F	5’-GGAATTCCATGGCGGAGGTGGGG-3’	62.1	~1500bp
mTip60-PCR-R	5’-GCTCTAGATCACCACTTTCCTCTCTTGCTC-3’	62.4	
mpCAF-PCR-F	5’-GCCTCTTCCAGAACCAGCTC-3’	61.2	~350bp
mpCAF-PCR-R	5’-TGGTTTCTGGTTCAGGGAGTT-3’	60.5	
mGCN5-PCR-F	5’-GGAATTCATGGCGGAACCTTCCCA-3’	60.8	~1500bp
mGCN5-PCR-R	5’-GCTCTAGACTACTTGTCGATGAGCCCTCC-3’	62.3	
mSmad4-PCR-F	5’-GGAATTCATGGAAAGTGAAACCTTTGCAAAAAGA-3’	63.4	~280bp
mSmad4-PCR-R	5’-GCTCTAGATCCAGGTGAGACAACCCG-3’	61.6	

m60SRP-PCR1-F	5'-ATGGTCCAGCGTTTGACATACCGCCG-3	75.4	
m60SRP-PCR1-F	5'-TGCTTTCTGACTCTGTGCTTGTGCC-3'	69.0	~348bp
m60SRP-PCR2-F	5'-ATGGTCCAGCGTTTGACATA-3'	59.0	
m60SRP-PCR2-F	5'-TTTCCCAACCTTCTTGGTGT-3'	59.4	~100bp

2.9.2 PCR

cDNA was synthesized from isolated total RNA as in Section 2.8, and used as template for PCR. Each reaction (Table 20) was cycled in the PE Biosystems DNA thermal Cycler 480 using the cycling parameters outlined in Table 21.

2.9.3 DNA Gel Electrophoresis

The PCR products (10 μL) were loaded on a 1.5 % agarose/EtBr gel after mixing with 2 μL of 6X loading dye. The electrophoresis was carried out at 80-100 V until the dye front reached two thirds of the gel. 1X TAE buffer was used as the running buffer for electrophoresis. A 100bp DNA ladder (GeneRuler^{TM}, MBI Fermentas) was used for size estimation of the DNA fragments. The EtBr-stained linear DNA fragments were observed under UV illumination and the gel was photographed using a digital camera.

Table 20. Components in each PCR reaction in RT-PCR analysis. A negative control without the DNA template was also included to ensure non-contamination.

Components	Volume (μL)
10X buffer	2.5
dNTP (10 mM)	0.5
Forward Primer (10 μM)	0.5
Reverse Primer (10 μM)	0.5
cDNA template	5
DyNAzyme II DNA Polymerase (Finnzymes)	0.5
Sterile distilled water	Made up to 25 μL

Table 21. Cycling parameters for PCR in RT-PCR analysis.

Cycle	Steps
1 (x1)	**Step 1**: 94 ^{o}C for 5 min
2 (x35)	**Step 1:** 94 ^{o}C for 30 s
	Step 2: 55 ^{o}C for 30 s
	Step 3: 72 ^{o}C for 30 s
3(x1)	**Step 1:** 72 ^{o}C for 5 min

2.10 Quantitative (Real-time) PCR Analysis

2.10.1 Primers

The primers used for amplification of fragments of murine FSHβ and LHβ cDNA in quantitative PCR (qPCR) are given in Table 22. Primers for the control, which amplified a fragment of the murine 60s ribosomal protein cDNA, are also given in Table 22.

Table 22. Primers used for qPCR Analysis

Primers	Sequence	Tm	Amplicon size
mFSHβ-RT-F	5'-TTCTGGTGCTGGAGAGCAA-3'	46.0	~120bp
mFSHβ-RT-F	5'-CCGCACACCAAGTGGTATT-3'	46.0	
mLHβ-RT-F	5'-TGCTGCTGAGCCCAAGTG-3'	47.5	~120bp
mLHβ-RT-F	5-ATGCAGACTGGGCAGAACT-3'	46.0	
m60SRP-RT-F	5'-ATGGTCCAGCGTTTGACATA-3'	44.6	~100bp
m60SRP-RT-F	5'-TTTCCCAACCTTCTTGGTGT-3'	44.6	

2.10.2 Real-time PCR

The components for the qPCR are given in Table 23, and cycling parameters outlined in Table 24. Briefly, the reaction mixtures were added to separate wells of the 96-well PCR v-bottom microplate (MJ Research Inc) and the microplate was sealed using an adhesive optical cover (Applied Biosystems). The qPCR was carried

out using ABI Prism 7000 (Applied Biosystems) accompanied with the ABI Prism 7000 SDS Software.

Table 23. Components in each qPCR reaction. Total volume for reaction is 20 μL. Components for each reaction are placed in a well of a 96-well PCR microplate.

Components	Volume (μL)
SYBR-Green® (Applied Biosystems)	5
Forward Primer (6 μM)	1
Reverse Primer (6 μM)	1
cDNA template	1
Sterile distilled water	12

Table 24. Cycling parameters for qPCR.

Stage	Steps
1 (x1)	**Step 1**: 50 °C for 2 min
2 (x1)	**Step 1:** 95 °C for 10 min
3 (x40)	**Step 1:** 95 °C for 15 s **Step 2:** 60 °C for 1 min

2.10.3 Calculations and Statistical Analysis

Comparative quantitative analysis was used. Calibrator samples (untreated or untransfected controls) were used to compare all of the test samples to determine the abundance of FSHβ and LHβ mRNA transcripts. The normalizer target was used to correct for variations in input template quantity. The normalizer is the housekeeping gene, murine 60s ribosomal protein gene.

In this method of analysis, the Ct values of the gene of interest and the normalizer in an unknown were compared to the Ct values of the calibrator sample in order to determine the fold difference between the unknown and the calibrator. The

ΔCt of all of the samples (unknown and calibrator) was calculated by subtracting the Ct value of the normalizer from the Ct value of the target as in the equation below:

$$\Delta Ct = Ct\ target - Ct\ normalizer$$

The ΔΔCt of the unknown samples was calculated by subtracting the ΔCt of the calibrator from the ΔCt of the target as given by the equation below:

$$\Delta\Delta Ct = \Delta Ct\ unknown - \Delta Ct\ calibrator$$

The fold induction was calculated by the formula:

$$Fold\ induction = 2^{-\Delta\Delta Ct}$$

ANOVA was carried out as a statistical analysis of the data generated from qPCR.

2.11 CAT Assays

2.11.1 Harvesting transfected cells

To harvest cells; the dish of cells was rinsed once with 3 mL HBSS (Hank's Balanced Salt Solution, Sigma). HBSS media (1 mL) was then added to the plates, which were then scraped. The cell suspensions were collected into a 1.5 mL Eppendorf tube. The cells were spun down at 3000 rpm for 5 min in 4 °C centrifuge. Without disturbing the pellet, the HBSS was aspirated from the tube. The cell pellet was resuspended using 135 μL of Harvest Buffer (0.5 % Triton X-100, 250 mM Tris pH 8) and vortexed thoroughly. The tubes were placed at –20 °C for 16 h before assay. After thawing, the cell suspensions were vortexed again. The cell debris was spun down at 14,000 rpm for 15 min in 4 °C centrifuge and the supernatant was used for assays.

2.11.2 CAT assays

The cell extract (110 μL) was placed in a 65 °C water bath for 10 min before vortexing and centrifugation at 14, 000 rpm for 5 min. For the CAT assay, 100 μL was added to 40 μL CAT mix (0.8 μl ^{3}H-acetyl CoA [Amersham Pharmacia Biotech], 0.5 μL acetyl CoA, 18.7 μL of 75 μM HCl, 0.5 μL 200 mM chloramphenicol and 19.5 μL of 250 mM Tris [pH 7.8]). The tubes were incubated at 37 °C for 3 h before 800 μL of 5 M urea was added and the contents were transferred to scintillation vials

with 4 mL Econofluor-2 (Amersham Pharmacia Biotech). The contents were mixed well and the radioactivity was counted for 5 min using the Winspectral^{TM} 1414 liquid scintilation counter.

2.11.3 Statistical Analysis

Differences in CAT activity were analyzed by a two-tailed Student's *t* test. Significant differences are taken at 95 % confidence level.

2.12 Western Blot Analysis

2.12.1 Cell Lysis

LβT2 cells were cultured as previously, and 1.5 mL of lysis buffer (62.5 mM Tris-HCL pH 6.8, 2 % SDS, 0.72 M β-mercaptoethanol and 7 % glycerol) was added with protease inhibitor cocktail (10 μL/mL: aprotinin, leupeptin, DMSO). The cells were left to lyse on ice for 10 min, before vortexing and protein concentration determined using the Bio-rad Bradford's protein assay. Lysates were stored at -20^{o}C.

2.12.2 Nuclear Protein Isolation

For western blot analysis requiring nuclear proteins, Nuclei EZ Prep Nuclei Isolation Kit (Sigma) was used following the manufacturer's instructions.

2.12.3 SDS-PAGE & Blotting

Gels of 5 % stacking and 10 % resolving were used. Each protein sample (15 μg/well) with equal volume of 2x loading buffer were boiled for 5 min and then loaded into each lane alongside protein molecular weight markers. The gel was run in the electrophoresis buffer at 60 V for stacking gel and 120 V for resolving gel until the dye reached the bottom. The separated proteins were transferred onto the PVDF membrane at 100 V for 1 h with ice pack and cold transfer buffer (Bio-rad Electrophoretic Transfer Apparatus).

2.12.4 Blocking

The membrane was immersed in 10 % non-fat dried milk with TBS-T (0.1 % Tween 20 in 1X TBS) for 1 h at room temperature on an orbital shaker. The membrane was washed three times with TBS-T for 10 min at RT.

2.12.5 Incubation with Antibodies and Visual Detection.

The membrane was incubated in primary antibodies [anti-mouse Smad4 (1:1000; Santa Cruz; #sc-7154); anti-mouse GCN5 (1:1000; Santa Cruz; #sc-20698); anti-mouse Tip60 (1:1000; Santa Cruz; #sc-25378); anti-mouse *c*-Fos (1:1000; Santa Cruz)] diluted in 1% non-fat dried milk with TBS-T, for 1 h on an orbital shaker and washed three times with TBS-T for 10 min at RT. The membrane was then incubated in diluted HRP labeled secondary antibody (1:2000; 1% non-fat dried milk with TBS-T) for 1 h on an orbital shaker followed by washing with TBS-T three times for 10 min at RT. The detection of the Smad4, GCN5 and Tip60 immunoreactive proteins was done using the ECL Plus^{TM} western blotting reagents in accordance with the manufacturer's instructions.

2.13 Immunofluorescence Confocal Microscopy

2.13.1 Fixing, Permeabilization and Blocking of Cells

LβT2 cells were grown on glass coverslips placed in 6-well plates with hormonal exposure and grown to 50-70 % confluency. The media was drawn off before rinsing twice with warm PBS (PBS). The cells were fixed with 2 % (w/v) paraformaldehyde (Sigma) in PBS for 15 min, followed by washing thrice with PBS for 5 min. After permeabilization with 5 % (v/v) Triton-x and 3 % (w/v) BSA in PBS for 10 min, washing with PBS for 5 min was done thrice. After blocking with 3% (w/v) BSA in PBS for 10 min, the cells were washed three times in PBS for 5 min.

2.13.2 Incubation with Antibodies, Mounting and Detection

After overnight incubation of the cells with antisera to mouse Sf-1 (30 μg/mL; Upstate Biotechnology), washing with PBS for 5 min was done thrice. The cells were then incubated with secondary antibody (anti-rabbit IgG (whole molecule) –TRITC antibody produced in goat; diluted 1:150 in PBS; Sigma) for 1 h. After washing thrice with PBS for 5 min, cells were mounted on glass slides with mounting media. Confocal microscopy (NUMI, Singapore) was used for detection of Sf-1 immunoreactive protein.

2.14 Chromatin Immunoprecipitation (ChIP)

LβT2 cells were grown to approximately 50-70 % confluency. They were transfected with constructs and/or treated accordingly. Dead cells were washed off from the culture dishes with HBBS, before addition of 10 mL of culture medium. The number of cells for a particular batch was counted using one of the culture dishes.

The proteins were cross-linked to DNA with formaldehyde, at a final concentration of 1 % (270 μL of 37 % formaldehyde in 10 mL of culture medium) and incubated at RT on a rotating platform for 10 min. Cross-linking was arrested by addition of glycine, to a final concentration of 0.125 M (1250 μL of 1 M glycine), and incubated at RT with rotation for 5 min. The culture medium was aspirated and the cells were washed twice with 3 mL of PBS containing protease inhibitors (1 mM phenylmethysulfonyl fluoride [PMSF], 1 μg/mL leupetin and 1 μg/mL pepstatin A). The cells were then scraped with an appropriate amount of PBS with protease inhibitors and 4 x 10^6 cells were transferred into 1.5 mL microfuge tubes as 1.4 mL cell suspensions.

Cells were afterwards pelleted by centrifuging for 10 min at 8,000 rpm at 4 ^oC. The supernatant was discarded and the cell pellet resuspended in 800 μL of SDS lysis buffer (1 % SDS, 10 mM EDTA and 50 mM Tris-HCl pH 8.1) with protease inhibitors, and incubated on ice for 10 min. The samples were sonicated on ice with a

MISONIX XL2020 sonifier to obtain DNA fragments of ~500 to 1000 bp, at setting 2 for 10 sec at 30% duty cycle, and then at setting 1 for 10 sec at 30% duty cycle with a 10-sec rest in between. The cells were then pelleted by centrifuging at 13,000 rpm at 4 ^{o}C for 10 min.

The supernatant was transferred to a fresh 1.5 mL microfuge tube. To quantify the input DNA in the samples, 10 % of the undiluted supernatant was removed and subsequently reverse cross-linked by incubating at 65 ^{o}C for 4 h in 12 µL of 5 M NaCl and 0.6 µL RNase (100 ng/µL). After which these samples were stored at -20 ^{o}C until recovery of the DNA together with those from the pull-down samples.

For the remainder of the supernatant, it was diluted with 2.25X volume of ChIP dilution buffer (0.01 % SDS, 1.1 % TritonX-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1 and 167 mM NaCl) with protease inhibitors. For pre-clearance of these pull-down samples, 40 µL of 50 % v/v protein A sepharose (PAS; CL 4B) was added and the samples were incubated for 2 h with rotation at 4 ^{o}C. The sepharose beads were collected by centrifuging briefly at 700 rpm for 1 min at 4 ^{o}C. The supernatant was transferred to fresh 1.5 mL microfuge tubes. Appropriate amounts of antibodies were added [GCN5 (25 µL; Santa Cruz; #sc-20698), Tip60 (25 µL; Santa Cruz; #sc-25378), Smad4 (25 µL; Santa Cruz; #sc-7154), HDAC1 (5 µL; Cell Signaling Technology) and HDAC7 (5 µL; Cell Signaling Technology)] and subsequently incubated overnight at 4 ^{o}C with rotation.

To collect the antibody-protein/DNA complex, 40 µL of PAS was added and was then incubated for 1 h at 4 ^{o}C with rotation. The sepharose beads were pelleted by

gentle centrifugation at 700 rpm for 1 min at 4 °C. The supernatant was discarded, and the PAS/antibody-protein/DNA complexes were washed for 4 min on a rotating platform with 1 mL of each of the buffers: (1) once with low salt wash buffer (0.1 % SDS, 1 % TritonX-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1 and 150 mM NaCl); (2) once with high salt wash buffer (0.1 % SDS, 1 % TritonX-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1 and 500 mM NaCl); (3) once with LiCl wash buffer (0.25 M LiCl, 1 % NP40, 1 % deoxycholate, 1 mM EDTA and 10 mM Tris-HCl pH 8.1); (4) twice with 1x TE. After each wash, sepharose beads were collected at 700 rpm for 2 min at 4 °C and the supernatant discarded.

Elution buffer (1 % SDS and 0.1 M $NaHCO_3$) was prepared fresh. The protein/DNA complexes were eluted by the addition of 250 µL of the elution buffer to the pelleted sepharose beads with shaking at RT for 15 min. The sepharose beads were pelleted at 700 rpm for 1 min at 4 °C and elution was repeated once more to obtain approximately 500 µL of eluant for each sample. The eluted samples were reverse cross-linked with 20 µL of 5 M NaCl and 1 µL RNase (100 ng/µL) and were incubated at 65 °C for 4 h. Following this, 10 µL of 0.5 M EDTA, 20 µL of 1 M Tris-HCl pH 6.5 and 2 µL of proteinase K (10 mg/mL) were added to the combined eluant and incubated for 1 h at 45 °C.

The DNA from the input and pull-down samples was then recovered by phenol/chloroform extraction and isopropanol/sodium acetate extraction. The DNA was resuspended in 50 µL of sterile distilled water, to be used later on for PCR.

The primers used for PCR are given in Table 25. The PCR components and cycling parameters are in Table 26 and Table 27 respectively.

Table 25. Primers used in ChIP

Primers	Sequence	Tm	Amplicon size
mLH promoter (2701) F	5'-CAATCTGGGGGTTCAGCGAG-3'	65.2	
mLH promoter (2941)R	5'-CCTTGGGCACCTGGCTTTAT-3'	63.4	~240bp
mFSH promoter (6072) F	5'-CCAAAGCAGTCTAAATGCCA-3'	58.9	
mFSH promoter (5792)R	5'-CACAGCCCATAGGAACAAGA -3'	58.7	~300bp

Table 26. Components in each PCR reaction (for ChIP). A negative control without the DNA template was also included to ensure non-contamination.

Components	Volume (μL)
10X buffer	2.5
dNTP (10 mM)	0.5
Forward Primer (10 μM)	0.5
Reverse Primer (10 μM)	0.5
DNA template	5
DyNAzyme II DNA Polymerase (Finnzymes)	0.5
Sterile distilled water	Made up to 25 μL

Table 27. Cycling parameters for PCR. *Annealing temperature depends on the Tm of the primers used.

Cycle	Steps
1 (x1)	**Step 1**: 94 °C for 5 min
2 (x35)	**Step 1:** 94 °C for 30 s
	Step 2*: 52 °C/58 °C for 30 s
	Step 3: 72 °C for 30
3(x1)	**Step 1:** 72 °C for 5 min

The PCR products (10 μL) were loaded on a 1.5 % agarose/EtBr gel after mixing with 2 μL of 6X loading dye. The electrophoresis was carried out at 80-100 V until the dye front reached two thirds of the gel. 1X TAE buffer was used as the running buffer for electrophoresis. A 100bp DNA ladder (GeneRuler^{TM}, MBI Fermentas) was used for size estimation of the DNA fragments. The EtBr-stained

linear DNA fragments were observed under UV illumination and the gel was photographed using a digital camera.

2.15 Mammalian Two-hybrid Assays

2.15.1 Cell Culture, Treatment and Transfection

Cells were grown in 96-well white luminescent microplates (NUNC) until 50-70 % confluency. The cells were then transfected with the appropriate pGLuc-Gal4 (100 ng), pM (200 ng), pVP16 (200 ng) and pRL-CMV (2 ng) constructs, keeping the amount of DNA transfected constant with PWS construct, and treated as described in Section 2.4.

2.15.2 Luciferase Assays

Following 48 h of transfection, cell medium was removed and cells were rinsed once with HBSS. Cell medium (20 μL) was replaced in each well before addition of 10 μL of Dual-Glo™ Luciferase Reagent (Promega) and the microplate was incubated at RT for 10 min with gentle agitation. Luminescent readings for the firefly luciferase (reporter gene in the pM and pVP16 constructs) were then measured using a luminometer (Veritas Microplate Luminometer; Turner Biosystems) accompanied by the Veritas Microplate Luminometer Software (Turner Biosystems). After which, 10 μL of Dual-Glo™ Stop & Glo® Reagent (Dual-Glo™ Stop & Glo® substrate and Dual-Glo™ Stop & Glo® Buffer in 1:100; Promega) was added, and the microplate was incubated at RT for 10 min with gentle agitation. Luminescent readings for the *Renilla* luciferase (reporter gene in the pRL-CMV construct as internal control) were then measured using the luminometer. The Dual-Glo™

Luciferase System together with the luminometer was optimized beforehand, and the details of this are given in Appendix A.

The firefly luciferase luminescent readings were normalized with the *Renilla* luciferase luminescent readings to correct variations in transfection efficiencies among samples. Statistical analysis was then carried out with the data generated using Student's t-test at 95% confidence level.

2.16 Promoter Studies

Cells were grown as in Section 2.15.1. The cells were then transfected with the appropriate expression vectors (200 ng each; Section 2.1.3) along with the reporter gene constructs [mouse FSHβ-Luc (100 ng) and pRL-CMV (2.5 ng)], keeping the amount of DNA transfected constant (~500 ng) with PWS construct, and treated as described in Section 2.4. Luciferase assays and statistical analysis were carried out as described in Section 2.15.2.

Chapter 3 Results

3.1 Effects of TSA and GnRH on the Regulation of Mouse Gonadotropin Genes in LβT2 and αT3 cells *Hyperacetylation of the nucleosomal histones has been widely accepted to be the impetus for gene activation, whereas the deacetylation of these histones has the reverse consequence. Furthermore, acetylation of other non-histone substrates has also been associated with the upregulation of certain genes.*

3.1.1 Regulation of Gonadotropin Genes through actions on the Chromatin

The gonadotropin gene FSHβ is not thought to be expressed in LβT2 cells, and both the FSHβ and LHβ genes are not expressed in the immature αT3 cells. In elucidating whether this repression of the gonadotropin genes is a result of the hypoacetylation of nucleosomal histones by the HDACs, we treated the cells with TSA, a HDAC inhibitor. Since GnRH is a critical regulator of the synthesis and release of the gonadotropins, we went on to test whether GnRH has the potential to overcome the repression of these genes.

TSA increases the endogenous mRNA level of FSHβ gene in LβT2 cells in a dosage dependent manner Northern blot analysis was carried out on total RNA from LβT2 cells treated with various TSA concentrations (20, 100 and 200 ng/mL). As shown in Figure 17, FSHβ mRNA was not detectable in the normal cells.

However, upon treatment with TSA, the FSHβ mRNA was detected, and its level increased with increasing concentration of TSA (from 20 to 200 ng/mL).

FSHβ mRNA is detected in LβT2 cells, and its level is increased by TSA and GnRH but the LHβ gene is unaffected We hypothesized that in the absence of physiological stimulators, FSHβ is possibly expressed at low levels in LβT2 cells. This was tested by the more sensitive RT-PCR, using the first-strand cDNA synthesized from the isolated total RNA. The RT-PCR analysis (Figure 18) was able to detect the low level of FSHβ mRNA in LβT2 cells, and by increasing the concentration of TSA from 20 to 100, and then to 200 ng/mL, the mRNA level was increased in a dosage dependent manner. Exposure to GnRH in LβT2 cells showed that the mRNA level of FSHβ gene was increased when compared to the level in the normal cells. Furthermore, the addition of TSA to the GnRH-incubated cells had no obvious effect on the FSHβ mRNA level even when TSA concentration was the highest at 200 ng/mL. The effects on the level of LHβ mRNA in LβT2 cells incubated with TSA and/or GnRH were not obvious. Similarly, the mRNA level of 60s ribosomal protein was unaffected by GnRH and/or TSA.

FSHβ and LHβ mRNAs are not detected in αT3 cells but their expression is induced by TSA and/or GnRH FSHβ is not expressed in αT3 cells (Figure 19). However, TSA (20 ng/mL) was able to induce FSHβ expression as observed by the detection of the FSHβ mRNA. The mRNA level was further increased with higher concentrations of TSA (100 and 200 ng/mL). GnRH was able to induce FSHβ expression. The effect on the FSHβ expression of adding TSA with GnRH to the cells was not obvious. LHβ is not expressed in αT3 cells, but again, TSA was able to

Figure 17. Northern Analysis of FSHβ mRNA level in LβT2 Cells. Total RNA (10 µg) from LβT2 cells treated with various TSA concentrations (20, 100 and 200 ng/mL) were run on formaldehyde agarose gel, and then blotted on to a nylon membrane. The Northern blot was probed with mouse FSHβ cDNA fragment (arrow). Hybridization was carried out at 65°C overnight, and blots were washed at 60°C, and then exposed to autoradiography film for 48 h.

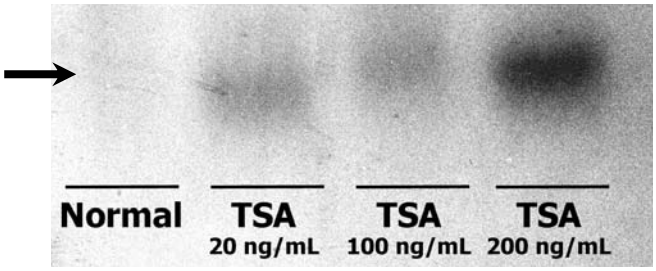
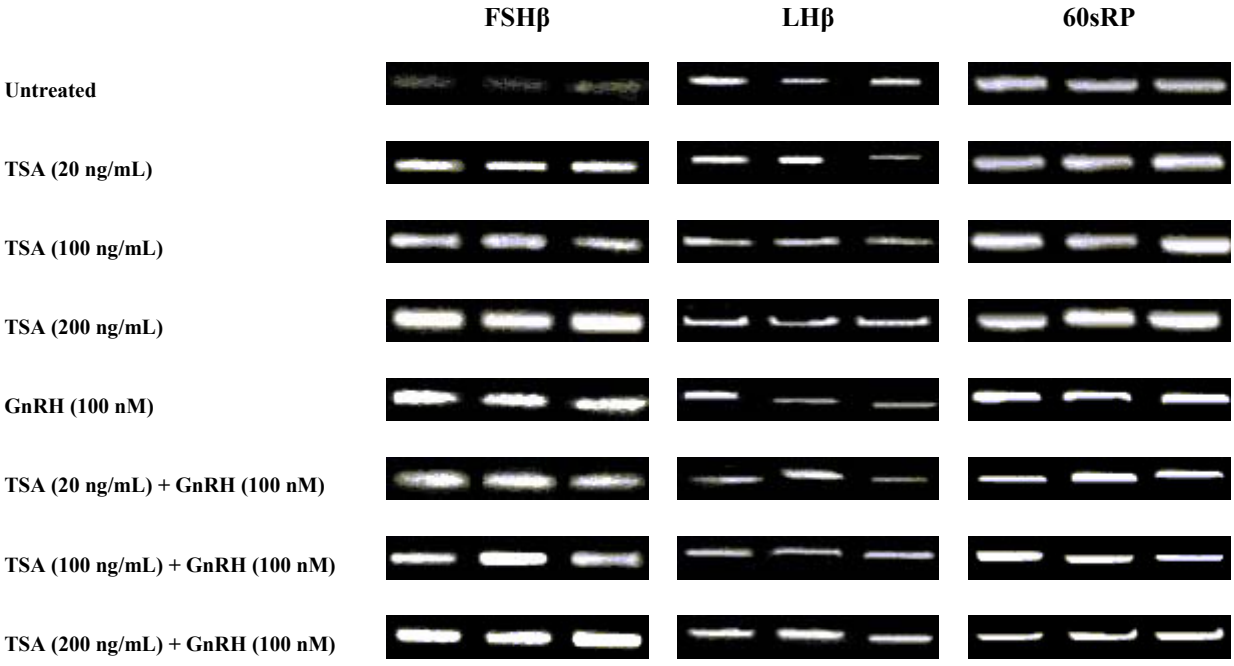


Figure 18. RT-PCR analysis of FSHβ and LHβ mRNA levels in LβT2 cells incubated with TSA and/or GnRH. Total RNA was isolated from LβT2 cells (60 mm plates) treated with TSA (20, 100 or 200 ng/mL) and/or GnRH (100 nM) for 24 h. cDNA was synthesized from the total RNA and used as template for PCR (25 cycles). The primers were designed to amplify 178 bp and 226 bp DNA fragments of the coding regions of the FSHβ and LHβ genes respectively. Amplification of a 348bp cDNA fragment of the mouse 60s ribosomal protein (60sRP) is shown as control. The experiment was carried out once.



induce LHβ expression; effects of higher concentrations of TSA (100 and 200 ng/mL) were not obvious. GnRH was also able to induce LHβ expression. In contrast, the mRNA level of 60s ribosomal protein were unaffected by TSA and/or GnRH.

FSHβ and LHβ mRNA levels are increased by TSA or GnRH in LβT2 cells at different time points To confirm our observations from the RT-PCR analysis, we employed the more accurate quantitation of the mRNA levels using quantitative (real-time) PCR (qPCR). We incubated the cells with TSA or GnRH for 8, 16, 24 or 36 h, keeping the concentrations of TSA and GnRH constant at 100 ng/mL and 100 nM respectively. Our qPCR analysis showed that the LHβ mRNA level is increased significantly ($P < 0.05$) by 24.53, 19.90 and 31.78 fold at 8, 24 and 36 h of incubation with TSA respectively (Figure 20A). Similarly, FSHβ mRNA level is increased significantly ($P<0.05$) by 40.46, 2.43, 16.80 and 83.00 fold at 8, 16, 24 and 36 h respectively. The general trend observed for LβT2 cells is that LHβ mRNA level increment by TSA is lesser compared to the increment in FSHβ mRNA level. The LHβ mRNA level in LβT2 cells is detected by this analysis to increase significantly ($P < 0.05$) by 56.69, 22.01 and 32.26 fold after 16, 24 or 36 h of incubation with GnRH respectively, with no significant ($P>0.05$) increase at 8 h (Figure 20B). Similarly, FSHβ mRNA level is increased significantly ($P<0.05$) by 64.82, 25.75 and 40.97 fold after 16, 24 or 36 h of incubation with GnRH (100 nM) respectively, with no significant ($P>0.05$) increase at 8 h.

Figure 19. RT-PCR analysis of FSHβ and LHβ mRNA levels in αT3 cells incubated with TSA and/or GnRH. Total RNA was isolated from αT3 cells treated with TSA and/or GnRH and analysed as in Figure 18. The experiment was carried out once.

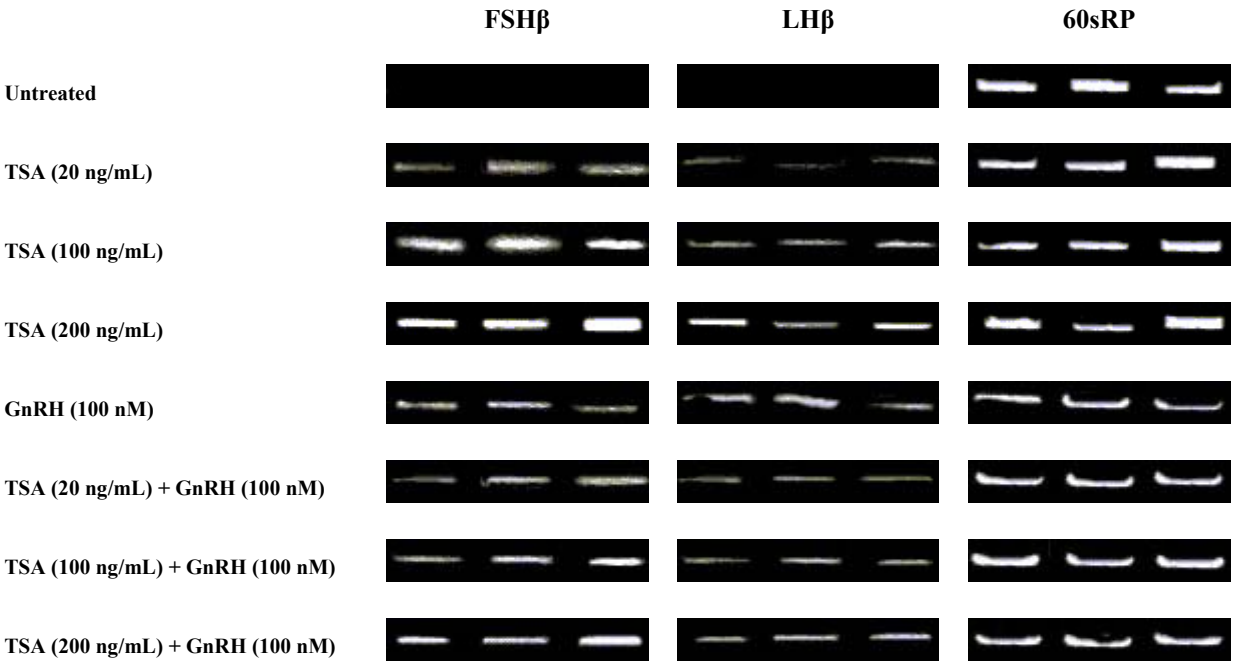
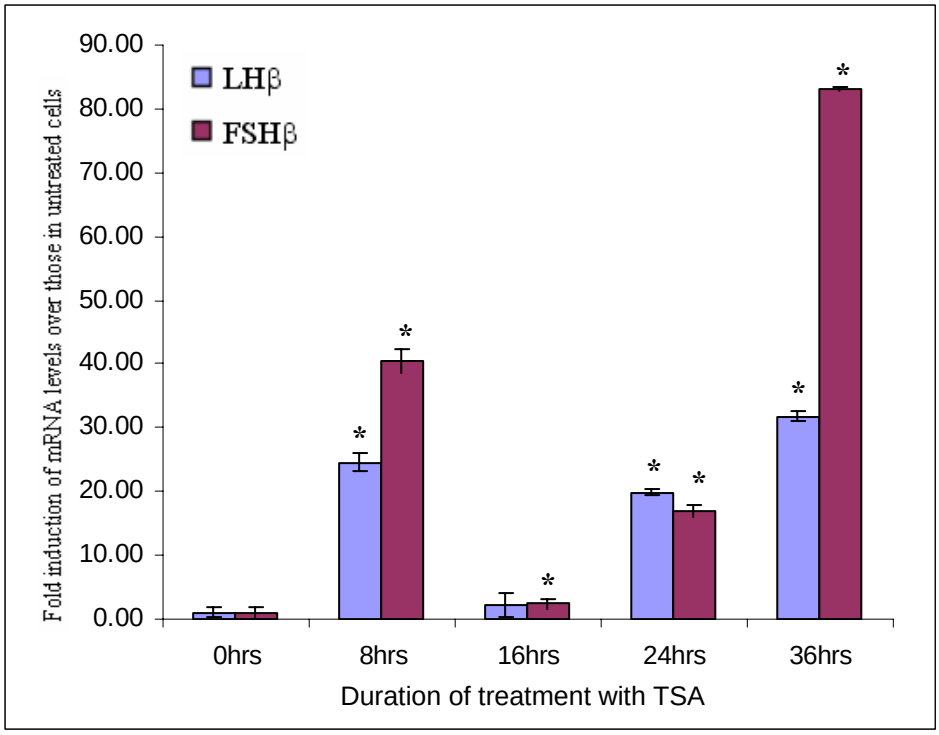
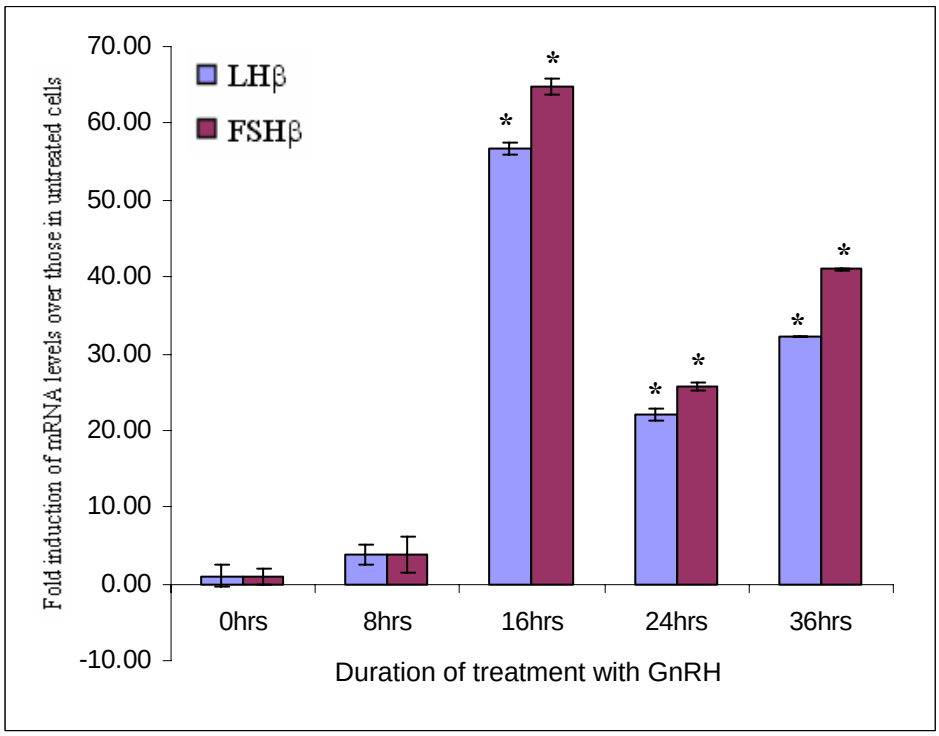


Figure 20. Quantitative PCR analysis of FSHβ and LHβ mRNA levels in LβT2 cells incubated with (A) TSA or (B) GnRH for various durations. Total RNA was isolated from LβT2 cells (60 mm plates) treated with TSA (100 ng/mL) or GnRH (100 nM) for 8, 16, 24 or 36 h. cDNA was synthesized from the total RNA and used as template for qPCR. The Ct values were normalized with those for 60sRP. Mean ± SEM, n = 3. Student's t-tests were carried out. Means designated by asterisks (*) are significantly different ($P < 0.05$) from the untreated samples. The experiment was repeated thrice and a representative graph is shown.

A



B



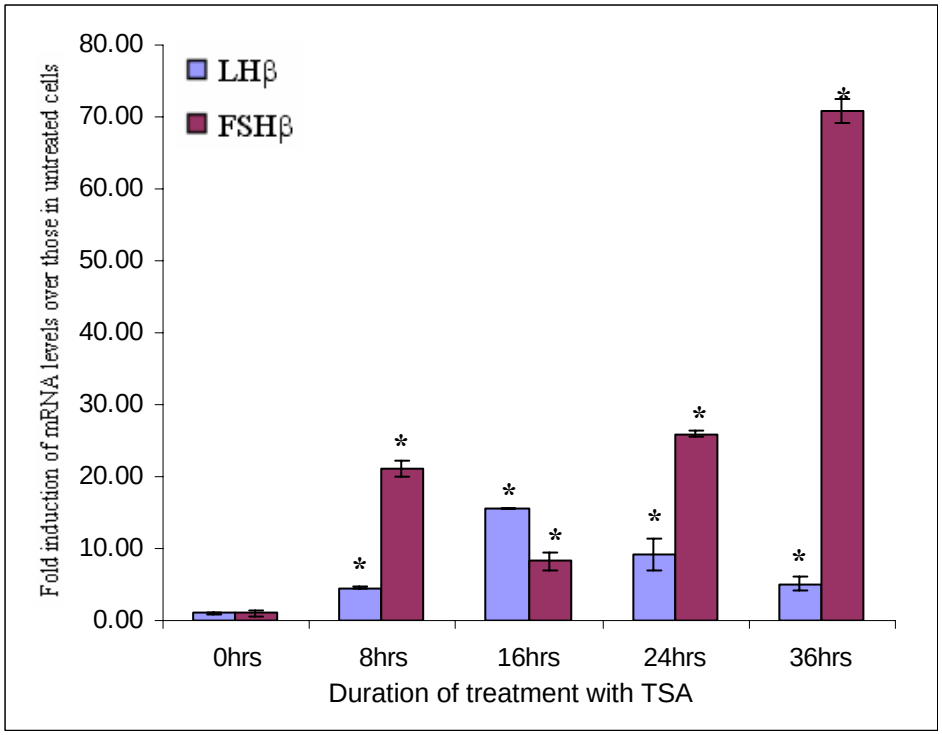
FSHβ and LHβ mRNA levels are increased by TSA or GnRH in αT3 cells at different time points The LHβ mRNA level in αT3 cells is induced significantly (P <0.05) by 4.51, 15.54, 9.17 and 16.89 fold after 8, 16, 24 or 36 h of incubation with TSA respectively (Figure 21A). Similarly, FSHβ mRNA level is induced significantly (P<0.05) by 21.11, 8.21, 25.84 and 70.93 fold after 8, 16, 24 or 36 h incubation with TSA respectively. Generally, LHβ mRNA level in αT3 cells is induced by TSA to a lesser extent compared to the increase in FSHβ mRNA level at the various time points. The LHβ mRNA level in αT3 cells is induced significantly (P <0.05) by 4.04, 2.02, 6.71 and 4.90 fold after 8, 16, 24 and 36 h of incubation with GnRH (100 nM) respectively (Figure 21B). Similarly, FSHβ mRNA level is induced significantly (P<0.05) by 4.27, 7.41 and 2.22 folds at 8, 24 and 36 h respectively. Generally, the increment in FSHβ and LHβ mRNA levels by GnRH is similar in these cells.

3.1.2 Regulation of Gonadotropin Genes through actions on Non-Chromatin Substrates

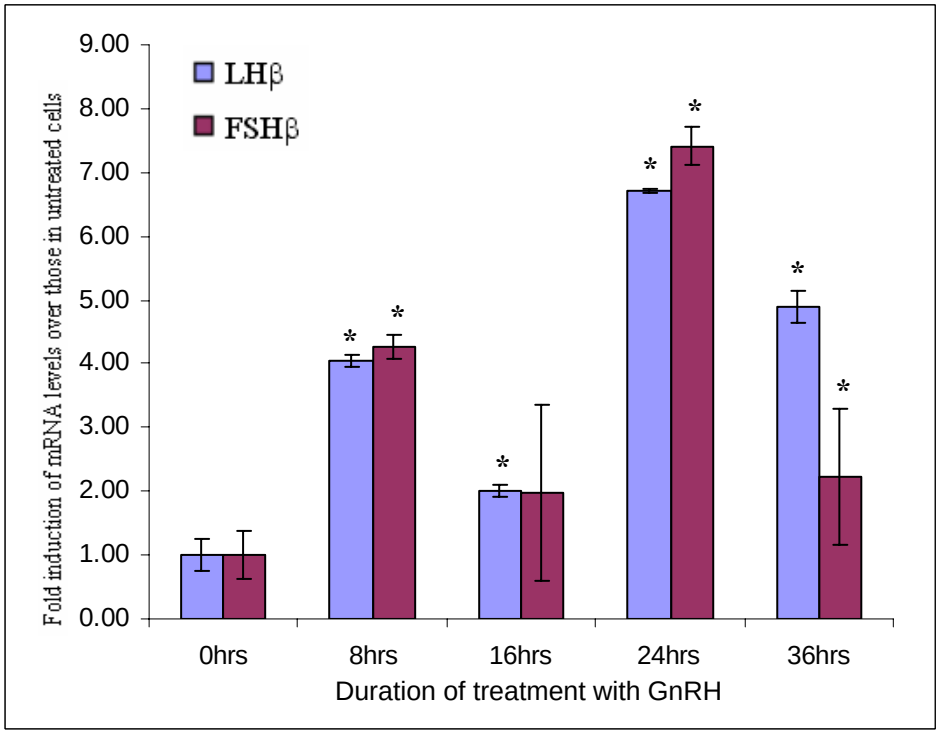
To investigate the extent of gonadotropin gene regulation following non-chromatin deacetylation by HDACs of targets other than the gonadotropin chromatin in both the mature and immature gonadotropes at basal and GnRH-modulated levels, we carried out: reporter gene analysis in both cell lines which were transiently transfected with Chinook Salmon FSHβ and LHβ promoter/reporter constructs; as well as detection of Sf-1 localization in αT3 cells upon GnRH-treatment.

Figure 21. Quantitative PCR analysis of FSHβ and LHβ mRNA levels in αT3 cells incubated with (A) TSA or (B) GnRH at different durations. Total RNA was isolated from αT3 cells treated with TSA or GnRH and analysed and presented as in Figure 20. The experiment was repeated thrice and a representative graph is shown.

A



B



TSA increases FSHβ gene promoter activity in LβT2 cells at both basal and GnRH-modulated levels but has no effect in αT3 cells The basal FSHβ gene promoter activity was significantly ($P<0.05$) increased in the LβT2 cells (Figure 22A) incubated with TSA by 2.86-fold. Similarly, when LβT2 cells were exposed to GnRH, the promoter activity increased by 10.6-fold ($P<0.05$). Upon exposure to TSA, the GnRH-mediated FSHβ gene promoter activity was increased to 27.2-fold. In contrast, the basal FSHβ gene promoter activity in αT3 cells (Figure 22B) was not significantly different when exposed to TSA ($P>0.05$) or GnRH ($P>0.05$). The GnRH-modulated promoter activity was also not affected by TSA ($P>0.05$).

TSA has no effect on LHβ gene promoter activity in LβT2 cells at either basal or GnRH-modulated levels The LHβ gene promoter activity in LβT2 cells was not affected by TSA ($P>0.05$) at either basal or GnRH-mediated levels (Figure 23A).

TSA decreases LHβ gene promoter activity in αT3 cells at GnRH-modulated levels TSA has no effect ($P>0.05$) on the basal LHβ gene promoter activity in αT3 cells. In contrast, GnRH caused the basal LHβ gene promoter activity to go up by 72.2-fold ($P<0.05$) and TSA reduced this increased LHβ gene promoter activity to 11.1-fold ($P>0.05$) over the controls (Figure 23B).

Sf-1 localizes in both the cytoplasm and nucleus of αT3 cells incubated with GnRH It is observed that the Sf-1 protein is localized not only in the nucleus, but also in the cytoplasm when exposed to 100 nM of GnRH for 24 h (Figure

Figure 22. Effects of TSA on FSHβ gene promoter activity in (A) LβT2 and (B) αT3 cells at basal and GnRH-modulated levels. The FSHβ promoter/reporter construct was transfected into the cells and 24 h later cells were incubated with TSA (100 ng/mL) and/or GnRH (100 nM) for 24 h. Relative promoter activities are indicated as fold induction over the promoter activity in the untreated control cells and expressed as mean ± SEM, where n = 4. A single asterisk on the bar indicates significant difference ($P<0.05$) from the control, and double asterisks indicate significant differences ($P<0.05$) from both the control and the GnRH- treated sample. The experiment was carried out once.

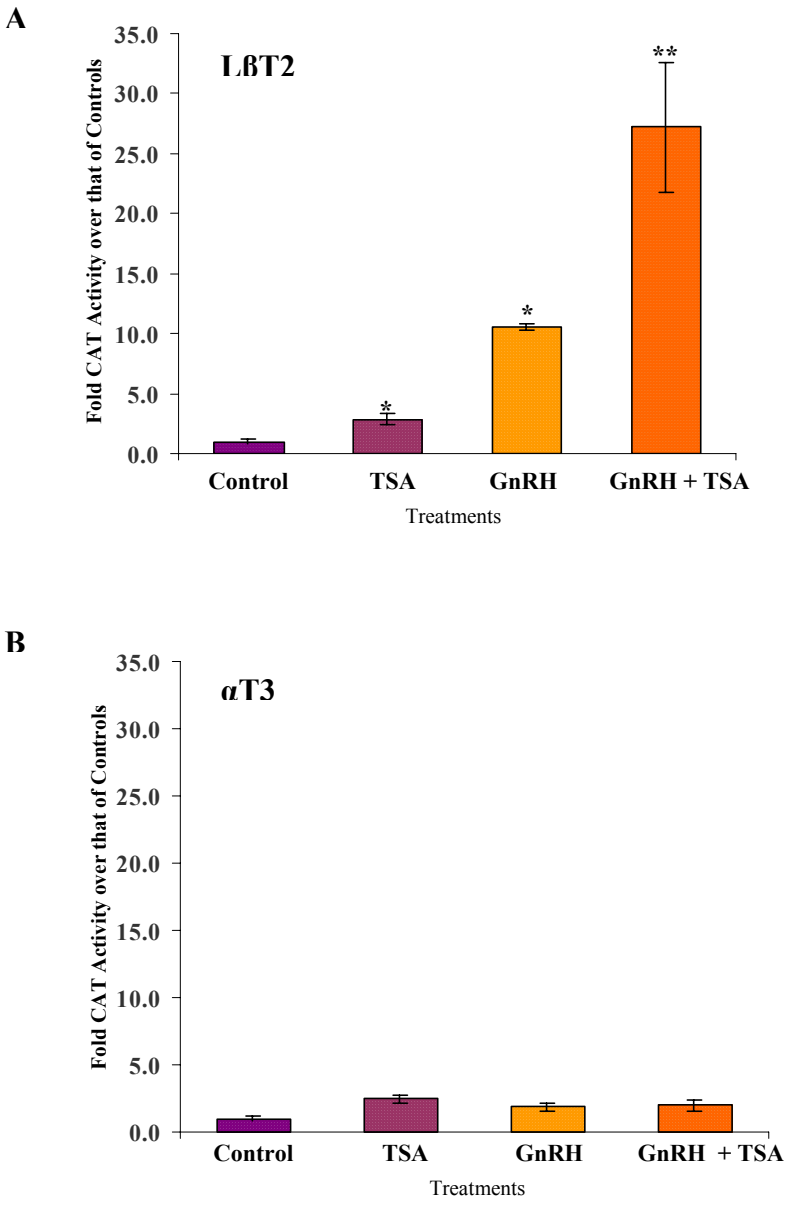
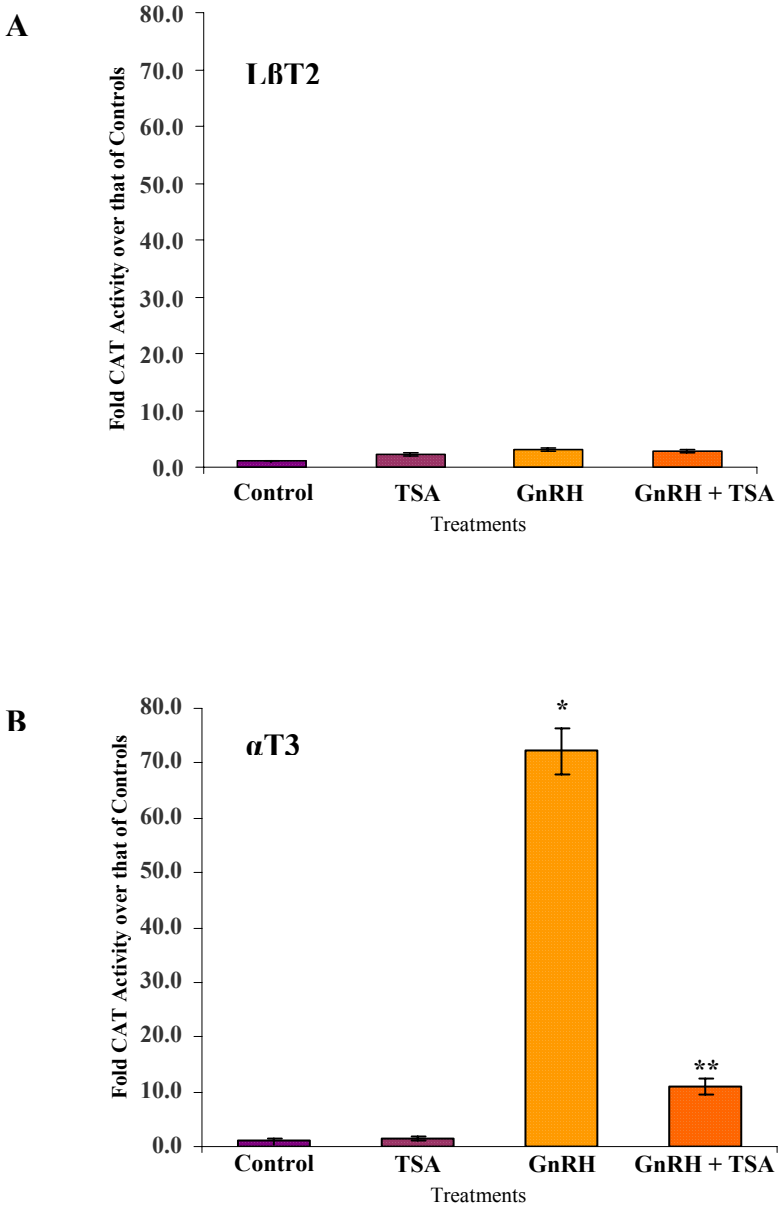
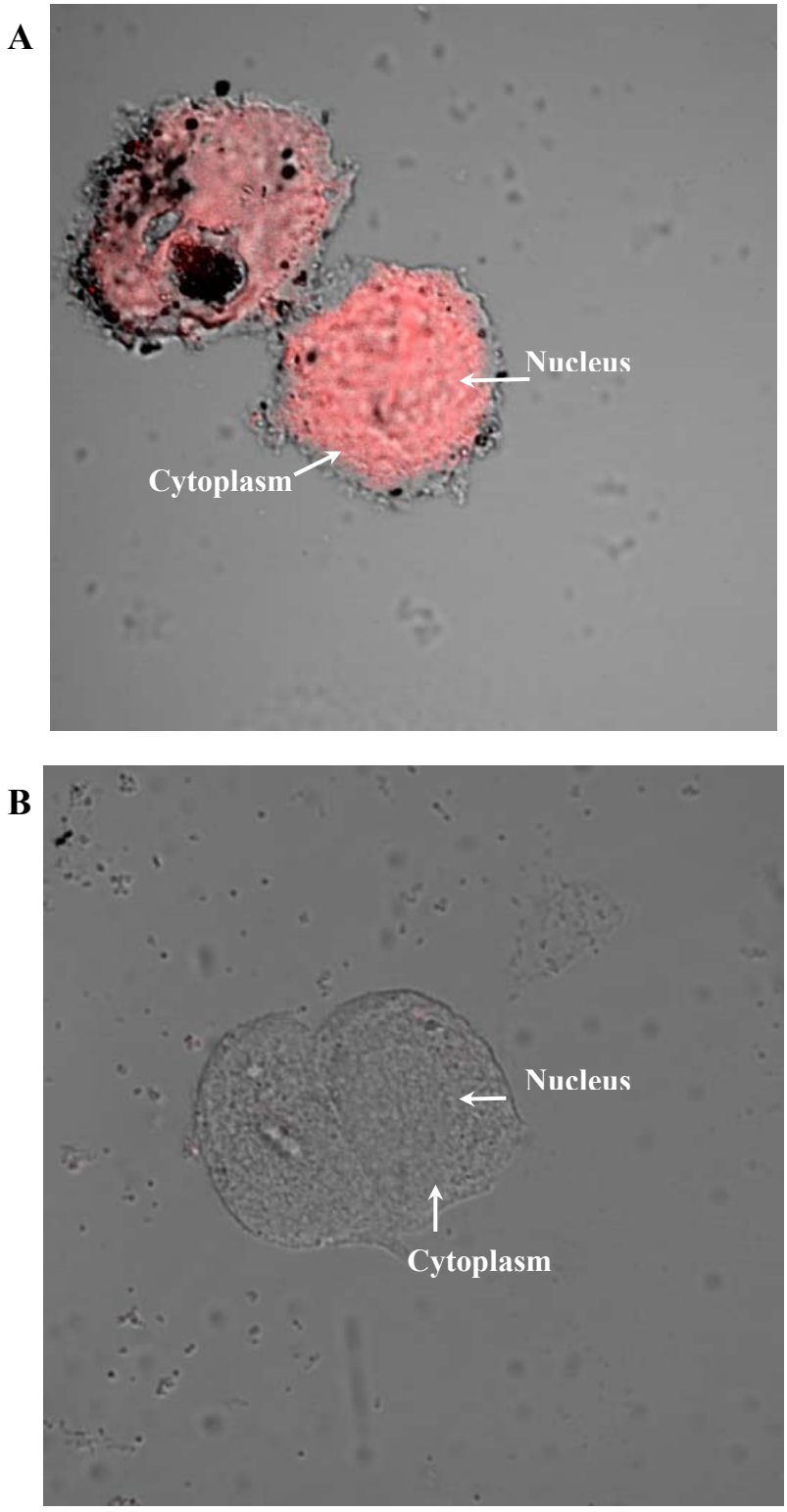


Figure 23. Effects of TSA on LHβ gene promoter activity in (A) LβT2 and (B) αT3 cells at basal and GnRH-modulated levels. The LHβ promoter/reporter construct was transfected into the cells, which are treated as in Figure 22. Promoter activity was measured and is represented also as in Figure 22. The experiment was carried out once.



24A). In contrast, the control without incubation with primary anti-Sf-1 antibodies did not detect Sf-1 in the cells (Figure 24B).

Figure 24. Localization of Sf-1 protein in the nucleus and cytoplasm of αT3 cell following GnRH exposure. αT3 cells, which express endogenous Sf-1, were transferred to glass coverslips and treated with 100 nM of GnRH for 24 h. They were then fixed with 2% paraformaldehyde for 15 min, permeabilized with 5% Triton-x for 10 min and blocked with 3% BSA for 10 min. The cells in **(A)** were incubated overnight with primary anti-Sf-1 antibody (30 µg/mL) and those in the control **(B)** were not. They were then incubated with goat anti-mouse TRIT-C (red) secondary antibody. The Sf-1 proteins were detected by confocal microscopy.



3.2 Involvement of HATs in the Regulation of Mouse Gonadotropin Genes via various Signaling Pathways *To comprehend further what chromatin modifying factors, apart from HDACs, are responsible for the regulation of mouse gonadotropin genes, a study on the involvement of HATs in the various signaling pathways in LβT2 cells was carried out by transfecting these cells with siRNA constructs designed to suppress the expression of CBP, Tip60, GCN5 and pCAF genes.*

Effects of Suppression of HATs on the Gonadotropin Gene Expression at Basal Level by Transfecting siRNA Constructs in LβT2 Cells The FSHβ mRNA levels in LβT2 cells are reduced markedly following transfections with siRNA constructs for the suppression of CBP, Tip60, GCN5 and pCAF (Figure 25). The FSHβ mRNA levels are almost undetectable for cells transfected with siRNA constructs to suppress CBP and Tip60. The basal level of the LHβ mRNA in LβT2 cells is unaffected when the cells were transfected with siRNA constructs for the suppression of CBP, Tip60 and GCN5, but the level is slightly decreased when transfected with pCAF-siRNA construct (Figure 25). The mRNA level of 60s ribosomal protein was unaffected by these transfections.

Figure 25. RT-PCR analysis of FSHβ and LHβ mRNA levels in LβT2 cells following the suppression of HATs. Total RNA was isolated from LβT2 cells (6-well plates) transfected for 48 h with siRNA constructs (6 μg/well) to knock down CBP, Tip60, GCN5 or pCAF. cDNA was synthesized from the total RNA and used as template for PCR. The primers were designed to amplify a 600bp DNA fragment of the coding region of the FSHβ gene and 300bp DNA fragment of the coding region of the LHβ gene. Amplification of a 100bp cDNA fragment of the mouse 60s ribosomal protein (60sRP) is shown as control. The experiment was repeated twice, and a representative is shown below.

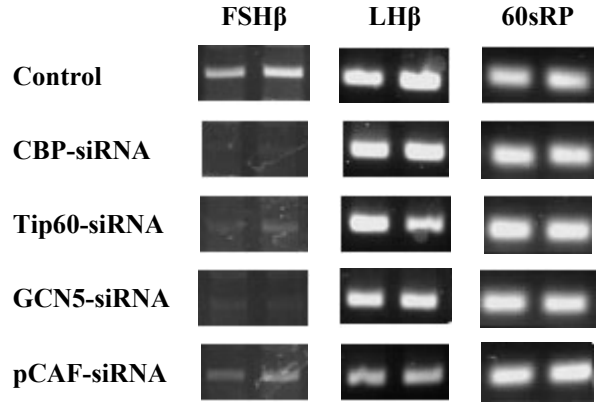
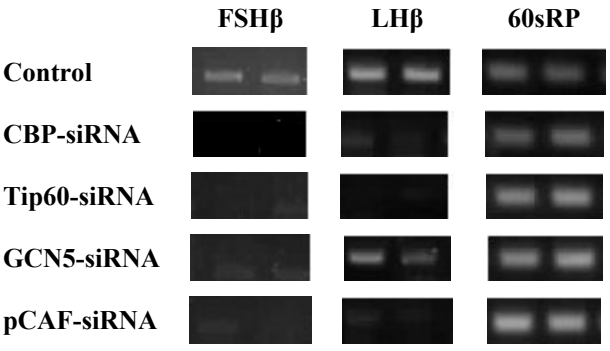


Figure 26. RT-PCR analysis of FSHβ and LHβ mRNA levels in GnRH-treated LβT2 cells following the suppression of HATs. Total RNA was isolated from GnRH-treated (100 nM; incubated for 24 h after 24 h of transfection) LβT2 cells transfected with siRNA constructs as in Figure 25 before analysis of mRNA levels, also as in Figure 25. The experiment was repeated twice, and a representative is shown below.



Effects of Suppression of HATs on the GnRH-stimulated Gonadotropin Gene Expression in LβT2 Cells The GnRH-stimulated levels of the FSHβ and LHβ mRNA in LβT2 cells are nearly undetectable when the cells were transfected with siRNA constructs for the suppression of CBP, Tip60, GCN5 and pCAF (Figure 26). In contrast, the mRNA level of 60s ribosomal protein was unaffected by these transfections.

Effects of Suppression of HATs on the Estradiol-stimulated Gonadotropin Gene Expression in LβT2 Cells The estradiol-stimulated levels of the FSHβ and LHβ mRNA in LβT2 cells are reduced when the cells were transfected with siRNA constructs for the suppression of CBP, Tip60, GCN5 and pCAF (Figure 27). Again, the mRNA level of 60s ribosomal protein was unaffected by these transfections.

Effects of Suppression of HATs on the GnRH-and-Estradiol-stimulated Gonadotropin Gene Expression in LβT2 Cells The level of the FSHβ mRNA in LβT2 cells treated with both GnRH and estradiol is reduced when the cells were transfected with siRNA constructs for the suppression of CBP and Tip60, and to a lesser extent for cells transfected with GCN5 and pCAF siRNA constructs (Figure 28). In contrast, the level of the LHβ mRNA in LβT2 cells is unaffected when the cells were transfected with any of these siRNA constructs. Similarly, the mRNA level of 60s ribosomal protein was unaffected by these transfections. A second unexpected band (~400 bp) of the LHβ gene was observed for GnRH-and-estradiol-treated cells transfected with GCN5 and pCAF siRNA constructs. Sequencing of this fragment confirmed that it contains intron-2 of the LHβ gene. This additional DNA band was

Figure 27. RT-PCR analysis of FSHβ and LHβ mRNA levels in estradiol-treated LβT2 cells following the suppression of HATs. Total RNA was isolated from estradiol-treated (10 nM; incubated for 24 h after 24 h of transfection) LβT2 cells transfected with siRNA constructs as in Figure 25 before analysis of mRNA levels, also as in Figure 25. The experiment was repeated twice, and a representative is shown below.

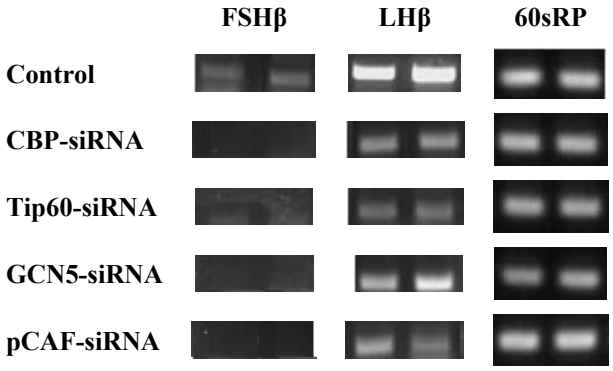
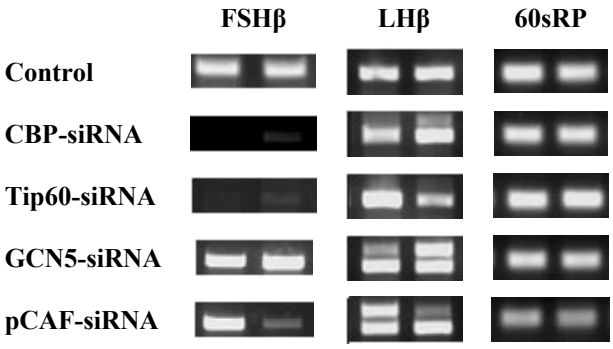


Figure 28. RT-PCR analysis of FSHβ and LHβ mRNA levels in LβT2 cells incubated with both GnRH and Estradiol following the suppression of HATs. Total RNA was isolated from LβT2 cells, which were treated with both GnRH and estradiol (100 nM and 10 nM respectively; incubated for 24 h after 24 h of transfection) transfected with siRNA constructs as in Figure 25 before analysis of mRNA levels, also as in Figure 25. The experiment was repeated twice, and a representative is shown below.



not detected for samples transfected with CBP or Tip60 siRNA constructs.

Effects of the Combinatorial Suppression of HATs on the Gonadotropin Gene Expression at Basal Level To test for the functional redundancy of HATs at basal level, we transfected LβT2 cells with a combination of siRNA constructs for the suppression of HATs (CBP and/or Tip60 and/or GCN5 and/or pCAF; 1.5 μg each) as shown in Figure 29. The LHβ mRNA levels were unaffected by any of the combinations of HAT suppression. On the other hand, those of FSHβ were reduced to different degrees for the different combinations of constructs.

Verification of effective HAT knock-down by siRNA Constructs To verify that the siRNA constructs are suppressing the expression of the HATs, Western blot and RT-PCR analyses were carried out. The protein levels of GCN5 and Tip60 in LβT2 cells are reduced markedly when the cells were transfected with these siRNA constructs (Figure 30A). The mRNA levels of CBP and pCAF have all decreased when the cells were transfected with these siRNA constructs (Figure 30B).

Figure 29. RT-PCR analysis of FSHβ and LHβ mRNA levels in LβT2 cells under the effect of combinatorial suppression of HATs. Total RNA was isolated from LβT2 cells (6-well plates) transfected with a combination of siRNA constructs (1.5 μg/well each; keeping the total DNA amount transfected constant with PWS constructs) as in Figure 25 before analysis of mRNA levels, also as in Figure 25. The experiment was carried out once.

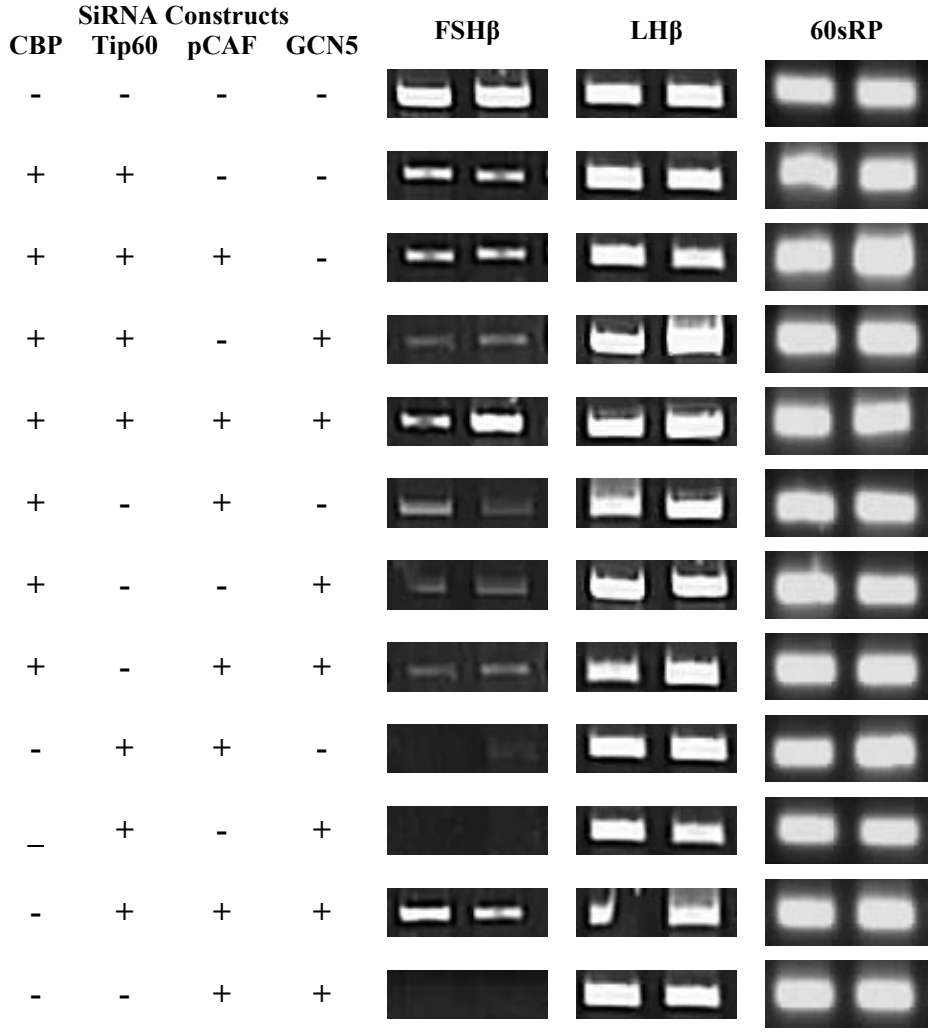
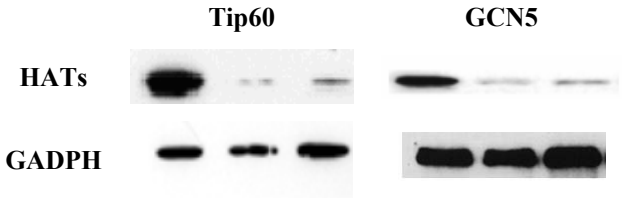
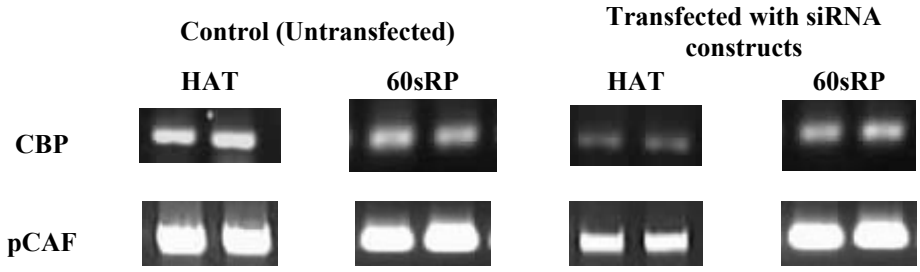


Figure 30. (A) Western blot and (B) RT-PCR analyses in verifying the suppression of HAT expression. Protein or total RNA was isolated from LβT2 cells (6-well plates) transfected for 48 h with siRNA constructs (6 μg) to knock down CBP, Tip60, GCN5 or pCAF as in Figure 25. (A) The protein samples (15 μg/lane) were run in 10 % SDS-PAGE, and the gel blotted on a PVDF membrane. The Western blot was probed with primary rabbit anti-mouse Tip60 and GCN5 antibodies and secondary goat anti-rabbit IgG antibodies. Primary goat anti-rabbit GADPH antibodies together with bovine anti-goat secondary IgG antibodies were used as controls to ensure uniform amount of proteins loaded in each well. Chemiluminiscence was detected by exposing the blot to film. Lanes (from left to right): 1, untransfected controls; 2 and 3, transfected with siRNA constructs. (B) For RT-PCR analysis, cDNA was synthesized from the total RNA and used as template for PCR. The primers were designed to amplify a DNA fragment of the coding regions of the CBP and pCAF genes. Amplification of a 100bp cDNA fragment of the mouse 60s ribosomal protein (60sRP) is shown as control. The experiments were repeated at least twice. The representatives are shown below.

A



B



3.3 Mechanisms of Recruitment of HATs to the Gonadotropin Gene Promoters via Various Signaling Pathways *The next step in gaining a more comprehensive insight of how HATs regulate the mouse gonadotropin genes is to study the mechanisms of the recruitment of HATs to the promoters. This study has a four-fold objective: (1) elucidate the occupancy of HATs at the promoter; (2) identify DNA-bound transcription factors responsible for occupancy; (3) investigate possible synergies between HATs and DNA-bound transcription factors in regulating promoter activity; and (4) study the protein-protein interactions between HATs and DNA-bound transcription factors.*

3.3.1 Occupancy of HATs at the Promoters

Chromatin immunoprecipitation (ChIP) was carried out to investigate the occupancy of the HATs, GCN5 and Tip60, on the gonadotropin FSHβ gene promoter following stimulation of various signaling pathways. HDAC1 occupancy in the promoter was also studied as a comparison to the HATs.

Occupancy of GCN5, Tip60 and HDAC1 at the FSHβ Gene Promoter in Hormonally-stimulated LβT2 Cells GCN5 occupies the FSHβ gene promoter in LβT2 cells at the basal level and is enhanced when the cells were treated with either

GnRH or activin (Figure 31). There was no occupancy when the cells were treated with estradiol. Interestingly, Tip60 occupies the promoter only when treated with GnRH and estradiol. HDAC1 occupies the promoter in the untreated cells and it seems that this is reduced when the cells were treated with estradiol. Conversely, HDAC1 is removed from the promoter following GnRH treatment (Figure 31).

3.3.2 DNA-bound Transcription Factors as Recruiters of HATs

To advance the understanding of the recruitment of HATs, siRNA constructs targeted to knock down expression of the DNA-bound transcription factors, ERα and *c*-Fos, were used in concert with ChIP.

Occupancy of GCN5 and Tip60 at the FSHβ Gene Promoter in Hormonally-stimulated LβT2 Cells with Suppression of ERα or *c*-fos

Following transfection of GnRH-treated LβT2 cells with a siRNA construct targeting either ERα or *c*-Fos, GCN5 no longer associates with the FSHβ gene promoter. On the other hand, transfection with the siRNA construct targeting ERα in GnRH-treated cells resulted in Tip60 not occupying the promoter, although Tip60 occupancy was unaffected by transfection with *c*-Fos siRNA construct. Moreover, Tip60 fails to occupy the promoter in estradiol-stimulated cells following transfection with siRNA construct against ERα. In contrast, Tip60 still occupies the promoter after transfection with *c*-Fos siRNA construct. Western blot analysis shows that the protein levels of *c*-Fos in LβT2 cells are reduced markedly when the cells were transfected with the siRNA construct (Figure 33).

Figure 31. Chromatin Immunoprecipitation (ChIP) Analysis of the association of FSHβ promoter region with GCN5, Tip60 and HDAC1 in hormonally stimulated LβT2 cells. LβT2 cells (100 mm plates) treated with GnRH (100 nM) and/or estradiol (10 nM) and/or activin (100 ng/mL) for 24 h were crosslinked with formaldehyde. The genomic DNA was then sonicated, and antisera added to pull-down the DNA fragments. PCR was carried out with the pulled-down DNA. The primers for PCR flank the FSHβ promoter region from -437 to -138 upstream from the start codon (~300 bp). The experiment was carried out once.

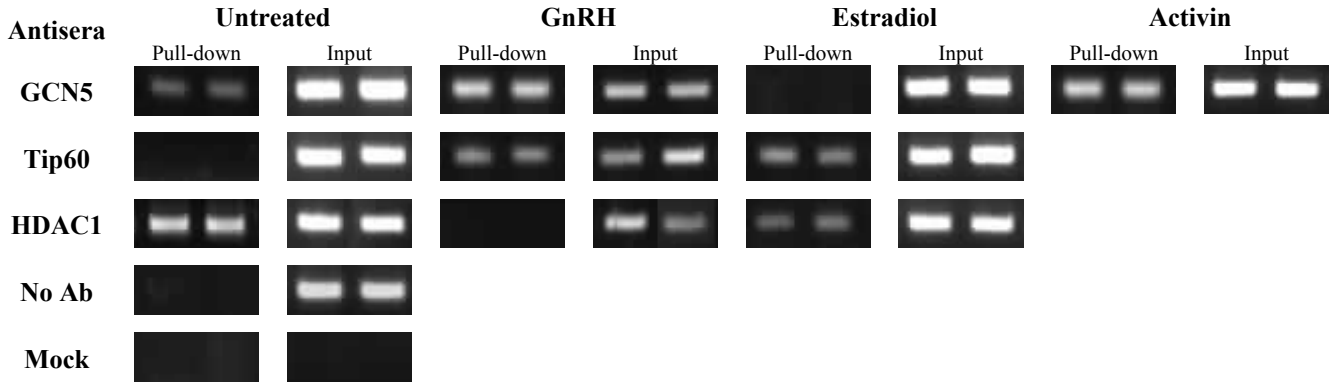


Figure 32.ChIP Analysis of the association of FSHβ promoter region with GCN5 and Tip60 in hormonally stimulated LβT2 cells transfected with siRNA constructs against ERα or *c*-Fos. ChIP was carried out with LβT2 cells (100 mm plates) treated with GnRH and/or estradiol following transfections with siRNA constructs (10 μg/plate each; 48 h) against ERα or *c*-Fos was as in Figure 31. ChIP results were analyzed and presented also as in Figure 31. The experiment was carried out once. Abbreviations: UT, untreated; E2, estradiol.

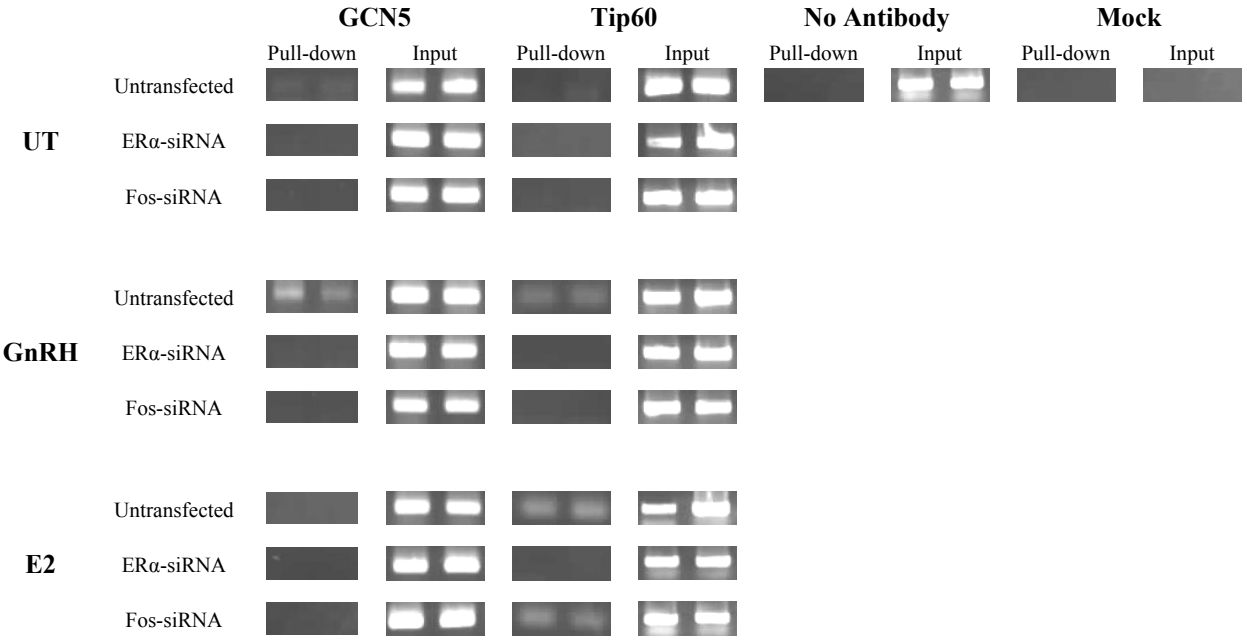
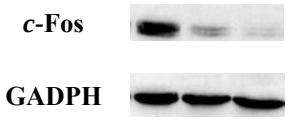


Figure 33. Western blot analysis verifies the suppression of *c*-Fos expression. Protein was isolated from LβT2 cells with siRNA construct to knock down *c*-Fos as in Figure 25. The protein samples were analyzed using Western blot as in Figure 30, with primary rabbit anti-mouse *c*-Fos. Lanes (from left to right): 1, untransfected controls; 2 and 3, transfected with siRNA constructs. The experiments were repeated at least twice. The representative is shown below.



3.3.3 Possible Synergies between HATs and DNA-bound Transcription Factors

Knowing which DNA-bound transcription factors are required for recruitment of HATs is not adequate in revealing the broad mechanism of HAT recruitment and action. To fully appreciate this aspect of gonadotropin gene regulation, studies on how different HATs and various DNA-bound transcription factors interplay with each other in regulating the promoter activity in various signaling pathways were carried out with reporter gene (luciferase) analysis. We carried out studies on the mouse FSHβ promoter with overexpression of the HATs GCN5 and Tip60 and a combination of DNA-bound transcription factors that include ERα, *c*-Jun, *c*-Fos, Sf-1, Egr-1 and Ptx-1 to elucidate such synergies.

GCN5 synergises with both *c*-Fos and *c*-Jun in activating the FSHβ promoter activity The FSHβ promoter activity following transfection with the expression vectors, GCN5, *c*-Fos and *c*-Jun have RLUs of 1.05, 1.05 and 1.68 respectively. Cotransfection of GCN5 with either *c*-Fos or *c*-Jun expression vectors gave a promoter activity of 1.15 and 2.10 RLUs, respectively, whereas cotransfection with both *c*-Fos and *c*-Jun resulted in 2.23 RLUs. Cotransfection with all three expression vectors stimulated promoter activity to 3.97 RLUs (Figure 34).

There is no synergy between GCN5 and/or Tip60 with ERα The FSHβ promoter activities in LβT2 cells following cotransfections with various combinations of expression vectors, did not show any synergism between GCN5 and/or Tip60 with ERα (Figure 35).

Figure 34. Effects of Overexpression of GCN5, *c*-Fos and *c*-Jun on the FSHβ Gene Promoter Activity. GCN5 expression vector (200 ng) was cotransfected for 48 h with mouse FSHβ firefly luciferase and *Renilla* reporter gene constructs (100 ng and 2.5 ng respectively), together with expression vectors for *c*-Fos and *c*-Jun (200 ng each) in LβT2 cells cultured in 96-well plates. The luciferase readings were normalized with the *Renilla* readings and the normalized readings of FSHβ construct alone was set at 1.00 relative light unit (RLU). The experiments were carried out once.

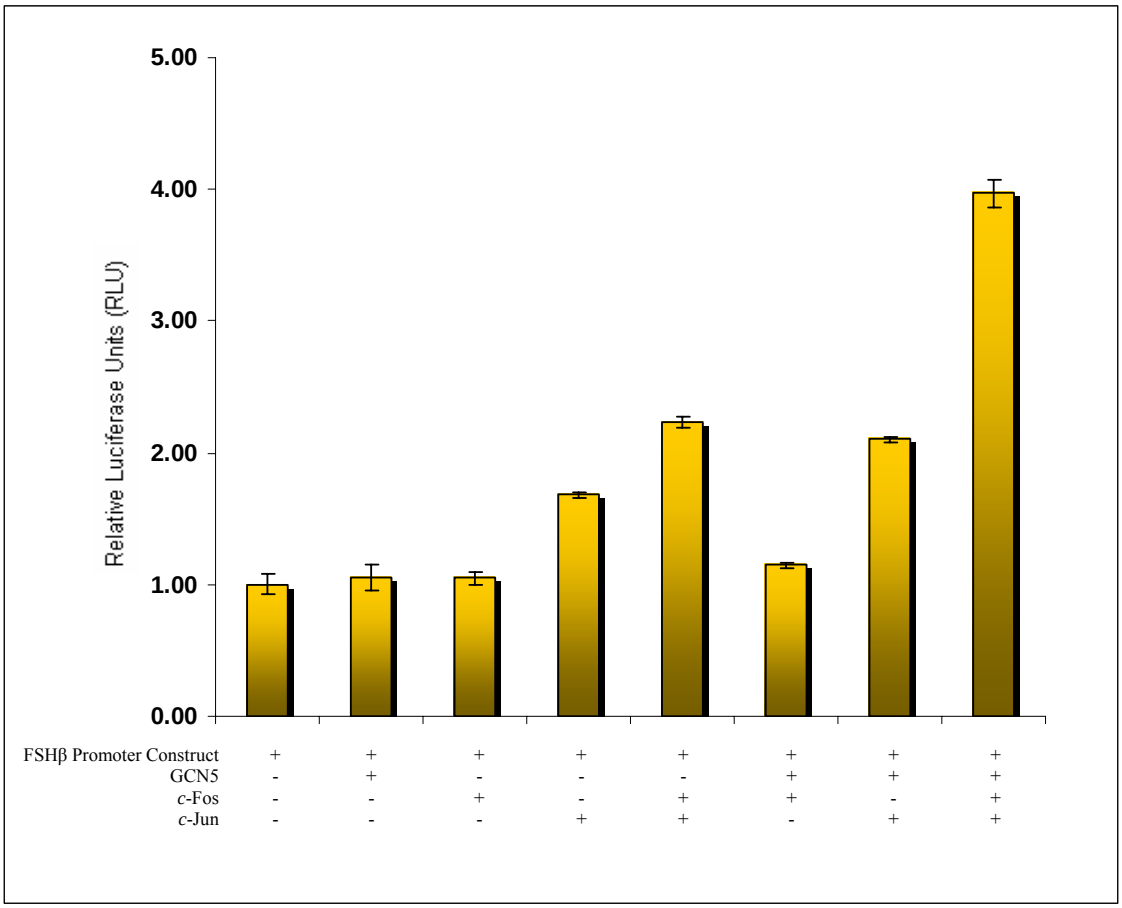
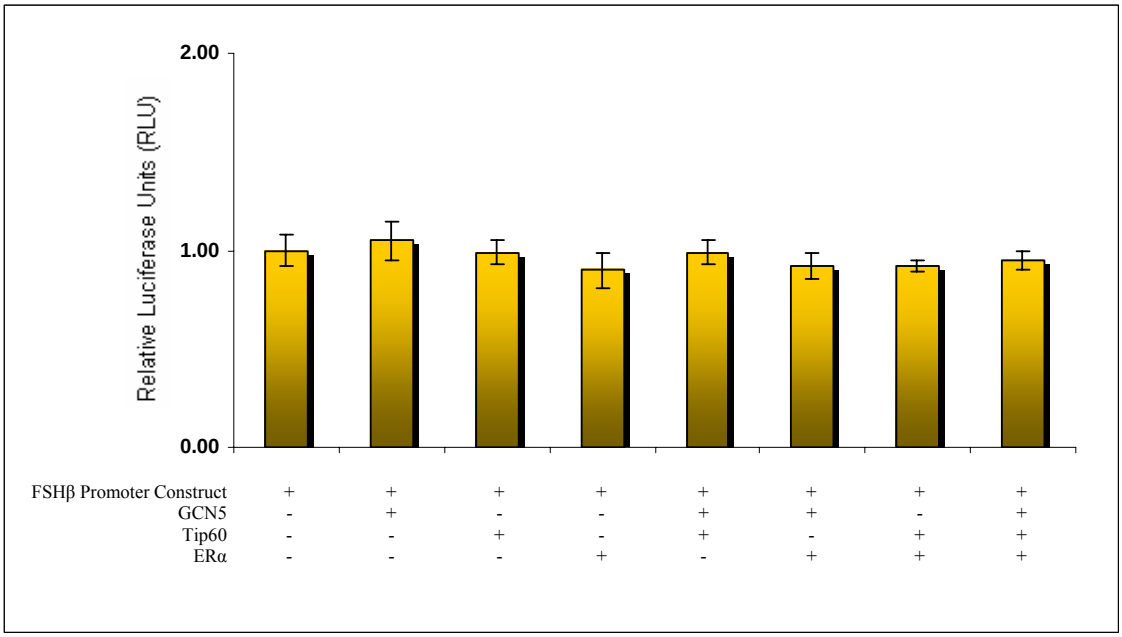


Figure 35. Effects of Overexpression of GCN5 and/or Tip60 with ERα on the FSHβ Gene Promoter Activity. GCN5 and/or Tip60 expression vector was cotransfected with mouse FSHβ firefly luciferase and *Renilla* reporter gene constructs, together with ERα expression vector in LβT2 cells as in Figure 34. The luciferase assays were analyzed and presented also as in Figure 34. The experiments were carried out once.



3.3.4 Protein-protein Interactions between HATs and DNA-bound Transcription Factors

Study of the dynamic protein-protein interactions amongst the HATs and between the diverse DNA-bound transcription factors in the various signaling pathways would enable us to fully elucidate the mechanisms of recruitment of HATs. Hence, mammalian two-hybrid assays were chosen for this purpose.

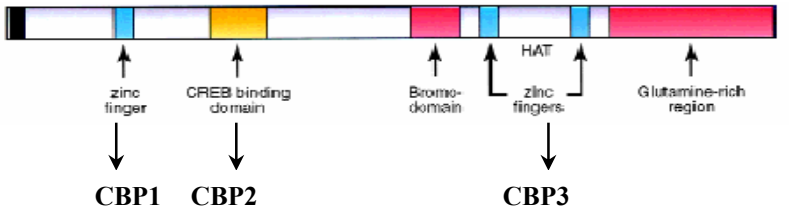
Tip60 interacts physically with CBP1 domain Following transfection with pVP16-Tip60 and pM-CBP1 constructs, along with the reporter gene constructs (pGal4-luc and pRL-CMV) in untreated, GnRH- and estradiol-treated LβT2 cells, the luciferase assay gave RLU values of 9.39, 10.48 and 8.63 respectively. In comparison, transfection with pVP16-Tip60 and pM, and pVP16 and pM-CBP1 in the cells gave luciferase readings of 0.80 and 4.73 RLUs (Figure 36B).

GCN5 has no physical interaction with either Tip60 or CBP Luciferase readings show that GCN5 does not interact with Tip60 or any of the CBP domains (Figure 36B).

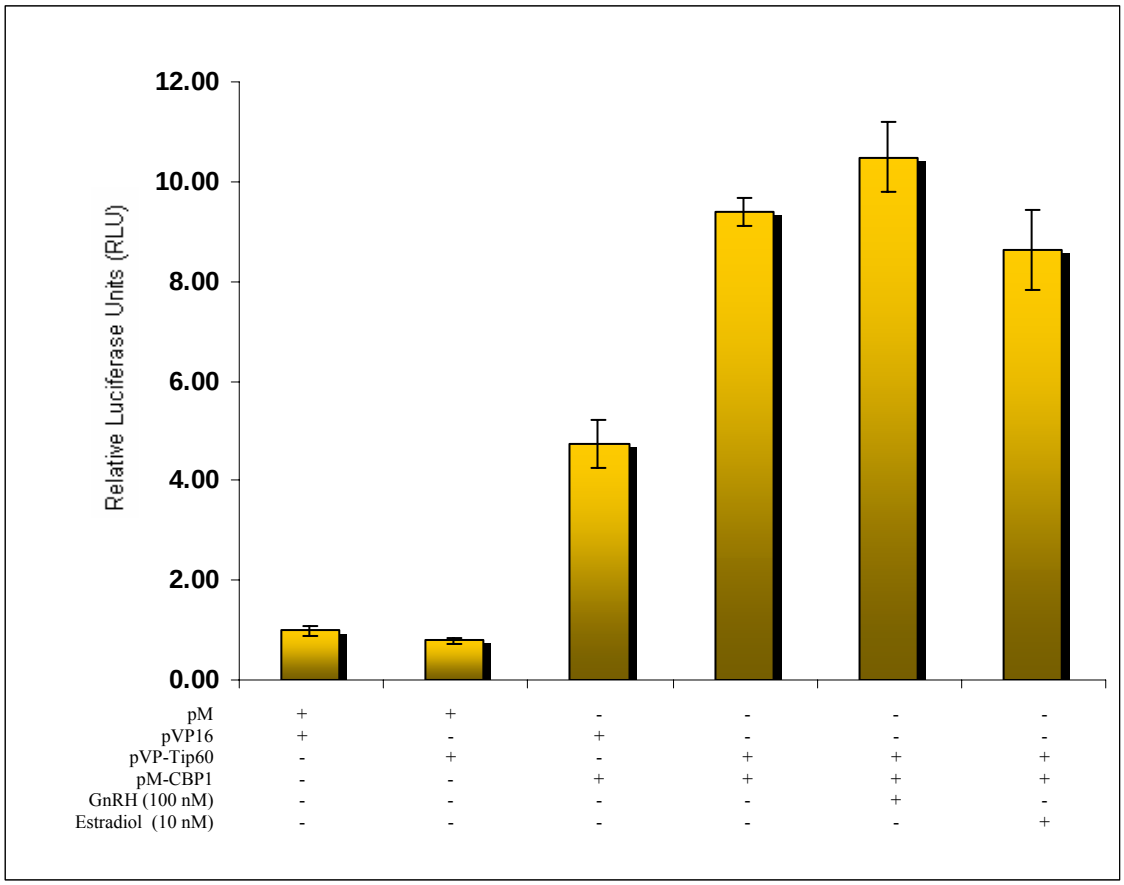
Tip60 interacts physically with the liganded-ERα Luciferase readings of transfections with pVP16-Tip60 and pM-ERα in estradiol-treated cells gave 2.34 RLU, compared with transfections with pVP16-Tip60 and pM, and pVP16 and pM-ERα, which gave 0.80 and 0.68 RLUs. This shows that Tip60 interacts with liganded ERα in LβT2 cells (Figure 37A).

Figure 36. Interactions amongst HATs. (A, B) pVP16 and pM constructs for CBP domains (CBP1, CBP2 and CBP3) were cotransfected with pM-GCN5 or pVP-Tip60 constructs in LβT2 cells as in Figure 34. The cells were treated with GnRH (100 nM) or estradiol (10 nM) 24 h before luciferase assays were carried out. The luciferase readings were normalized with the *Renilla* readings. The normalized readings for the basal interaction (between pM and pVP16) were set at 1.00 relative light unit (RLU). (C) Proteins found not to interact are given also in a table format with their RLU values and standard errors. The experiments were carried out once. Abbreviations: E2, estradiol.

A



B



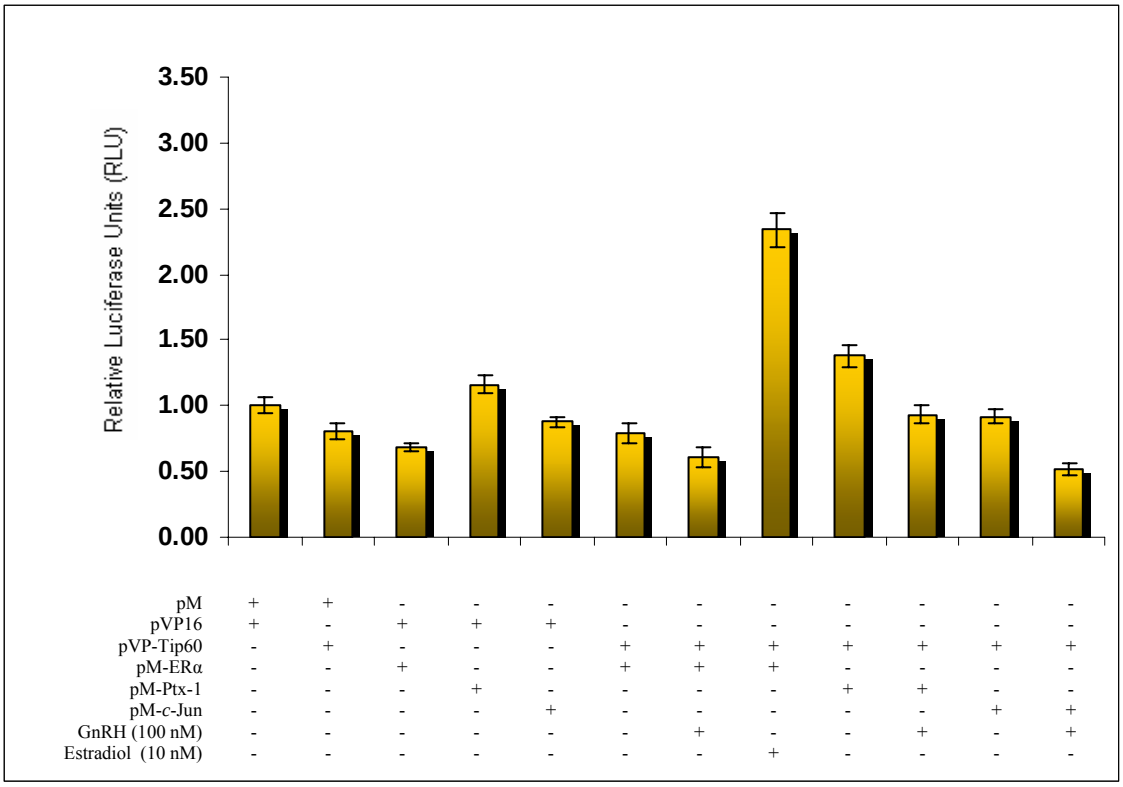
C

pM	pVP16	Treatments	RLU	S.E.
GCN5	Tip60	-	0.31	0.02
GCN5	Tip60	GnRH	0.16	0.00
GCN5	Tip60	E2	0.27	0.02
GCN5	CBP1	-	0.55	0.03
GCN5	CBP1	GnRH	0.31	0.02
GCN5	CBP1	E2	0.47	0.02
GCN5	CBP2	-	0.31	0.03
GCN5	CBP2	GnRH	0.16	0.00
GCN5	CBP2	E2	0.29	0.03
GCN5	CBP3	-	0.53	0.02
GCN5	CBP3	GnRH	0.17	0.02
GCN5	CBP3	E2	0.32	0.02
CBP2	Tip60	-	0.82	0.02
CBP2	Tip60	GnRH	0.51	0.08
CBP2	Tip60	E2	0.85	0.11
CBP3	Tip60	-	0.82	0.10
CBP3	Tip60	GnRH	0.54	0.03
CBP3	Tip60	E2	1.18	0.07

CBP but not GCN5 interacts physically with gonadotropin gene-specific transcription factors at its various domains Luciferase readings show that GCN5 does not interact with any of the transcriptions factors tested (Figure 37B). In contrast, the CBP domain, CBP1, interacts with ERα, *c*-Jun, Sf-1, Egr-1 and Smad3. Both CBP2 and CBP3 domains also interact with Sf-1 (Figure 38B and C).

Figure 37. Interactions of Tip60 or GCN5 with transcription factors. (A) pVP16-Tip60 or (B) pM-GCN5 constructs was cotransfected with the pVP16 or pM constructs for ERα, *c*-Jun, *c*-Fos, Sf-1 and Ptx-1 in LβT2 cells as in Figure 36. The luciferase assays were analyzed and presented also as in Figure 36. Proteins found not to interact with GCN5 are given in a table with their RLU values and standard errors. The experiments were carried out once.

A

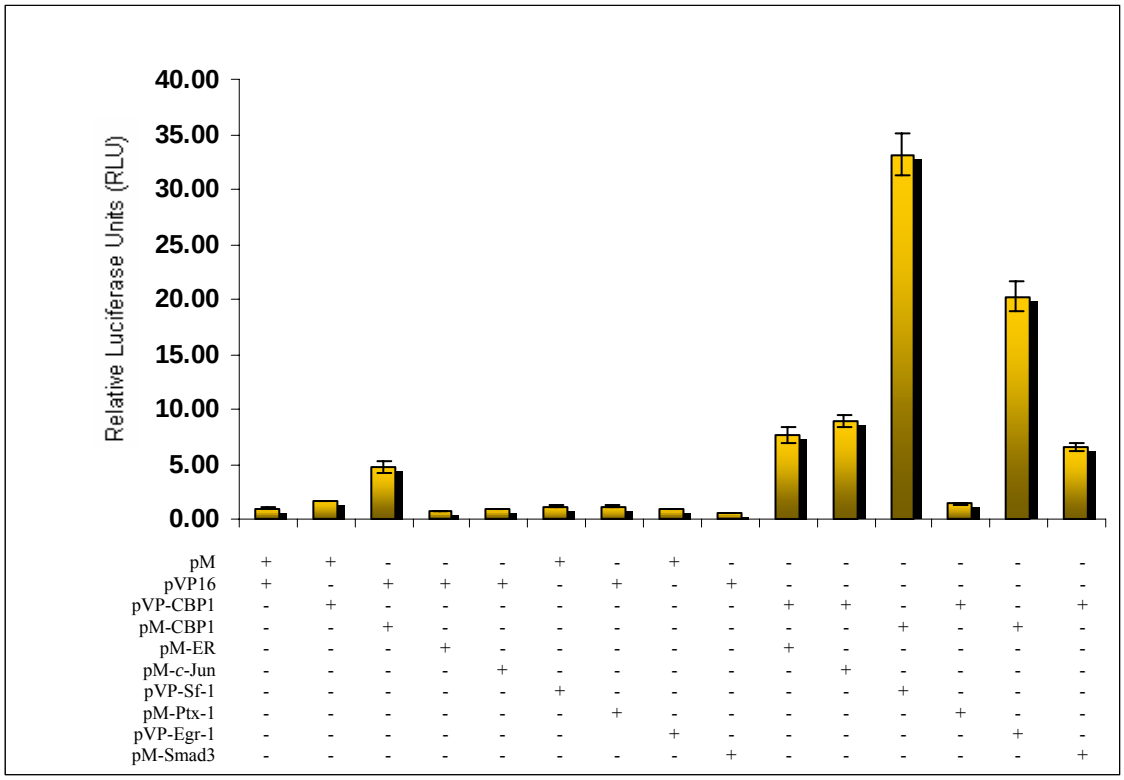


B

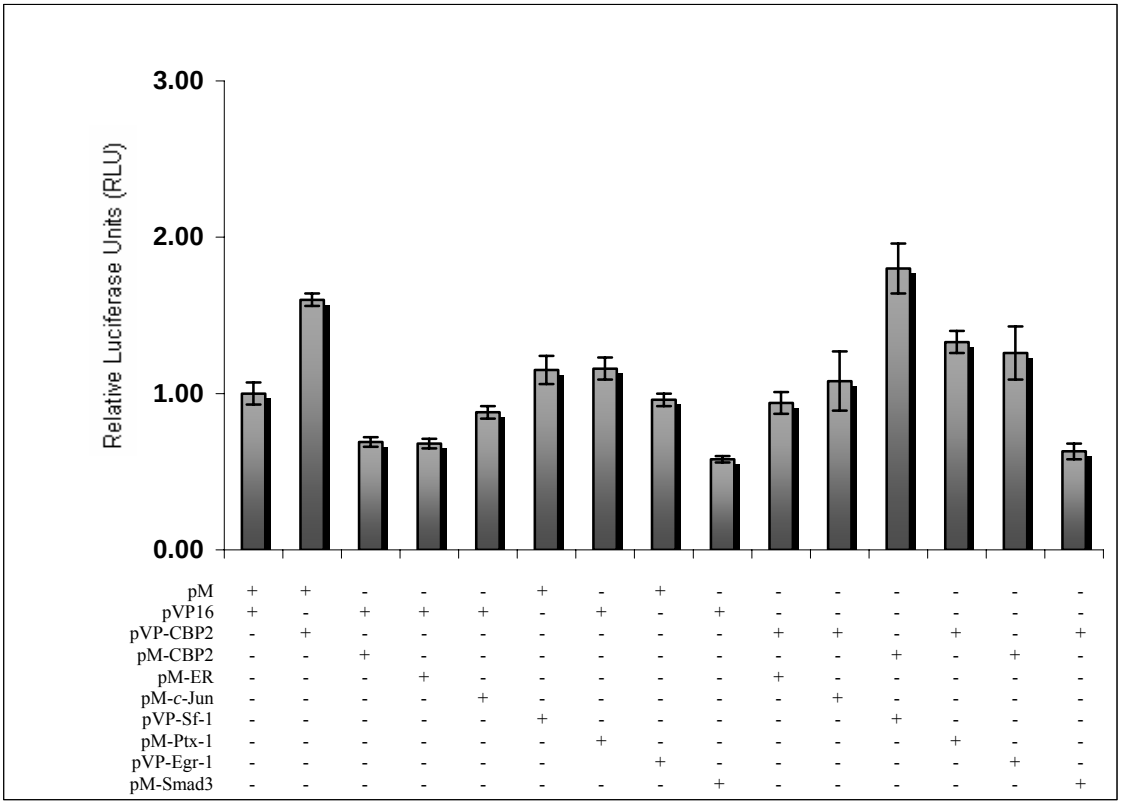
pM	pVP16	Treatments	RLU	S.E.
GCN5	pVP16	-	0.41	0.01
pM	ERα	-	0.72	0.05
pM	Ptx-1	-	0.86	0.04
pM	Sf-1	-	1.15	0.09
pM	*c*-Jun	-	0.81	0.09
pM	*c*-Fos	-	0.74	0.01
GCN5	ERα	-	0.29	0.02
GCN5	ERα	GnRH	0.17	0.01
GCN5	ERα	E2	0.36	0.04
GCN5	Ptx-1	-	0.41	0.02
GCN5	Ptx-1	GnRH	0.22	0.01
GCN5	Sf-1	-	0.50	0.03
GCN5	Sf-1	GnRH	0.19	0.01
GCN5	*c*-Jun	-	0.30	0.03
GCN5	*c*-Jun	GnRH	0.18	0.01
GCN5	*c*-Fos	-	0.36	0.03
GCN5	*c*-Fos	GnRH	0.17	0.01

Figure 38. Interactions of CBP with transcription factors. pVP16 or pM constructs of CBP domains: (A) CBP1, (B) CBP2 and (C) CBP3 were cotransfected with the pVP16 or pM constructs for ERα, *c*-Jun, Sf-1, Egr-1 and Ptx-1 in LβT2 cells as in Figure 36. The luciferase assays were analyzed and presented also as in Figure 36. The experiments were carried out once.

A



B


Relative Luciferase Units (RLU)
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1.00
0.00
pM + + - - - + - + - - - - - - -
pVP16 + - + + + - + - + - - - - - -
pVP-CBP2 - + - - - - - - - + + - + - +
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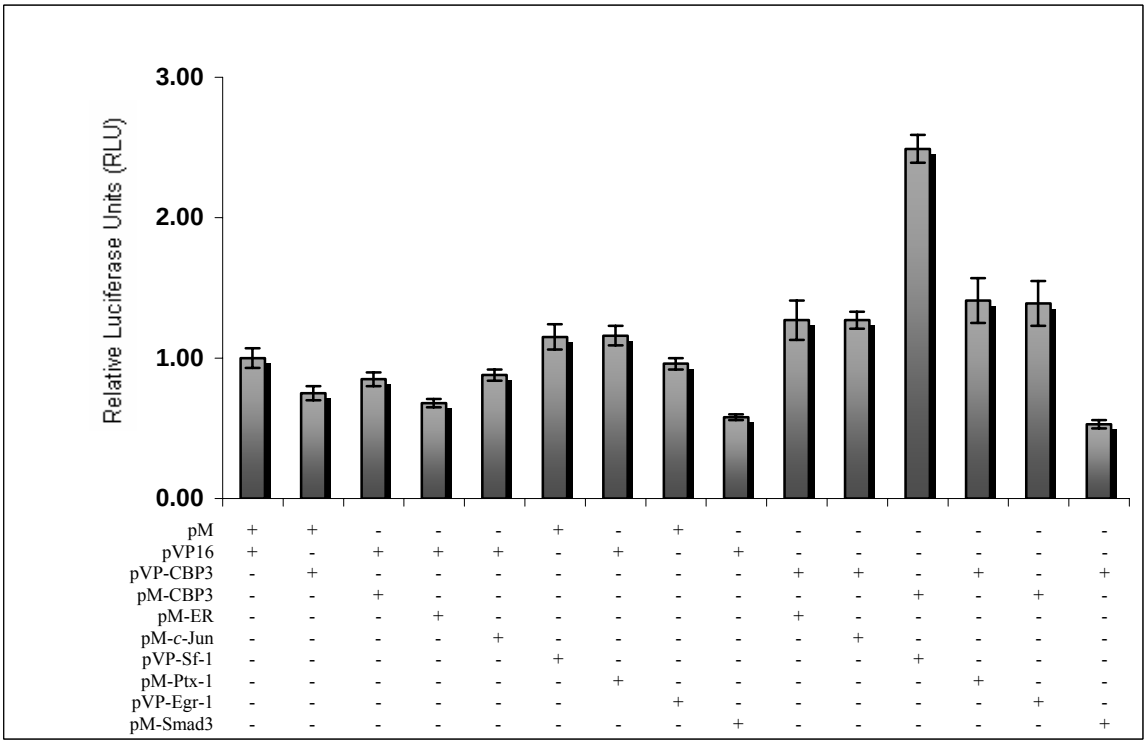
pM-ER - - - + - - - - - + - - - - -
pM-c-Jun - - - - + - - - - - + - - - -
pVP-Sf-1 - - - - - + - - - - - + - - -
pM-Ptx-1 - - - - - - + - - - - - + - -
pVP-Egr-1 - - - - - - + - - - - - + -
pM-Smad3 - - - - - - - - + - - - - - +

C


Relative Luciferase Units (RLU)
3.00
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pM + + - - - + - + - - - - - - -
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pVP-CBP3 - + - - - - - - - + + - + - +
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pM-ER - - - + - - - - - + - - - - -
pM-c-Jun - - - - + - - - - - + - - - -
pVP-Sf-1 - - - - - + - - - - - + - - -
pM-Ptx-1 - - - - - - + - - - - - + - -
pVP-Egr-1 - - - - - - + - - - - - + -
pM-Smad3 - - - - - - - - + - - - - - +

3.4 Possible Crosstalk between the Signaling Pathways and its Relation to the Regulatory Functions of HATs and HDACs

Crosstalk between different signaling pathways is common in signal transduction and a number of studies have shown how the different components of TGF-β signaling pathway crosstalk with the GnRH-signaling pathway. To further understand this, a study on how Smad proteins affect the GnRH- and estrogen-signaling in gonadotropes was carried out. The relation of the regulatory functions of HATs and HDACs to such crosstalk was also considered.

3.4.1 Combinatorial and Differential Treatments

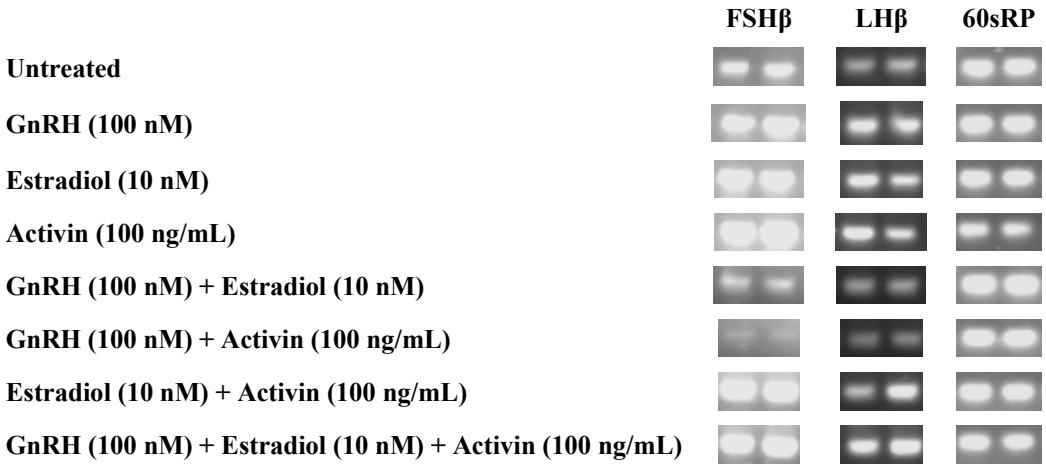
Before embarking on the study of how Smad proteins affect GnRH- or estrogen-signaling in gonadotropes, the consequence of combinatorial and differential treatments of LβT2 cells was studied. To achieve this, the cells were treated with GnRH and/or estradiol and/or activin. RT-PCR and qPCR analyses were done to measure the mRNA levels of FSHβ and LHβ genes, whereas Western blot analysis was carried out to measure the amounts of acetylated H3 histones: an indication of global gene activation.

The levels of FSHβ and LHβ mRNAs are increased by GnRH, Estradiol or Activin GnRH (100 nM) increased the mRNA levels of FSHβ and LHβ genes in LβT2 cells, as did estradiol (10 nM). Similarly, activin (100 ng/mL) increased the mRNA level of FSHβ gene and also that of LHβ gene. Apparently, the increase by activin treatment was greater for the FSHβ gene than for the LHβ gene. In contrast, the mRNA level of 60s ribosomal protein was unaffected by any of the treatments (Figure 39).

The levels of FSHβ and LHβ mRNAs are unaffected following treatment with GnRH and Estradiol or GnRH and Activin The levels of FSHβ and LHβ mRNAs are unaffected when the cells were treated with both GnRH (100 nM) and estradiol (10 nM) simultaneously. When this result is compared with those of the cells treated with either GnRH or estradiol, it seems that the combined treatment suppresses the activation of the FSHβ and LHβ gene expression by either factor alone. Similarly, the levels of FSHβ and LHβ mRNAs are unaffected by treatment with both GnRH (100 nM) and activin (100 ng/mL; Figure 39). In comparison with the effects of either of these treatments alone, the combined treatment seems to suppress the activation of the FSHβ and LHβ gene expression. The mRNA level of 60s ribosomal protein was unaffected by the treatments (Figure 39).

The levels of FSHβ and LHβ mRNAs are increased following treatment with Estradiol and Activin or with a combination of GnRH, Estradiol and Activin A combination of estradiol (10 nM) and activin (100 ng/mL) increased both the expression levels of FSHβ and LHβ genes in LβT2 cells. Apparently, the increase was greater for FSHβ gene than for the LHβ gene. A combination of GnRH

Figure 39. RT-PCR analysis of FSHβ and LHβ mRNA levels in LβT2 cells incubated with GnRH and/or Estradiol and/or Activin. Total RNA was isolated from LβT2 cells (60 mm plates) treated with GnRH (100 nM) and/or Estradiol (10 nM) and/or Activin (100 ng/mL) for 24 h. cDNA was synthesized from the total RNA and used as template for PCR. The mRNA analysis was done as in Figure 25. The experiment was repeated once.



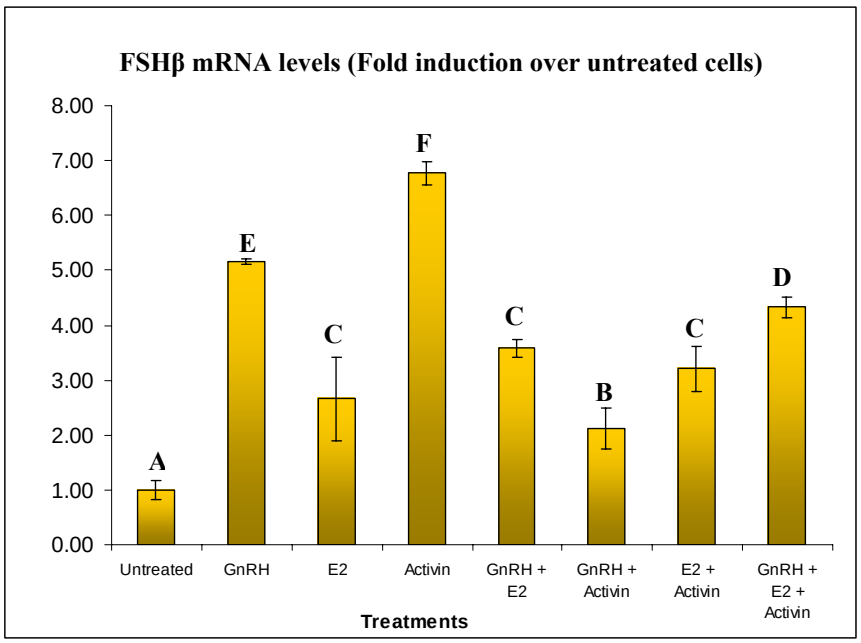
(100 nM), estradiol (10 nM) and activin (100 ng/mL) increased both the expression levels of FSHβ and LHβ genes in LβT2 cells as well. The mRNA level of 60s ribosomal protein was unaffected by these treatments (Figure 39).

Effects of Combinatorial and Differential Treatments on the FSHβ mRNA levels Quantitative PCR analysis revealed that the FSHβ mRNA level is increased significantly ($P < 0.05$) by 5.16, 2.66 and 6.77 fold by GnRH (100 nM), estradiol (10 nM) or activin (100 ng/mL) treatment for 24 h. The fold induction of FSHβ mRNA levels treated with GnRH is significantly ($p<0.05$) reduced from 5.16 to 3.58 fold when estradiol was added together with the GnRH. The induction of FSHβ mRNA levels by GnRH is significantly ($p<0.05$) decreased from 5.16 to 2.12 when activin was added. The fold induction of FSHβ mRNA levels following combined treatment with all three factors is 4.33 fold (significant as compared to the untreated samples; $p<0.05$). However, this increment is not synergistic in nature (Figure 40A).

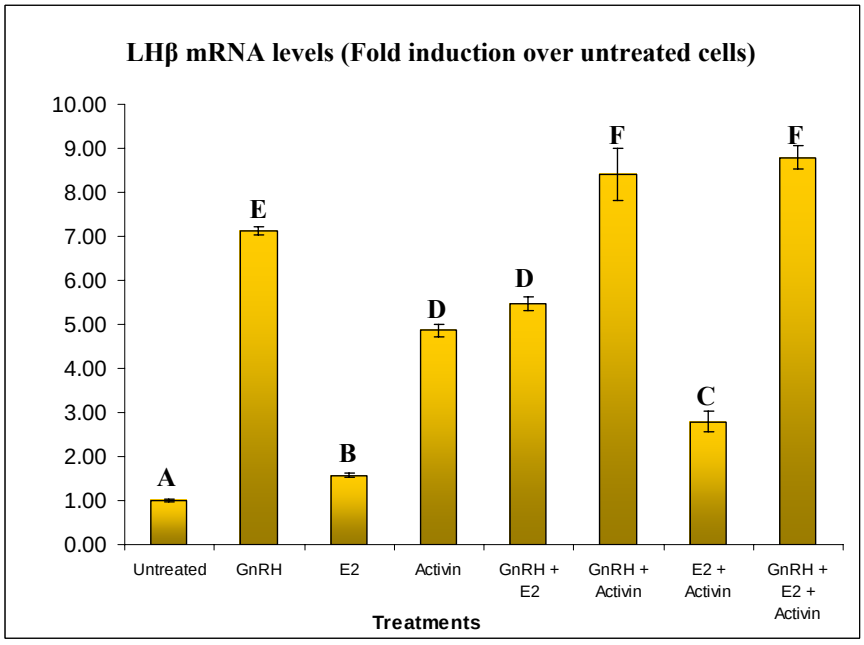
Effects of Combinatorial and Differential Treatments on the LHβ mRNA levels The LHβ mRNA level is increased significantly ($P < 0.05$) by 7.13, 1.53 and 4.86 fold by GnRH (100 nM), estradiol (10 nM) or activin (100 ng/mL) incubation for 24 h respectively. The fold induction of LHβ mRNA levels treated with GnRH is significantly ($p<0.05$) reduced from 7.13 to 5.48 when estradiol was added together with the GnRH. The induction of LHβ mRNA levels by GnRH is significantly ($p<0.05$) increased from 7.13 to 8.41 when activin was added. However, this increment is not synergistic in nature. The fold induction of LHβ mRNA levels following combined treatment with all three factors is 8.79 (significant as compared

Figure 40. Quantitative PCR analysis of (A) FSHβ and (B) LHβ mRNA levels in LβT2 cells incubated with GnRH and/or Estradiol and/or Activin. Total RNA was isolated from LβT2 cells (6-well plates) treated with GnRH (100 nM) and/or Estradiol (10 nM) and/or Activin (100 ng/mL) for 24 h. cDNA was synthesized from the total RNA and used as template for qPCR. Mean ± SEM, n ≥ 2. Student's t-tests were carried out. Means designated by different letters are significantly different (P < 0.05) from each other. The experiment was repeated once. Abbreviations: E2, estradiol.

A



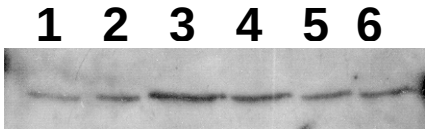
B



to the untreated samples; $p<0.05$). Again, this increment is not synergistic in nature (Figure 40B).

Extent of Histone (H3) Acetylation by Combinatorial and Differential Treatments Estradiol (10 nM; lane 3) or activin (100 ng/mL; lane 4) treatment of LβT2 cells for 24 h increases the amounts of acetylated H3 histones (acetylated H3-Lys27 residue), but GnRH treatment (100 nM; 24 h; lane 2) does not appear to have the same effect. However, when GnRH treatment was paired with estradiol (lane 5) or activin treatment (lane 6), the amounts of acetylated H3 histones appear to decrease when compared to the treatment with either estradiol (lane 3) or activin alone (lane 4; Figure 41).

Figure 41. Western blot analysis of acetylated H3 histone nuclear proteins abundance when exposed to GnRH or Estradiol or Activin. Nuclear proteins were isolated from LβT2 cells (60 mm plates) treated with GnRH (100 nM) or estradiol (10 nM) or activin (100 ng/mL). The nuclear proteins (15 µg/lane) were run on SDS-PAGE, and then blotted on to a PDVF membrane. The Western blot was probed with primary rabbit anti-mouse acetylated H3-Lys27 antibodies and secondary goat anti-rabbit IgG antibodies. Chemiluminiscence was detected by exposing the blot to film. The experiment was carried out once. Lanes: 1-Untreated; 2-GnRH (100 nM); 3-estradiol (10 nM); 4-activin; (100 ng/mL); 5-GnRH and estradiol; 6-GnRH and activin.



3.4.2 Effects of Smads on the GnRH- and Estrogen- Signals in Gonadotropes

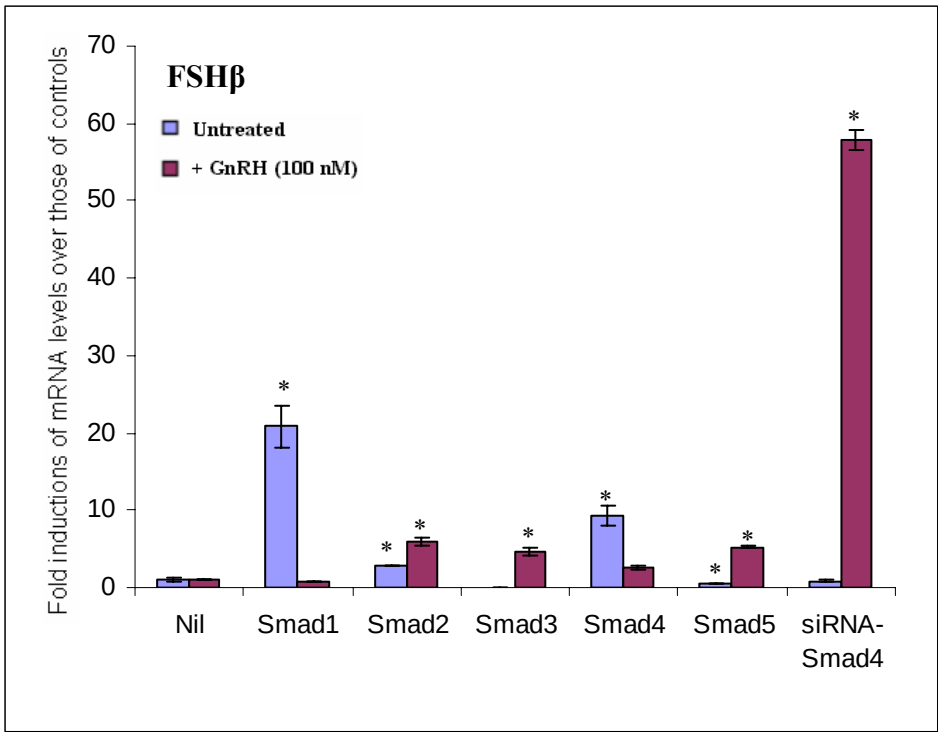
To understand the molecular mechanism of the crosstalk between activin and GnRH or estrogen signaling pathways, several Smad proteins were overexpressed, or Smad4 was suppressed in LβT2 cells. The mRNA levels of FSHβ and LHβ were analyzed by qPCR. ChIP was employed to study the occupancy of Smad4 protein on the FSHβ gene promoter, under various conditions, and also that of GCN5 and HDAC7 to study their possible role in the crosstalking between activin signaling pathway and GnRH or estrogen signaling pathways.

Effects of Overexpression or Suppression of Smads on the FSHβ mRNA levels FSHβ mRNA level in LβT2 cells transfected with Smad1, Smad2 and Smad4 expression vectors is increased significantly ($P<0.05$) by 20.80, 2.78 and 9.20 fold respectively over levels in control cells. The FSHβ mRNA level, however, is apparently unaffected ($P>0.05$) following transfection with Smad3 expression vector or Smad4 siRNA construct. In contrast, the transfection with Smad5 expression vector caused a decrease ($P<0.05$) in the FSHβ mRNA levels by nearly half (from 1.00 to 0.59; Figure 42A).

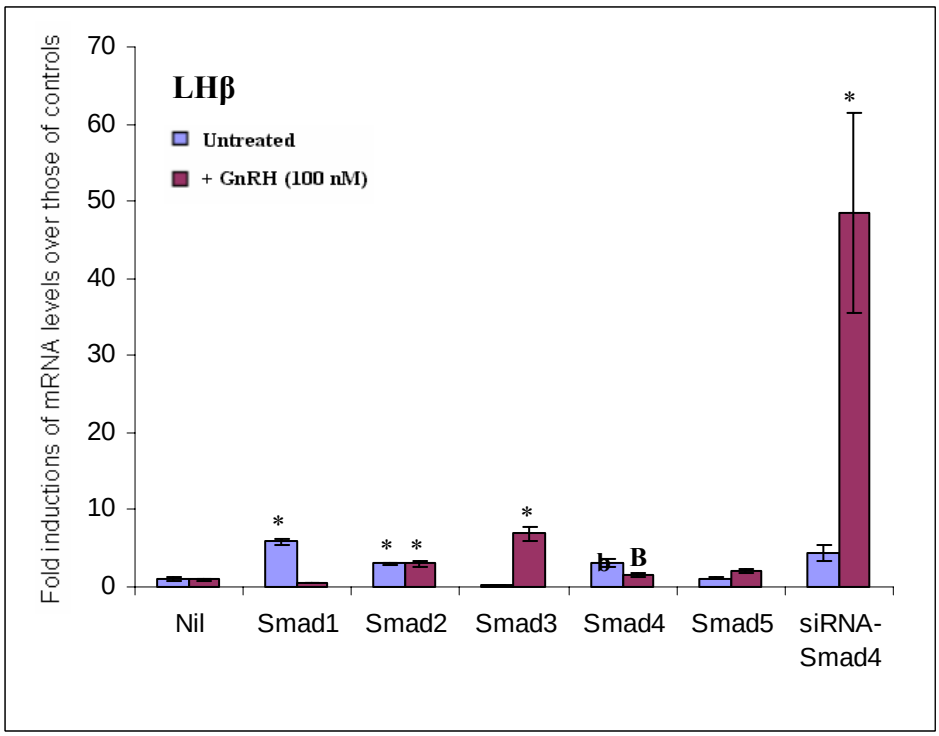
Effects of Overexpression or Suppression of Smads on the LHβ mRNA levels The LHβ mRNA level in LβT2 cells is apparently unaffected ($P>0.05$) by transfection with Smad3, Smad4 or Smad5 expression vectors. However, transfection with Smad1 or Smad2 expression vectors or Smad4 siRNA construct significantly ($P<0.05$) increases the LHβ mRNA level by 5.84, 3.01 and 4.41 fold respectively (Figure 42B).

Figure 42. Quantitative PCR analysis of (A) FSHβ and (B) LHβ mRNA levels in LβT2 cells following overexpression of Smad proteins or suppression of Smad4 protein without and with GnRH treatment. Total RNA was isolated from LβT2 cells (6-well plates) transfected with Smad1, Smad2, Smad3, Smad4, Smad5 expression and Smad4 siRNA constructs (6 µg), and treated with or without GnRH (100 nM) for 24 h. cDNA was synthesized from the total RNA and used as template for qPCR. Mean ± SEM, n = 3. Student's t-tests were carried out for samples in the same treatment group. Means designated by a single asterisk (*) are significantly different ($P < 0.05$) from the untransfected samples. ANOVA with Bonferroni tests were carried out between treatment groups. Means designated by different letters are significantly different ($P<0.05$). The experiment was repeated twice and representative graphs are shown. Abbreviations: Ctrl, control; Smad1/2/3/4/5, Smad1/2/3/4/5 overexpression; Smad4-siRNA, Smad4 suppression by Smad4-siRNA construct.

A



B



Effects of Overexpression or Suppression of Smads on the FSHβ mRNA level in GnRH-treated LβT2 cells FSHβ mRNA levels are apparently unaffected ($P>0.05$) in GnRH-treated (100 nM; 24 h) LβT2 cells by transfection with Smad1 and Smad4 expression vectors. However, transfection with Smad2, Smad3 or Smad5 expression vectors or Smad4 siRNA construct significantly increased ($P<0.05$) the FSHβ mRNA level by 5.98, 4.66, 5.26 and 57.88 fold respectively. The fold induction of FSHβ mRNA level transfected with Smad4 expression vector is lessened significantly ($P<0.05$) from 9.20 to 2.57 (about by a factor of 3.4) fold by GnRH. In contrast, the FSHβ mRNA level following transfection with Smad4 siRNA construct is increased significantly ($P<0.05$) from 0.90 to 57.88 (about by a factor of 60) fold when incubated with GnRH (Figure 42A).

Effects of Overexpression or Suppression of Smads on the LHβ mRNA level in GnRH-treated LβT2 cells LHβ mRNA levels are apparently unaffected ($P>0.05$) in GnRH-treated (100 nM; 24 h) LβT2 cells following transfection with Smad1, Smad2, Smad4 or Smad5 expression vectors or Smad4 siRNA construct Transfection with Smad3 expression vector significantly increased ($P<0.05$) the LHβ mRNA level by 6.88 fold. However, the fold induction of LHβ mRNA level in LβT2 cells transfected with Smad4 expression vector is almost halved ($P<0.05$; from 3.28 to 1.51 fold) by GnRH. In contrast, the LHβ mRNA level in cells transfected with Smad4 siRNA construct is increased significantly ($P<0.05$) from 4.41 to 48.50 (about by a factor of 12) fold when incubated with GnRH (Figure 42B).

Effects of Smad4 Overexpression or Suppression on the FSHβ mRNA level The FSHβ mRNA level in LβT2 cells is increased significantly following transfection with Smad4 expression vector ($P<0.05$) by 3.49 fold and following transfection with Smad4 siRNA construct, the increase is only 1.70 fold ($P<0.05$;). The FSHβ mRNA level in GnRH-treated (100 nM; 24h) cells transfected with Smad4 expression construct is decreased significantly ($P<0.05$) from 4.92 to 1.33 fold whereas its level is increased significantly from 4.92 to 6.93 fold ($P<0.05$) by transfection with Smad4 siRNA construct. Similarly, the FSHβ mRNA level in estradiol-treated (10 nM; 24 h) cells transfected with Smad4 expression vector decreased significantly ($P<0.05$) from 9.82 to 4.06 fold and its level increased significantly from 9.82 to 11.73 fold ($P<0.05$) by transfection with Smad4 siRNA construct (Figure 43).

Effects of Suppression of HDAC1 or HDAC2 on the Smad4-mediated FSHβ mRNA level In identifying the HDACs which may account for the repression observed by Smad4 in the GnRH- and estrogen-mediated induction of the FSHβ gene in LβT2 cells (Figures 42 and 43), we suppressed HDAC1 or HDAC2 by transfecting the cells with siRNA constructs targeting these HDACs. The FSHβ mRNA levels in GnRH-treated (100 nM; 24 h) cells are increased (in comparison to untransfected GnRH-treated cells) by transfection with Smad4 expression vector and either HDAC1 or HDAC2 siRNA constructs. On the other hand, it seems that these levels in estradiol-treated (10 nM; 24 h) cells are unaffected (in comparison to untransfected estradiol-treated cells) by transfection with Smad4 expression vector and either HDAC1 or HDAC2 siRNA constructs (Figure 44).

Figure 43. Quantitative PCR analysis of FSHβ mRNA levels in LβT2 cells following overexpression or suppression of Smad4 protein incubated with GnRH or Estradiol. Total RNA was isolated from LβT2 cells transfected with Smad4 expression construct or Smad4 siRNA construct, and treated with GnRH or estradiol as in Figure 42, and presented also as in Figure 42 with statistical analysis carried out separately for untreated (small letters), GnRH-treated (capital letters) and estradiol-treated (italicized letters) cells. The experiment was repeated twice and a representative graph is shown. Abbreviations: Ctrl, control; Smad4, Smad4 overexpression; Smad4si, Smad4 suppression by Smad4-siRNA construct.

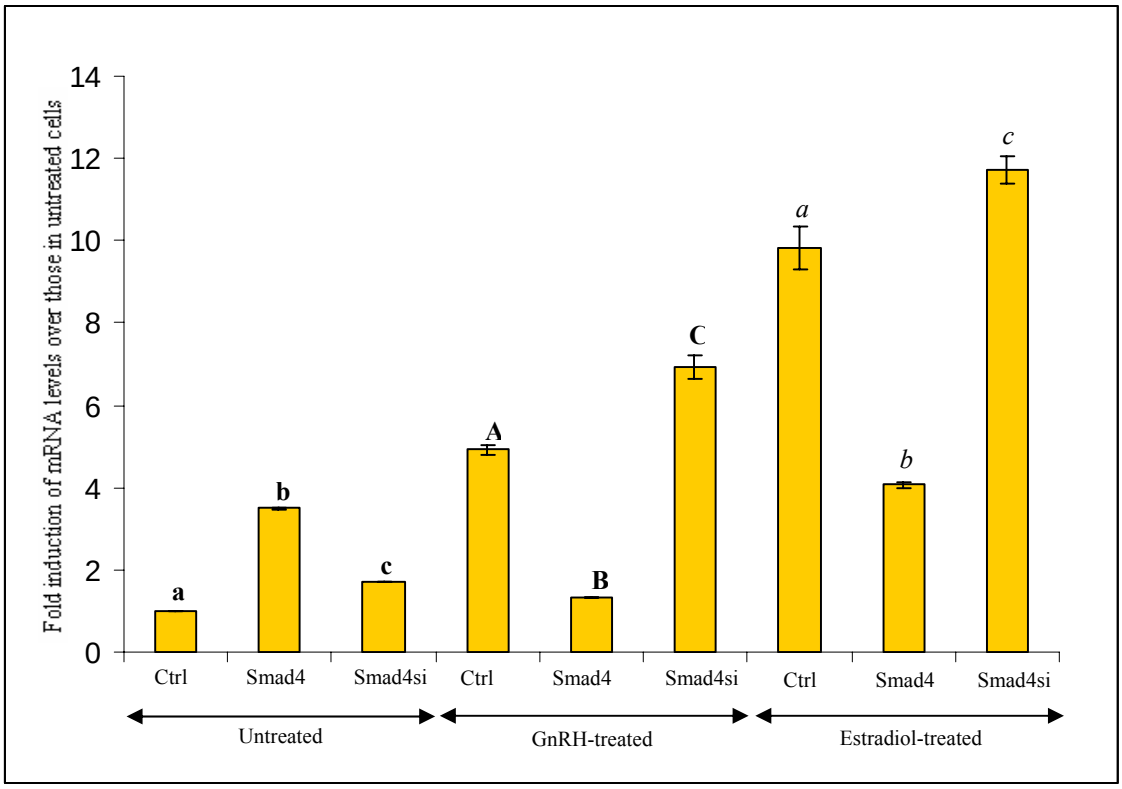
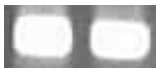
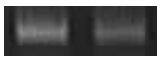
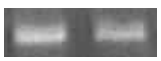
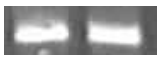
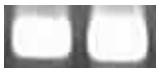
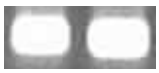
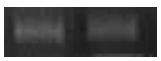
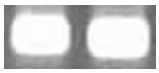
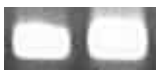
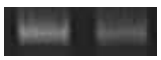
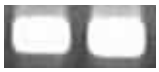


Figure 44. RT PCR analysis of FSHβ mRNA levels in LβT2 cells following overexpression or suppression of Smad4 protein or suppression of HDAC1 and HDAC2 incubated with GnRH or Estradiol. Total RNA was isolated from LβT2 cells (6-well plates) transfected with Smad4 expression construct (6 µg) or HDAC1 or HDAC2 siRNA construct (6 µg), and treated with GnRH (100 nM) or estradiol (10 nM) for 24 h. cDNA was synthesized from the total RNA and used as template for RT-PCR as in Figure 25. The experiment was carried out once. Abbreviations: '-', absent; '+', present; Smad4, Smad4 overexpression; HDAC1si, HDAC1 suppression by HDAC1-siRNA construct; HDAC2si, HDAC2 suppression by HDAC2-siRNA construct.

Treatments		Constructs			FSHβ	60sRP
GnRH	Estradiol	Smad4	HDAC1si	HDAC2si		
-	-	-	-	-		
+	-	-	-	-		
-	+	-	-	-		
-	-	+	-	-		
-	-	-	+	-		
-	-	-	-	+		
+	-	+	-	-		
+	-	+	+	-		
+	-	+	-	+		
-	+	+	-	-		
-	+	+	+	-		
-	+	+	-	+		

Occupancy of Smad4, GCN5 and HDAC7 on the FSHβ Gene Promoter of LβT2 Cells To elucidate the molecular mechanism of Smad4 in the possible crosstalk between activin signaling pathway and GnRH/estrogen signaling pathways, ChIP was carried out with antisera immunoreactive to the Smad4, GCN5 and HDAC7 proteins in LβT2 cells treated with activin and/or GnRH/estradiol. Smad4 was found to occupy the FSHβ gene promoter following treatment with activin (100 ng/mL; 24 h). However, when either GnRH (100 nM; 24 h) or estradiol (10 nM; 24 h) treatment is paired with the activin treatment, Smad4 no longer occupies the FSHβ promoter. In the untreated cells, GCN5 does not occupy the promoter, but HDAC7 does. However, upon treatment with activin alone, GCN5 occupies the promoter, but not HDAC7. When either GnRH or estradiol treatment is paired with activin treatment, GCN5 does not occupy the promoter, but HDAC7 does (Figure 45).

Verification of effective Smad4 knock-down by siRNA Construct To verify that the siRNA construct is suppressing the expression of the Smad4, Western blot analysis was carried out. This revealed that the protein levels of Smad4 in LβT2 cells are reduced markedly when the cells were transfected with the siRNA construct (Figure 46).

Figure 45. ChIP Analysis of the association of FSHβ promoter with Smad4, GCN5 and HDAC7 in hormonally stimulated LβT2 cells. ChIP was carried out, as in Figure 31, with LβT2 cells treated with activin and/or GnRH and/or estradiol as shown below. ChIP results were analyzed and presented also as in Figure 31. The experiment was carried out once.

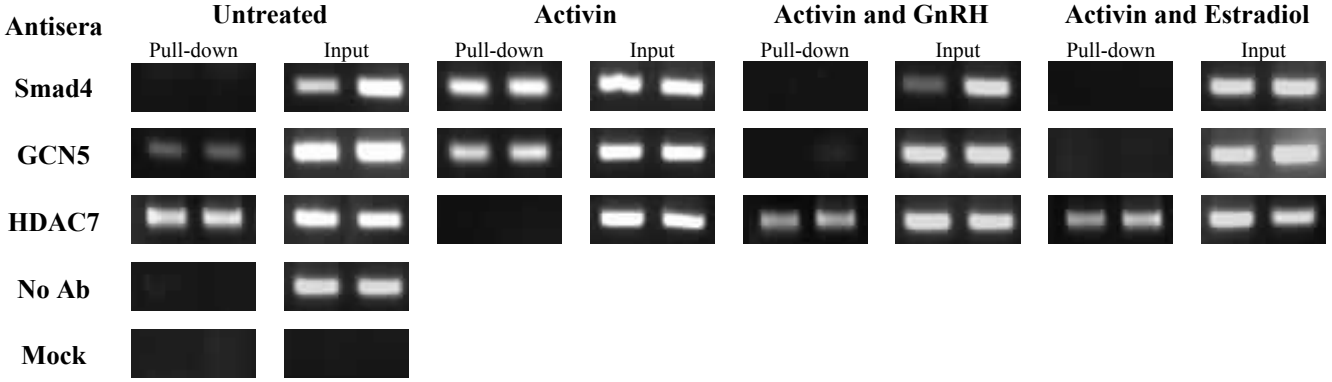
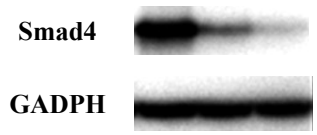


Figure 46. Western blot analysis in verifying the suppression of Smad4 expression. Protein was isolated from LβT2 cells with siRNA construct to knock down Smad4 as in Figure 25. The protein samples were analyzed using Western blot as in Figure 30, with primary rabbit anti-mouse Smad4. Lanes (from left to right): 1, untransfected controls; 2 and 3, transfected with siRNA constructs. The experiments were repeated at least twice. The representative is shown below.



3.4.3 Synergistic Effects of Smad4 with other Transcription Factors regulating the Gonadotropin Gene Promoter Activity

To have a clearer understanding of how the various the Smad proteins may affect activation by other signaling pathways, reporter gene (luciferase) analysis was carried out. In this analysis, combinations of expression vectors for Smad4, ERα, *c*-fos, *c*-jun, Ptx-1, Egr-1 and Sf-1, were cotransfected into LβT2 cells together with promoter construct for the mouse FSHβ gene. The cells were also treated with GnRH and/or estradiol to test the differential effects of stimulations by these various activators.

There is no synergism between Smad4 and GnRH or estradiol The FSHβ promoter activity in LβT2 has an RLU of 2.02 with Smad4 overexpression (transfected with expression vectors for 48 h). Following stimulation by GnRH (100 nM; 24 h) or estradiol (10 nM; 24 h) with Smad4 overexpression, RLUs of below 2.02 were recorded. This indicates that there is no synergism between Smad4 and GnRH or estradiol (Figure 47).

There is no synergism between Smad4 and other signaling mediators except with Egr-1 The FSHβ promoter activity following transfection with the following expression vectors, Smad4, ERα, *c*-Fos, *c*-Jun, Sf-1, Egr-1 and Ptx-1 have RLUs of 2.02, 1.11, 1.25, 2.52, 4.66, 2.86 and 1.04 respectively. Cotransfection of Smad4 expression vector with these expression vectors, ERα, *c*-Fos, *c*-Jun, Sf-1, Egr-1 and Ptx-1 gave promoter activities of 1.91, 1.77, 2.09, 4.53, 4.38 and 1.56 RLUs respectively. This showed that Smad4 (2.02 RLU) synergises with Egr-1 (2.86 RLU) but not with the other factors in activating the promoter (Figure 48).

Figure 47. Effects of Smad4 overexpression under GnRH or Estradiol stimulation on the FSHβ Gene Promoter Activity. Smad4 expression vector was cotransfected for 48 h with mouse FSHβ and *Renilla* reporter gene constructs in LβT2 cells cultured in luminescent 96-well plates. GnRH (100 nM) or estradiol (10 nM) was added 24 h prior to luciferase assays. The luciferase readings were normalized with the *Renilla* readings. The experiment was carried out once.

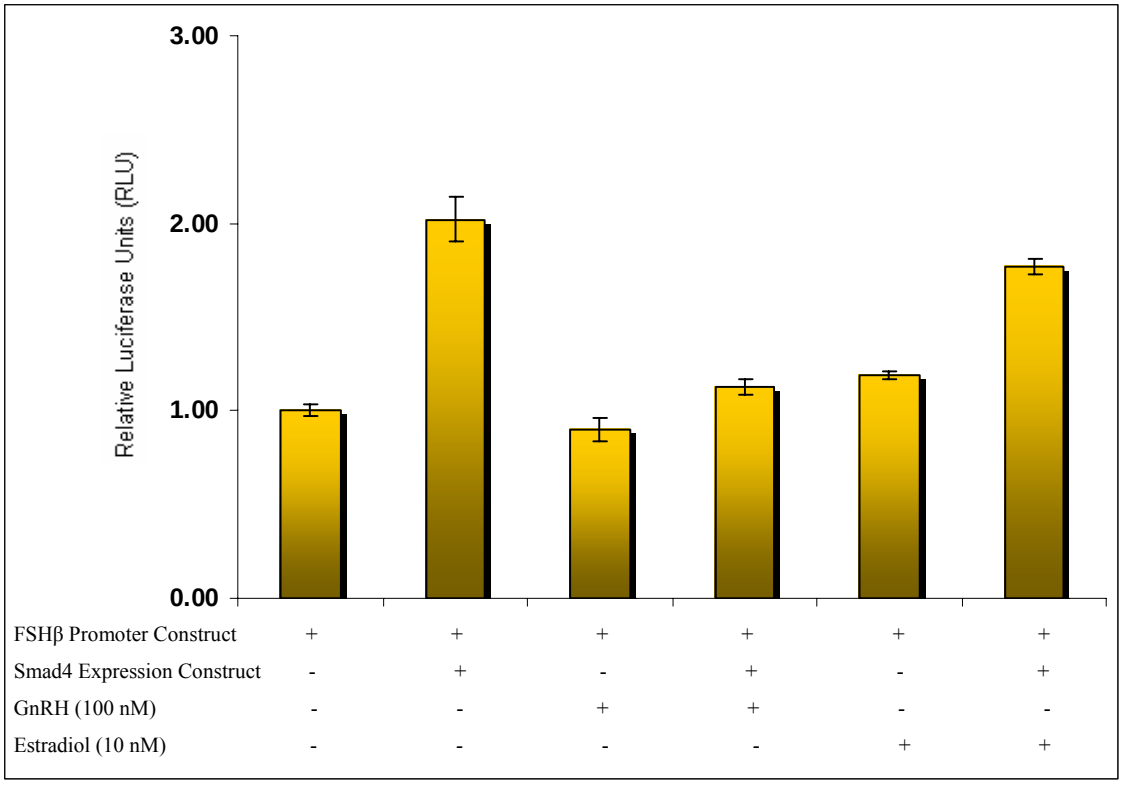
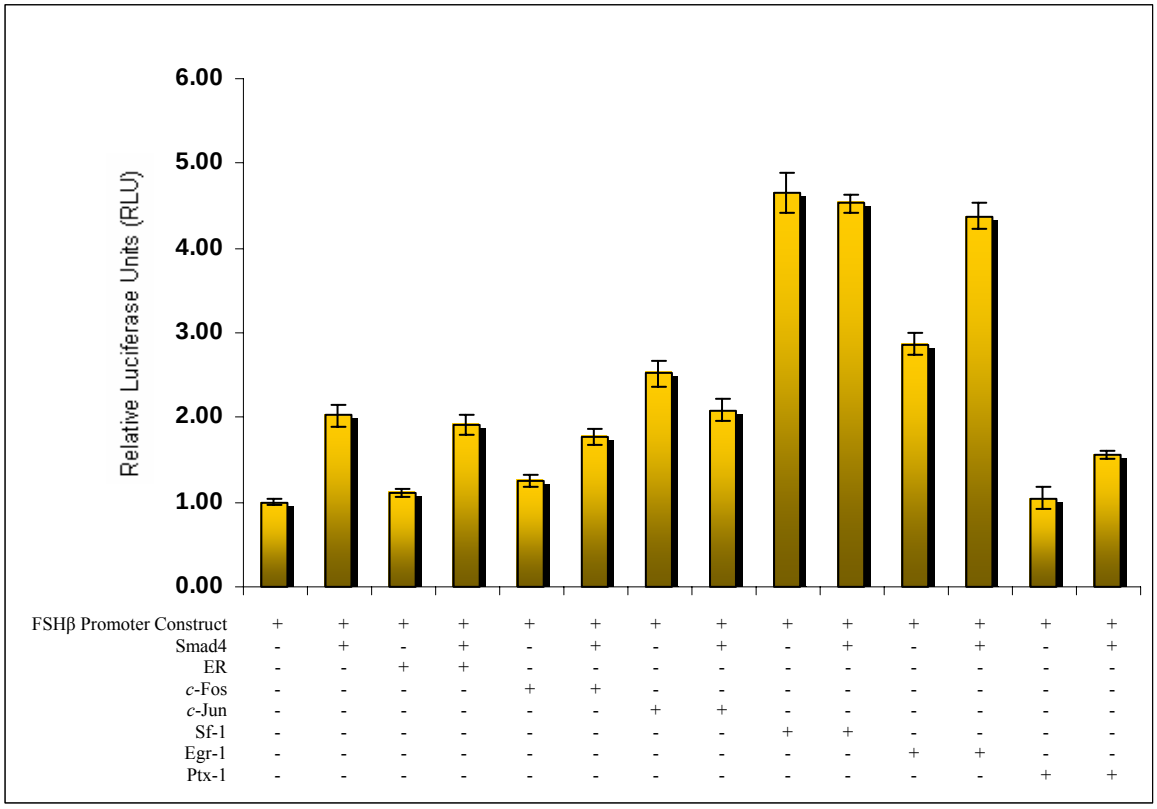


Figure 48. Effects of Overexpression of Smad4 and other Transcription Factors on the FSHβ Gene Promoter Activity. Smad4 expression vector was cotransfected for 48 h with mouse FSHβ and *Renilla* reporter gene constructs, together with expression vectors for ERα, *c*-Fos, *c*-Jun, Sf-1, Egr-1 and Ptx-1 in LβT2 cells as in Figure 47. The luciferase assays were analyzed and presented also as in Figure 47. The experiment was carried out once.



3.4.4 Protein-protein Interactions between Transcription Factors

Protein-protein interactions between the transcriptional factors involved in various signaling pathways may help to reveal how Smads may have a role in the crosstalk between signaling pathways. Mammalian two-hybrid assays were carried out to study these interactions between in LβT2 cells treated with GnRH or estradiol. Interactions between Smad4 and GCN5 were also assayed.

Smad4 interacts physically with the liganded-ERα at its MH1 domain, but not with other signaling mediators LβT2 cells were transfected with pairs of pM and pVP16 mammalian two-hybrid constructs, along with reporter gene constructs (pGal4-luc and pRL-CMV) under various hormonal stimulation (GnRH, 100 nM, 24 h; estradiol, 10 nM, 24 h) to assay for protein-protein interactions. The relative light unit (RLU) measured for pVP-Smad4 and pM-ERα constructs transfected in estradiol-treated cells is 3.71 ($P<0.01$; Figure 49). The RLUs for the pairs of pVP-MH1 and pM-ERα, and PVP-SAD and pM-ERα constructs transfected in estradiol-treated cells as 3.12 and 1.80 ($P<0.01$; Figure 50). Others not mentioned are considered non-interactions (RLU<1.50) and are also found in Figures 49 and 50.

There is no physical interaction between Smad4 and GCN5 Smad4 does not interact with GCN5 whether with or without hormonal stimulation (RLU<1.00; Figure 51).

Figure 49. Interactions between Smad4 and other Transcription Factors. pVP16-Smad4 construct was cotransfected for 48 h with the pM constructs for ERα, *c*-Jun and Ptx-1 in LβT2 cells as in Figure 47. The luciferase readings were normalized with the *Renilla* readings. The normalized readings for the basal interaction (between pM and pVP16) were set at 1.00 relative light unit (RLU). The experiment was carried out once.

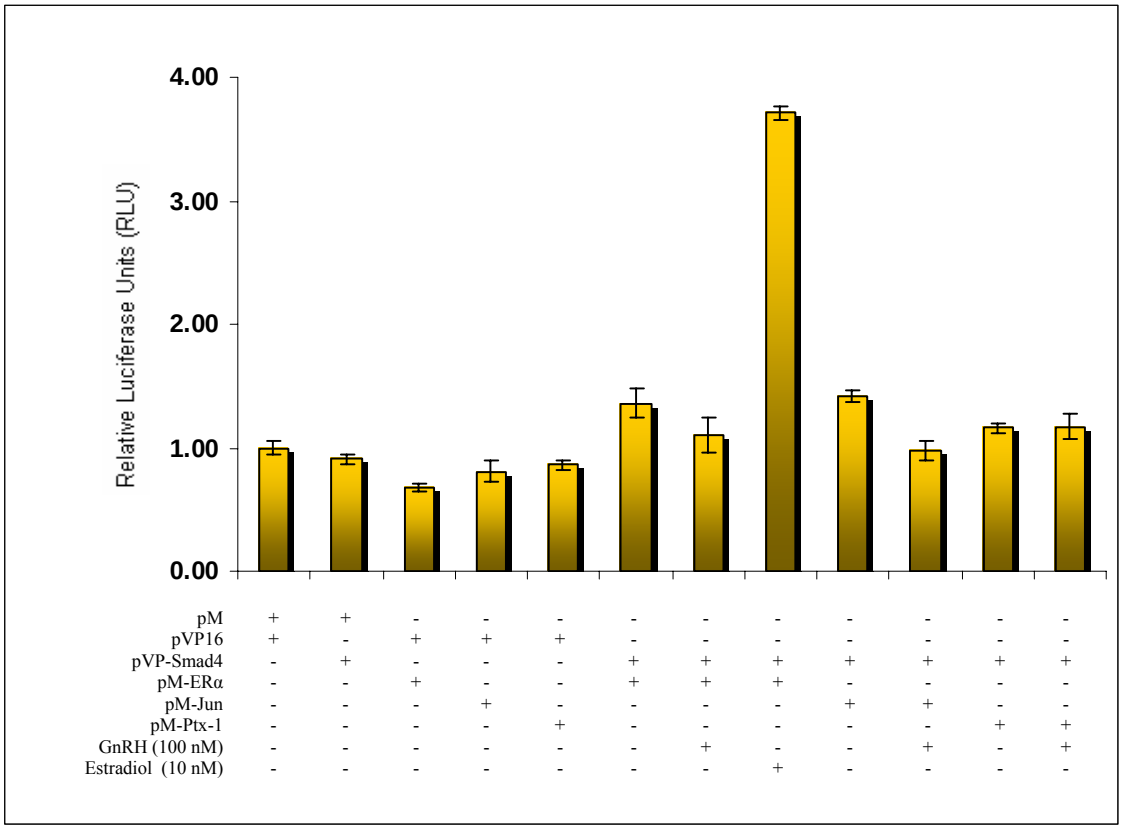
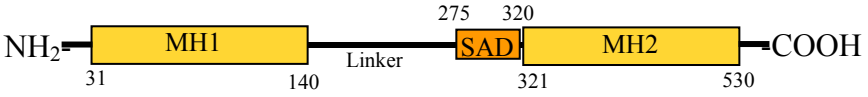


Figure 50. Interactions between Domains of Smad4 and ERα. pVP16 constructs for (A) Smad4 domains (MH1, MH2 and SAD) were cotransfected for 48 h with the pM-ERα construct in LβT2 cells as in Figure 47. (B) The luciferase assays were analyzed and presented also as in Figure 49. The experiment was carried out once.

A



B

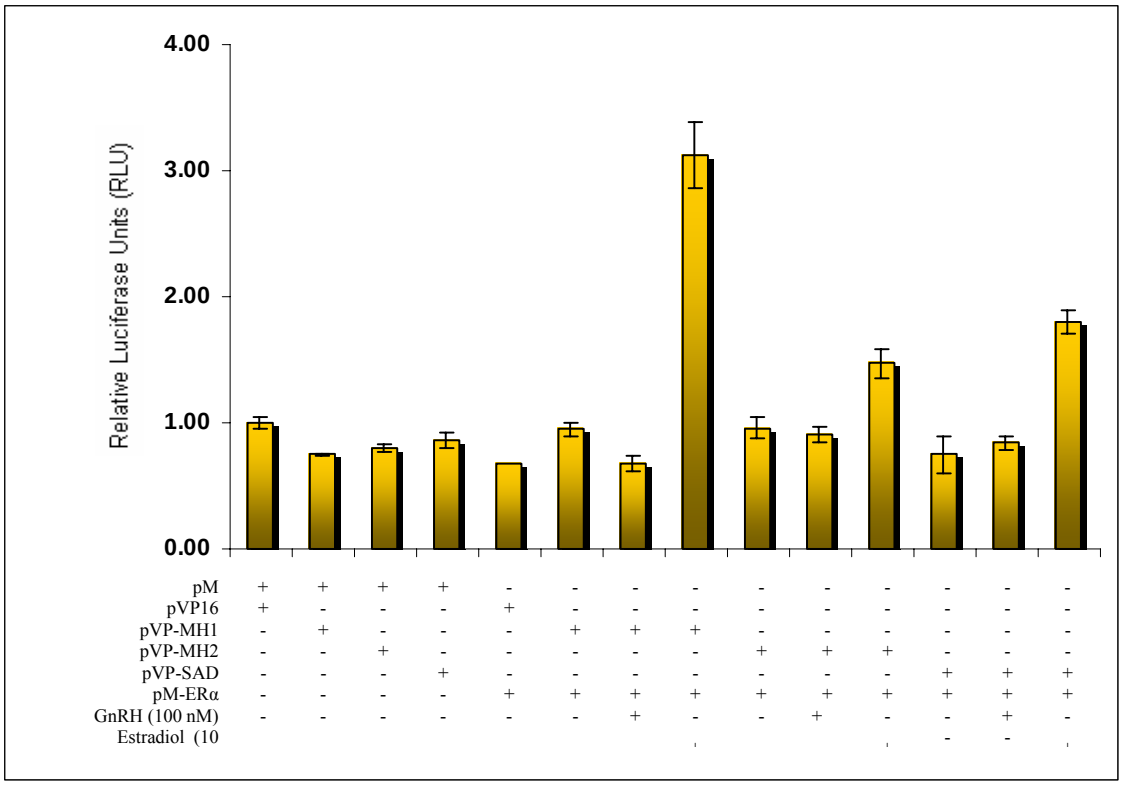
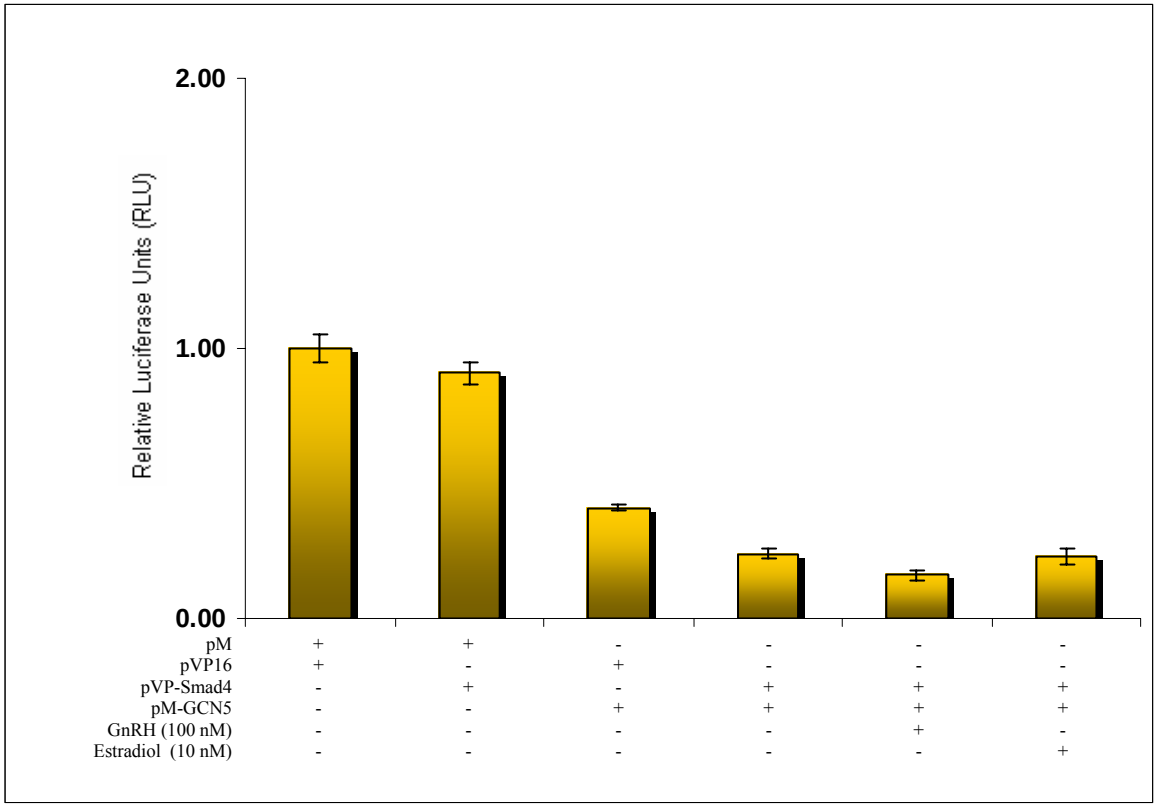


Figure 51. Interactions between Smad4 and GCN5. pVP16-Smad4 construct was cotransfected for 48 h with the pM-GCN5 construct in LβT2 cells as in Figure 47. The luciferase assays were analyzed and presented also as in Figure 49. The experiment was carried out once.



Chapter 4 Discussion

4.1 Effects of TSA and GnRH on the Regulation of Mouse Gonadotropin Genes in LβT2 and αT3 cells

To assess whether the repression of gonadotropin genes in the mouse gonadotrope is due to the restrictive local chromatin structure brought about by HDACs, TSA was exposed to the gonadotropes to inhibit these HDACs. GnRH, being a paramount regulator of the gonadotropin genes, was also found to overcome this repression.

Gonadotropin genes are repressed by nucleosomal-histone hypoacetylation Northern and RT-PCR analysis of the endogenous FSHβ mRNA levels revealed that the FSHβ gene is expressed in the murine mature gonadotrope LβT2 cells although levels are extremely low. Following this finding, we had the notion that LHβ and FSHβ mRNAs might also be present at such low levels in the immature αT3-1 gonadotrope. Hence we proceeded with RT-PCR analysis using total RNA extracted from αT3-1 cells treated with TSA, which has been shown to cause the accumulation of hyperacetylated histone tails leading to gene transcription (Section 1.4). The results of RT-PCR demonstrate that the expression of the repressed gonadotropin genes in the immature gonadotrope is induced by TSA (inhibition of HDAC). To confirm these observations, we employed the more accurate quantitation of mRNA levels using qPCR. Again, TSA is demonstrated to markedly induce the

expression of the repressed LHβ and FSHβ genes in the αT3-1 cells and the repressed FSHβ gene in the LβT2 cells.

There is evidence that acetylation of non-chromatin substrates can also facilitate gene activation (Section 1.4). Therefore, to rule out the effects of acetylation on the non-chromatin substrates leading to gene expression of LHβ and FSHβ genes in the immature αT3-1 gonadotrope, we employed promoter studies using Chinook salmon LHβ and FSHβ gene promoter/CAT-reporter constructs. Our assay results show that the activities of both the LHβ and FSHβ gene promoters are unaffected by TSA.

Corroborating the results from our mRNA-level analyses and promoter studies, we noted that TSA is only able to affect the chromatin template, suggesting that the HDAC inhibition resulted in hyperacetylation of the nucleosomal histone tails leading to the activation of the LHβ and FSHβ genes in the immature gonadotrope. This strongly suggests that the repressed states of the gonadotropin genes are primarily due to the nucleosomal histone-hypoacetylation. Hence, we have clearly demonstrated that chromatin remodeling via histone-hypoacetylation indeed has a role in the regulation of murine gonadotropin genes.

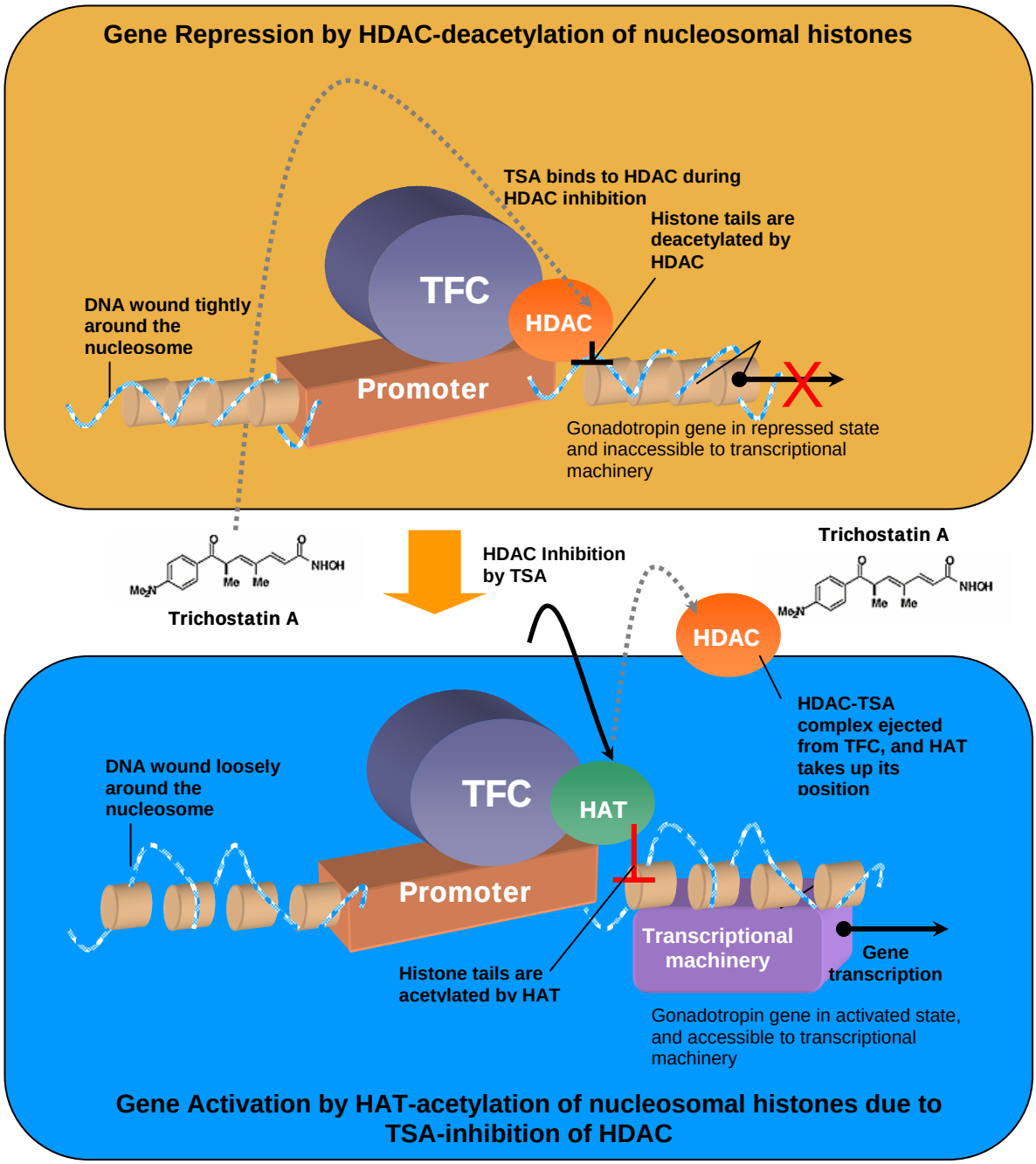
Following this discovery, we are able to propose a mechanism through which HDACs might inhibit gonadotropin gene expression via chromatin remodeling, and how this is relieved by TSA (Figure 52). We propose that in the repressed state, the histone tails are hypoacetylated causing the local chromatin structure where the gene promoter is located, to be highly compact mainly due to the tight associations between

the highly basic HDAC-deacetylated histones and the acidic phosphate backbones of DNA. The HDAC is recruited possibly by a transcription factor complex (TFC), which in turn is recruited by specific sites in the promoter. This compaction refuses accessibility to the transcriptional machinery, and henceforth, gene expression is repressed.

The TSA molecule probably binds to the binding site of the HDAC, as was demonstrated by Finnin *et al* (1999), causing a conformational change that ejects the entire HDAC-TSA complex from the TFC. The initial release of the HDAC would provide the exposed histone tails the opportunity to be acetylated by unbound HATs. As the histones get acetylated, additional HATs would be recruited by TFC, and further acetylation would occur until the accumulation of hyperacetylated histones results in a complete chromatin remodeling. This would lead to the local chromatin structure, where the gonadotropin gene promoter is found, becoming accessible for transcriptional machinery to recognize and bind to the DNA molecule. Subsequently, gene transcription would take place.

The HDAC-TSA complex would break down into separate units with time. The acetylated lysine residues of the histone tails would then become the substrates for the HDAC molecules, recruited by the gene-specific TFC; restoring the repressive states of the gonadotropin genes. The proposed mechanism of gene regulation by chromatin remodeling via HDAC-deacetylation is highly plausible, as many studies have shown that chromatin remodeling as a result of nucleosomal histone tail post-translational modifications does regulate gene expression (Kouzarides *et al*, 2000; Strahl and Allis, 2000; Roth *et al*, 2001).

Figure 52. Proposed Mechanism through which HDACs inhibit gonadotropin gene expression and how this is relieved by TSA. In the repressed state, acidic DNA interactions with the basic HDAC-deacetylated histone tails result in a compact local chromatin structure, making the gonadotropin gene inaccessible to transcriptional machinery. However, TSA binds to the HDAC, and causes a conformational change in HDAC causing the HDAC-TSA complex to be released from the transcription factor complex (TFC), and its replacement by a HAT, which catalyzes the acetylation of the histone tails, and relieves the DNA-histone associations. Hence, the local chromatin is accessible to the transcriptional machinery, and the gene is activated.



Acetylation of non-chromatin substrates may enhance transcription of the FSHβ gene Northern and RT-PCR analysis showed that increasing the concentration of TSA from 20 to 100, then to 200 ng/mL enhanced the transcription of the FSHβ gene in both types of gonadotrope at the basal level. We know that TSA, caused the histones to be acetylated, and subsequently the chromatin template becomes accessible to the transcriptional machinery. However our results showed that further increase in HDAC inhibition, elevated the FSHβ mRNA level further. The qPCR data also points to this analogous observation: the longer the cells were incubated with TSA (0, 8, 16, 24 and 36 h) the greater the FSHβ mRNA level. We suggest that once the local chromatin structure is 'open' for transcription, it will remain 'open' for as long as the histone tails are kept hyperacetylated by HATs. This means that the local chromatin structure has two states: 'open' and 'close'. It is likely, therefore, that there is a threshold for which the structure will 'open' by acetylation and 'closed' by deacetylation, and any further acetylation or deacetylation would not affect its structure.

It is highly probable therefore that increasing TSA concentrations or exposure durations leads to more acetylation of non-chromatin substrates, which enhances the FSHβ gene transcription in the gonadotropes. Studies have shown that acetylation can also facilitate the binding of transcription factors to their binding sites in isolated nucleosomes (Di Mauro *et al.*, 1993; Di Mauro *et al.*, 2002). In addition, there is growing evidence to support the mechanism of gene transcription by activating transcriptional coactivators (Jacob *et al.*, 2001), or by inhibiting their transcriptional corepressors via acetylation (Bereshchenko *et al.*, 2002). Therefore, we suggest that increasing TSA concentration or incubation duration consolidates all downstream

effects of acetylation, which not only includes histone modifications, but also transcription factor-binding facilitation, coactivator activation and corepressor inhibition. Noting that the increase in the mRNA levels of the FSHβ gene was obvious with greater amounts or longer duration of TSA exposure, we propose that the transcription of FSHβ gene may involve these mechanisms of gene transcription by acetylation.

GnRH regulates both transcription factors and chromatin remodeling complexes for the expression of FSHβ From our RT-PCR and qPCR analyses, we found that GnRH overcomes the repression of the gonadotropin genes in the immature gonadotrope. This led us to hypothesize that the GnRH-mediated signal transduction pathway (Figure 6; Section 1.2) may contain components which are able to render modifications to the chromatin structure, making it accessible for transcription factors to activate gene transcription. GnRH probably plays dual roles in gonadotropin gene expression: firstly, it regulates the DNA-bound transcription factors essential for gonadotropin gene expression namely, AP-1 (comprising *c*-fos and *c*-jun*)*, SP-1, Sf-1, Egr-1 and Ptx-1 (Brown and McNeilly, 1999); secondly, it regulates chromatin modifying factors, causing the chromatin to be accessible. However, the latter role has yet to be established, until this study was initiated (discussed in Section 4.2).

Sf-1 is localized in the cytoplasm and nucleus of the αT3-1 cells after GnRH exposure Sf-1 is an essential transcription factor for the expression of the LHβ gene both basally and in response to GnRH. This study has shown that Sf-1 is localized in both the cytoplasm and nucleus of the αT3-1 cells after GnRH exposure.

Jacob and colleagues reported that Sf-1 is specifically exported out from the nucleus into the cytoplasm when exposed to TSA (Jacob *et al*, 2001). They also demonstrated that Sf-1 is acetylated by TSA exposure. The nuclear export has been found to be a possible reason for Sf-1's increased half-life and stability. Our mRNA-level analyses showed that in the immature gonadotrope, LHβ gene is repressed, but is activated when exposed to GnRH alone. Furthermore, the LHβ promoter activity was greatly increased at GnRH-modulated level. Reconciling our observations, we suggest that GnRH causes the translocation of Sf-1 into the cytoplasm indirectly; thereby prolonging the GnRH effect through increasing the stability of Sf-1. Further, we suggest that GnRH could possibly bring about the localization of Sf-1 through perhaps attaching to a microtubule-associated HDAC, such as HDAC6, which is implicated in regulating microtubule stability and function, and it is localized exclusively in the cytoplasm (Hubbert *et al.,* 2002, Kawaguchi *et al.*, 2003, Kovacs *et al.,* 2004).

4.2 Involvement and Mechanistic Actions of HATs in the Regulation of Mouse Gonadotropin Genes via various Signaling Pathways

Our discovery that the mouse gonadotropin genes are repressed by nucleosomal-histone hypoacetylation, and can be overcome by TSA and GnRH, led us to investigate the involvement of the antagonists of HDACs, the HATs, in the various signaling pathways regulating both FSHβ and LHβ genes in LβT2 cells.

Basal expression of the FSHβ gene but not the LHβ gene involves CBP, Tip60, GCN5 and pCAF Data from our RT-PCR analyses of FSHβ and LHβ mRNA levels in LβT2 cells following transfection with siRNA constructs targeting the HATs, CBP, Tip60, GCN5 and pCAF, suggests that the co-activator complex containing these HATs may facilitate the FSHβ gene expression, and that an entirely dissimilar complex may be implicated for the basal expression of LHβ gene (Figure 25). Functional redundancy amongst these HATs in regulating the LHβ gene expression was ruled out as our findings demonstrated that the LHβ mRNA levels were unaffected even with different combinatorial suppressions of these HATs (Figure 29). However, there is evidence that LHβ basal expression requires HATs not presently included in the study. One such HAT is p300, which was implicated in regulating the synergy of Egr-1 and Sf-1 activation of the LHβ gene expression (Mouillet *et. al.*, 2004). Even though p300 and CBP share extensive homology (Arany

et. al., 1994) and genetic and molecular analyses suggest that they perform overlapping functions (Arany *et. al.*, 1995, Lundbiad *et. al.*, 1995; Petrij *et. al.,* 1995; Tanaka *et. al.,* 1997*;* Yao *et. al.*, 1998), our results indicate that each may function uniquely on different genes. It is highly probable that LHβ basal gene expression involves p300, while FSHβ gene expression requires CBP, considering that a recent study has also shown that p300, but not CBP, stimulates transcription of the cyclin D1 promoter (Giordano and Avantaggiati, 1999). Clearly, similar studies utilizing siRNA technology suppressing p300 should be carried out to ascertain this possibility.

GnRH- and estrogen-modulated expression of both gonadotropin genes involves CBP, Tip60, GCN5 and pCAF Our results show that GnRH- and estrogen-mediated expression of the LHβ gene involves CBP, Tip60, GCN5 and pCAF. It demonstrates that the LHβ gene expression is regulated differentially when hormonally induced by either GnRH or estrogen (Figures 26 and 27). Similarly, FSHβ gene expression, under these conditions, also involves these HATs. The discovery that GnRH- and estrogen-modulated expression of both genes involves a common set of HATs indicates the parallel effects GnRH and estrogen have on both of these genes. It is possible that GnRH and estrogen orchestrate activation of these genes via similar co-activator complex recruitment comprising these HATs. Our data further indicated that mRNA splicing may be involved in the regulation of LHβ expression as knocking-down pCAF or GCN5 in GnRH-and-estrogen-treated LβT2 cells produced an unspliced variant of the LHβ mRNA containing intron-2 (Figure 28). Suppression of CBP or Tip60 did not have similar outcome. In contrast to LHβ gene expression, FSHβ mRNA levels were reduced by CBP or Tip60 suppression. It is likely that knocking-down GCN5 and pCAF may have indirect effects on the splicing

machinery, as these HATs are known to regulate gene expression ubiquitously (Roth *et. al*., 2001).

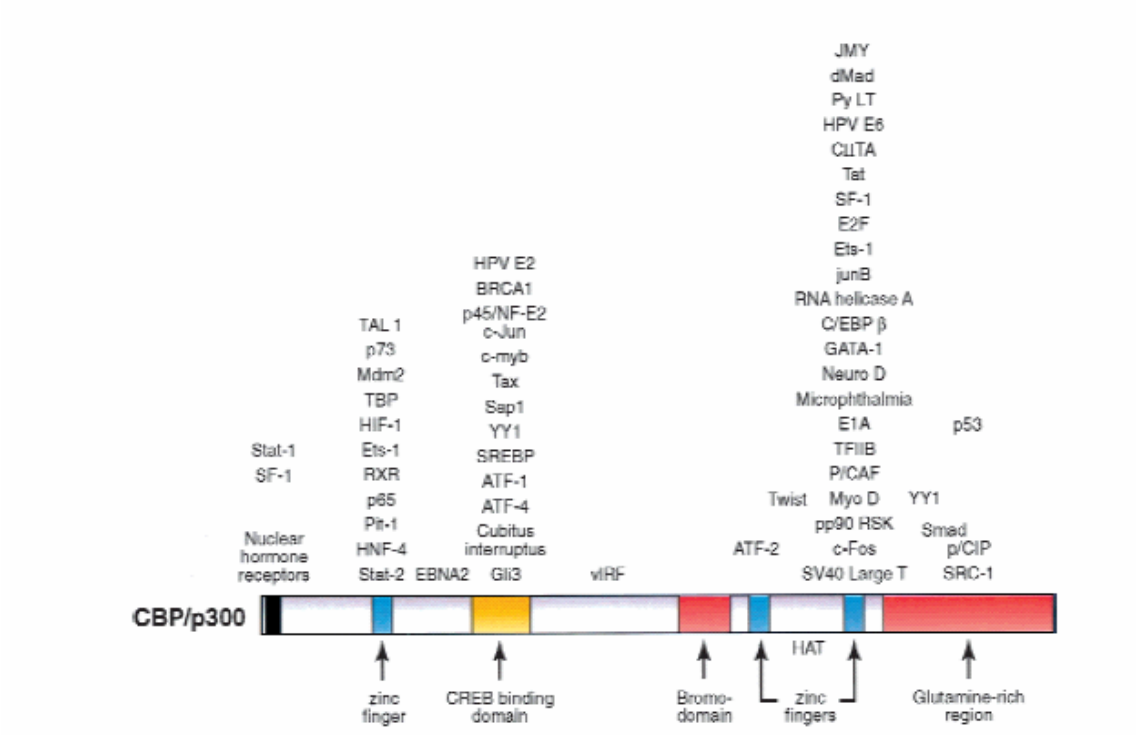
GCN5 is recruited to the FSHβ gene promoter by the Unliganded ERα and AP-1 but does not directly interact with them The ChIP analysis of the association of the FSHβ gene promoter with GCN5 in LβT2 cells revealed that GCN5 is recruited to the promoter following GnRH or activin treatment but not with estradiol treatment (Figure 31). Our ChIP-siRNA data clearly demonstrates that the recruitment of GCN5 to the promoter under GnRH stimulation does not occur without ERα or *c*-Fos (Figure 32). This coincides with the observation by Metivier and colleagues (2003) that both GCN5 and ERα are associated to a natural target promoter. Furthermore, our promoter studies showed that GCN5 regulates the synergy of *c*-Fos and *c*-Jun in activating the mouse FSHβ gene promoter (Figure 34). Moreover, GCN5 does not physically interact with ERα, *c*-Fos or *c*-Jun in the recruitment, as shown by our mammalian two-hybrid interaction assays (Figure 37B). Based on our HAT-HAT interaction data, GCN5 appears not to interact with Tip60 or CBP (Figure 36). Nonetheless, a separate study has shown that GCN5 is found in a complex with TRRAP, which is also a component of Tip60 complex consisting of Tip60, p400, Tip48 and Tip49 (Vassilev *et. al*., 1998; Brand *et. al.*, 1999; Ikura *et. al*., 2000; Amati *et. al*., 2001). Taken together it is likely that GCN5 associates with the promoter via interactions with other HATs or co-activators, such as TRRAP, which possibly provide physical links to the DNA-bound factors, ERα and AP-1.

Unliganded and Liganded ERα recruits Tip60 to the FSHβ gene promoter when stimulated by GnRH and Estradiol respectively Our ChIP experiments

demonstrate that Tip60 is associated with the FSHβ gene promoter in LβT2 cells treated with GnRH or estradiol (Figure 31). Tip60 fails to occupy the promoter when ERα is knocked down in both cases. Suppression of *c*-Fos has no effect on the occupancy of Tip60 on the estradiol-stimulated promoter, but loses its ability to associate to the GnRH-stimulated promoter (Figure 32). We found that Tip60 and ERα have no synergistic effect on the promoter activity (Figure 35). Moreover, we also showed that Tip60 physically interacts with the liganded ERα and CBP (Figures 36 and 37). This all may explain the mechanism of how Tip60 is recruited to the promoter under the various signaling pathways. In the GnRH-modulated expression of FSHβ gene, Tip60 is recruited to the promoter via interaction with CBP, which has been shown to have physical links with the unliganded ERα and *c*-Fos (Bannister *et. al.*, 1995; Janknecht *et. al.,* 1995; Janknecht *et. al.,* 1996; Kamei *et. al.*, 1996). As for the estrogen-modulated expression, Tip60 is most likely recruited to the promoter by direct physical interaction with the DNA-bound liganded ERα homodimer, which then probably recruits other HATs and co-activators, including CBP, GCN5 and pCAF.

CBP is a versatile co-activator Our protein-protein interactions assays of CBP with HATs and DNA-binding factors show that CBP physically interacts with Tip60 (at CBP1 domain; Figure 36B), ERα, *c*-Jun, Egr-1 (a novel interaction), Smad3 (at CBP1 domain; Figure 38A) and Sf-1 (at CBP1, CBP2 and CBP3 domains; Figures 38A, B and C). Our data and those from other studies (Figure 53) indicate that CBP is a versatile co-activator in the regulation of gonadotropin genes as it is able to interact with both HATs and gonadotropin gene-specific DNA-bound transcription factors, making it suitable as a molecular bridge linking the gonadotropin gene-specific DNA-

Figure 53. CBP/p300 interactions. Interacting proteins are shown at the top of the figure; functional domains are depicted below. Many known interactions are not included due to space limitations (adapted from Goodman and Smolik, 2000)



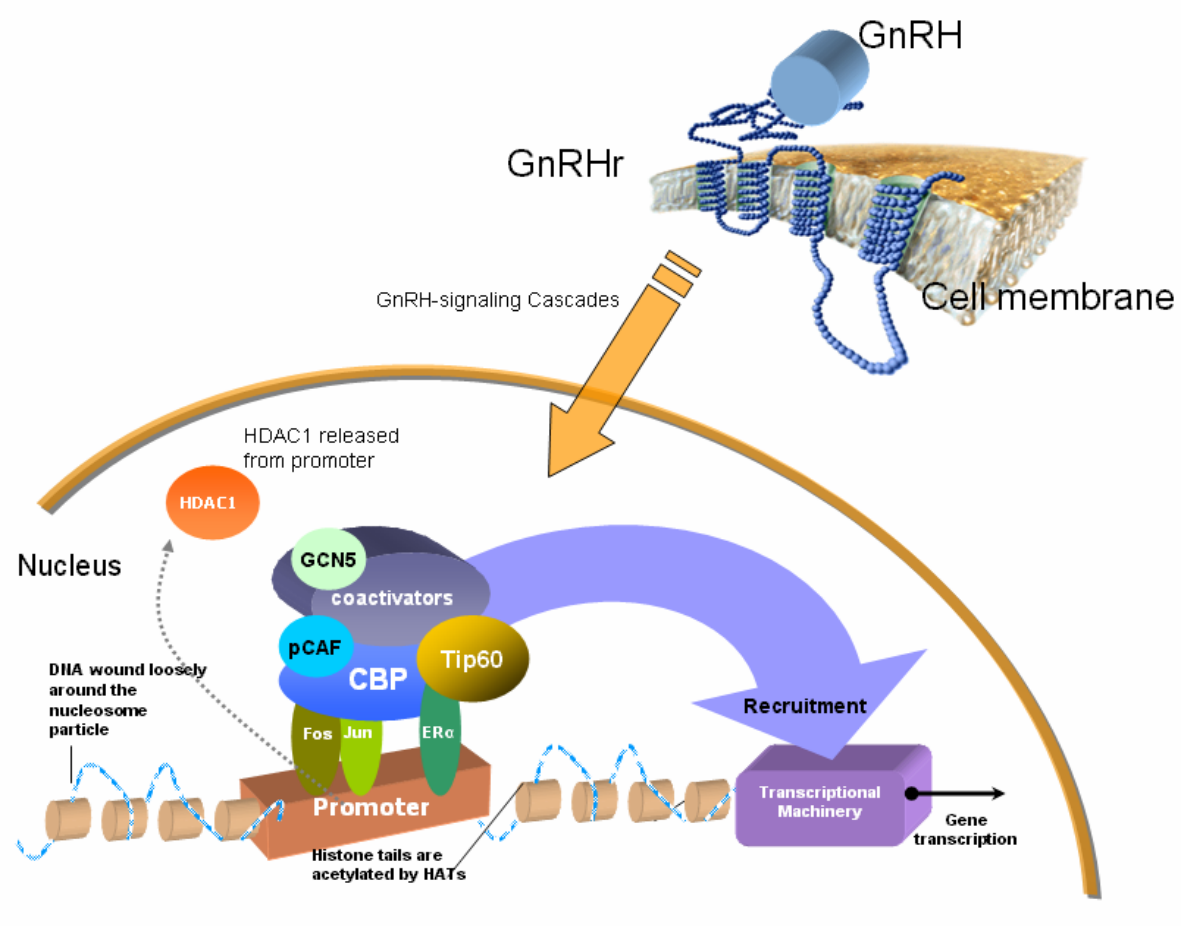
bound factors to co-activators.

HDAC1 is removed from the promoter by either GnRH or Estradiol Stimulation In comparison to the HATs, ChIP analysis of association of the FSHβ gene promoter with HDAC1 in LβT2 cells showed that it is associated with the unstimulated promoter, but is removed following either GnRH or estradiol stimulation, with a lesser degree for the latter (Figure 31). This observation coincides with the traditional view that HAT-HDAC exchanges take place at the promoters (Section 1.4).

The Dynamic Interplay of HATs, HDACs and DNA-bound Transcription Factors in Hormonally Induced Expression of the FSHβ Gene Consolidating our observations; we propose a model (Figure 54) that depicts the dynamic interplay of HATs, including CBP, Tip60, GCN5 and pCAF, HDAC1 and gonadotropin gene-specific DNA-bound transcription factors inclusive of ERα, *c*-Fos and *c*-Jun in the GnRH-mediated expression of the FSHβ gene. In this model, GnRH causes the release of HDAC1 from the promoter, and the subsequent recruitment of CBP by AP-1 (via physical interactions with *c*-Fos and *c*-Jun), Tip60 by the unliganded ERα (via physical interactions), and other HATs and co-activators to the promoter. This coactivator complex then recruits the general transcriptional machinery that drives gene expression.

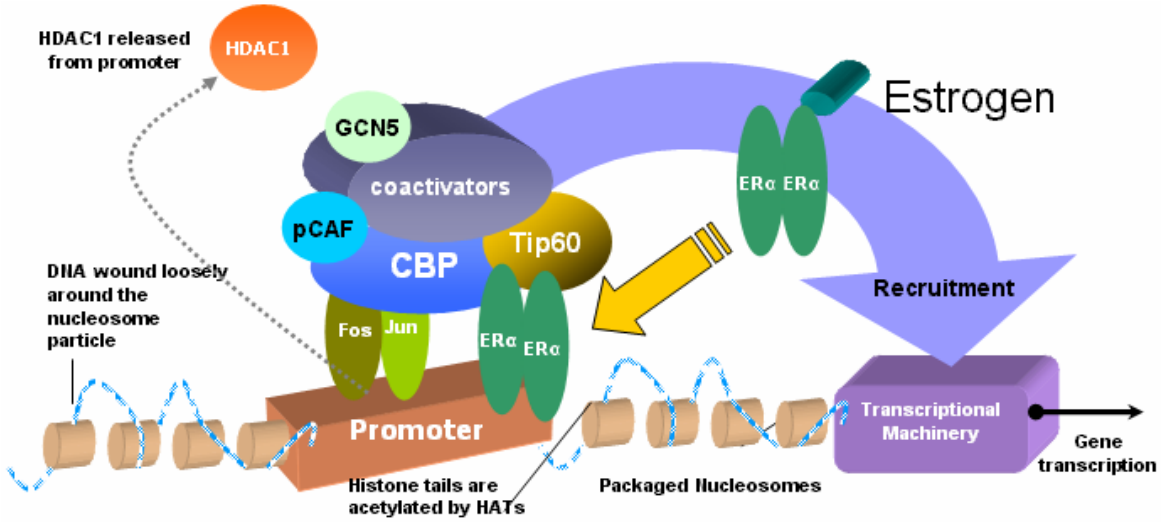
In the estrogen-mediated expression of the FSHβ gene, estrogen binds to ERα, and causes ERα to dimerize and bind the promoter. This causes the release of HDAC1 from the promoter and shortly the recruitment of Tip60 by the liganded homodimer

Figure 54. A proposed model for the Dynamic Interplay of HATs, HDACs and DNA-bound Transcription Factors in GnRH-stimulated FSHβ gene expression. GnRH binds to GnRHr on the cell membrane and the signaling cascade potentiates the release of HDAC1 from the FSHβ gene promoter. This is followed by the recruitment of CBP by AP-1 (via physical interactions with *c*-Fos and *c*-Jun) and Tip60 by the unliganded ERα (via physical interactions with each other). Other HATs, including GCN5 and pCAF, and co-activators are also recruited to the promoter. This coactivator complex then links itself to the general transcriptional machinery that drives gene expression.



ERα (via physical interactions) takes place together with other HATs and co-activators. This then activates the transcription of the gene (Figure 55).

Figure 55. A proposed model for the Dynamic Interplay of HATs, HDACs and DNA-bound Transcription Factors in Estrogen-stimulated FSHβ gene expression. Estrogen binds to ERα in the nucleus, causing it to dimerize and bind to the promoter. This then potentiates the release of HDAC1 from the FSHβ gene promoter, followed by the recruitment of Tip60 by the liganded ERα homodimer (via physical interactions). Other HATs, including CBP, GCN5 and pCAF, and co-activators are also recruited to the promoter. This coactivator complex then links itself to the general transcriptional machinery that drives gene expression.



4.3 Possible Crosstalk between the Signaling Pathways and its Relation to the Regulatory Functions of HATs and HDACs

In understanding the involvement and mechanistic actions of HATs in the regulation of gonadotropin genes via multiple signaling pathways, the commonly occurring crosstalk between signal transductions must be considered. In the case of the gonadotropin genes, the regulatory functions of HATs and HDACs were related to the possible crosstalk between the activin- and GnRH- or activin- and estrogen-signaling pathways, with emphasis on the Smad proteins.

Crosstalk between Activin-, GnRH- and Estrogen-induced signals The consequence of combinatorial and differential treatments of LβT2 cells was studied to understand the gonadotropin gene regulation in the physiological context of the cells. The cells were treated with GnRH and/or estradiol and/or activin. RT-PCR and qPCR analyses were carried out to measure the mRNA levels of FSHβ and LHβ genes, whereas Western blot analysis was performed to measure the amounts of acetylated H3 histones: an indication of global gene activation. We have found that GnRH represses the activation by activin and estrogen on both the gonadotropin genes at the transcriptional level (Figures 39 and 40) and also at the global histone H3 acetylation levels (Figure 41). These observations clearly display the occurrence of crosstalk

between these pathways in the gonadotropes, leading to effects on the global acetylation status of nucleosomal histones. This is an indication that HATs and HDACs may be involved in the crosstalk.

Smads crosstalk with GnRH-induced signal In dissecting the mediators that are involved in the crosstalk between the signaling pathways, Smad proteins, Smad1, 2, 3, 4 and 5 were overexpressed and Smad4 was suppressed (by transient transfections with Smad4 siRNA construct) in LβT2 cells treated with and without GnRH. Our qPCR data showed that overexpression of Smad1, 2, 4 and 5 increased the FSHβ mRNA levels and Smad1 and 2 increased the LHβ mRNA levels in untreated LβT2 cells (Figure 42A). More importantly, Smad4 overexpression is observed to be repressing the GnRH-activation of both the FSHβ and LHβ genes when compared to the levels in the untreated LβT2 cells (Figures 42A and B). To confirm this observation, we transfected the cells with Smad4 siRNA construct to suppress its expression levels. Indeed, suppressing Smad4 expression in GnRH-treated cells markedly increased the mRNA levels of both gonadotropin genes (Figure 42B). This implies that Smad4 may be acting as a transcriptional corepressor for the GnRH-signaling pathway.

Smad4 as a transcriptional corepressor for GnRH-mediated and Estrogen-mediated FSHβ Gene Expression When we discovered the potential role of Smad4 as a transcriptional corepressor in the GnRH-signaling pathway, we focused our attention to its repressive effects on the FSHβ gene. We transfected Smad4 expression vectors and siRNA constructs in LβT2 cells treated with either GnRH or estradiol, and monitored the FSHβ mRNA levels using qPCR. The qPCR

data supports our previous observations that Smad4 is a transcriptional corepressor. Its repressive effects were obvious for both GnRH- and estrogen-mediated FSHβ gene expression: smad4 overexpression represses the GnRH- or estrogen-mediated activation of FSHβ mRNA levels while its suppression activates these levels (Figure 43). Moreover, our studies of the mouse FSHβ promoter with Smad4 overexpression stimulated by GnRH or estrogen point to the fact that Smad4 is a transcriptional corepressor for both the GnRH- and estrogen-mediated signaling pathways (Figure 47). We also show that there is no synergism between Smad4 and other signaling mediators (ERα, *c*-Fos, *c*-Jun, Sf-1, and Ptx-1) in activating the promoter, with Egr-1 as an exception.

Following our discovery that Smad4 is a corepressor for GnRH- and estrogen-mediated FSHβ gene expression, we investigated its mechanistic repressive actions by first looking at its association with the FSHβ promoter in LβT2 cells. We found that Smad4 is associated with the promoter only when stimulated with activin, but loses this association following simultaneous stimulation by activin and GnRH or activin and estrogen (Figure 45). We also looked at the association of GCN5 and HDAC7 to this promoter. We found out that activin enhances the association of GCN5 to the promoter. However, following simultaneous stimulation by activin and GnRH or activin and estrogen, GCN5 is no longer associated to the promoter (Figure 45). Additionally, we found out that the association of GCN5 to the promoter is not physically linked to Smad4 (Figure 51). In contrast HDAC7 is associated to the unstimulated promoter, but loses this association following activin stimulation. Conversely, following simultaneous stimulations by activin and GnRH or activin and estrogen, HDAC7 is associated to the promoter again (Figure 45).

Recently, complexes consisting of HDACs have been implicated as mediators of the effects of TGF-β on gene expression (Nomura *et. al.*, 1999; Wotton *et. al.*, 1999; Ng and Bird, 2000; Frederick and Wang, 2002). In view of this, we went on to investigate the cause of such repression by Smad4 in relation to HDAC involvement. In identifying the HDACs which may account for the repression observed by Smad4 in the GnRH- and estrogen-mediated induction of FSHβ gene in LβT2 cells, we suppressed HDAC1 or HDAC2 by transfecting the cells with siRNA constructs targeting these HDACs. Our RT-PCR analysis of FSHβ mRNA levels following the suppression of these HDACs indicated that HDAC1 and HDAC2 are involved in the repression by Smad4 for the GnRH-mediated gene expression, but not in the repression for estrogen-mediated gene expression (Figure 44). Corroborating these observations with the ChIP analysis associating HDAC7 with the promoter (Figure 45), it is highly probable that a corepressor complex comprising of HDAC1, 2 and 7 causes the repression by Smad4 in the GnRH-mediated signaling pathway, and a complex with HDAC7 as its component, causes the repression in the estrogen signaling.

We went on further to elucidate the possible interactions between Smad4 and other transcription factors to understand its molecular mechanism of repression by using mammalian two-hybrid system. We found that the domains MH1 and SAD of Smad4 physically interact with the liganded ERα (Figures 49 and 50). Studies have shown that the Zn2+-dependent binding of Smad4 to the DNA is via the MH1 domain (Shi and Massague, 2003), while its interaction with p300/CBP is via its SAD domain (Dijke and Hill, 2004). Therefore, it is plausible that Smad4 corepression is brought about by the subjugation of its MH1-mediated DNA binding and SAD-mediated

p300/CBP binding by interaction of its MH1 and SAD domains with ERα respectively in the estrogen-modulated FSHβ gene expression.

Possible molecular mechanism of Smad4 Corepression of Estrogen-mediated FSHβ Gene Expression Based on our observations, we proposed a possible mechanistic model of Smad4 corepression of estrogen-mediated FSHβ gene expression. In this model (Figure 56), activin signaling activates the R-Smads (Section 1.2), which then interact with Smad4. Estrogen binds and dimerizes the ERα. At this point, the MH1 and SAD domains of Smad4 interact with the liganded ERα, hence, failing to bind to the DNA and also to coactivators, such as p300/CBP. This maintains the gene in its repressive state as a result of hypoacetylation of nucleosomal histones by HDACs, inclusive of HDAC7. We present a model of activin-mediated FSHβ gene expression as a comparison (Figure 57; refer to Figure 55 also, as a comparison).

Figure 56. A possible molecular mechanism of Smad4 Corepression of Estrogen-stimulated FSHβ gene expression. Activin signaling activates the R-Smads, which then interacts with Smad4. Estrogen binds and dimerizes the ERα. At this point, the MH1 and SAD domains of Smad4 interact with the liganded ERα, preventing it binding to the DNA and also to coactivators, such as p300/CBP. This maintains the gene in its repressive state as a result of hypoacetylation of nucleosomal histones by HDACs, inclusive of HDAC7.

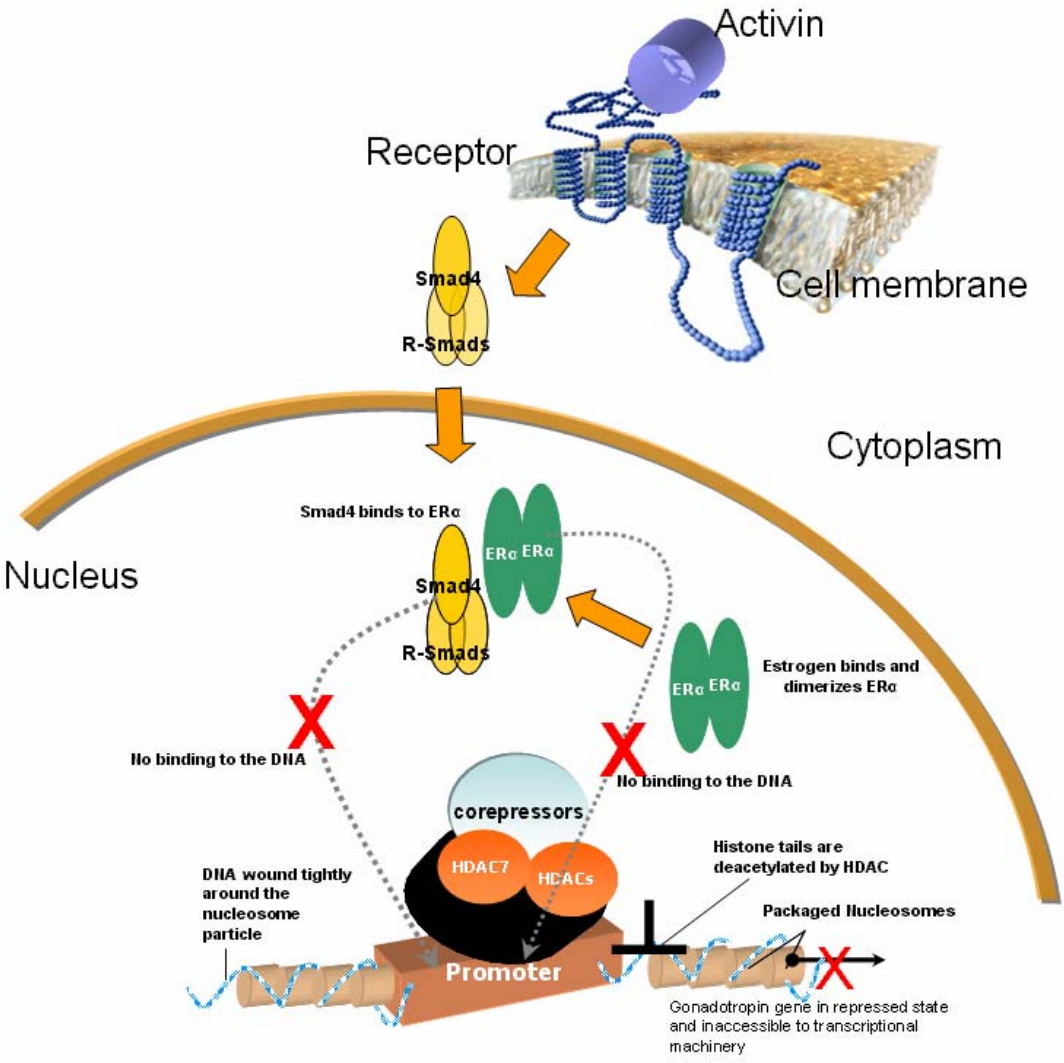
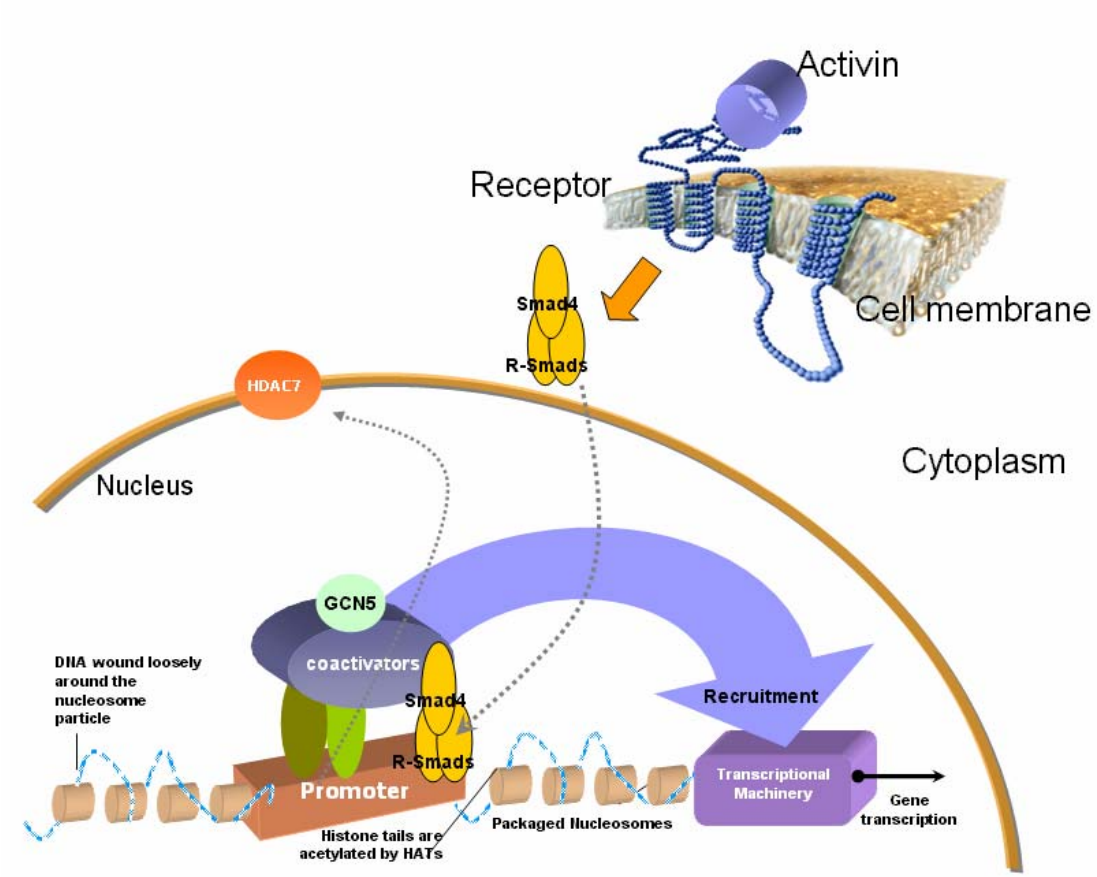


Figure 57. A proposed model for the Dynamic Interplay of HATs, HDACs and DNA-bound Transcription Factors in Activin-stimulated FSHβ gene expression. Activin binds to the receptor on the cell membrane, activating the R-Smads and causing them to complex with Smad4. This complex then binds to the promoter, potentiating the release of HDAC7 from the FSHβ gene promoter, followed by the recruitment of GCN5 and other co-activators to the promoter. This coactivator complex then recruits itself to the general transcriptional machinery that drives gene expression.



4.4 Conclusion

This present study has shown that the pituitary gonadotropin genes are repressed by nucleosomal-histone hypoacetylation brought about by the HDACs. We also established that dissimilar assemblages of HATs are recruited to the gene promoters by different DNA-bound transcription factors, as orchestrated by the various signaling pathways. The interplay between these chromatin modifying factors and transcription factors is important in understanding how the varying physiological states of the pituitary gonadotropes define gonadotropin gene transcription. The findings presented in this study will lead to a greater understanding of mammalian reproductive genetics.

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Appendix

A: Optimization of Luciferase Reporter Gene Assay

A1 Materials and Method

LβT2 cells were grown as described (Section 2.2). The cells were transfected with 10 ng of pRL-CMV construct (Section 2.1) and with varying quantities of pCMV-Luc construct (Section 2.1) ranging from 12.5 ng to 1,600 ng (Section 2.3). Keeping the total DNA amount constant, appropriate amounts of PWS constructs were cotransfected. Luciferase assays were carried out subsequently (Section 2.15.2) and the data generated is presented in Section A2.

A2 Results

Figure 58. Optimization of Luciferase Reporter Gene Assay. A logarithmic plot of normalized Relative Light Units (RLUs) versus DNA amounts of pCMV-Luc (in ng). Normalized RLUs are derived by dividing the firefly luciferase luminescent readings by the *Renilla* luciferase luminescent readings.

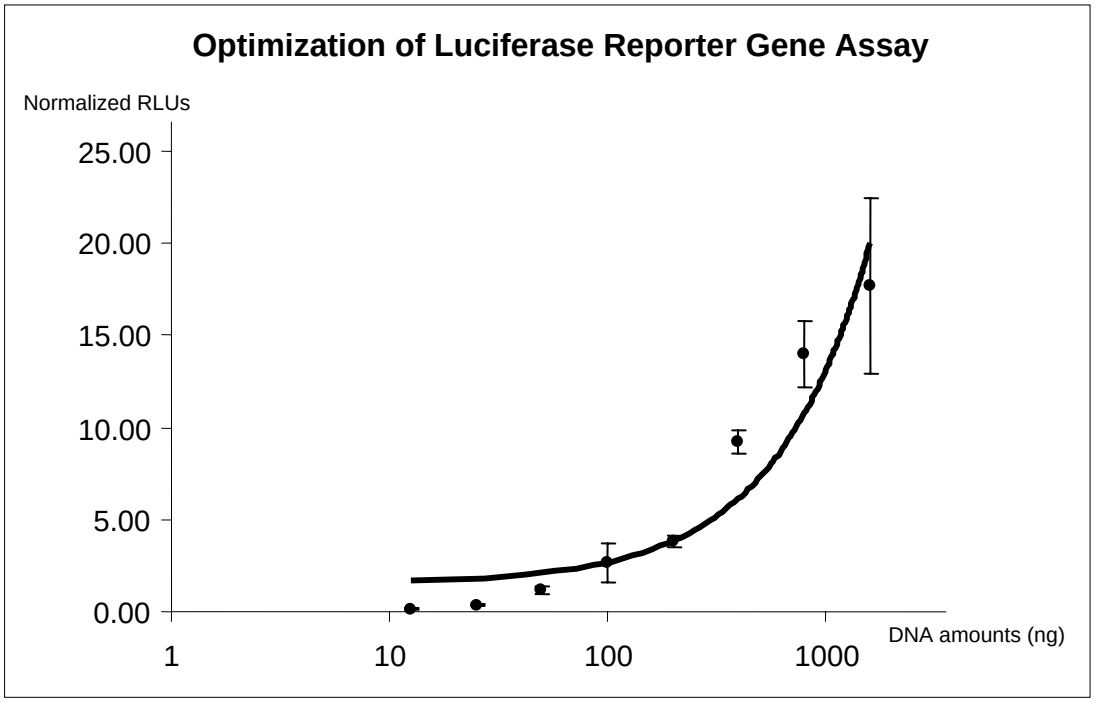

Figure 58 showed a linear relationship between the DNA amounts of pCMV-Luc (which contains an enhanced promoter for the firefly luciferase reporter gene) and the normalized RLUs. This indicates that the system is stable, and able to assay the luciferase reporter gene expression accurately and in a linear fashion i.e. as the DNA amount increases, the normalized RLU increases in the same manner. Thus, the luciferase reporter gene assay system has been optimized and could be used subsequently for promoter studies or mammalian two hybrid assays involving the firefly/*Renilla* luciferase reporter gene constructs.