Regulation of LHβ transcription by WT1 and DAX1 proteins in the pituitary gonadotrope

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Abstract

Luteinizing hormone (LH) is secreted throughout the reproductive cycle from the gonadotrope cells of the anterior pituitary, and is required for steroidogenesis and ovulation. LH contains an α-subunit common with FSH, and a unique LHβ subunit that defines biological activity. Basal LHβ transcription is low and stimulated by the hypothalamic hormone GnRH. GnRH induces synthesis of Egr1 (early growth response protein-1) and stimulates the cyclic binding of transcription factors Egr1 and SF1 (steroidogenic factor-1) on the LHβ promoter. By blocking proteasomal degradation using the inhibitor MG132, our lab previously demonstrated that proteasomal inhibition hampered the cyclic binding of Egr1 and SF1 on the LHβ promoter, and we hypothesized that there could be a DNA-bound or transcription factor-bound inhibitory protein that hindered this cyclic association. These inhibitory proteins might require removal by proteasomal degradation to recruit transcriptional activators. Our candidate for the DNA binding repressor protein was WT1 (Wilms tumour1) and a potential candidate for the transcription factor-bound inhibitory protein was DAX1. WT1 (Wilms tumor protein1) is a zinc finger transcription factor with an essential role in urogenital system development. It regulates several reproductive genes via interactions with SF1 or binding to GC-rich elements such as Egr1 binding sites. DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1), regulates several reproductive and steroidogenic genes by interacting with SF1 and has been suggested to have both co-activator and co-repressor

properties. In this thesis we investigated the potential roles for WT1 and DAX1 in LHβ transcription using clonal mouse gonadotrope LβT2 cells. We demonstrate the presence of WT1 in LβT2 and mouse pituitary cells, and that the protein bound to the endogenous LHβ promoter. The mRNAs for WT1(+KTS), which contains a three amino-acid insertion between the 3rd and 4th zinc-fingers, and the WT1 (-KTS) variant were both expressed at significant levels. WT1 mRNAs and protein were decreased approximately 50% by GnRH treatment, under conditions where Egr1 mRNA and protein, and LHβ transcription, were stimulated. Decreasing expression of mRNA for WT1(-KTS) decreased stimulation of LHβ and Egr1 by GnRH, whereas decreasing both WT1(-KTS) and (+KTS) increased endogenous LHβ transcription, and prevented LHβ but not Egr1 stimulation by GnRH, suggesting differing biological activities for the WT1 isoforms. Overexpression of WT1 showed that WT1(-KTS) enhanced LHβ promoter GnRH stimulation 2-to-3-fold and required only the 3'Egr1 site. WT1(+KTS) repressed both basal and GnRH-stimulated LHβ promoter activity by approximately 70%, and required both Egr1 and SF1 sites. Our data suggest that WT1 can modulate LHβ transcription, with differential roles for the two WT1 variants; WT1 (-KTS) enhances and WT1 (+KTS) suppresses transcription.

We investigated the role of DAX1 in LHβ transcription and the effect of proteasomal degradation in the cyclic binding of the co-regulatory proteins on the LHβ promoter. Chromatin immunoprecipiation showed that GnRH stimulates binding of DAX1 to the LHβ promoter, suggesting it acts on the endogenous gene. Inhibition of proteasomal

activity prevents association of the SF1 co-activators SRC1 and GCN5, and regulatory proteins WT1 and DAX1, but sustained binding of the co-repressor SMRT to the LHβ promoter. DAX1 overexpression increased GnRH-stimulated LHβ promoter activity in a dose dependent manner. Decreasing endogenous DAX1 levels by siRNA decreased LHβ mRNA primary transcripts. Thus, DAX1 appears to be an endogenous activator of LHβ expression. To better understand DAX1 functions, we used full length and truncated forms of the LHβ promoter and found that the proximal GnRH response region is sufficient for DAX1 activity. We found that mutation of either SF1 site in the proximal region eliminated DAX1 enhancement of GnRH-stimulated promoter activity. However, subsequent experiments using siRNA against SF1 and measurement of DAX1 association with the LHβ promoter, or DAX stimulation of promoter activity, suggests that DAX1 can still bind and stimulate the LHβ promoter in the absence of SF1.

Overall our data shows that WT1 (+KTS) could serve as the DNA-bound inhibitory protein as per our hypothesis, and that proteasome activity regulates cyclic binding of coregulators to the LHβ promoter, suggesting exchange of negative and positive regulators is required for cyclic transcription factor association and transcription. In the process, we also found that DAX1 is a dose dependent, positive or negative co-regulator of GnRH stimulated LHβ transcription, and that the WT1 (–KTS) stimulates LHβ transcription.

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Chapter 1:Background

Hypothalamic-Pituitary-Gonadal axis and female reproduction

The hypothalamic-pituitary-gonadal (HPG) axis controls the female reproductive system. The HPG axis is a tightly regulated system comprised of three major endocrine organs, including the hypothalamus, pituitary and ovary. Neurons in the hypothalamus secrete the decapeptide hormone, gonadotropin releasing hormone (GnRH) in a pulsatile manner that varies across the female menstrual cycle (1,2). Gonadotrope cells in the anterior pituitary secrete LH and FSH hormones in response to specific GnRH pulse frequency and amplitude patterns. Slow GnRH pulses (1pulse/120mins) favor FSH synthesis and secretion, which is required for egg maturation in the ovary and faster GnRH pulses (1pulse/30mins) favor LH synthesis and secretion (1,2). Variations in gonadotropin secretory patterns in turn impact sex steroid secretion from the ovary and the ovarian sex steroids can feed back to the hypothalamus and the pituitary. These complex feedback loops drive the normal menstrual cycle. The menstrual cycle can be divided into 3 main phases, from beginning to end, based on ovarian biology: the follicular phase, the ovulatory phase and the luteal phase (1,3,4). Slow GnRH pulses stimulate FSH synthesis and secretion. FSH stimulates the development and maturation of the ovarian follicles during the late luteal and early follicular phases. During the follicular phase, FSH contributes to the establishment of one dominant follicle. The dominant follicle produces the hormone 17β-estradiol (E2), which feeds back to the hypothalamus to increase the GnRH pulse frequency that enhances LH synthesis and secretion. At the pituitary level, high concentrations of E2 sensitize the gonadotrope cells for GnRH responsiveness by increasing GnRH receptor expression. Together,

these phenomena provoke an LH surge, which triggers the ovulation- the release of egg/oocyte (from the dominant follicle) into the fallopian tube. After ovulation, the remnant follicle is luteinized to form a structure called the corpus luteum. This marks the beginning of the luteal phase, during which the corpus luteum synthesizes and secretes progesterone along with E2. Progesterone feeds back to the hypothalamus to slow down GnRH pulse frequency. Slower GnRH pulses result in FSH secretion and initiates a new cycle. In the absence of human chorionic gonadotropin, the corpus luteum collapses, and E2 and P levels fall – this allows the next cycle to proceed. However, in the presence of human chorionic gonadotropin from a fertilized conceptus, the corpus luteum is maintained, which allows continued E2 and P secretion that suppresses GnRH/gonadotropin secretion to prevent cycle initiation and to support pregnancy.

The feedback loop, whereby steroid hormones feed back to the hypothalamus to alter GnRH pulse frequency is an important component of the regulation of the cycle. In response to cyclic changes in the ovarian hormone secretion (estrogen and progesterone) corresponding changes occur in the uterus. Estrogen stimulates endometrial cell proliferaton (endometrial proliferative phase) and thus the thickening of endometrium/uterine wall. After ovulation, progesterone from the corpus luteum converts the proliferative endometrium to a secretory endometrium (endometrial secretory phase), with blood vessel formation and mucoid secretions - changes that are necessary to support possible implantation. In the absence of continuous or chorionic gonadotropin stimulation of the corpus luteum, progesterone and estrogen secretion wanes; in response to sex steroid withdrawal, the uterine lining is shed as menstrual bleeding (3–6).

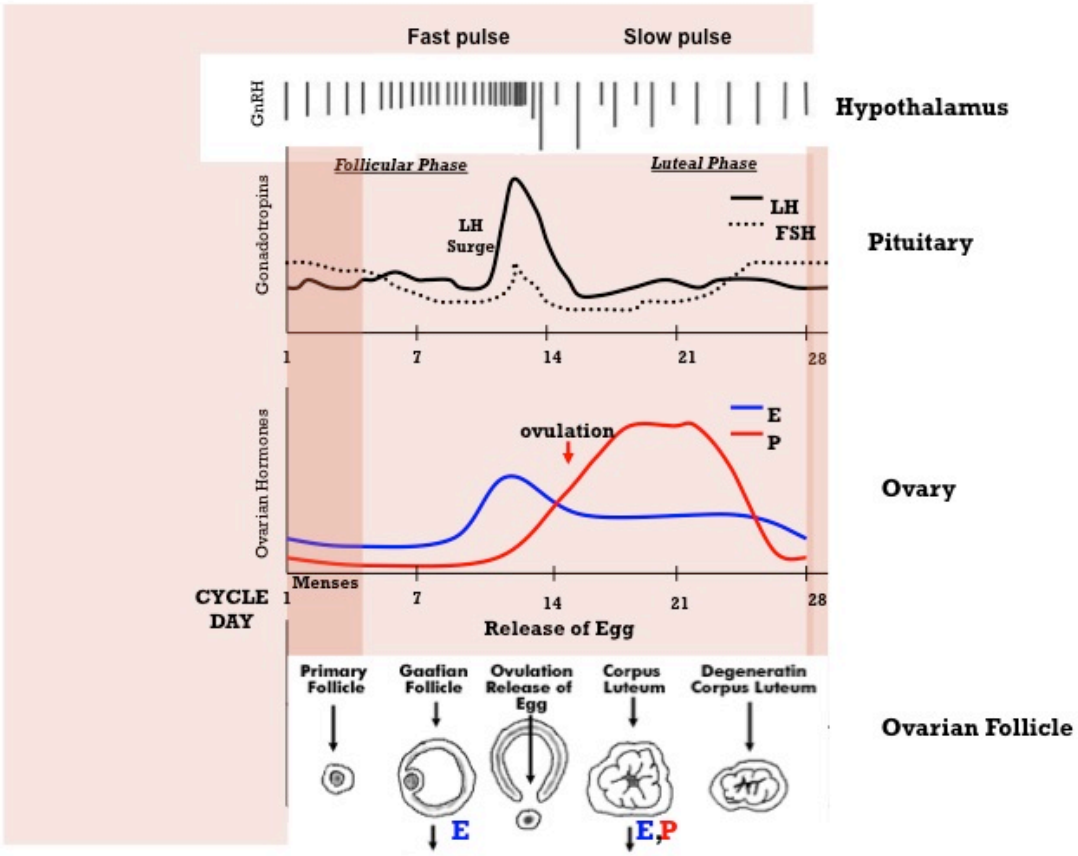

Figure 1 Pulsatile GnRH release and hormonal regulation controlling menstrual cycle 'E' denotes estrogen and 'P' denotes progesterone hormones

Figure 1 Pulsatile GnRH release and hormonal regulation controlling menstrual cycle ‘E’ denotes estrogen and ‘P’ denotes progesterone hormones.

Fast and slow GnRH pulses favors LH and FSH hormone synthesis respectively in the pituitary as shown by the continuous and dotted lines. FSH and LH controls follicular maturation and ovulation in the ovary, along with directing sex steroid hormone (estrogen and progesterone) synthesis. These steroid hormones then feed back to the hypothalamus and the pituitary to regulate GnRH pulses and synthesis of gonadotropin hormones. Figure adapted from thesis work by Heidi Walsh 2009.

H-P-G feedback by non-steroidal hormones

FSH levels may be selectively altered in pituitary cells by non-steroid hormones like activin, inhibin and follistatin produced in the ovarian follicles and the pituitary (7,8). Inhibin and follistatin have inhibitory effects on FSH and activin has a stimulatory effect on FSH. Activin can be a homo- or heterodimer, composed of same or different sub-type of β subunits (β_A, β_B) subunits linked by a di-sulphide bond. Inhibin is a heterodimer made in combination of an α–subunit and different subtype of β subunits (β_A, β_B), according to which it is named inhibin A (α β_A) and inhibin B (α β_B). Follistatin, on the other hand, has no similarity with activin or inhibin subunits .It is a glycoprotein hormone that binds to activin, and exerts its suppressive effect on FSH by neutralizing the stimulatory effects of activin. In the early follicular stage, E2 is produced from the developing ovary, which feeds back to the hypothalamus to increase GnRH pulse frequency, causing increased LH synthesis and secretion. The rise in Inhibin B expression in the ovary during mid-follicular phase helps account for the reduction of FSH at this time. At mid-cycle LH secretion is increased in response to rising GnRH, resulting in and ovulation. Although inhibin is produced in the ovary, it inhibits FSH action by a negative feedback to the pituitary. In addition to stimulation of FSH synthesis and secretion, activin also stimulates the expression of GnRH receptor, FSH receptor and proliferation of ovarian granulosa cells, where androgen is converted to estrogen by aromatase enzyme. (9,10).

PCOS: Elevated LH in PCOS

Any kind of hormonal dysregulation in the HPG axis might lead to certain infertility syndromes such as polycystic ovarian syndrome. Polycystic Ovarian Syndrome (PCOS) affects many women estimated approximately 5-12% of women of reproductive age, and is a common cause of infertility (11,12). Common features of PCOS are anovulation, hyperandrogenism and in some cases metabolic problems like insulin insensitivity that leads to compensatory hyperinsulinemia; ovarian cysts are also often observed. In approximately 75-95% of PCOS patients, elevated levels of LH and/or high LH/FSH ratios enhance ovarian steroidogenesis. Additionally relatively low FSH levels hinder follicular development (and thus ovulation), which contributes to the subfertility of PCOS. Increased levels of LH stimulate ovarian theca cells to produce androgen at higher than normal levels. In addition, low FSH levels (relative to LH) result in low levels of aromatase enzyme (enzyme catalyzing the conversion of androgen to E2) production in the granulosa cells of the ovary. As a consequence, ovarian follicles in PCOS have reduced ability to covert androgen precursors to estrogens; this contributes to increased androgen production. These two processes synergistically elevate androgen levels in PCOS patients. Elevated androgen levels can then interfere with progesterone negative feedback on the hypothalamus, thus preventing the slowing of GnRH pulses and the resulting increase in FSH (11,13).

Neuroendocrine dysregulation causes continuous induction of rapid GnRH pulse frequency that increases LH levels resulting in inappropriate LH /FSH ratio. Incessant rapid-GnRH pulse frequency occurs due to reduced hypothalamic sensitivity to negative

feedback by progesterone. Under normal circumstances progesterone reduces GnRH pulse frequency after ovulation, which in turn reduces LH secretion and favors FSH secretion. Exogenous progesterone treatment to control and PCOS women restored normal LH levels. However, higher progesterone was required for PCOS patients to achieve a similar suppressive effect on GnRH/LH pulses (14). This suggests that in PCOS patients, the hypothalamus is less responsive to progesterone-negative feedback, possibly due to elevated androgens. In support of this theory, anti-androgen (flutamide) administration restored progesterone feedback in PCOS women (15). Androgen antagonizes the negative progesterone feedback, which is required to slow down GnRH pulse frequency and suppress LH secretion, after ovulation. Androgen interferes with progesterone feedback, indirectly increasing LH levels and high LH increases androgen production in the ovary; this further perpetuates the hormonal dysregulation (12,16,17).

Development of PCOS cannot be accounted for by a single factor, rather, combinations of various factors are likely to be responsible for the syndrome, including genetic contributions, obesity and other unknown causes (18,19) . My work is focused in understanding the detailed transcriptional mechanism of LH, as dysregulation of LH is a major contributor in PCOS development. GnRH is the most critical regulator of gonadotropin secretion and subunit gene transcription. Among the gonadotropin subunit genes, the LHβ subunit is most dependent on GnRH pulses, and is stimulated to the greatest extent (6).

GnRH receptor and signaling

GnRH receptors, a seven helical transmembrane protein belonging to the G-protein coupled receptor family, are present on cells of the anterior pituitary. GnRH receptor, upon stimulation by GnRH, initiates signaling cascades to effectively transcribe its target genes (gonadotropin genes). GnRH receptors transduce intracellular signals via G-proteins (GTP binding proteins), which in turn, activates other downstream signaling cascades like MAPK (mitogen activated protein kinase). G-proteins are trimeric proteins made up of a α-subunit and a heteodimeric β-γ subunit. The α-subunit can be of three main subtypes - Gs, Gi or Gq. GnRH signaling, for gonadotropin gene transcription in the clonal gonadotrope line, LβT2 cells, occurs via Gs and Gq subtypes (20). Gs protein signals via activating adenylate cyclase to catalyze cAMP production and in turn activating protein kinase A. However, Gq upon stimulation dissociates from the β-γ dimer and gets activated by binding GTP (20). It then activates Phospholipase C-β which cleaves PI biphosphate into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (21,22). DAG activates protein kinase C (PKC) and IP3 causes rise in intracellular calcium ions, which also activate PKC. Activated PKC then phosphorylates and hence activates target proteins like MAPK (mitogen-activated protein kinase) to drive the signaling cascade further (22). GnRH signaling via MAPK members ERK (extracellular signal-regulated kinase) and JNK (c-Jun N-terminal kinase) is important for gonadotropin gene transcription, including both stimulation of transcription factor levels, and post-translational modifications of transcription factors. ERK is involved in transcription of the alpha subunit gene and FSHβ, however JNK signaling is involved specifically for GnRH-stimulated LHβ transcription(23). GnRH stimulated LHβ promoter

activity remained unaffected by ERK inhibitor or presence of catalytically inactive ERK however, JNK inhibitor and dominant negative JNK completely inhibited GnRH stimulated LHβ transcription (23). Moreover, GnRH induction of ERK was found to be PKC and intracellular or extracellular calcium ion dependent unlike JNK, suggesting a differential regulation of these two signaling cascades by GnRH (21–24). Studies using specific protein kinase inhibitors and measuring GnRH regulated Egr1 promoter activity revealed that GnRH stimulation of Egr1 occurs through PKC and ERK signaling pathways (25). A recent study reported GnRH signaling via GnRH-induced activation of AMP-activated protein kinase (AMPK) to be involved in LHβ transcription. AMPK inhibitor compound C or siRNA against AMPK inhibited GnRH stimulated endogenous LHβ transcription and Egr1 synthesis (26). GnRH also activates and signals via Calcium/calmodulin-dependent kinase II (Ca/CaMK II), a mediator in calcium signaling, to influence LHβ gene transcription. Blocking the calcium influx and depleting the intracellular calcium levels inhibited GnRH activation of CaMK II. A specific CaMK II inhibitor resulted in suppressed GnRH stimulated LHβ transcription (27). GnRH signaling has also been reported to work in concert with cAMP signaling via activation of Protein Kinase A to influence LHβ transcription through 3'SF1 binding site on the LHβ promoter (28).

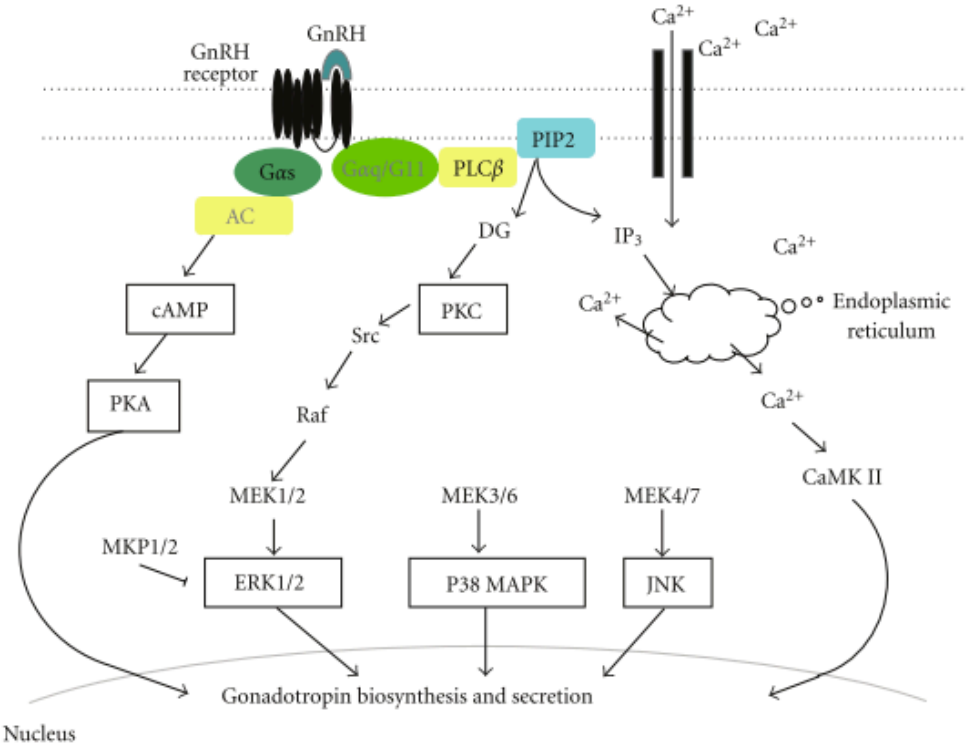

Figure 2 GnRH Signaling

Figure 2 GnRH Signaling Upon GnRH binding, GnRH receptors transduce intracellular signals via coupling with Gs and Gq subunits of the G-proteins to regulate gonadotropin gene transcription. GnRH receptor coupling with Gq activates phospholipase Cβ (PLCβ) and in response two messager molecules 1,4,5-triphosphate (IP3) and diacylglycerol (DG) are produced. IP3 stimulates the release of intracellular calcium from endoplasmic reticulum and DG along with the intracellular calcium activates protein kinase C pathway. PKC then activates MAPK family proteins extracellular signal- regulated kinase (ERK), P38 MAPK, c-Jun N-terminal kinase (JNK). Calcium signaling also influences PKC signaling pathway. However, GnRH differentially regulates these pathways to differentially regulate gonadotropin genes. GnRH also activates calcium-calmodulin kinase II (CaMK II) via IP3. GnRH receptor coupling with Gs subunit of G-protein stimulates cAMP production via adenylate cyclase. cAMP then activates protein kinase A (PKA) pathway which influences gonadotropin gene transcription. MAP kinase phosphatase 1/2 (MKP1/2) regulates ERK activity by dephosphorylation.

LHβ promoter and transcription

LH is composed of two subunits, a common α–subunit that it shares with FSH, and a distinct β subunit. GnRH, secreted from the hypothalamus in a pulsatile manner, regulates transcription of these subunit genes in the anterior pituitary. The pulsatile release of GnRH, 1/8-30mins pulse favors alpha subunit synthesis, 1/30-60min favors LHβ and 1/120-240 min pulse favors FSHβ synthesis (1). Among all the 3 subunit genes, LHβ is most dramatically and precisely regulated by GnRH (6).The rat LHβ promoter (Figure 3) is comprised of two GnRH responsive regions, the distal (-617 to -366 bp relative to transcription start site) and the proximal region (-127 to -50 bp) . The distal region contains two SP1 sites and a CArG box. The rat promoter is nearly identical to the mouse promoter, but the Sp1 binding site is slightly different and has a lower affinity for this transcription factor. The proximal region of LHβ promoter is conserved across all mammalian species and consists of GnRH responsive elements that includes two binding sites for the transcription factors Egr1 (early growth response protein), SF1 (orphan nuclear receptor) and one binding site for Ptx1 (homeobox protein). GnRH stimulates the cyclic and coordinated binding of these transcription factors on LHβ promoter (29). These transcription factors, along with their co-regulatory proteins, regulate LHβ transcription. Full transcriptional activation requires interactions and synergy between the distal and proximal response elements (30–32).

Examination of the gonadotropin subunit genes shows that all three subunits bind the orphan nuclear receptor SF1, and this transcription factor is critical for expression of all three genes, as well as the GnRH receptor gene (33–35). However, the GnRH

response regions are very divergent, and each contains binding sites for different early-response proteins; for LHβ, this is Egr-1 (30). Egr-1 is rapidly stimulated by GnRH several 100-fold from undetectable levels; ERK, JNK, and AMPK signaling cascades are all required for this stimulation (26,36). After the rapid induction of Egr1 synthesis by GnRH, the three transcription factors Egr1, SF1 and Ptx1 synergistically enhance LHβ transcription through protein-protein interactions on the promoter (37) . In addition to these transcription factors, co-regulatory proteins that associate with the transcription factors play a significant role in regulating transcription. Several proteins that were shown to co-activate LHβ transcription includes β-catenin, P300, SNURF and CBP (CREB binding protein) (31,38–40). Overexpression and siRNA studies showed β-catenin to be positively regulating the functional interaction between SF1 and Egr1, with maximal promoter activity during GnRH stimulated LHβ transcription. β-catenin directly interacts with SF1. By mutating the β-catenin binding site on SF1 (235-4 AA, in the ligand binding domain), Egr1-SF1 synergy was compromised (38). In vitro and in vivo studies revealed that phosphorylated CBP protein interacts with Egr1 to enhance GnRH responsiveness of the LHβ promoter in LβT2 cells. Female mice with a mutated CBP-phosphorylation site (Serine 436 mutated to Alanine) were less responsive to GnRH and had dysregulated estrous cycles (40). The small nuclear ring finger protein SNURF acts as a co-activator by facilitating interaction between the distal and proximal GnRH-sensitive regions of the LHβ promoter by associating with Sp1 via the SNURF Ring finger, and with SF1 via charged amino acids near the SNURF N-terminal (31). SNURF binds to TATA binding protein (TBP) on the promoter and serves as a bridge between

the transcription factors and the general transcriptional machinery complex (41). Additional coactivators bind to SF1, described later, and additional activating proteins may yet be identified for the LHβ gene.

Proteins that suppress LHβ transcription include co-repressor proteins that bind to regulatory transcription factors, and additional co-repressor proteins that directly suppress LHβ gene transcription. Co-repressor proteins of Egr1 called NGFI-A-binding protein, Nab1 and Nab2 repress GnRH stimulated LHβ transcription in a GnRH pulse frequency dependent manner. At higher GnRH pulse frequency Egr1 synthesis is favored which activates LH transcription, on the contrary at low GnRH pulse frequency Nab1, Nab2 mRNAs are synthesized (42). Zfhx1a, or ZEB, zinc finger homeodomain protein, acts as a negative regulator of LHβ transcription. It binds to the promoter through E-box elements at -381 and -182bp sites. Zfhx1a overexpression decreased GnRH stimulated LHβ transcription and mutation of the three E-box (binding sites for Zfhx1a) sites on the promoter increased GnRH stimulated transcription (43). SNIP1 (smad nuclear interacting protein) was also shown to suppress LHβ promoter activation by sequestering co-activator P300, which acts as a stabilizer of SF1-Egr1 interaction required for LHβ promoter activation. P300 overexpression stimulated LHβ transcription only in presence of both Egr1 and SF1, and SNIP1-mediated suppression was released by P300 overexpression(39). SF1, as an orphan nuclear receptor, can also associate with several proteins that are typical co-suppressors for the nuclear receptor family, including SMRT and NCOR (44,45).

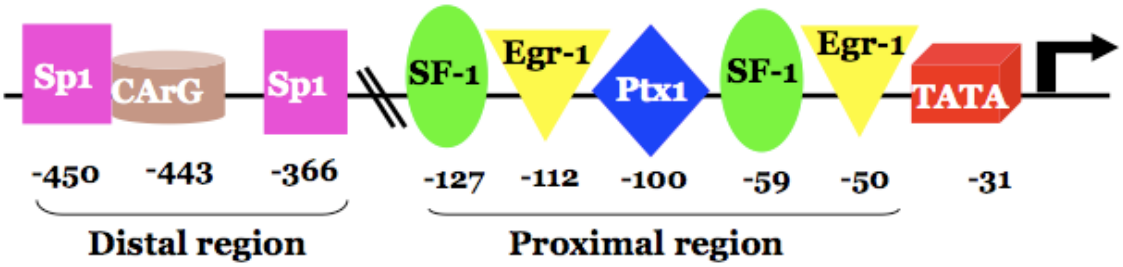

Figure 3 Rat LHβ subunit gene promoter showing binding sites for different proteins and transcription factors.

LHβ promoter has distal and proximal GnRH responsive elements. The distal region consist of two SP1 binding site and a CArG box, the proximal region is conserved across mammalian species and consists of two binding sites for Egr1 and SF1 and one homoebox protein Ptx1 binding site. Figure adapted from thesis work by Heidi Walsh, 2009.

Nuclear receptors and transcription

Nuclear receptors: Nuclear Receptors (NR) are a group of transcription factors that upon receiving external signals (Ligand binding or post-translational modification) can act on target gene transcription by directly binding to DNA. NRs regulate the expression of a wide array of genes involved in development, homeostasis, reproduction and metabolism (46). Most nuclear receptors are ligand-activated and common ligands are hormones, including steroids, Vitamin D and others. However there are some orphan nuclear receptors with unidentified ligands, including SF1, which is regulated primarily through posttranslational modification as opposed to ligand activation. Certain phospholipids like phosphatidyl inositols have been suggested to serve as SF1 ligands, since mass spectrometry results show that they bind within the SF1 ligand binding pocket (47–49). Sphingosine has been shown to act as an inhibitory ligand of SF1 during the transcription of CYP17 gene in the adrenal cortex and is suggested to be exchanged by some unidentified activator ligand (49).

A Typical NR structure includes (Fig.4):

a) N-terminal ligand independent activation domain called AF1.

b) DNA binding domain (DBD)

c) Hinge region (imparts flexibility)

d) C-terminal Ligand Binding Domain (LBD): which is involved in

ligand recognition and binding, cofactor interaction and dimerization

LBD domain (composed of 12 helices), AF2 domain or 12^{th} helix upon ligand binding changes its conformation and the NR becomes active (46).

Figure 4 General structure of a nuclear receptor

A typical nuclear receptor structure comprising of a N-terminal ligand independent activation domain (AF1), a DNA binding domain (DBD) containing two zinc fingers that allows the receptor to bind to it's target gene promoter, Hinge region (imparts flexibility), a C-terminal Ligand Binding Domain (LBD) involved in ligand recognition and binding, cofactor interaction and dimerization LBD domain (composed of 12 helices), AF2 domain or 12th helix upon ligand binding changes its conformation and the NR becomes active (46).

In the basal, inactivated state, nuclear receptors are bound to co-repressors that directly or indirectly repress transcription (50,51). For example some of these proteins (SMRT, NCOR) interact with additional proteins such as histone de-acetylases, which modify histones and keeps the chromatin in a compressed configuration that does not allow access to transcription factors or the transcriptional machinery. The co-repressors interact with the LBD domain of the NR through via the CoRNR (variations of the motif, I/L-x-x-I/V-I) box present near its C-terminal end (52)

In ligand activated NRs, the AF2 domain (in the LBD domain) undergoes a conformational change upon ligand binding which facilitates release or the exchange of the co-repressors with co-activators. In the case of cytosol located NR, ligand activation signals the NR to move to the nucleus where it binds to target gene DNA with the help of zinc finger domains in the DNA binding domain. Once bound to their target DNA, a typical nuclear receptor recruits co-activator complexes like SRC/P160, CBP/p300. Some of these proteins have histone acetylase (HAT) activity that helps in unwinding the chromatin structure to facilitate gene transcription, and that forms a transcriptionally active complex interacting with required basal transcriptional machinery that includes RNAPol II to carry on transcription. These co-activators contain LXXLL motifs that interact with the AF2 domain of the NR. The interplay between the co-repressors and the co-activators of the NRs regulate the NR response to the cellular signaling commands for gene expression. These co-regulatory proteins might have diverse enzymatic activities that include phosphorylation, methylation, acetylation, ubiquitination and chromatin remodeling; these co-regulatory proteins might also be targeted for several of the above-mentioned modifications. Several co-activators, co-repressors and

the proteins of the basic transcriptional machinery are often ubiquitinated and targeted for proteasomal degradation. Proteasome mediated degradation of the proteins involved in transcriptional complex facilitates to clear off the promoter after each transcriptional cycle to initiate a new rounds of transcription (46,53–55).

SF1

SF1 is an orphan nuclear receptor and an important transcription factor regulating genes related to steroidogenesis and reproduction. SF-1 knockout mice fails to develop of the adrenal glands, gonads, ventromedial hypothalamus and proper functioning of pituitary gonadotropes is also hampered (56). SF1 is expressed in steroidogenic tissue of adrenal cortex, ovaries and leydig cells of the testis, and regulates enzymes pertaining to steroidogenesis. In the pituitary, SF1 is involved in the transcription of the common α and unique β subunit genes of LH and FSH and also the GnRH receptor (33–35). In addition to DNA binding ability, SF1 regulation of its target gene also involves forming a protein complex by interaction with other transcription factors and co-regulatory proteins. SF1 is phosphorylated at serine 203 residue at the C-terminal region of the hinge, near the AF-1 domain. SF1 phosphorylation signals it to recruit and interact with the co-activators SRC1, GCN5 and CBP through the activation domain2 (AF2) located in the putative ligand-binding domain. Both these domains are also required for interaction of SF1 with the co-repressor SMRT(44,57,58).

In response to GnRH, cyclic and coordinated binding of the transcription factors EGR1 and SF1 occur on the chromatin associated with the LHβ promoter (29). In addition to

these transcription factors, relevant co-activators (increase transcription) or co-repressors (decrease) could also be recruited by these transcription factors.

Egr1

In response to GnRH, synthesis of early zinc-finger transcription factor Egr1 (early growth response1) is stimulated to initiate LHβ transcription. Egr1 knock-out mice are infertile, primarily due to lack of LHβ expression (59). Mutation of any of the two Egr1 sites in the LHβ promoter has been shown to significantly decrease both basal and GnRH stimulated LHβ transcription (29,37). Egr1 acts in concert with the orphan nuclear receptor SF1 to induce LHβ transcription (60,61). The Egr1 family of early growth response proteins includes Egr2, Egr3 and Egr4 are rapidly synthesized and transiently activated in response to cellular signals/stimuli. They are DNA binding proteins containing three zinc finger motifs that binds to the GC rich region, GCG (G/T) GGGCG, on the target promoter. Egr1 transcriptional activity is negatively regulated by its repressor proteins NAB1 and NAB2 through direct interaction on the repressor domain (42). CBP (a transcriptional activator protein) is phosphorylated in response to GnRH and phosphorylated CBP directly interacts with Egr1 to co-activate LHβ transcription (40). Egr1 shares binding motifs similar to that of WT1 and SP1(62,63). Egr1 has been shown to be post transcriptionally modified by phosphorylation, sumoylation and ubiquitination (40,64). The phosphorylated form(s) of Egr1 binds DNA more efficiently and in a dose dependent manner (65). EGF (epidermal growth factor) induced expression of Egr1 has been found to be regulated by sumolytion and ubiquitination of Egr1(66). Immunoprecipitation and western blot assays were performed using antibodies against Egr1 and SUMO in the presence or absence of sumoylation inhibitor

NEM. Under these conditions, Egr1 was found to be sumoylated in response to 30mins of EGF treatment. However, 60mins of EGF treatment caused polyubiquitination of Egr1. Overexpression of SUMO1 and ubc9 (SUMO conjugating enzyme) decreased Egr1 mRNA and protein levels in response to EGF. When treated with proteasome inhibitor MG132 in similar conditions, as stated above, Egr1 protein levels were increased, suggesting SUMOlaytion mediated decrease of Egr1 levels induced by EGF is possibly through Egr1 ubiquitination and proteasome mediated degradation (66).

DAX1

SF1 has been found to co-express and interact with another orphan nuclear receptor protein, DAX1. DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) is encoded by the gene NR0B1 and is expressed throughout the entire HPAG axis (67). Mutations in this gene have been associated with the disorder adrenal hypoplasia congenita (AHC), which causes underdevelopment of the adrenal cortex and other secondary effects including hypogonadotropic hypogonadism (HH), a combined defect of both pituitary and the hypothalamus with low gonadotropin and steroid levels (68). Gene duplication causes dosage sensitive sex reversal. The structure of DAX1 differs from other general nuclear receptors (Fig. 5) in the sense that it does not have the typical DNA binding domain and therefore it acts mainly through protein-protein interactions (68,69).DAX1 physically interacts with SF1 through its LXXLL-related motifs (70). DAX1 and SF1 have similar expression patterns and knock-down studies revealed that these two proteins take part in several common pathways related to reproduction, such as urogenital ridge development during embryogenesis, endocrine development, steroid synthesis in adrenal cortex and gene

regulation in testis, ovary and anterior pituitary (45,69,71–75)**.** Studies depicted DAX1 to be acting primarily as a repressor of SF1 target genes by physically interacting with SF1 (45). However, Xu et al showed, DAX1 functions as a co-activator along with SRA (Steroid Receptor RNA Activator) to regulate the SF1 target gene, Mc2R (melanocortin 2 receptor, adrenalin glands), during steroidogenesis (75). Reporter assays showed that DAX1 acts as a co-activator in a dose dependent manner only when co-transfected with SRA and SF1(75) . DAX1 mutants **(**R269P and N422I; human AHC linked naturally occurring mutation**)** decreased this co-activation significantly (75). In our study, we investigated the role of DAX1 in LHβ transcription. Our overexpression and siRNA studies showed DAX1 to be a positive regulator of LHβ transcription.

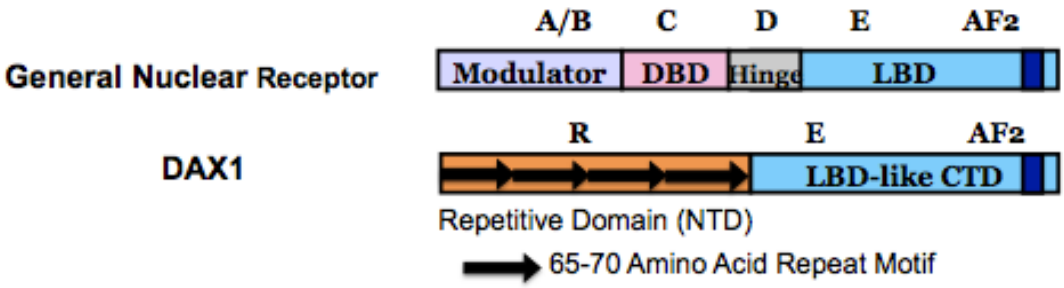

Figure 5 A structural comparison between a general nuclear receptor with DAX1

DAX1 lacks the usual AF1 domain, a defined DNA binding domain and the hinge region as present in a typical nuclear receptor. Instead, the N-terminal domain of DAX1 consists of a 3.5 alanine/glycine rich repeats of a 65–70 amino acid motif. However, it has a carboxy terminal domain similar to LBD domain and AF2 domain similar to a typical NR (69).

WT1

Wilms tumor suppressor gene (*WT1*) is essential for early urogenital development, and homozygous mutations in WT1protein result in embryonic lethality due to a failure in the development of kidneys and gonads (76,77). WT1 is a DNA-binding protein and has four zinc finger domains; it binds to GC–rich elements. The WT1 protein (Wilms Tumor 1) associates with Sp1, SF1 and DAX1, to exert influence on many reproductive gene promoters including SF1 itself, and is essential for mammalian urogenital development and gonadogenesis prior to sexual differentiation (63,74). In addition to protein-protein interactions, WT1 binds to GC-rich motifs similar to those for Egr-1 or Sp1 (63,78). Time dependent differential binding of the transcription factors WT1, Egr1, and SP1 on the same promoter motif regulates the α Isoform of the Human Thromboxane A2 Receptor, during megakaryocyte differentiation (63). In spite of these intriguing associations with transcription factors involved in LHβ transcription, the potential role of WT1 in LHβ gene transcription had not previously been examined.

WT1 has a broad range of target genes and can act as either a transcriptional repressor or activator, in a cell and promoter specific manner (79). For example, it has been found to transcriptionally repress the human PDGF a chain(80), human telomerase reverse transcriptase (81), proto-oncogenes bcl-2 and c-myc (82) genes and transcriptionally activate several target genes including SF1 (83), DAX1 (84) , erythropoietin (85) and amphiregulin (86) genes. The WT1 gene has ten exons that encode a transcription factor with a proline-glutamine rich amino terminal involved in protein-protein interaction, and four zinc-finger domains towards the carboxy-terminal end that are involved in DNA-binding. There are several splice variants of WT1 including +KTS and –KTS

variants generated by mRNA splicing and resulting in the presence or absence of a three amino acid (KTS) insertion between the third and fourth zinc finger (87,88). WT1 binds to the Egr1 DNA consensus sequence - 5-GCGGGGGCG-3'; the +KTS variant has very low affinity for this site (88,89). The role of –KTS (wild-type) protein as a transcriptional regulator has been well established, including its ability to activate the SF1 gene promoter during early gonad development (83). The +KTS variant has been shown to be involved in RNA processing and RNA metabolism (90–92), but a recent report showed that +KTS can also bind to a specific DNA sequence (5′-ACCAAGCGGGATGCGGAGCCGCCGCCGCCGCCG-3′on the planar cell polarity gene promoter SCRIBBLE and regulate its transcription in developing kidney (93). Within the same cell or tissue, the two variants appear to have distinct roles to play, and maintaining a proper ratio (+KTS/-KTS 2:1) between them is also crucial. For example, a mutation causing a reduced WT1 (+KTS) to WT1 (-KTS) ratio has been shown to give rise to Frasier syndrome (sex reversal carrying developmental defects in kidney and gonads) (94–96). In this thesis work, I investigated the biological role of WT1 in LHβ transcription and addressed the individual roles of the WT1 (+KTS) and WT1 (-KTS) variants. Our data shows WT1 to be a novel regulator of LHβ transcription.

Interplay between transcription factors on the LHβ Promoter

The synergy between Egr1-SF1 plays a significant role in LHβ transcription in the pituitary (31,38) similarly SF1 interacts with proteins like DAX1 and WT1 during early gonadogenesis (74). SF1 plays a role in determining male gonad development by regulating the expression of mullerian inhibiting substance (MIS). MIS is a polypeptide hormone that inhibits the formation of the mullerian duct. Continued development of the

mullerian duct results in the formation of the female reproductive tract (74). SF1 has been found to synergize with WT1 (-KTS splice variant) through direct interactions to favor MIS expression during gonadal differentiation; however, DAX1 interrupted the WT1-SF1 synergistic activation of MIS gene. The dosage sensitivity of DAX1, relative to other factors like SF1 and WT1, has been suggested to play a role in MIS expression. For example, in normal males the gene dosage ratio of WT1: DAX1 is 1:2, since DAX1 is an X-linked gene and WT1 is autosomal. In Denys Drash syndrome (WT1 mutation in human) the WT1: DAX1 ratio is altered and due to DAX1 being more abundant, interrupts the WT1-SF1 synergy that promotes MIS gene transcription (74). Such interactions of SF1 with DAX1 and WT1 led us to question if the DAX1 and WT1 proteins play a role in LHβ transcription.

Post translational modification and ubiquitination of transcription factors:

Modulation of transcription factor activity by various posttranslational modifications (PTM) adds another level of regulatory mechanism to gene expression. The common PTMs on transcription factors and their co-regulators are phosphorylation, glycosylation, acetylation, methylation, ubiquitylation and sumoylation. These PTMs can affect the protein activity (activation or repression), stability, sub-cellular localization, degradation, DNA binding capacity and other protein-protein interactions (97).

Among the first targets of ubiquitination and proteasomal degradation to be discovered were the histone proteins (98). The ubiquitin proteasome system (UPS) has since been

shown to act on many other proteins. UPS plays a significant role in transcription by ensuring rapid turnover of proteins (transcription factors, signaling molecules, co-regulatory proteins) and promoter clearance to start a new cycle of transcription. UPS is also responsible for recognizing and degrading misfolded proteins, degrading the RNApol II at the DNA damage site, and regulating the cellular localization of proteins (99–101). Ubiquitination can increase or decrease transcriptional activity of specific proteins. For example, the presence of proteasome inhibitor (MG132) increases transcriptional activity of glucocorticoid receptor (102); however, estrogen receptor mediated transactivation was reduced by proteasome inhibition (103). PTMs and ubiquitination regulate transcription in a cell and promoter-specific manner (104).

The Ubiquitin–proteasome system regulating protein turnover has an array of implications involving the cell cycle, signaling pathways, and transcription. Recently, mutations related to dysregulation of this system have been linked to various neurodegenerative diseases and cancer (105–107). Mutation in the RING domain of BRCA1 (E3 ligase) has been found to predispose to cancer and is also linked to lack of ubiquitin ligase activity. Wild type BRCA1 was able to ubiquitinate target substrate but cancer predisposing-mutations in the RING domain eliminated its E3 ligase activity (105). Accumulation of misfolded proteins due to dysregulation of UPS has been linked to Parkinson disease. Mutations in several genes give rise to Parkinson disease, and two of them serve as UPS enzymes - Parkin protein which has a E3 ubiquitin ligase activity, and Ubiquitin C-terminal Hydrolase L1 (UCHL1) (106,107). There are several steps in targeting and attaching ubiquitin to proteins (Figure 6). Ubiquitin, an 8.5 KD

protein, is first activated in an ATP-dependent manner by an enzyme called E1, which then transfers ubiquitin to E2, a conjugating enzyme. E3 ligase enzymes exhibit substrate specificity, and bind to both E2 and the substrate protein (which is to be ubiquitinated). E3 then transfers the ubiquitin from E2 to the appropriate lysine residue on the protein substrate. The substrate protein can be both mono- and poly-ubiquitinated. Mono-ubiquitination (one ubiquitin on a lysine) plays a role in protein-protein interactions whereas, a polyubiquitinated protein (more than 4 ubiquitin residues on a growing chain on the same lysine residue) is recognized and degraded by the 26s proteasome machinery, consisting of a 20s proteolytic core protein complex and two 19s regulatory protein complex caps at the ends (99,104,108,109). Many lysines may be ubiquinated within a single protein; however, specific motifs favor specific lysine ubiquitination, and post-translational modifications around these motifs can favor ubiquitination (110).

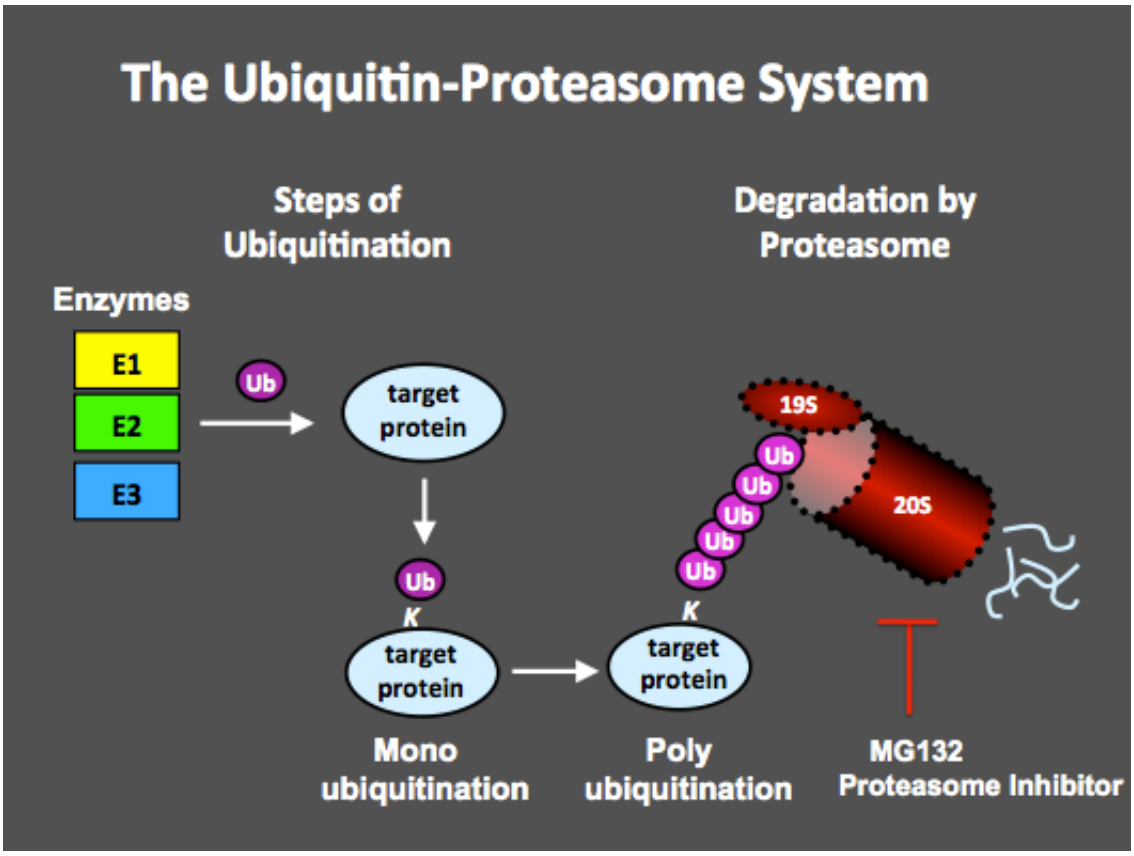

Figure 6 Ubiquitin Proteasome System

The enzymes E1, E2 and E3 tag the target protein with ubiquitin molecules at the appropriate lysine residue. E1 is ubiquitin-activating enzyme, which by ATP dependent process binds to the ubiquitin molecule. The ubiquitin molecule is then transferred to the enzyme E2 (which is ubiquitin conjugating enzyme). The third enzyme, which is an ubiquitin ligase, then transfers the ubiquitin from E2 to the target substrate or protein. A monoubiquitinated protein has been shown to take part in protein-protein interaction and polyubiquitinated protein is degraded by the 26s proteasome machinery.

Role of ubiquitination in transcription

The ubiquitination and proteasome system have been shown to regulate transcription by affecting the degradation of nuclear receptors, other transcription factors, co-regulatory proteins and signaling molecules (104). Proteasome degradation also helps in promoter clearance after one cycle of transcription to start another cycle. (Fig.7). Phosphorylation often marks the protein as a target for ubiquitination and degradation. The stability of β-catenin in the Wnt signaling pathway is regulated by phosphorylation-dependent ubiquitination and degradation (111). Mutation of the serine37 residue within the six amino-acid ubiquitin-target-sequence, compromises its ability to get degraded by the proteasome machinery. Treatment with proteasome inhibitor ALLN resulted in accumulation of heavy molecular weight ubiquitin-tagged, β-catenin proteins, and treatment with a PKC inhibitor also abrogates β-catenin ubiquitination, proving that the ubiquitination of β-catenin is PKC-mediated and phosphorylation-dependent (111). A crucial transcription factor in LHβ transcription, SF1, is phosphorylated at the ser203 residue as a result of GnRH stimulation, which marks SF1 as an ubiquitintion target; proteasome inhibition by MG132 caused accumulation of ubiquitin tagged SF1 in LβT2 cells and this process was stimulated by GnRH (29,112). Pin1 **(**peptidyl-prolyl *cis-trans* isomerase**),** which is stimulated by GnRH, regulates gonadotropin β-subunit gene transcription by facilitating conformational changes in SF1 that favor ubiquitination. Monoubiqutination of SF1, in turn enhances the SF1-Pitx1 interaction and hence favors the SF1 mediated transcription of the gonadotropin β -subunit genes (112). Regulation of the SRC-3 co-activating function is also regulated by ubiquitin-proteasome system.

Mono-ubiquitination promotes SRC-3 binding to the estrogen receptor and the monoubiquitinated SRC-3 was found to enhance transcriptional activity of ER (109). SRC-3 is eventually poly-ubiquitinated and degraded after its action as a co-activator is completed, however this polyubiquitination process was shown to progress along with transcription, and the hormone estradiol stimulated the polyubiquitination of SRC-3 (109). The turnover of the liganded and unliganded human ERα on ER response elements is maintained by the ubiquitin proteasome system. Proteasome inhibition in this case immobilized the ERα on nuclear matrix preventing it from taking part in transcription (113). The Ubiquitin-proteasome system is also utilized by TGFβ to stimulate the degradation of its negative regulator Ski in malignant human cancer cells, which actually promotes cancer metastasis. Phosphorylated smad proteins along with specific E3 ligase, arkadia, perform Ski degradation process in response to TGFβ (114). Cells use efficient ways of converging different PTMs, in this case phosphorylation and ubiquitination to regulate gene expression. In the next section we will discuss the role of ubiquitination specifically in LHβ transcription.

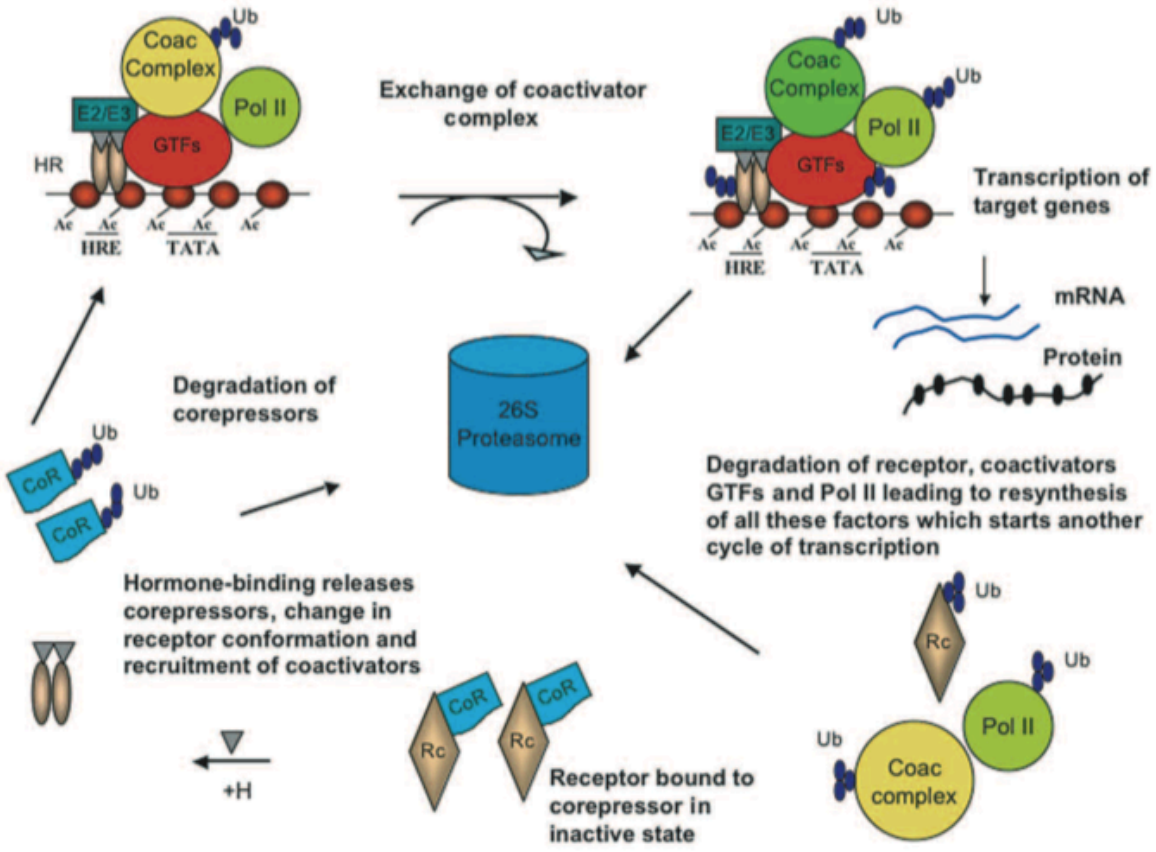

Figure 7 Role of Ubiquitin-Proteasome systems in general nuclear-receptor mediated transcription

Figure 7 Role of Ubiquitin-Proteasome systems in general nuclear-receptor mediated transcription. In absence of ligand the NRs are bound to co-repressors and are inactive. Upon ligand/hormone (denoted by 'H') binding, a conformational change occurs detaching the co-repressor. The proteasome machinery degrades the negative co-regulators/co-repressors. The ligand activated dimers then bind to their target gene, recruit co-activators, and carry on transcription. During transcription, co-activators are targeted for mono-ubiqutination, which first stimulates protein-protein interactions; eventually they are poly-ubiquitinated and targeted for proteasome-mediated degradation. Finally, the UPS enzymes degrades the co-activators and proteins of general transcription machinery including RNApol II to clear the promoter and initiate another round of transcription (55).

Role of the proteasome on LHβ transcription

As stated above, posttranslational modifications and the UPS system play a significant role in regulating activity of a protein and gene transcription. The role of proteasome activity and other posttranslational modifications regulating the transcription factors involved in LHβ transcription has been explored.

Previous studies published by our lab (29) showed that proteasome inhibition severely inhibited GnRH-stimulated LHβ transcription (29). Treatment of LβT2 cells with the proteasome inhibitor, MG132, drastically reduced the GnRH stimulated expression of endogenous LHβ primary transcript (Fig.8). ChIP assays demonstrated that GnRH stimulated cyclic binding of the transcription factors Egr1 and SF1 as well as the of phosphorylated RNA polymerase II which correlates with the active transcription on the LHβ promoter; this cyclic association was abolished by proteasome inhibition with MG132 (Fig.9). Immunoprecipitation and western blot analysis showed that GnRH stimulated the poly-ubiquitination of Egr1 and SF1, and consecutive Chromatin immunoprecipitation assays revealed that poly-ubiquitinated Egr1 and SF1 were associated with the LHβ promoter. This suggests that proteasome-directed Egr1 and SF1 degradation might be required for LHβ transcription (29).

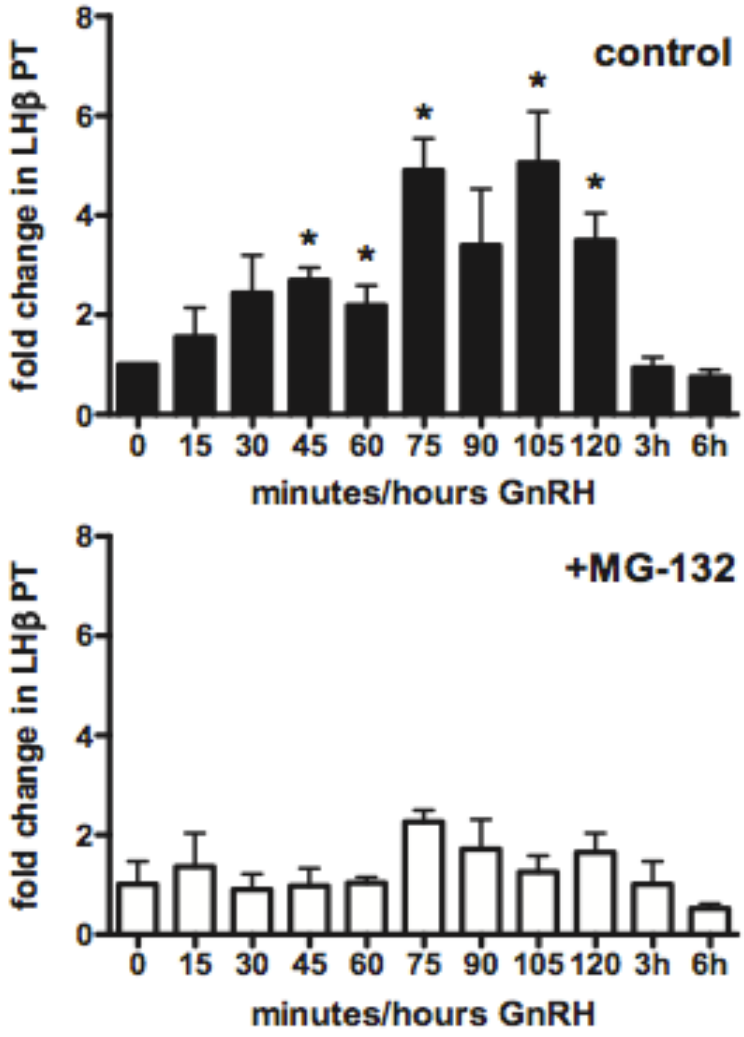

Figure 8 Proteasome inhibition by MG132 (50 μM) inhibits GnRH (50 nM) stimulated expression of the endogenous LHβ primary transcript

Figure 8 Proteasome inhibition by MG132 (50 μM) inhibits GnRH (50 nM) stimulated expression of the endogenous LHβ primary transcript. LβT2 cells were treated with 50μM MG132 and 50nM GnRH and cells were collected at the indicated time points. RNA was extracted and LHβ primary transcript was measured by RT-PCR using primers spanning the intron/exon border. The primary transcript mRNA levels were normalized by GAPDH mRNA levels.(29)

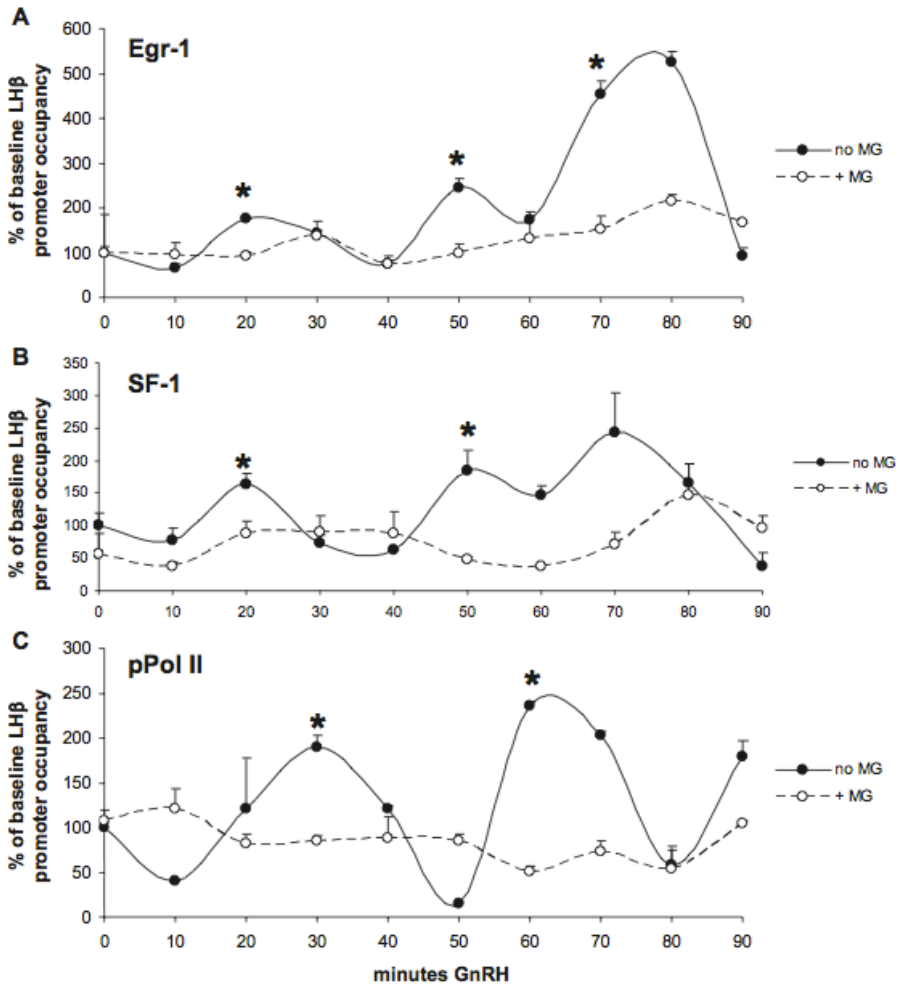

Figure 9 Chromatin immunoprecipitation assay showing the effect of proteasomal inhibition on the LHβ promoter occupancy.

ChIP assay showing the effect of proteasomal inhibition on the LHβ promoter occupancy by Egr1, SF1 and pRNA pol II during GnRH stimulated LHβ transcription. LβT2 cells were treated with or without MG132 (50 μM) and incubated with 50 nm GnRH and collected every 10mins for 90mins.ChIP assays were performed using antibody against Egr1, SF1 and pRNApol II. LHβ promoter occupancy was measured by quantitative real time PCR using primers for LHβ promoter (29).

Several post-translational modifications occur on SF1 that could modulate its activity and degradation, in turn influencing LHβ transcription, has been explored.

SF1 is phosphorylated on Serine203 in response to GnRH via GnRH induced MAPK signaling pathway. As an orphan nuclear receptor, SF1 phosphorylation (or other post transcriptional modifications) regulates its interaction with other proteins such as GRIP1, SMRT and Pitx1 (44,112). This serine203 phosphorylation has been shown to prime SF1 for ubiquitination. Mono-ubiquitination occurs at the lysine119 residue, which enhances SF1 transcriptional activity on the LHβ subunit gene, as demonstrated using LβT2 cells (112). Transfection of wild type and mutated SF1 at S203A followed by immunoprecipitation and western blot analysis detected the mono-ubiquitinated form of wild-type SF1 but not the mutant S203A, suggesting SF1 ubiquitination requires the S203 phosphorylation site. Similarly, mutation of the L119 site on SF1 prevented polyubiquitination. Together this shows phosphorylation of SF1 is required for its ubiquitination. Ubiquitination of SF1 favors SF1-Pitx1 binding which is required for LHβ transcription. SF1 was also sumoylated at sites K119 and K194 Fig.10 (112). GnRH stimulates expression of the ubiquitin conjugation enzyme, ubc4, that increases ERα mediated transactivation of LHβ promoter (115). GnRH stimulated mRNA expression of ubc4 and transfection of LβT2 cells with siubc4 increased ERα protein levels and decreased ERα transactivation of the LHβ promoter in response to GnRH. ChIP assays showed that siubc4 transfection (to decrease ubc4 levels) decreased promoter occupancy by ERα. The enzyme ubc4 is suggested to mono-ubiquitinate ERα, which is ultimately polyubiquitinated and degraded after one cycle of transcription to clear ERα

off of the promoter and to begin a new transcriptional cycle (115). Together, these results demonstrate that proteasome mediated degradation is vital to GnRH-stimulated *LH*β expression, and this occurs in part by allowing proper transcription factor association with the *LH*β promoter.

We hypothesized that MG132 mediated inhibition of the cyclic binding of the transcription factors Egr1 or SF1 on the *LH*β promoter (29) could be due to proteins that hinder the cyclic association of the transcription factors. These inhibitory proteins might required to be removed by proteasomal degradation in order to recruit transcriptional activators and eventually clear off the promoter to maintain several rounds of transcription. Such proteins could be an inhibitor protein or an activator protein either bound directly to the DNA, or co-regulatory proteins associated with the transcription factors. Our candidate for the DNA binding repressor protein was WT1 (Wilms tumour 1) which has been shown to bind to the Egr1 binding sites (62). Our results we show that MG132 prevented the binding of WT1 to *LH*β promoter as depicted by ChIP assays. WT1 protein accumulation was also observed in the presence of MG132 and GnRH.The other type of inhibitory protein that could disrupt the cyclic binding of the transcription fators could be a coregulatory protein bound to the transcription factors. Since DAX1 and SMRT (a co-repressor) have been shown to physically interact with SF1 (44,70,73) and DAX1 has also been shown to act as co-repressor of SF1 by recruiting repressor protein NCoR onto SF1(45). DAX1 and SMRT were possible candidates for the transcription factor (SF1) bound inhibitory protein.However, our data shows DAX1 to be a dose dependent positive regulator of LHβ transcription. WT1 splice variants can serve as a repressor or activator of LHβ transcription playing distinct roles

in modulating transcription. Proteasome inhibition gives distinct profiles of GnRH-stimulated coactivator and corepressor occupancy of the LHβ promoter.As suggested by previous studies along with our observation, proteasomal activity seems to regulate transcription by degrading the repressor proteins so that transcriptional activators could be recruited.After each transcription cycle the proteasome degrades the proteins involved to clears off the promoter and begin a new transcriptional cycle.

Overall, GnRH stimulation results in association and disassociation of several proteins on the promoter, including those with stimulatory and inhibitory roles.

GnRH regulation of the LHβ transcription occurs at several levels that involve stimulating the synthesis of relevant proteins (Egr1), controlling the cyclic association of the regulatory proteins with the LHβ promoter and their degradation via the UPS system after each cycle of transcription. In this work we have explored the role of proteasome in regulating the cyclic association of the co-regulatory proteins SRC1, GCN5, SMRT, DAX1 on the promoter and identified WT1 as a novel regulator and DAX1 as a dose dependent positive regulator of LHβ transcription.

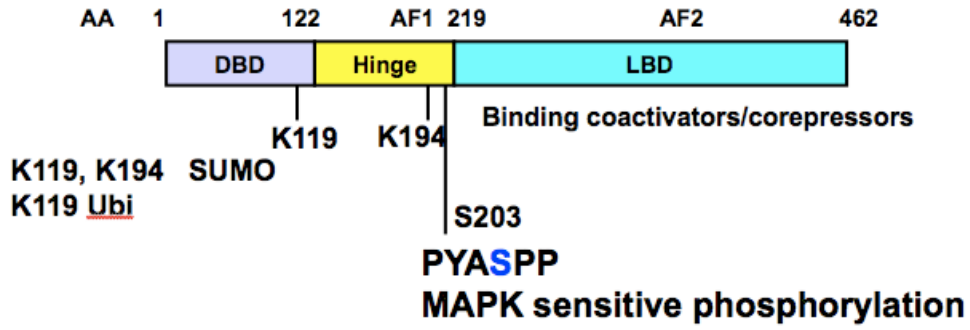

Figure 10 Structure of SF1 showing sites of post-translational modifications

Several post-translational modifications occur on SF1 that controls its activity as a transcription factor. SF1 is phosphorylated at serine 203 and ubiquitinated at lysine 119 and sumoylated at lysine 119 and 194.

CHAPTER 2: A new role for Wilms Tumor protein 1: differential activities of + KTS and –KTS variants **to regulate LHβ transcription**

INTRODUCTION

Gonadotropin hormones secreted from the anterior pituitary control female reproduction, and Luteinizing Hormone (LH) specifically is necessary for ovulation and steroidogenesis (1,11). LH consists of two subunits, an alpha subunit shared with FSH, and a unique beta subunit, which is limiting for the intact hormone (5). Hypothalamic GnRH is a crucial modulator of the gonadotropin subunit genes, and among all the subunits LHβ is most dramatically and precisely regulated by GnRH (6,116). The LHβ promoter includes two GnRH responsive regions. The distal region contains two SP1 sites and a CArG box. The proximal GnRH response element, conserved across all mammalian species including humans, consists of two Egr1 (Early Growth Response 1), two SF1 (Steroidogenic Factor 1) binding sites, and a binding site for the homeobox protein Ptx1. Full transcriptional activation requires interactions and synergy between the distal and proximal response elements (30,32). Synthesis of the zinc-finger transcription factor Egr1 (early growth response1) occurs rapidly in response to GnRH and is a critical component of increased LHβ transcription (29,37,59,61). SF1 is a nuclear receptor that regulates the transcription of several genes involved in steroidogenesis and reproduction, including the pituitary gonadotropin subunit genes and the GnRH receptor (56,60,117).

In response to GnRH, coordinated binding of transcription factors occurs on the LHβ promoter (29,31) . These proteins in turn may associate with additional stimulatory and

suppressive regulatory proteins, including SNURF (31), SRC-1 (118) and DAX-1 (45,74) that influence the response of reproductive genes to hormonal and physiological challenges. The WT1 protein (Wilms Tumor protein 1) associates with Sp1, SF1 and DAX-1 to exert influence on many reproductive gene promoters including SF1 itself, and is essential for mammalian urogenital development and gonadogenesis prior to sexual differentiation (74,83,87,88). In addition, WT1 binds directly to DNA at GC-rich motifs similar to those for Egr1 or Sp1 (62,63). In spite of these intriguing associations with the transcription factors involved in LHβ gene transcription, the potential role of WT1 in LHβ gene transcription has not previously been examined.

WT1 has a broad range of target genes and can act as either a transcriptional repressor or activator, in a cell and promoter specific manner. For example, WT1 represses transcription of the human PDGF A chain (80), human telomerase reverse transcriptase (81) , and proto-oncogenes bcl-2 and c-myc (82) genes, but stimulates the SF1 (74)(119), DAX-1 (84), erythropoietin (85) and amphiregulin (86) genes. The WT1 gene has ten exons that encode a proline-glutamine rich amino terminal involved in protein-protein interactions, and four zinc-finger domains towards the carboxy-terminal end that bind DNA (87,88). There are several splice variants of WT1, the most common of which include +KTS and –KTS, variants resulting in the presence or absence of a three amino acid (KTS) insertion between the third and fourth zinc finger near exon 9 (88). In this paper, we investigated the role of WT1 in LHβ transcription, addressing the individual roles of the WT1 (+KTS) and WT1 (-KTS) variants under basal and GnRH-stimulated conditions. Our data shows WT1 to be a novel regulator of LHβ and that the splice

variants differentially regulate LHβ transcription. The +KTS variant represses both basal and GnRH stimulated LHβ transcription whereas the –KTS variant activates GnRH-stimulated LHβ transcription.

MATERIALS AND METHODS

Cell culture, transient transfection and luciferase assay

Experiments were performed using the clonal mouse gonadotrope cell line, LβT2 as previously described (29). Cells were maintained in Dulbeccos Minimal Essential Medium (DMEM) with 10%FBS (fetal bovine serum) and 1% antibiotic/antimycotic (GIBCO,Grand Island, New York). For experiments, cells were plated in phenol red-free DMEM with 5% charcoal stripped serum and 2%L-glutamine. GnRH (50 nM, Bachem Biosciences Inc, King of Prussia, PA under the name LHRH) was used as indicated. LβT2 cells were plated using DMEM plus 5% charcoal stripped newborn calf serum at the concentration of 500,000 cells per well in 12-well (20 mm diameter) dishes. After 24h, the cells were transfected with a luciferase reporter construct driven by the rat LHβ promoter using Lipofectamine 2000 (Invitrogen; Carlsbad,California). For transfection experiments, 0.33 μg per reaction of the rat LHβ gene promoter from -617 to +44 bp containing both distal and proximal GnRH responsive elements or 1μg per reaction of truncated promoter -245 to +44 region containing only the downstream GnRH response element fused to luciferase were used. In WT1 overexpression/dose response studies, increasing concentrations of WT1 splice variants +KTS and –KTS plasmid DNA up to 0.33 μg were transfected along with the reporter plasmid. Empty vector pCB6+ plasmid at various concentrations was transfected to keep total DNA constant. WT1 expression

plasmids were a generous gift from Dr. Nicholas Webster, UCSD (120). In some studies, the rat LHβ gene promoter from -617 to +44 bp with previously described mutations in either the SF1 sites or the Egr1 sites (30)(28) were used to define the requirement for these DNA regions in WT1 actions. After 48 h of transfection, the cells were treated with or without GnRH for 6 hrs and the cell lysates were collected in 200ul of 1x passive lysis buffer (Promega, Madison, WI, cell culture lysis reagent). The samples were centrifuged at 13,000 rpm for 1 min and supernatant was collected. Luciferase activity was measured using a Turner TD-20e luminometer (Turner Designs, Mountain View, CA). Total proteins of each sample were measured using the Bradford Protein assay (Bio-Rad dye; Hercules, CA). Luciferase activity was normalized as described (26,29). Mean and standard error were calculated for 6 samples. Statistical significance was determined using paired student T-test and ANOVA and with differences between treatment groups determined by Bonferroni multiple comparison test (28).

In some experiments, normal mouse pituitaries were used to measure WT1 mRNAs. Female mice were ovariectomized between 2-3 months of age; approximately 10-14 days post-ovariectomy, animals were treated with oil or 300 ng 17β–estradiol for 3 days as previously described (121,122). Animals were killed at 9AM and pituitaries collected for RNA purification and mRNA measurement as previously described (26,28). For *in vitro* GnRH treatment, pituitary cells from adult mice were treated in culture with 5 nM GnRH; WT1 mRNAs were measured and normalized for GAPDH mRNA (26).

siRNA delivery and primary transcript assay

To decrease expression of mRNAs and protein, siRNA was delivered into LβT2 cells using nucleofection technology according to the manufacturer's instructions (Amaxa Corp., Gaithersburg, MD) and as previously described (26). The LβT2 cells were nucleofected with siGENOME SMART pool siRNA (0.2nM;Dharmacon RNA Technologies, Lafayette, CO) directed against mouse WT1 or a non-targeting negative control siRNA (0.2nM,siCON #1; Dharmacon), in Solution T and using Program A-020. Each reaction contained 5 $X10^6$ cells and was divided between three wells in a 35-mm plate containing 2 ml 5% SNCS. Cell lysates were collected after 72 h for primary transcript assay for LHβ, RT-PCR to detect WT1 mRNA isoforms, and immunoblotting to confirm WT1 knockdown. Experiments were performed in triplicate three times. Protein levels were analyzed on immunoblots.

To measure the mRNAs, RNA was isolated from the cell lysates using the QIAGEN (Valencia, CA) RNeasy kit and was briefly treated with DNase (Roche, Indianapolis, IN) to remove DNA contamination. Total RNA was subjected to reverse transcription of the mRNA using the iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed using cDNA as template, as described (26,28). Primers were designed against the first intron/exon border of the mouse LHβ gene to detect unspliced mRNA primary transcript (PT) (forward primer sequence, 5'-AGAGGCTCCAG-GTAAGATGGTA-3'; reverse primer sequence, 5'-CCACTCAGTATA- ATACAGAAAC-3'). Egr1 primary transcript mRNA was measured as previously described (26,29). Both Egr1 and LHβ primary transcript mRNAs were normalized to GAPDH mRNA levels.

Samples without the reverse transcriptase enzyme during cDNA synthesis were used as negative controls. In results, five representative experiments among seven experiments are shown. To measure expression of WT1 (splice variants +KTS and –KTS) mRNA, cells were treated with vehicle or 50 nM GnRH for 90 min, RNA was isolated and quantitative PCR was performed. The WT1 primers used were: forward primer sequence, 5-CATCTGAAACCAGTGAGAAACG-3; reverse primer sequence for -KTS, 5-CTCATACAGGTGAAAAGCCCTT-3, reverse primer sequence for +KTS, 5-CTCATACAGGTAAAACAAGTGAAAAGCCCTT-3. All mRNAs were normalized to GAPDH mRNA levels. Averages and SEM were calculated from PCR replicates.

Western Blot

For Western blot analysis, 2 X 10^6 cells per well were plated in 6-well 35 mm dishes. After 24h, cells were treated with 50 nM GnRH and collected every 30 min for 3.5 h. Cells were lysed and collected using 2x gel loading buffer [100 mM Tris-HCL (pH 6.8), 4% SDS, 20% glycerol] plus protease inhibitors as described by Andrade et al (26), and the protein concentration was measured using the Pierce (Rockford, IL) BCA Kit. Cell lysates were heated for 5 min at 95 C and equal amounts of proteins of each sample were separated by 10% SDS-PAGE using 140 constant voltage for approximately 2 h. Proteins were transferred to a nitrocellulose membrane using 35V constant voltage for 3 h. Membranes were then blocked using 10% non-fat dry milk in Tris-buffered saline plus 1% Tween 20 (TBST) for 1h in room temperature. Membranes were then incubated with a WT1 primary antibody (C-19: SC-192 Santa Cruz Biotechnologies, Santa Cruz CA) overnight (1:500) or Egr1 primary antibody (Cell Signaling Technology) overnight

(1:1000) at 4C followed by three 5 min washes with TBST and another incubation with secondary antibody, horseradish peroxidase-conjugated donkey anti-rabbit Fab fragment IgG (1:5000;GE HealthCare; Piscataway, New Jersey) for 2h. Relative levels of proteins were detected with ECL, Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and X-ray film autoradiography. Monoclonal anti-β-actin primary antibody (Sigma) was used as a loading control to re-probe the membrane. Band intensity was measured by densitometry and normalized against β-actin in the same samples.

Chromatin Immunoprecipitation

Assays were performed as previously described (28,29)**.** To measure WT1 promoter occupancy under basal conditions (without GnRH treatment), LβT2 cells were pre-treated with 2.5 μM of α-amanitin for 1h followed by thorough washing with PBS and incubation with phenol red-free DMEM with 5% charcoal stripped serum and 2%L-glutamine for 20 min. For GnRH studies, LβT2 cells were first pre-treated with 2.5 μM of α-amanitin for 1h to synchronize protein occupancy on the promoter, washed, then incubated in fresh media with or without 50 nM GnRH and collected every 10 min for 2h. Chromatin immunoprecipitation (ChIP) assays were performed by cross-linking the chromatin with 1% formaldehyde for 10 min; the reaction was stopped by addition of 1.25 M glycine and cells were collected in cold PBS plus protease inhibitors (28,29). Cross-linked chromatin was sonnicated to approximate lengths of 1000 bp, using a cup horn sonicator (Misonix, Farmingdale, NY). Whole-cell extract was diluted with ChIP sonication buffer plus protease inhibitors, divided into aliquots from each sample at

each time point to measure input chromatin DNA, and for immunoprecipiation with specific antibodies. Aliquots were incubated with and without primary antibody overnight at 4 C. Antibodies for WT1 (SC-192), Egr1 (SC-189X), or RNA polymerase II (CTD4H8) were obtained from Santa Cruz Biotechnology (WT1 and Egr1), or Millipore, respectively. Protein G PLUS agarose beads (SC-2002; Santa Cruz Biotechnology) were then added for 2 h at 4C to precipitate the antibody bound chromatin. Agarose beads were washed with sonication buffer and Tris-EDTA buffer. DNA-protein complex was released with elution buffer and cross-links were reversed by incubation with NaCl at 65 C overnight. DNA was purified using the QIAGEN PCR purification kit and promoter occupancy was measured with quantitative real-time PCR (iCycler; Bio-Rad) as described (29). The primers used were located at - 102bp (Forward 5-CTGTGTCTCGCCCCCAAAGAGATTA-3) and -1bp (reverse 5-CCTGGCTTTATACCTGCGGGGTT-3) to detect the LHβ promoter. Each individual sample was corrected for background and normalized for total chromatin input.

RESULTS

WT1 is expressed in LβT2 cells and associates with the LHβ promoter

To determine if the common WT1 variants WT1 (-KTS) and WT1(+KTS) are expressed in LβT2 cells, we first measured WT1 mRNAs by RT-PCR, using specific primers to detect the splice variants. The amplified products, separated on a 1% agarose gel, (Fig. 11 A) show bands that correspond to expected sizes for both +KTS (301 bp) and –KTS (292 bp) products; PCR product was not detected without reverse transcriptase either on a gel (not shown) or by incorporation of Syber Green into PCR product (Fig. 11). WT1 protein is also expressed under basal (without GnRH) conditions in LβT2 cells,

whereas Egr1, a zinc-finger transcription factor proven to associate with the LHβ promoter, is not expressed at detectable levels under the same conditions (Fig. 11B). Only one specific protein band for WT1 was observed, as expected, as the three amino acid difference cannot be detected by electrophoresis. To determine if WT1 could bind to the endogenous LHβ promoter, chromatin immunoprecipitation assays were performed in untreated LβT2 cells. Under basal conditions (Fig. 11C), both WT1 and phosphorylated RNA Polymerase II associate with the promoter, suggesting that WT1 may play a role in regulating transcription.

GnRH decreases WT1 mRNA and Protein

The most critical regulatory pathway for LHβ is through GnRH, which induces synthesis of the early response gene and transcription factor Egr1; Egr1 then binds to GC-rich DNA motifs similar to those for Sp1 and WT1 and stimulates transcription (29,123,124) . To investigate if GnRH also regulates WT1, LβT2 cells were treated with or without GnRH for 90min and WT1 (+KTS, -KTS) mRNA levels and WT1 protein levels were measured. Fig. 12 shows that GnRH actually reduces the mRNA levels of both the WT1 splice variants by approximately 50%. Over 10-12 experiments, the relative amounts of +/-KTS variants that were expressed varied somewhat between experiments, as did the degree of GnRH suppression. However, overall the two mRNA variants were expressed under basal conditions and regulated by GnRH.

We also compared GnRH effects on the protein expression levels of both WT1 and Egr1 in the same experiment. LβT2 cells were treated with GnRH over a period of 0-3.5

h. WT1 or Egr1 proteins were measured by immunoblotting, then normalized to β–actin levels and quantified by densitometry analysis. Fig. 12 showed that WT1 was easily detected in cells without GnRH stimulation, when Egr1 was not expressed. In the presence of GnRH, WT1 protein levels were decreased by approximately 50%, within 30- 60 min but were maintained at this level between 3-3.5h. In contrast, Egr1 protein was transiently and robustly stimulated within 30 min of GnRH treatment, with highest expression levels by 1 h of GnRH. Thus, GnRH differentially regulates WT1 and Egr1, by suppressing WT1 and stimulating Egr1 expression.

GnRH stimulates WT1 and Egr1 association with the LHβ promoter

GnRH stimulates the binding of transcription factors and co-regulatory proteins on the LHβ promoter (29). Since GnRH regulates the mRNA and protein expression levels of Egr1 and WT1, we investigated how GnRH might regulate WT1 association with the LHβ promoter by performing chromatin immunoprecipitation assays in the presence of GnRH (Fig 13). In the presence of GnRH, transcription factors and RNA Pol II associate with the promoter in a rhythmic fashion, with an interval time of approximately 30 min (29); both WT1 and pRNA Pol II show this pattern of association. Egr1 was recruited to the promoter with a similar pattern, but with a somewhat delayed association. Maximal Egr1 association required more than 30 min, reaching highest values at 60 min, correlating with the time needed for GnRH-stimulated synthesis, between 30 and 60 min (as in Fig. 12). Thus, WT1 might modulate LHβ transcription at times when Egr1 is present or absent on the promoter.

Reduced levels of endogenous WT1 influence basal and GnRH-stimulated endogenous LHβ gene transcription

To further investigate a potential role for WT1 on endogenous LHβ gene transcription, we decreased endogenous WT1 expression via targeted knockdown by siRNA. A non-targeting siRNA was used in parallel as a control. After 72 h of siRNA treatment, cells were incubated with GnRH for 90 min, and LHβ primary transcript mRNA was measured. As shown in Fig. 14A, knockdown of WT1 (-KTS) mRNA alone reduced endogenous WT1 protein by approximately 50%. Under these conditions, basal LHβ mRNA transcription was not significantly affected, but GnRH-stimulated transcription was suppressed by approximately 50%. When both mRNA isoforms for WT1 were decreased, with endogenous WT1 protein decreased by >90% (Fig. 14B), basal LHβ transcription was significantly increased (approximately 2-fold) compared to siControl, and GnRH treatment resulted in lower LHβ primary transcript levels compared to siControl GnRH, or siWT1 cells treated with vehicle. Because WT1 is a transcriptional regulator, and because Egr1 expression is important for LHβ transcription, we also tested the effects of WT1 variant knockdown on the basal and GnRH-stimulated expression of Egr1 mRNA. Interestingly, knockdown of WT1 (-KTS) alone reduced GnRH stimulation of Egr1 mRNA by approximately 60%, similar to the suppression of GnRH-stimulated LHβ transcription. In contrast, knock-down of both WT1 isoforms does not suppress Egr1 expression and GnRH stimulation of Egr1 occurs even though LHβ transcription is not stimulated by GnRH. These data suggest that there may be

different, and perhaps even opposing, biological roles for the two WT1 variants on LHβ transcription.

WT1 +KTS and WT1-KTS variants differentially regulate LHβ promoter activity

To directly test the role of the WT1 +KTS and –KTS splice variants in LHβ transcription, basal and GnRH-stimulated activity of the LHβ promoter (-617 to +44 bp) was measured in the absence or presence of increasing amounts of WT1 (+KTS) or WT1 (–KTS) expression vectors. WT1 (-KTS) overexpression significantly increased the GnRH stimulation of LHβ promoter activity by approximately 3-to 4-fold at the highest concentrations (Fig 15A). The -617LHβ promoter contains two regions that might be influenced by WT1, including the distal enhancer that contains Sp1 binding sites, and the proximal enhancer containing Egr1 binding sites; both are required for effective GnRH-stimulated transcription to occur (30,32). We thus assessed whether WT1 was able to influence LHβ transcription through the proximal region by testing the LHβ luciferase (-245 to +44 bp) construct containing only proximal GnRH response elements. As shown in Fig 15B, WT1 (-KTS) overexpression significantly increased the basal (up to 3-fold) and GnRH stimulated-LHβ promoter activity of the shorter construct up to 4-fold. In contrast, WT1 (+KTS) overexpression significantly decreased the basal and GnRH-stimulated LHβ promoter activity of both the -617 LHβ (Fig 16 A) and the shorter -245 bp constructs (Fig. 16B), by up to 70% at the highest WT1 concentrations.

Our overexpression data suggests that WT1 splice variants +KTS and –KTS could differentially regulate LHβ transcription, with WT1 (–KTS) being a positive regulator and

WT1 (+KTS) being a negative regulator. In both cases, the proximal promoter containing the Egr1 sites is sufficient to impart WT1 regulation. The proximal GnRH-responsive region also contains binding sites for Ptx1 and SF1; SF1 and Egr1 bind to adjacent sites and all three proteins can form complexes that play an important role in basal and GnRH stimulated LHβ transcription (37). Thus, protein-protein interactions on DNA at Egr1 or SF1 sites may also be important for WT1 effects. To directly test if the WT1 variants act through the same or different DNA elements, LHβ promoter (-617 to +44bp) luciferase constructs containing mutations specifically in both 5'- and 3'-SF1 sites, or individual 3'- or 5-'Egr1 sites, were tested for the response to WT1 variant overexpression in the presence or absence of GnRH. As seen in Figure 17 and 18, mutation of the 3'-Egr1 site, but not the SF1 sites, in the LHβ promoter abrogated the effects of the WT1 (-KTS) variant (Fig.17). In contrast, mutation of the SF1 sites or the 3'-Egr1 site eliminated the effects of the WT1 (+KTS) variant on the LHβ promoter (Fig.18). Thus, WT1 (-KTS) appears to mediate its effects exclusively via Egr1 sites, whereas the WT1 (+KTS) variant requires both Egr1 sites and SF1 sites for its biological effects.

Expression and regulation of WT1 variants in normal mouse pituitary

To test if WT1 variants are expressed and regulated in normal gonadotrope cells in addition to the clonal mouse gonadotrope cell line, mRNA was collected from mouse pituitary cells under different physiological conditions and WT1 mRNA variants were measured. As shown in Figure 19, both WT1 variants were expressed in pituitary glands from ovariectomized female mice (upper panel). WT1 (-KTS) expression was

highest in ovariectomized mice treated with 17β-estradiol compared to those treated with vehicle alone, whereas WT1 (-KTS) mRNA was not significantly changed between the two groups although the trend was to lower expression in pituitary cells from ovariectomized mice. In mouse pituitary cells treated in culture with GnRH (lower panel) , WT1 (-KTS) mRNA levels were decreased approximately 75% by GnRH, while WT1 (+KTS) mRNA was not significantly changed with GnRH treatment.

DISCUSSION

In this work, we show that WT1 regulates LHβ transcription, and that the –KTS and +KTS have differential roles in this regulation. The function of WT1 is well established in the urogenital (kidney and gonads) system (74,83,87,88), but its role has been largely unexplored in the pituitary. We demonstrate that WT1 is expressed in LβT2 cells, an immortalized mouse gonadotrope cell line, is regulated by GnRH (Fig.11 and Fig. 12), binds to the LHβ promoter, and regulates promoter activity (Fig.13, 15 and16). Both WT1 mRNA variants are expressed in normal mouse pituitary (Fig 19) and the WT (-KTS) variant also appears to be significantly regulated by GnRH

As a Zinc-finger transcription factor, WT1 binds to GC-rich regions common to Egr1 and SP1, but often under different physiological conditions or with different biological outcomes (62,63,88,124). For example, WT1 and Egr1 have been shown to regulate the expression of the STIM1 gene by competing for one of the common binding sites on the STIM1 promoter. Egr1 stimulates STIM1 expression and WT1 antagonizes this effect by binding to Egr1 binding sites (124). In addition, co-ordination of the differential

binding pattern of the transcription factors WT1, Egr1, and Sp1 in time-dependent manner was shown to be involved in the regulation of the α Isoform of the human thromboxane A2 receptor during megakaryocyte differentiation (63). In response to PMA, there is an initial induction of Egr1 and reduction of WT1 association with this promoter, followed by SP1 occupancy after sustained PMA treatment (63).

Our data shows mRNA expression of the two most prevalent WT1 splice variants, –KTS and +KTS, and presence of the WT1 protein in untreated cells (Fig 11). Chromatin immunoprecipitation (ChIP) assays showed the association of WT1 to LHβ promoter in untreated cells, suggesting that WT1 could modulate basal LHβ expression (Fig 11). The differential regulation of WT1 and Egr1 by GnRH in LβT2 cells (Fig. 12), suggests that Egr1 and WT1 may play somewhat different roles in LHβ expression. WT1, but not Egr1, was expressed in untreated cells, and while GnRH dramatically induces Egr1 protein, WT1 protein is reduced by approximately 50%. GnRH stimulates LHβ transcription, and induces cyclic binding of the transcription factors Egr1 and SF1, and RNAPol II, on the LHβ promoter to drive transcription (29). In this work, we showed that GnRH stimulates the cyclic association of both WT1 and Egr1 to the LHβ promoter, but Egr1 occupancy lags that of WT1, as GnRH must first stimulate Egr1 protein synthesis (Fig 13). These data suggest involvement of WT1 in GnRH-stimulated LHβ transcription as well. Antibodies are not available that can distinguish between the WT1 (-KTS) and WT1 (+KTS) variants, and we cannot distinguish if both variants are binding to the promoter, or if one variant binds preferentially under basal or GnRH-stimulated conditions, by this method.

siRNA mediated knockdown of WT1 (-KTS) mRNA alone did not significantly alter basal LHβ mRNA transcription, measured by levels of LHβ primary transcript mRNA, but decreased GnRH-stimulated transcription by approximately 50%. This decrease correlated with the decreases in Egr1 mRNA primary transcript under the same conditions (Fig. 14A). In comparison, overexpression of WT1 (-KTS) significantly increased GnRH stimulation of LHβ promoter activity by approximately 3-to 4-fold at the highest concentrations (Fig 15); a small but significant increase in basal expression was also noted with the smaller promoter construct containing Egr1 sites and the proximal GnRH response region. Based on mutation data (Fig. 17) only the 3'-Egr1 site, and not SF1 sites, is required for WT1 (-KTS) activity on LHβ. This is in keeping with a positive role for WT1 (-KTS) in LHβ transcription, via Egr1 expression and Egr1 binding sites. The Egr1 promoter contains GC-rich motifs that bind Egr1 (125) and potentially other related transcription factors such as WT1, so WT1(-KTS) regulation of this gene may be direct.

In contrast, when both mRNA isoforms for WT1 were decreased, and endogenous WT1 protein decreased by >95% (Fig. 14B), basal LHβ transcription was significantly increased (approximately 2-fold), and GnRH treatment decreased transcription below basal levels (Fig 14B). GnRH-stimulated Egr1 primary transcript mRNA was not reduced, as was the case when only WT1 (-KTS) was knocked down. WT1 (+KTS) may antagonize the effects of WT1 (-KTS) on Egr1 expression directly, or act via other genes and pathways. Overexpression of WT1 (+KTS) alone decreased both basal and

GnRH-stimulated LHβ promoter activity (Fig. 16), and required both the 3'Egr1 site and SF1 sites (Fig. 18).

These data suggest a scenario in which WT1 (+KTS) acts to suppress LHβ, while WT1 (-KTS) acts primarily to enhance GnRH stimulation of LHβ promoter activity . WT1 binding to the LHβ promoter under basal (no GnRH) conditions could help maintain promoter activity at low levels, and WT1 is associated with the promoter in the absence of GnRH (Fig 11). WT1 (-KTS) would have positive effects on the promoter, while WT1 (+KTS) would be suppressive, and there may be competition between the two isoforms for association with the LHβ promoter at the same gene site. Because WT1 exerts its actions only via the 3'-Egr1 site, stimulated Egr1 expression in the presence of GnRH would presumably result in more effective promoter occupancy at both Egr1 sites, and greater LHβ transcription. This is consistent with the lesser ability of WT1 (-KTS) alone to increase LHβ promoter activity compared to promoter stimulation with GnRH (Fig. 15), and the ability of Egr1 overexpression to effectively substitute for GnRH in stimulating LHβ promoter activity (26,29). Interestingly, WT1 (-KTS) appears to be more tightly regulated in normal pituitary cells (Fig. 19). In LβT2 cells, GnRH treatment would decrease expression of the repressor WT1 (+KTS), as well as the stimulator WT1 (-KTS), but because Egr1 is more effective on LHβ than WT1 (-KTS), overall LHβ promoter activity will be much higher. It is expected that WT1 (-KTS) and WT1 (+KTS) could compete for binding to the LHβ promoter at the same sites.

The failure of GnRH to stimulate LHβ with complete WT1 protein knockdown in Fig. 14 is less straightforward. Increased basal activity may occur due to loss of suppression by WT1(+KTS), but changes in Egr1 are not sufficient to explain this result and both WT1 isoforms may influence other genes or pathways that could influence LHβ transcription. The +KTS variant was shown to be involved in RNA processing and RNA metabolism (90), but a recent report showed that +KTS can also bind to DNA on the planar cell polarity gene promoter SCRIBBLE and regulate its transcription in developing kidney (93). WT1 acts synergistically with the transcription factor SF1 to regulate the expression of the mullerian inhibiting substance gene during development of male gonads (74), and to regulate the expression of α-inhibin in sertoli cells (119). Both DNA binding and protein-protein interactions with SF1 may play a role in LHβ promoter regulation by WT1 (+KTS). Additional protein-protein interactions with WT1 (+KTS) could also contribute to these biological effects.

Differential regulation of transcription by the two WT1 splice variants has been noted for some other genes. For example, WT1+KTS strongly represses the insulin receptor promoter, whereas repression by WT1–KTS is more moderate and occurs only in the presence of additional C/EBPβ or a dominant negative p53 (120). The –KTS splice variant of WT1 has been shown to stimulate α-inhibin expression in sertoli cells of the testis but the +KTS variant had no such effect (119). For LHβ, the two variants appear to have opposing roles in transcription, and the relative balance between the two forms may be crucial. Interestingly, mutations in intron 9 of the WT1 gene, where alternative

splicing to generate WT1+KTS and –KTS occurs, result in Frasier syndrome, including sex reversal and developmental defects in kidney and gonads (94,95,126,127). Mutations in some Frasier syndrome patients result in the predicted decrease of the WT1 (+KTS) isoform and diminution of the WT1 (+KTS/-KTS) isoform ratio (94,95). Given the crucial role of WT1 in development of the reproductive organs and urogenital tract, and the necessary feedback between steroids on the hypothalamus and pituitary, studies to evaluate a potential role of *WT1* mutations on pituitary function are difficult. However, a WT1 mutation (IVS9+5G>A) that causes Frasier syndrome has also been linked to hypergonadotropic hypogonadism and increased serum levels of gonadotropins (LH and FSH) in patients (126). In at least one patient with a WT1 mutation (IVS9+4C>T) and high basal LH, both a decrease in the WT1 (+KTS) and an increase in the WT1 (–KTS) isoforms were observed (127). These observations are in agreement with the increase in basal LH transcription observed in our siRNA studies when knockdown of both WT1 (+KTS) and WT1 (–KTS) occurred, and not when only the WT1 (-KTS) variant was reduced.

Overall our data suggests that the WT1 (+KTS) and (-KTS) splice variants play a differential and opposing role in regulating LHβ transcription. The role of WT1 as an activator or repressor seems to be context and promoter specific, and could also be influenced by the ratio of its splice variants if they exert opposing effects on the same promoter. We observed that the proximal GnRH responsive element region containing the two Egr-1 binding sites, and the two SF1 binding sites, is sufficient to exert the effects of WT1. Both direct WT1 binding to DNA and protein-protein association with

SF1 could play a role in WT1 function in this system. The two splice variants (-KTS and +KTS) of WT1 acting as a positive and negative regulator, respectively, to regulate LHβ gene transcription, defines a novel regulatory role of WT1 in pituitary gonadotropes and the reproductive system.

FIGURES:

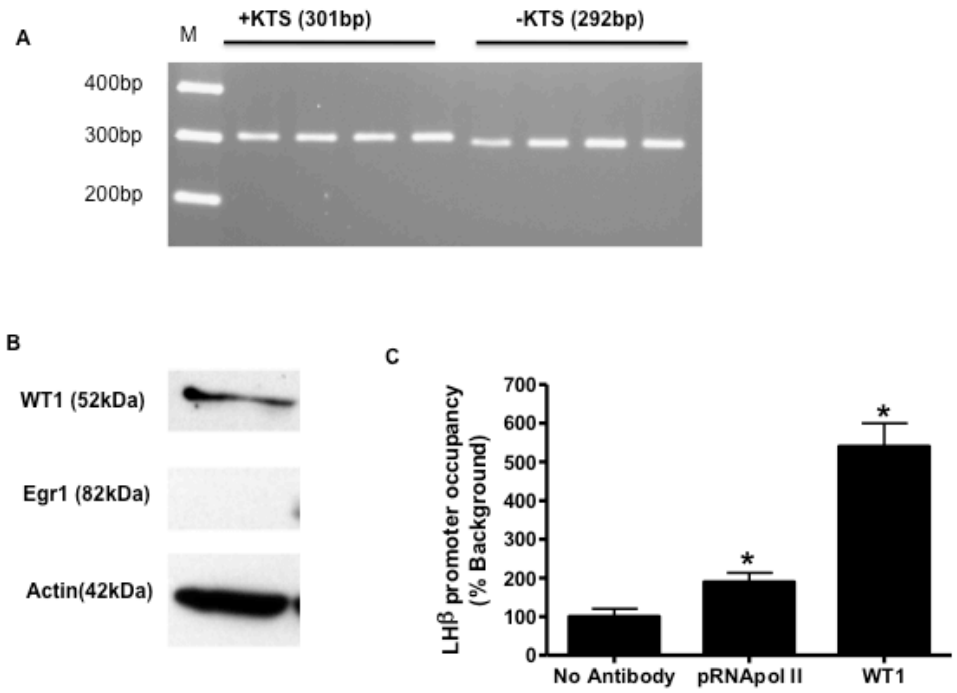

Figure 11 WT1 (-KTS and +KTS) expression and chromatin association in LβT2 cells.

Figure 11 WT1 (-KTS and +KTS) expression and chromatin association in LβT2 cells.

A.WT1 mRNA (+KTS and –KTS) splice variant PCR products expressed in LβT2 cells. Whole cell RNA was extracted from LβT2 cells, reverse transcribed to cDNA and quantified by real-time PCR, using specific primers to detect +KTS and –KTS splice variants, then displayed on a 1% agarose gel. Bands corresponding to the products for +KTS (301bp) and –KTS (292 bp) WT1 were detected in 4 independent samples of mRNA. B. WT1 and Egr1 protein expression in LβT2 cells. Cell proteins (30μg) were separated on 10% polyacrylamide-SDS gels, and then analyzed by immunoblotting with specific antibodies for WT1, Egr1 and β-actin. Specific proteins were detected in the same samples of untreated LβT2 cells. C. Chromatin association of WT1 with the endogenous LHβ promoter in LβT2 cells. The association of WT1 and RNA Polymerase II with the LHβ promoter in untreated LβT2 cells was measured by Chromatin immunoprecipitation assays with antibodies against WT1 and phosphorylated RNApol II, as well as control (no Antibody). LHβ promoter occupancy was measured by quantitative real time PCR using primers specific for the LHβ promoter, and normalized for chromatin input in each sample. In this study, background binding (no Antibody) was set at 100% and association of RNA Polymerase II and WT1 are expressed relative to background values. Association was measured in 3 independent experiments with duplicate samples.

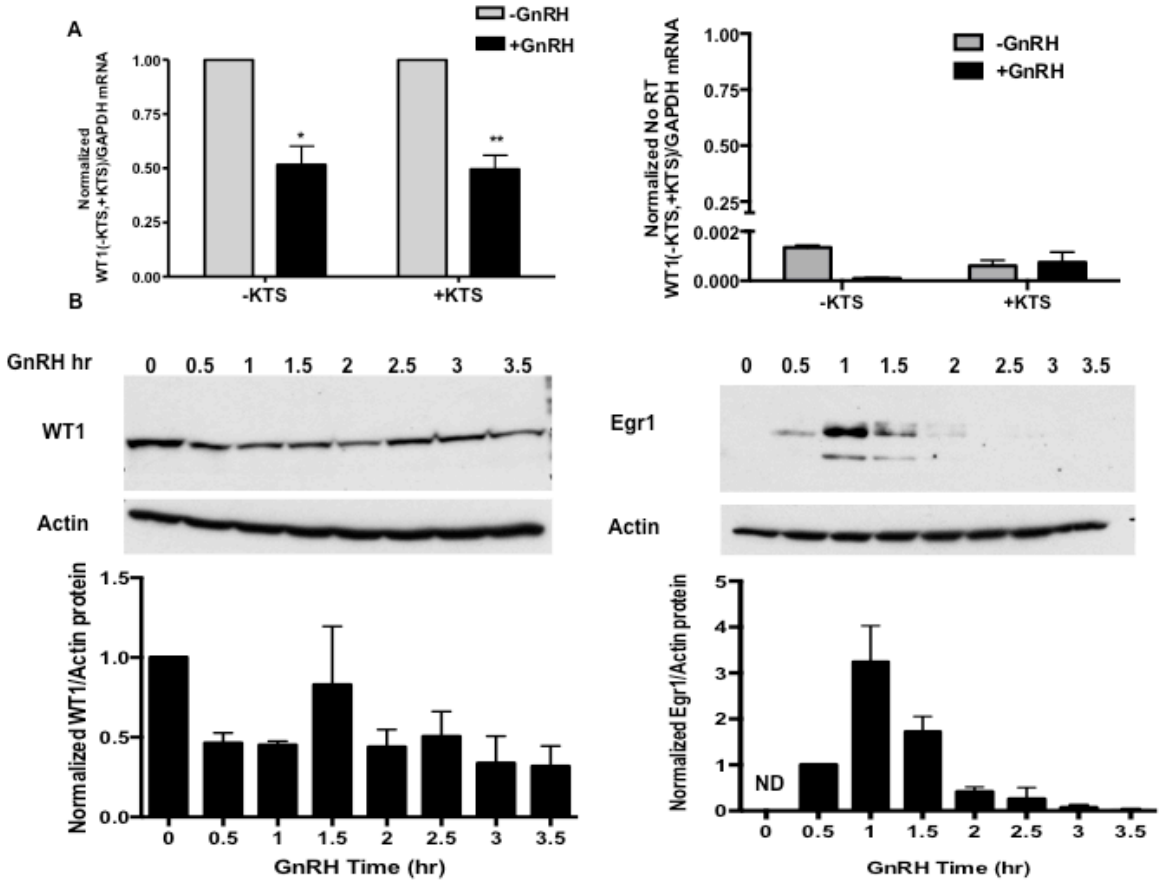

Figure 12 WT1 mRNA and protein levels are decreased by GnRH.

Figure 12 WT1 mRNA and protein levels are decreased by GnRH.

A. WT1 (- KTS) and (+ KTS) mRNA variant levels in LβT2 cells treated with 50nM GnRH for 90min. RNA was extracted and mRNAs levels measured by RT-PCR and normalized against GAPDH mRNA. Values for WT1 mRNAs are shown for both Reverse Transcriptase (RT) and no RT (negative control) conditions. Note that for no RT, Y-axis is interrupted and expanded to show the low values. Data is the mean ± SEM from 5-7 experiments. *= P<0.05, -GnRH vs +GnRH; ** = P<0.001, -GnRH vs +GnRH

B. WT1 and Egr1 protein levels in LβT2 cells treated with 50nM GnRH for 0 to 3.5 h. The experiment was performed three times. Upper panel: Cells were lysed after GnRH treatment and proteins (30 μg) were separated by 10% SDS-PAGE, then detected with antibodies against WT1 or Egr1. Immunoblotting for β-actin was performed on the same blots as WT1 and Egr1, and used for normalization of these proteins quantified by densitometry analysis. A representative blot is shown. Lower panel: Quantification of protein bands was performed with densitometry, and normalized protein levels are shown from combined experiments. Bands for Egr1 protein were not detected (ND) in any blot at time zero without GnRH.

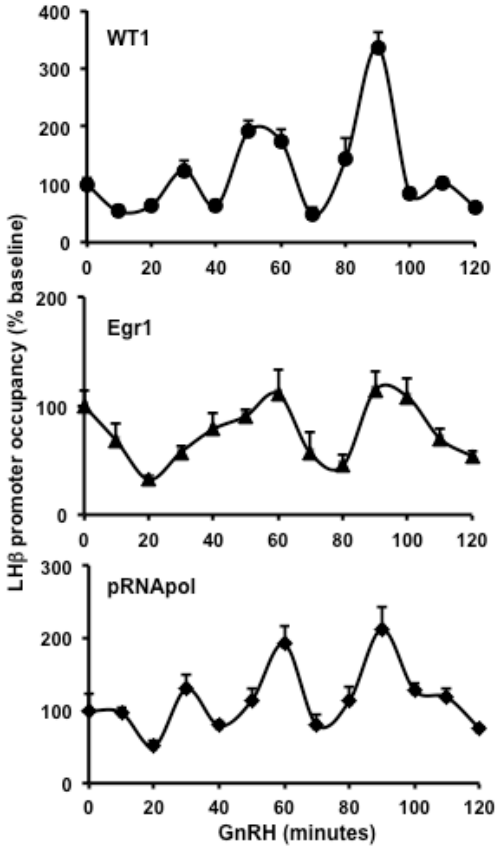

Figure 13 WT1 and Egr1 occupancy of the endogenous LHβ promoter in response to GnRH.

Figure 13 WT1 and Egr1 occupancy of the endogenous LHβ promoter in response to GnRH.

LβT2 cells were incubated with or without 50 nM GnRH and collected every 10 min for 120 min. ChIP assays were performed using antibody against WT1, Egr1 and phosphorylated (active) pRNA Pol II. LHβ promoter occupancy was measured by quantitative real time PCR using primers for the LHβ promoter. The experiment was performed three times with duplicate samples and replicate PCR measurements in each sample. Data are presented as the mean + SEM and expressed as LHβ promoter occupancy relative to basal (no GnRH at time zero) binding.

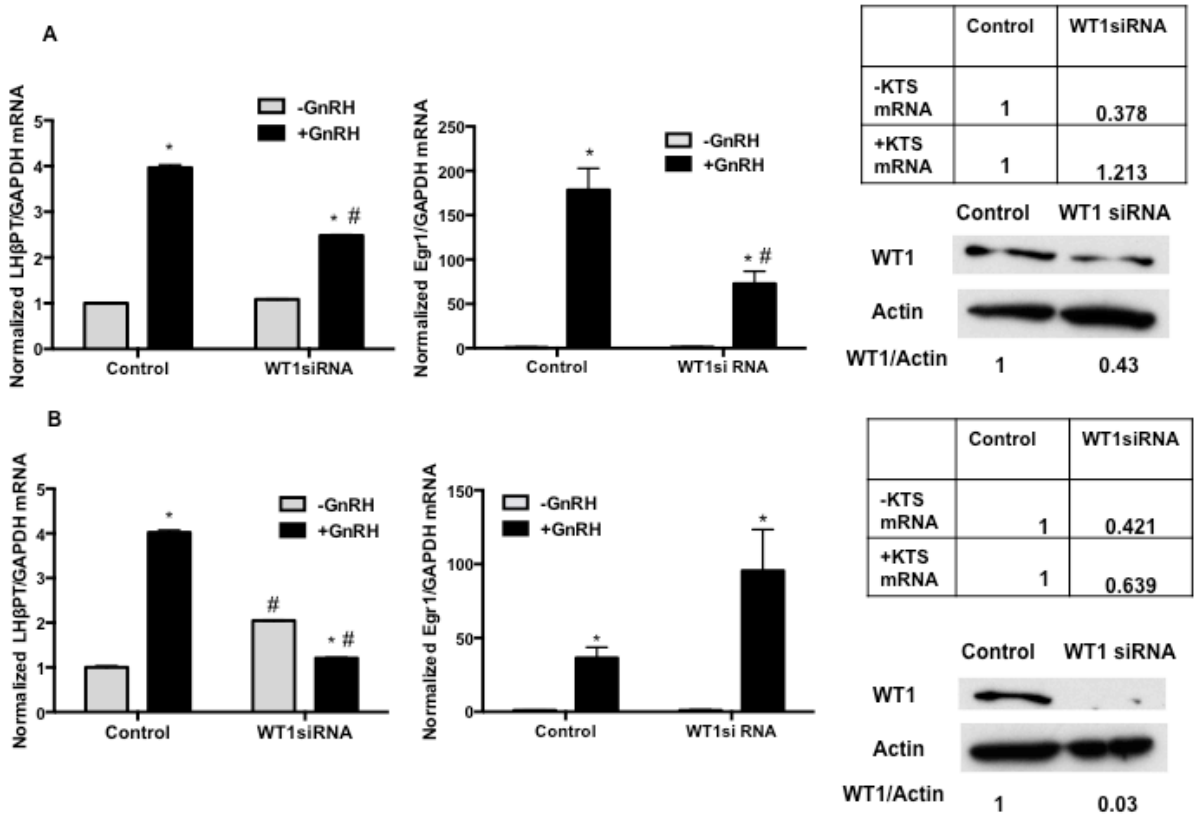

Figure 14 WT1 siRNA increases the basal and decreases GnRH stimulated expression of endogenous LHβ primary transcript.

Figure 14 WT1 siRNA increases the basal and decreases GnRH stimulated expression of endogenous LHβ primary transcript.

LβT2 cells were transfected with siRNA against WT1 and a non-targeting siRNA as a control. After 72 h of incubation, cells were treated with or without GnRH for 1.5 h, followed by cell lysate collection, RNA extraction and western blot analysis (30 μg protein) on 10% PAGE-SDS gels. WT1 protein was detected by immunoblotting with specific antibodies for WT1, and normalized for β-actin on the same blot. A. Expression of the endogenous LHβ primary transcript, Egr1 primary transcript mRNA, and WT1 protein under conditions where the WT1 (-KTS) variant mRNA was reduced. B. Expression of the endogenous LHβ primary transcript, Egr1 primary transcript mRNA, and WT1 protein under conditions where both the WT1 (-KTS) and WT1 (+KTS) variant mRNAs were reduced. For each condition, the experiment was performed twice with 5 replicates. LHβ and Egr1 primary transcript mRNAs were normalized for GAPDH mRNA in the same sample. Control samples contained non-targeting siRNA. * P<. 05 -GnRH vs +GnRH, in either Control siRNA or WT1 siRNA treatments. Values are mean ± SEM for 5 replicates. # = P<. 05 -GnRH Control siRNA vs –GnRH WT1 siRNA, or p<. 05 +GnRH Control siRNA vs +GnRH WT1 siRNA.

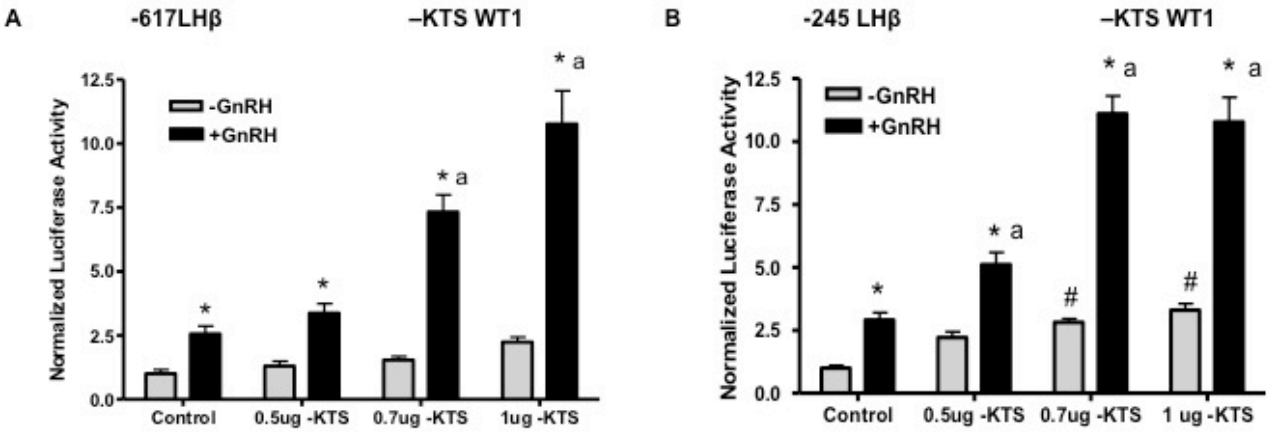

Figure 15 WT1 (-KTS) enhances GnRH-stimulated LHβ promoter activity

Figure 15 WT1 (-KTS) enhances GnRH-stimulated LHβ promoter activity.

LβT2 cells were transfected with either A: A luciferase reporter construct driven by the rat LHβ promoter (-617 to +41 bp) including both distal and proximal GnRH responsive promoter regions, or B: A luciferase reporter construct driven by the rat LHβ promoter (-245 to +44 bp), including only the proximal GnRH response region of the promoter. Constructs were cotransfected with or without 0.5, 0.7, or 1 μg of WT1 (-KTS) plasmid, or control plasmid to normalize total DNA. After 48 h post-transfection, cells were treated with 50nM GnRH for 6hrs and collected in lysis buffer. Luciferase activity was measured, and data expressed as mean ± SE for 6 samples; the experiment was performed 3 times. Statistical significance was determined using ANOVA (confidence interval determined by the Bonferroni multiple comparison test). * P<.05 -GnRH vs +GnRH, # P<.05 control,-GnRH vs –GnRH+WT1 , a P<.05 control,+GnRH vs +GnRH +WT1

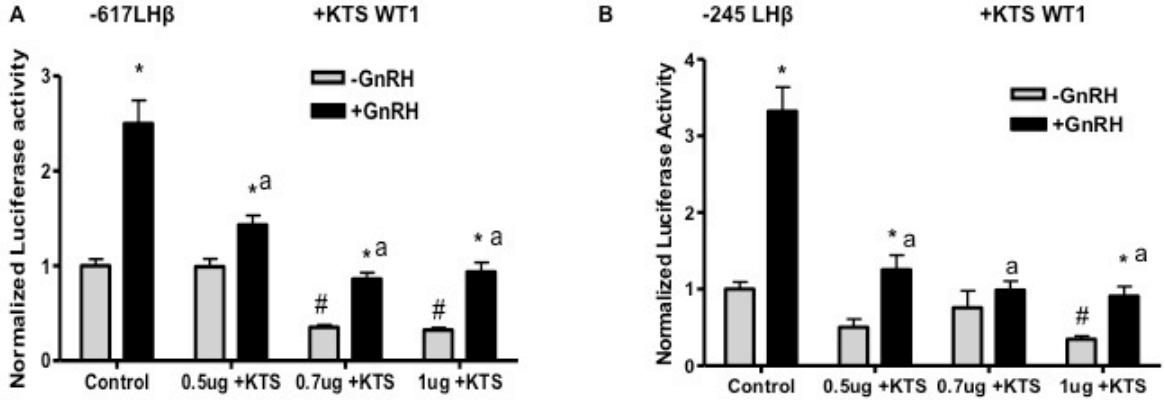

Figure 16 WT1 (+KTS) decreases basal and GnRH-stimulated LHβ promoter activity.

Figure 16 WT1 (+KTS) decreases basal and GnRH-stimulated LHβ promoter activity.

LβT2 cells were transfected with either **A)** A luciferase reporter construct driven by the rat LHβ promoter (-617 to +41 bp) including both distal and proximal GnRH responsive promoter regions, or **B)** A luciferase reporter construct driven by the rat LHβ promoter (-245 to +44 bp), including only the proximal GnRH response region of the promoter. Constructs were cotransfected with or without 0.5, 0.7,1 μg of WT1 (+KTS) plasmid or control plasmid to normalize DNA. At 48 h post-transfection, cells were treated with 50nM GnRH for 6 h and collected in lysis buffer. Luciferase activity was measured, and data expressed as mean ± SE for 6 samples; the experiment was performed 3 times each. Statistical significance was determined using ANOVA (confidence interval determined by the Bonferroni multiple comparison test). * P< .05 -GnRH vs +GnRH , #= P<.05 control,-GnRH vs –GnRH+WT1 , a = P<.05 control,+GnRH vs +GnRH +WT1

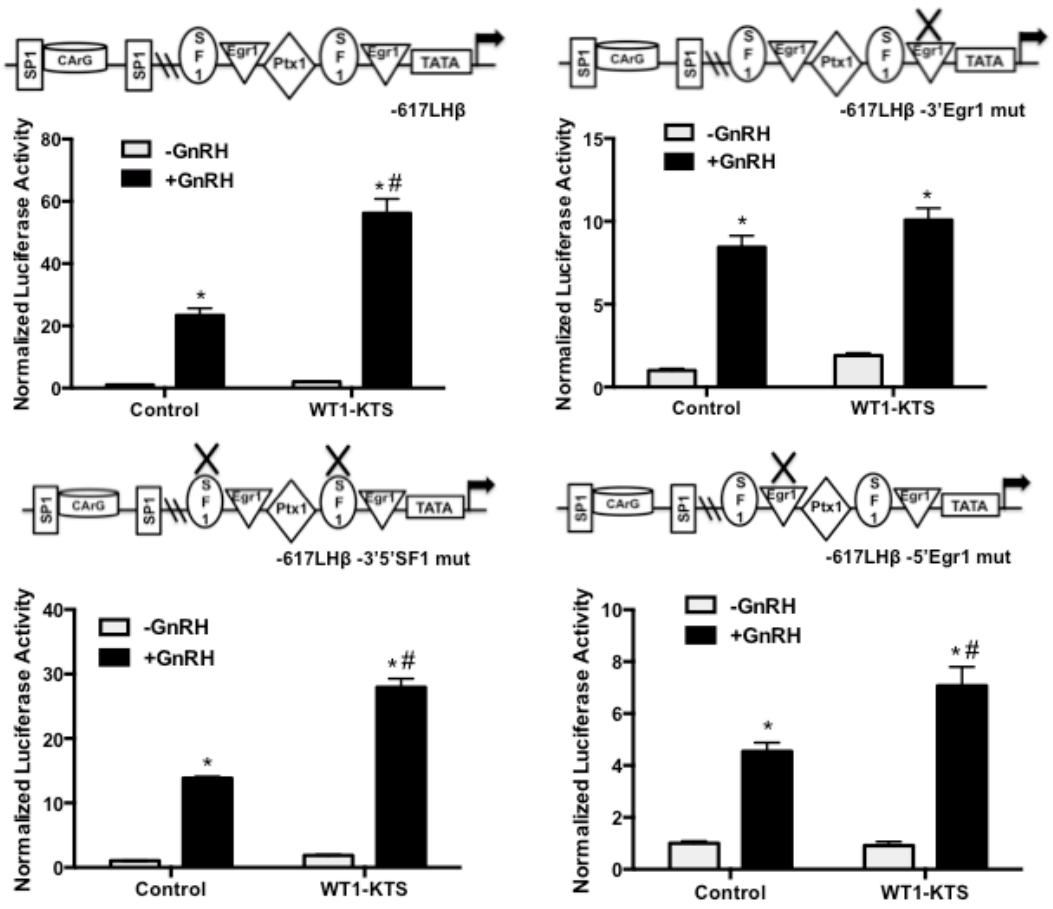

Figure 17 Differential responses of LHβ promoter mutants to WT1 (-KTS)

Figure 17 Differential responses of LHβ promoter mutants to WT1 (-KTS)

LβT2 cells were transfected with luciferase constructs containing either the wild type (-617 to +44bp) LHβ promoter, or the same construct mutated at both SF1 sites, or the individual 5'Egr1 or 3'Egr1 sites. Constructs were cotransfected with or without 1 μg of WT1(+KTS; upper panel) or WT1(-KTS, lower panel) plasmid or control plasmid to normalize DNA. At 48 h post-transfection, cells were treated with 50nM GnRH for 6 h and collected in lysis buffer. Luciferase activity was measured, and data expressed as average ± SE for 6 samples; the experiment was performed 3 times each. Statistical significance was determined using ANOVA (confidence interval determined by the Bonferroni multiple comparison test). * P< .05 -GnRH vs +GnRH, #= P<.05 control,-GnRH vs –GnRH+WT1 , a = P<.05 control,+GnRH vs +GnRH +WT1

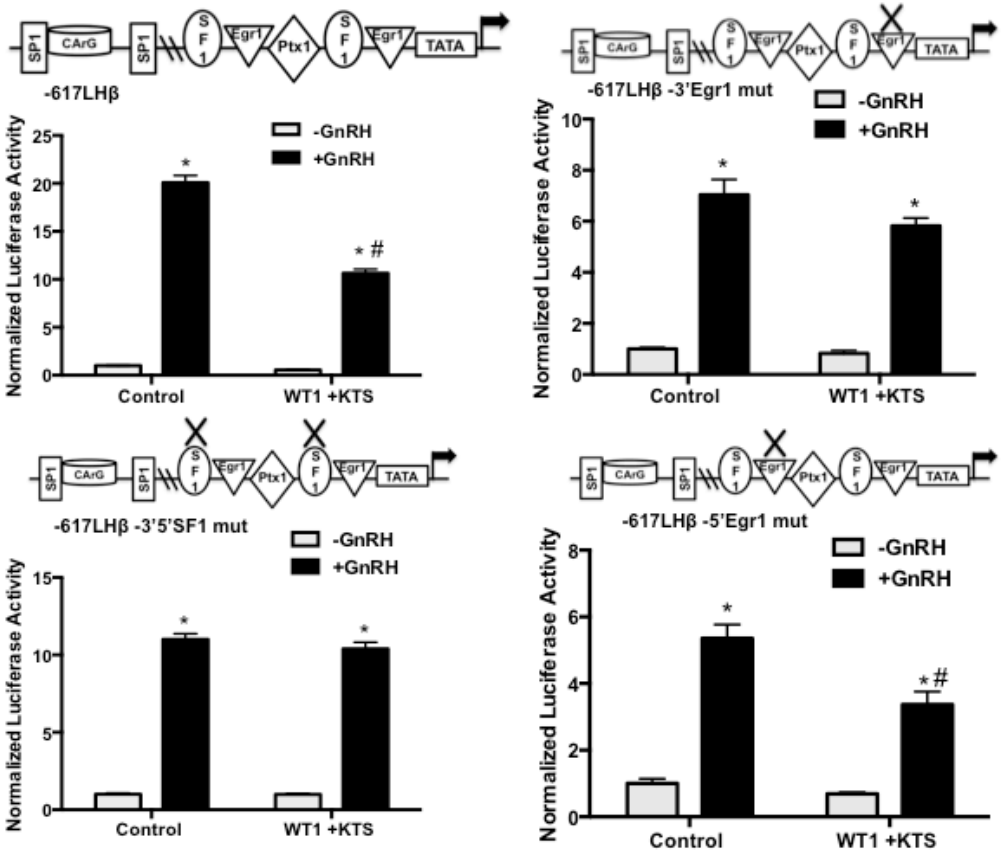

Figure 18 Differential responses of LHβ promoter mutants to WT1 (-KTS)

LHβ promoter mutants were transfected into LβT2 cells with or without 1μg of WT1 (+KTS) plasmid or control plasmid to normalize DNA. Data were analyzed and expressed as in Figure 15.

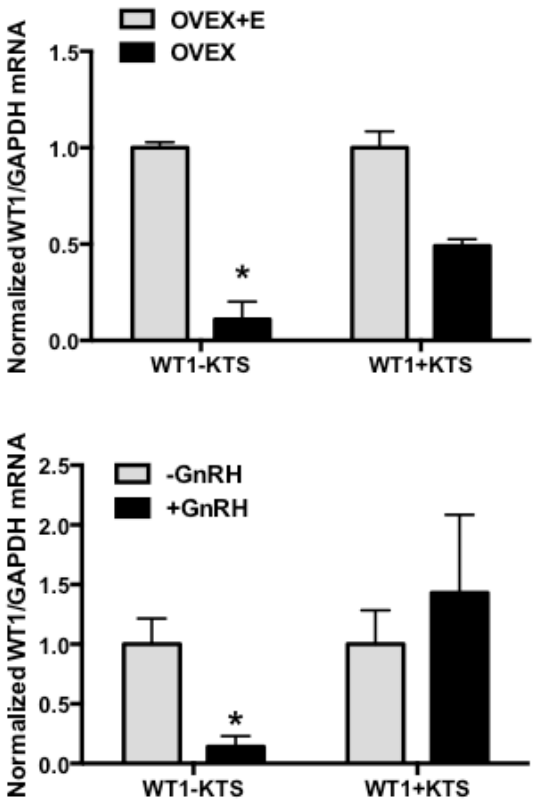

Figure 19 Expression and regulation of WT1 variant mRNA in mouse pituitary cells

WT1 variant mRNAs were measured by quantitative RT-PCR and normalized to GAPDH mRNA in the same samples in pituitary glands from: (Upper panel) Ovariectomized (OVEX) mice treated *in vivo* with vehicle or E2 for 3 d or (Lower panel) Pituitary glands from intact mice treated in culture with 5 nM GnRH for 30 min. Data are from 4 independent determinations and expressed as the mean $\pm$ SEM. * $P < 0.05$ OVEX vs OVEX + E, or Control vs GnRH.

Chapter 3: Proteasomal regulation and role of transcription co-regulator DAX1 in LHβ transcription.

INTRODUCTION

Gonadotropin hormones, secreted from the anterior pituitary, control female reproduction. Luteinizing hormone (LH) is necessary for ovulation and steroidogenesis while Follicle Stimulating Hormone (FSH) is required for follicle maturation (1,5,11). Hormonal dysregulation causing an incorrect ratio of LH/FSH synthesis/secretion might lead to infertility syndromes such as polycystic ovarian syndrome, which is characterized by high LH levels, elevated androgens, and erratic menstrual cycles in women (11). Understanding the transcriptional regulation of the gonadotropin genes can be beneficial in developing future therapies. LH and FSH consist of two subunits, a shared alpha subunit, and unique beta subunits, which are limiting for the intact hormones. Hypothalamic GnRH is a crucial modulator of these three gonadotropin genes. Among the three subunit genes, GnRH most dramatically and precisely regulates LHβ, although there are always high levels of the α-subunit present in gonadotrope cells (1,5,6,116).

The LHβ promoter is comprised of two GnRH responsive regions. The distal region has two SP1 binding sites and a CArG box (30,31). The proximal GnRH response element, conserved across all mammalian species including humans, consists of two Egr1 (Early Growth Response 1) and SF1 (Steroidogenic Factor 1) binding sites and a binding site for the homeobox protein Ptx1. Previous studies show that for full transcriptional activation of the rat promoter, an interaction between the distal and proximal response elements is required (31,32,61).

In response to GnRH, synthesis of early zinc-finger transcription factor Egr1 (early growth response1) occurs and is important for LHβ transcription. Egr1 knock-out mice are infertile primarily due to lack of LHβ expression (59), and mutation of either Egr1 site has been shown to significantly decrease both basal and GnRH stimulated LHβ transcription (30). Egr1 acts in concert with the orphan nuclear receptor SF1 to induce LHβ transcription (29,59,61,128). SF1 is an important transcription factor regulating genes related to steroidogenesis and reproduction. SF-1 knock-out mice showed failure of development of the adrenal glands, gonads, ventromedial hypothalamus and proper functioning of pituitary gonadotrophs (34,56,67,117). In the pituitary, SF1 is involved in the transcription of α and β subunit genes of LH and FSH, and also the GnRH receptor (33,35). In response to GnRH, cyclic and co-ordinated binding of the transcription factors EGR1 and SF1 occur on the chromatin associated with the LHβ promoter (29,60). GnRH-stimulated proteasomal degradation is required for the cyclic binding of SF1 and Egr1 on the promoter (29,112,128). These transcription factors recruit co-activators (increase transcription) or co-repressors (decrease transcription) to the promoter and ultimately regulate LHβ transcription. SRC1 and GCN5 are the typical co-activators of SF1 and SMRT is a typical co-repressor of SF1 (44,57,58). The cyclic binding of these co-regulators could also be influenced by proteasomal degradation, as is the case with transcription factor binding.

The co-regulator DAX1 was typically thought to be a repressor of SF1, but recently DAX1 has been shown to act either as a co-activator or co-repressor of SF1 depending on the cell and the promoter type (45,75). DAX1 is an orphan nuclear receptor encoded

by the gene NR0B1 and expressed throughout the entire HPAG axis (69). Mutation in this gene has been associated with the disorder AHC that causes underdevelopment of the adrenal cortex; secondary effects include hypogonadotropic hypogonadism (HH), a combined defect of both pituitary and the hypothalamus. Gene duplication causes dosage sensitive sex reversal (68,69). The structure of DAX1 differs from other general nuclear receptors in the sense that it doesn't have the typical DNA binding domain, and therefore acts mainly through protein-protein interactions (69). Evidence of physical interaction, similar expression patterns, and knock down defects of DAX1 and SF1 revealed that these two proteins take part in several common pathways (67,129). Several studies have depicted DAX1 to be acting as a repressor for SF1 target genes by physically interacting with SF1 (57,70). However, Xu et al showed DAX1 acting as a co-activator along with SRA (Steroid Receptor RNA Activator) for the SF1 target gene Mc2R (melanocortin 2 receptor) in the adrenal glands (75). The dosage of DAX1 relative to other factors like SF1 and WT1 has been implicated to play a role in human sex determination, based on interactions between the three factors (74). In this work, we investigated the role of the proteasome in the cyclic association of co-regulator proteins, including DAX1, to the LHβ promoter, and further explored the biological role of DAX1 in LHβ transcription. Our data showed that proteasomal degradation was necessary to maintain the proper cycling of the co-regulatory proteins on the promoter, and that DAX1 could act as a positive or negative regulator of GnRH regulated LHβ transcription in a dose dependent manner.

MATERIALS AND METHODS:

Cell culture, transient transfection and luciferase assay: All the experiments were performed using a clonal mouse gonadotrope cell line, LβT2 (originally obtained from Dr. Pamela Mellon, University of California, San Diego) as described in (29). Cells were maintained in Dulbecco's Minimal Essential Medium (DMEM) with 10%FBS and 1% antibiotic/ antimycotic (GIBCO, Grand Island, New York). For experiments, cells were plated in phenol red-free DMEM with 5% charcoal stripped newborn calf serum (SNCS) and 2%L-glutamine. Cells were treated with 50nM GnRH (Bachem Biosciences Inc, King of Prussia, PA under the name LHRH) as indicated. LβT2 cells were plated using DMEM plus 5% SNCS at the concentration of 500,000 cells per well in 12-well (20mm diameter) dishes. After 24h cells were transfected with a luciferase reporter construct driven by the rat LHβ promoter from -617 to +41 bp relative to the transcription start site (consisting of both distal and proximal GnRH response elements) or truncated promoter, 245 to +44 region (only the proximal region, contains only the downstream GnRH response element), 0.33 ug per reaction using Lipofectamine 2000 (Invitrogen, Carlsbad, California). In DAX1 overexpression/dose response studies constant (0.3ug) or several doses 0.1,0.3,0.5,1ug per reaction of DAX1 plasmid DNA was transfected along with the reporter plasmid. Empty vector pcDNA 3.1 plasmids were transfected in the control sets. To introduce mutations in the SF1 binding sites of the promoter, site-directed mutagenesis was performed using the QuikChange kit (Stratagene, La Jolla, CA) as described in (28). Bold letters in the following sequences indicate mutated residues: 3'NR5A1GCCTCTGCTTAGTGG**AA**TTCCCACCCCCACAACCCG, 5'NR5A1GTCCCTGGCTTTTCTGA**AA**TTGTCTGTCTCGCCCCC. After 48 h of

transfection, the cells were treated with or without GnRH for 6 h and the cell lysates were collected in 200ul of 1x passive lysis buffer (Promega, Madison, WI, cell culture lysis reagent). The samples were centrifuged at 13,000 rpm for 1 min and supernatant was collected. Luciferase activity was measured using a Turner TD-20e luminometer (Turner Designs, Mountain View, CA). Total proteins of each sample were measured using Bio-Rad (Hercules, CA) protein dye and used for normalizing the Luciferase activity of respective samples. Mean and standard error were calculated for 6 samples. Statistical significance was determined using paired student T-tests.

siRNA delivery and primary transcript assay:

siRNA were delivered into LβT2 cells using nucleofection technology according to the manufacturer's instructions (Amaxa Corp., Gaithersburg, MD) as previously described in (26). The LβT2 cells were transfected with siGENOME SMARTpool siRNA (0.2nM;Dharmacon RNA Technologies, Lafayette, CO) directed toward mouse DAX1 or SF1 or a non-targeting negative control siRNA (0.2nM,siCON #1; Dharmacon), in Solution T and using Program A-020. Each reaction contained 5 $X10^6$ cells and was divided between three wells in a 35-mm plate containing 2 ml 5% SNCS. 48h post-transfection cells were treated with GnRH for 90mins and cell lysates were collected for primary transcript assay and immunoblotting to confirm DAX-1 knockdown. Incase of siRNA-targeted knockdown of SF1 cells further were subjected to ChIP assay or luciferase assay after 72h. Experiments were performed in triplicate three times. Protein levels were analyzed on immunoblots. To measure specific mRNAs RNA was isolated

from the cell lysates using the QIAGEN (Valencia, CA) RNeasy kit and was briefly treated with DNase (Roche, Indianapolis, IN) to remove DNA contamination. Total RNA was subjected to reverse transcription of the mRNA using the iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed using cDNA as template as described in (26,29). Primers were designed against the first intron/exon border of the mouse LHβ gene to detect unspliced mRNA Primary transcript (PT), forward primer sequence, 5'-AGAGGCTCCAG- GTAAGATGGTA-3'; reverse primer sequence, 5'-CCACTCAGTATAATACAGAAAC-3'. All LHβ PT mRNA was normalized to GAPDH mRNA levels. Samples without the reverse transcriptase enzyme during cDNA synthesis were performed as a negative control. In the result a representative experiment among 4 experiments has been shown. Mean and SEM were calculated from PCR replicates.

Western Blot: For Western blot analysis, 2 X 10^6 cells per well were plated in 6-well 35 mm dishes. Cells were lysed and collected, using 2x gel loading buffer. [100 mM Tris-HCL (pH 6.8), 4% SDS, 20% glycerol] plus protease inhibitors after 48h or 72h or as indicated. Protein concentration was measured using the Pierce (Rockford, IL) BCA Kit. Cell lysates were heated for 5 min at 95 C and equal amounts of proteins of each sample were separated by 10% SDS-PAGE using 140 constant voltage for about 2 h. Proteins were transferred to a nitrocellulose membrane using 35v constant voltage for 3hrs. Membranes were then blocked using 10% non-fat dry milk in Tris-buffered saline plus 1% Tween 20 (TBST) for 1hr in room temperature. Membranes were then incubated with a DAX1 primary antibody (Santa Cruz Biotechnologies, Santa Cruz CA)

overnight (1:500) followed by three 5min washes with TBST and another incubation with secondary antibody, horseradish peroxidase-conjugated donkey anti-rabbit Fab fragment IgG (1:5000;GE HealthCare, Piscataway, New Jersey) for 2hrs. Relative levels of proteins were detected with ECL, Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and X-ray film autoradiography. Monoclonal anti-β-actin primary antibody (Sigma) was used, as a loading control, to re-probe the membrane. Band intensity was measured by densitometry and normalized against β-actin for respective samples.

Chromatin immunoprecipitation assay (ChIP): ChIP assays were performed as previously described in (29). LβT2 cells were plated in 10cm dishes ($10x10^6$ cells per plate) .The cells were first pre-treated with 2.5μm of α-amanitin for 1hr to synchronize the promoter, then thoroughly washed with PBS and incubated with or without 50 μm GnRH and collected every 10mins for 60mins or as indicated. Incase of proteasome studies the cells were also pretreated with vehicle (ethanol) or 50uM of MG132 (proteasome inhibitor from Sigma St.Louis, MO, dissolved in ethanol) and collected every 10mins for 60mins.Chromatin immunoprecipitation (ChIP) assay was performed by cross-linking the chromatin with 1% formaldehyde for 10 min, and the reaction was stopped by addition of 1.25 M glycine. Cross-linked chromatin was sonicated to approximate lengths of 1000 bp, using a cup horn sonicator (Misonix, Farmingdale, NY). Whole-cell extract was diluted with ChIP sonication buffer plus protease inhibitors, divided and incubated with and without primary antibody overnight at 4^0 C temperature.

Antibody for GCN5, WT1, DAX1 (Santa Cruz Biotechnology), SF1, SRC1 (Upstate) and RNA polymerase II antibody (CTD4H8, Milipore) were used. Protein G PLUS agarose beads (SC-2002; Santa Cruz Biotechnology) were then added for 2 h to precipitate the antibody bound chromatin. Agarose-Beads were washed with sonication buffer and Tris-EDTA buffer. DNA-protein complex was released with elution buffer and cross-links were reversed by incubation with NaCl at 65 C overnight. DNA was purified using the QIAGEN PCR purification kit and promoter occupancy was measured with quantitative real-time PCR (iCycler; Bio-Rad) as described in (29). The following primer sequences were used to detect LHβ promoter, 102bp (forward - CTGTGTCTCGCC CCCAAAGAGATTA) and 1bp (reverse-CCTGGCTTTATACCT-GCGGGGTT). Each group was normalized using 10% input sample for that treatment group.

RESULTS

Rapid association of DAX1 with the LHβ promoter in response to GnRH

GnRH stimulates the cyclic binding of transcription factors and co-regulatory proteins on the LHβ promoter (29,112,128). SF1 and Egr-1 transcription factors are stimulated to associate on LHβ promoter in response to GnRH (29). Since, DAX1 lacks the DNA binding domain (69), and unable to directly bind to DNA ,DAX1 mostly acts through protein-protein interactions.DAX1 has been shown to physically interact with SF1 and therefore ,it is likely that DAX1 binds to LHβ promoter via SF1, or other transcription factors like ER and AR (69,70,74,129–131). To check if DAX1 associates with the promoter in response to GnRH, we performed ChIP assay in the presence and absence

of GnRH. The cell lysates were immunoprecipitated with antibodies for pRNApol II, SF1 and DAX1. LHβ promoter occupancy was measured by quantitative real time PCR with primers specific to LHβ promoter (-617 to +44 bp). In Fig.20, ChIP assay results shows that GnRH stimulates the binding of DAX1 to LHβ promoter along with SF1 and pRNA pol II (indicator of transcription) suggesting a possible role of DAX1 LHβ transcription.

Cyclic association of LHβ co-regulators requires proteasome activity

The ubiquitin-proteasome system has been shown to regulate transcription by influencing post-translational modification, protein-protein interactions, and ensuring proper degradation and recycling of the proteins involved in transcription (29,112). Our previous work demonstrated that proteasome inhibition by MG132 reduced the GnRH-stimulated expression of endogenous LHβ primary transcript along with hindering the cyclic binding of the crucial transcription factors Egr1 and SF1 on the LHβ promoter (29). We therefore investigated the possible role of proteasome degradation in regulating the association of the co-regulatory proteins on LHβ promoter. Our results (Fig.21) show that proteasome inhibition compromises the binding of SF1 as well as pRNA POLII. Similarly, inhibition of proteasome activity hinders the cyclic association of the co-activators SRC1 and GCN5 as well as DAX1, which can act as a positive or negative regulator of SF1 (69). In contrast, MG132 treatment sustains the binding of the co-repressor SMRT. Studies by another group (132) shows that the protein mSiah2 targets the repressor protein NCoR for proteasomal degradation by direct binding and as a consequence half life of NCoR decreases from 14 to 3 hrs. By reducing the endogenous NCoR level mSiah2 regulates the repressive activity of nuclear receptor RevErb, which uses NCoR as a co-repressor, to repress its target gene (132) . Our

proteasomal study suggests that the repressor protein needs to be degraded before any transcription activator protein could be recruited to the promoter. Moreover, resemblance of the DAX1 binding pattern with those of the co-activators (GCN5 and SRC1) suggested that DAX1 could be a positive regulator of LHβ transcription.

Reduced levels of endogenous DAX1 decrease GnRH-stimulated expression of endogenous LHβ primary transcript (PT)

To further explore the role of DAX1 in LHβ transcription, endogenous DAX1 was decreased by siRNA. Cells were transfected with siRNA against DAX1 to bring down its cellular level and a non-targeting siRNA was used as a control. After 48hr of incubation, cells were treated with GnRH for 90 min and endogenous LHβ primary transcript was measured using real-time PCR. LHβ PT was normalized to GAPDH mRNA levels. Fig.23 demonstrates a 30% reduction of DAX1 protein levels in the cells transfected with siDAX1. In this experiment, reduced DAX1 levels increased the basal but decreased the GnRH stimulated expression of LHβ primary transcript, the nascent and unspliced form of RNA, by approximately 50% relative to the control set. Thus, under endogenous cell conditions, DAX1 appears to be a positive regulator of GnRH stimulated LHβ transcription.

DAX1 overexpression increases GnRH stimulated LHβ transcription

To further investigate the positive role of DAX1 in LHβ transcription, we overexpressed DAX1 in LβT2 cells and measured the LHβ promoter (-617 to 44bp) driven luciferase reporter gene activity in presence or absence of GnRH (50nM). Western blot analysis

shows DAX1 level in control and DAX1 transfected cells, showing an increase in DAX1 levels with DAX1 transfection. DAX1 overexpression significantly increased the GnRH stimulated LHβ promoter activity by approximately 4-fold; however, DAX1 did not affect the basal transcription (Fig.22). To evaluate how increasing concentrations of DAX1 could effect LHβ transcription, a DAX1 dose-response experiment was performed. LβT2 cells were transfected with increasing concentration of DAX1 (0.1,0.3,0.5 and 1 ug concentration per reaction) and promoter activity was measured for each dose. Results showed significant enhancement, then inhibition of the GnRH stimulated promoter activity at 0.3ug and 1ug of DAX1 respectively. (Fig.22) Our overexpression data suggests that DAX1 could be a positive or negative regulator of LHβ transcription in a dose dependent manner.

The proximal region of the LHβ promoter is sufficient for DAX1 mediated increase of GnRH stimulated LHβ transcription

The LHβ promoter has two major GnRH response elements and the combined effect of both the distal and the proximal region is required for effective transcription to occur (30,31). Since DAX1 modulates GnRH stimulated LHβ transcription, we assessed whether DAX1 is able to enhance LHβ transcription through the proximal GnRH response region, or required both regions. LβT2 cells were transfected with both the full-length (-617 to +44bp) and the truncated promoter (-245 to +44bp) and the effect of DAX1 overexpression was measured in presence or absence of GnRH with luciferase reporter assays. Fig.24 shows DAX1 was able to increase the GnRH stimulated LHβ transcription via both full-length and the truncated promoter region. Thus, the proximal

region of the promoter is sufficient to impart DAX1 activity.

Potential role of SF1 sites in DAX1 mediated GnRH stimulation of LHβ transcription

DAX1 cannot bind directly to the DNA and has been found to trans-activate SF1 target genes by direct protein-protein interaction with SF1. We mutated either of the two SF1 binding sites (3' or 5') by site directed mutagenesis and transfected these mutated promoters along with DAX1 to measure DAX1 activation of GnRH stimulated LHβ transcription with luciferase assay. We used the full-length (-617 to +44bp) promoter as control for DAX1 activation and 0.3ug of DAX1 plasmid. Our results show that DAX1 activated the GnRH stimulated LHβ transcription through the full-length promoter, however, DAX1 could not significantly activate or further enhance the GnRH stimulation of LHβ transcription through the promoter containing 5'SF1 and 3'SF1 mutations respectively. This suggests that SF1 sites could play a role in DAX1 activation of GnRH stimulated LHβ transcription (Fig.25).

Cyclic association of DAX1 is independent of SF1 association on the LHβ promoter

DAX1, as suggested by our results, can act through the proximal region and requires 3'SF1 site for its ability to activate GnRH stimulated LHβ transcription. To understand the mode of action of DAX1, LβT2 cells were transfected with control siRNA or siRNA targeted against SF1. After 72h of post transfection SF1 protein levels were decreased by 70%. Our results (Fig.26) show that DAX1 is associated with the LHβ promoter in

presence or absence of SF1 (siSF1) indicating that the cyclic association of DAX1 on LHβ promoter can occur independently of SF1.

DAX1 mediated regulation of LHβ promoter is not dependent on SF1

Although DAX1 association to LHβ promoter occurs independently of SF1, it is possible that DAX1 activity might require SF1 protein. To examine this possibility we transfected LβT2 cells with siRNA against SF1 to reduce endogenous SF1 levels and also overexpressed DAX1 using a plasmid dose of 0.1ug. We then measured the basal and GnRH stimulated LHβ promoter activity by reporter gene assay. siRNA against SF1 reduced the endogenous SF1 levels to about 60%. Under these conditions, DAX1 enhanced GnRH-stimulated LHβ transcription in the presence or absence of SF1 (Fig.27).

GnRH and proteasome activity regulate WT1 but not DAX1 protein levels.

Since DAX1 binding to LHβ promoter was regulated by proteasome activity and GnRH, we examined whether GnRH and proteasome regulates DAX1 protein levels. DAX1 has been suggested to interact with another protein, WT1 to regulate several reproductive genes including one essential for gonadogenesis prior to sexual differentiation (74,133). We therefore, investigated if GnRH and proteasome regulates DAX1 or WT1 protein levels. To measure the protein levels LβT2 cells were treated with 50nM GnRH for 0 to 6 h in presence or absence of proteasome inhibitor MG132.Immunoblotting for β-actin was performed on the same blots used as loading control. Our results (Fig.28) suggest that GnRH and proteasome activity regulates WT1 protein but not DAX1 protein levels. As demonstrated in the previous chapter, GnRH reduces WT1 mRNA and protein

levels, however in Fig.28 we show that the rapid GnRH mediated decrease of WT1 protein is prevented by MG132, causing accumulation of WT1 protein.

Proteasome activity is required for the cyclic association of WT1 on LHβ promoter

DAX1 interacts with WT1 to regulate several reproductive and steroidogenic genes (74,133). Since GnRH and proteasome activity were found to regulate WT1 protein levels, we investigated the role of proteasome activity on WT1 association with the LHβ promoter.WT1 protein occupancy on LHβ promoter was measured by quantitative real time PCR using primers specific for the LHβ promoter. Our results in the previous chapter show that WT1 is bound to LHβ promoter under basal conditions, and GnRH stimulates the cyclic association of WT1 to the LHβ promoter. In Fig.29 we demonstrate that proteasome inhibition prevents the GnRH-stimulated association. This suggests that proteasome degradation facilitates the GnRH stimulated cyclic association of WT1 on the LHβ promoter.

DISCUSSION

Gene transcription requires the cyclic binding of transcription factors, co-regulatory proteins and basic transcriptional machinery including pRNApol II to the gene promoter. After each cycle of transcription, the proteins need to be removed from the promoter so that a new transcription cycle can begin. During transcription, the ubiquitin-proteasome system has been closely linked to alterations in protein–protein interactions, association of co-activators and co-repressors on transcription factors, turnover of transcription factors/co-regulators, and clearing proteins from the promoter to facilitate new rounds of transcription (55,101,103,104,109). In this work, we show proteasome activity regulates

the binding of the co-regulators GCN5, SRC1 (co-activators), SMRT (co-repressor) and WT1, DAX1 (both co-activator and co-repressor activities) on the LHβ promoter. We also show that the orphan nuclear receptor DAX1 acts as either a positive or negative regulator of LHβ transcription in a dose dependent manner.

Previous studies have shown that association of the transcription factors SF1 and Egr-1 with the LHβ promoter is cyclic, and coordinated by GnRH (29). Proteasomal inhibition has been shown to prevent the cyclic recruitment of these transcription factors on the LHβ promoter, (29) and we questioned if the association of the co-regulatory proteins were also affected by proteasome or not. Co-regulatory proteins that associate with SF1 include the typical co-activators SRC1 and GCN5, the co-repressor SMRT (44,57,58), and DAX1, which can act as a co-activator or co-repressor in a cell- and promoter-specific manner (69,130,131,134). Our results (Figs 20 and 21,) show that GnRH stimulates the binding of the transcription factor SF1 and several co-regulators including co-activators (SRC1, GCN5) co-repressor (SMRT), and DAX-1, as well as pRNApol II, which indicates transcription. Proteasomal inhibition with MG132 prevents the association of the co-activators SRC1, GCN5, as well as co-regulators DAX1 and WT1 (Figs. 21 and 29). However, proteasome inhibition sustained the binding of the co-repressor SMRT to the LHβ promoter, suggesting that the repressor protein may be required to be removed before transcriptional activators can be recruited. As previously shown by the O'Malley group, mono-ubiquitination of the coactivator SRC3 on promoters enhances its interaction with ER and stimulates ER-mediated transactivation (109). As transcription progresses, SRC3 is poly-ubiquitinated and ultimately degraded to assist promoter clearance (109). SF1, phosphorylated in response to GnRH, then

becomes mono-ubiquitinated; this is believed to favor interactions with Egr1 and Ptx-1 (112). Thus, protein-protein interactions and their modulation by the ubiquitin-proteasome system may influence the transcriptional activation of SF1 and thus stimulate LHβ gene transcription (112).

GnRH and proteasome activity regulates Egr1 protein levels as well as SF1 and Egr1 ubiquitination (29). As demonstrated in the previous chapter, GnRH rapidly reduces WT1 mRNA and protein levels. In this work (Fig.28), we show that the GnRH-mediated decrease of WT1 protein is prevented by MG132, suggesting that proteasome activity regulates WT1 protein levels. However, DAX1 protein levels remained unaffected by GnRH or proteasome inhibition (Fig. 28). The failure to see changes in DAX1 levels under these conditions does not mean, however, that DAX1 is not a target of the proteasome, as high basal expression of protein and/or long protein half-life might make such changes difficult to detect. For example, SF1 ubiquitination is highly stimulated by GnRH, but SF1 levels are not significantly changed after MG132 treatment (29). Ubiquitination studies were not preformed on DAX1.

The orphan nuclear receptor DAX1 interacts with SF1 on several reproductive and steroidogenic genes (74,75,133). DAX1 and SF1 are co-expressed throughout the Hypothalamic-Pituitary-Adrenal-Gonadal axis (69,72,135) and are both involved in regulation of several reproductive and steroidogenic genes (73–75,130,133). DAX1 has been shown to act as a co-repressor of SF1 by recruiting the repressor protein NCoR onto SF1(45). As opposed to this well-known role of DAX1 as co-repressor of many

SF1 target genes (44,69,70), a recent report has shown DAX1 to be a co-activator of some SF1 target genes (75). DAX1 interacts with nuclear-receptor coregulator SRA (steroid receptor RNA activator) and p160 family co-activator TIF2 (transcription intermediary factor-2), to form a co-activator complex to increase SF1-mediated ACTH receptor (Mc2R) gene expression (75). Moreover, DAX1 knockdown also decreases the expression of the CYP11A1 and StAR genes in adrenal and leydig cells, suggesting DAX1 endogenously stimulates those genes(75). SF1 and DAX1 synergistically induce expression of several testis specific genes in developing mice testis (73). Interestingly, WT1, DAX1 and SF1 have been shown to regulate the transcription of the MIS gene, which determines male reproductive tract formation during gonadogenesis (74), suggesting that the ratio of WT1 to DAX1 to SF1 can be important in determining gene expression. DAX1 interacts with other nuclear receptors such as androgen receptor (AR) and estrogen receptor (ER) in addition to SF1 (130,131).Reports show that DAX1 directly interacts with AR and inhibits AR mediated transactivation. DAX1 has also been suggested to sequester the dimerized AR in the cytoplasm, preventing it from nuclear localization or preventing AR from recruiting co-activators by direct interaction inside the nucleus (130). DAX1 interacts with ERα and ERβ to co-repress their transactivation via the LXXLL motif at the N-terminal repeat domain of DAX1. The LXXLL domain is usually a distinctive feature of nuclear receptor co-activators; however DAX1 can also uniquely act as an LXXLL-containing co-repressor (131).

Considering that DAX1 can act as negative or positive regulator and the pattern of DAX1 binding to LHβ promoter resembled the co-activator (GCN5 and SRC1) binding pattern in our experiments, we wanted to understand the biological role of DAX1 in LHβ

transcription. Our results (Fig.20) show that rapid binding of DAX1 occurs with 20 mins of GnRH treatment, this suggests that DAX1 binding is stimulated in response to GnRH. Moreover, increasing doses of DAX1 increased GnRH-stimulated LHβ transcription, but high dose of DAX1 repressed LHβ transcription, suggesting a dose-dependent regulation of DAX1 on LHβ transcription (Fig.22). To better understand the role of DAX1, siRNA was targeted against DAX1 to reduce endogenous DAX1 levels and LHβ primary transcript (PT) was measured (Fig.23). The GnRH stimulation of LHβ PT was decreased by 50% when compared to the control set; however, the basal LHβ PT level was increased 2 fold by DAX1 siRNA. Moreover DAX1 overexpression increased LHβ transcription as depicted by our reporter gene assay (Fig. 22).

The proximal GnRH response element, consisting of the Egr1 and SF1 binding sites, on the LHβ promoter was sufficient to impart DAX1 stimulation (Fig.24). Since DAX1 has been shown to interact with SF1 on several reproductive and steroidogenic genes, there is a possibility that DAX1 is recruited to the LHβ promoter via SF1.To better understand the role of SF1 in DAX1 activation of LHβ transcription, we used promoter constructs carrying SF1 mutations at the 3' and 5' SF1 binding sites on the proximal promoter (Fig.25). DAX1 stimulation of LHβ transcription was not significant when 5' SF1 site was mutated. Moreover with a 3' SF1 mutated construct, DAX1 was unable to further enhance the GnRH stimulation of LHβ transcription, suggesting the SF1 sites, individually or together, could be aiding DAX1 stimulation (Fig.25). However, in cells treated with siRNA targeted against SF1 to reduce endogenous SF1 levels, GnRH-stimulated cyclic binding of DAX1 on LHβ promoter remained unaffected by the

absence of SF1 (Fig.26). Under similar experimental conditions (reduced level of endogenous SF1), we also measured LHβ transcription with a reporter gene assay (Fig.27). These results show that siSF1 treatment did not disrupt the DAX1 enhancement of GnRH-stimulated LHβ transcription. This suggests the role of DAX1 to enhance LHβ transcription is independent of SF1, at least under our experimental conditions. However, one caveat is that there was only a partial knockdown of SF1 in the transcription studies, and a much larger reduction in SF1 may be needed to observe a biological effect. In addition, the zero time point of SF1 binding was very high in the SF1 knockout study, even though subsequent points were low. Further experiments are required to investigate the involvement of SF1 in DAX1 mediated activation of LHβ transcription including a better and efficient knockdown of SF1 by siRNA.

DAX1 also interacts with the nuclear receptor LRH1 (Liver Receptor Homolog-1), which shares structural similarity to SF1 and binds to SF1 binding sites (134,136,137). DAX1 and SRA synergistically coactivate LRH1-mediated transactivation of Oct4 gene expression in mouse embryonic stem cells (134). LRH1 is expressed in LβT2 cells, and induces the basal and GnRH stimulated LHβ transcription (136). The SF1 knockout mice is able to produce LH when treated with GnRH, but mutation of both SF1 sites on LHβ promoter significantly reduces basal and GnRH stimulated LHβ transcription (136,138,139). Mutation of either 3' or 5' SF1 sites on the proximal GnRH responsive region of the LHβ promoter decreases the transactivation of LRH1 on LHβ promoter transcription, as shown by reporter gene assay using cells lacking endogenous SF1 (136,140). This study suggests the importance of SF1 sites, more than the protein. Our

experiment suggests SF1 to be dispensable in DAX1 binding to the LHβ promoter, and DAX1 stimulation of LHβ transcription. However, the inability of DAX1 to further enhance GnRH response significantly in the presence of mutated 3'SF1 or 5'SF1 sites suggests a possibility that DAX1 might be acting through LRH1 to regulate LHβ transcription. Moreover, as the SF1 binding sites are adjacent to Egr1 and Ptx1 binding sites, protein binding to the SF1 sites could be providing stability to transcriptional complexes involving Egr1, Ptx1 and other co-regulatory proteins.

A few previously published studies (42,60) suggest DAX1 to be a negative regulator of LHβ transcription. The variation may occur from the experimental conditions used; we used different doses of DAX1 as opposed to a single constant dose of DAX1. In studies using a human placental cell line it was shown that SF1 site mutations did not effect DAX1 regulation of LHβ transcription unless Egr1 was added, suggesting DAX1 regulation is context dependent and might vary in the presence of other proteins (60). Our studies did not identify how DAX1 enhances GnRH stimulation, but it may occur through recruitment of additional co-activator, prevention of co-repressor recruitment, or stabilization of protein-protein interactions such as Egr1-LRH1/SF1.

Overall, our studies show that the ubiquitin and proteasome system plays a crucial role in controlling the GnRH stimulated cyclic association of the regulatory proteins (inhibitory and stimulatory) on the LHβ promoter, and their degradation after each cycle of transcription. DAX1 and WT1 were identified as regulators of LHβ transcription. Modulated of DAX1 and WT1 by GnRH and proteasome activity maintained their dynamic association on the chromatin during LHβ transcription.

FIGURES

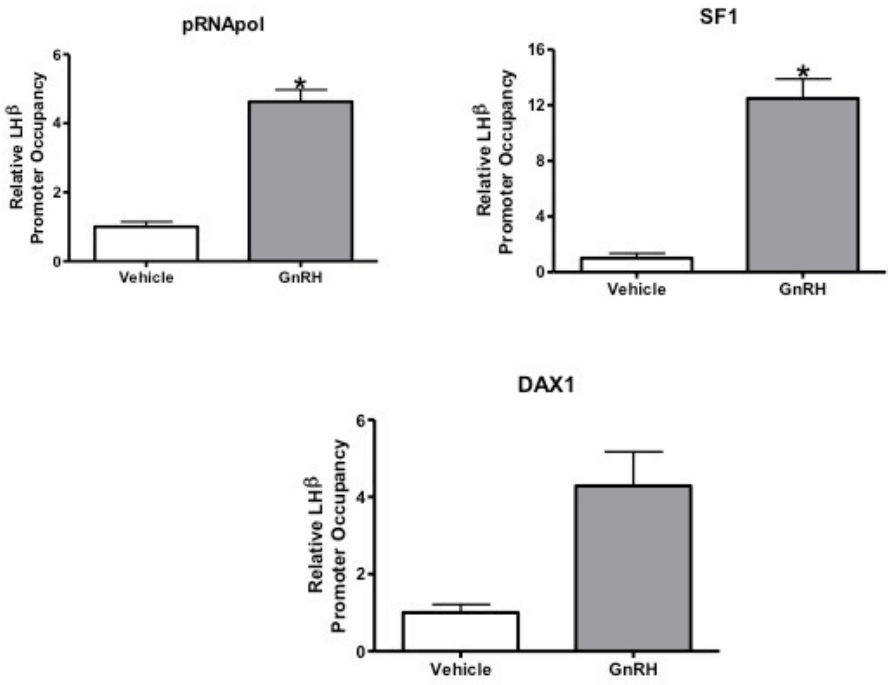

Figure 20 Rapid association of DAX1 with LHβ promoter in response to GnRH

Figure 20 Rapid association of DAX1 with LHβ promoter in response to GnRH: LβT2 cells were first pre-treated with 2.5μm of α-amanitin for 1hr to clear the promoter of proteins and to synchronize further protein association with the promoter. Cells were then incubated with or without 50 nM GnRH and collected after 20mins. The ChIP assay was performed using antibodies for pRNApol, SF1 and DAX1, and LHβ promoter occupancy was measured by quantitative real time PCR using primers specific for the LHβ promoter (-102 to -1bp). Results show DAX1 associates with the LHβ promoter at the same time point as SF1 and pRNApol II. *P<0.05 control vs 20mins GnRH

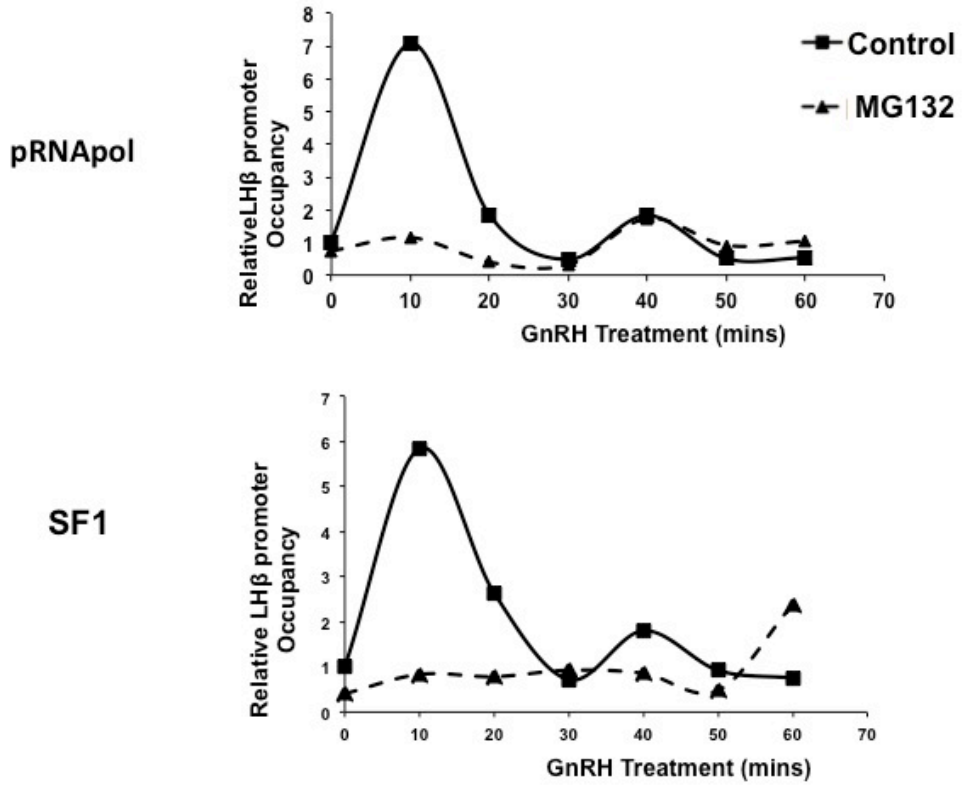

Figure 21 Cyclic associations of coregulators to the LHβ promoter requires proteasomal degradation

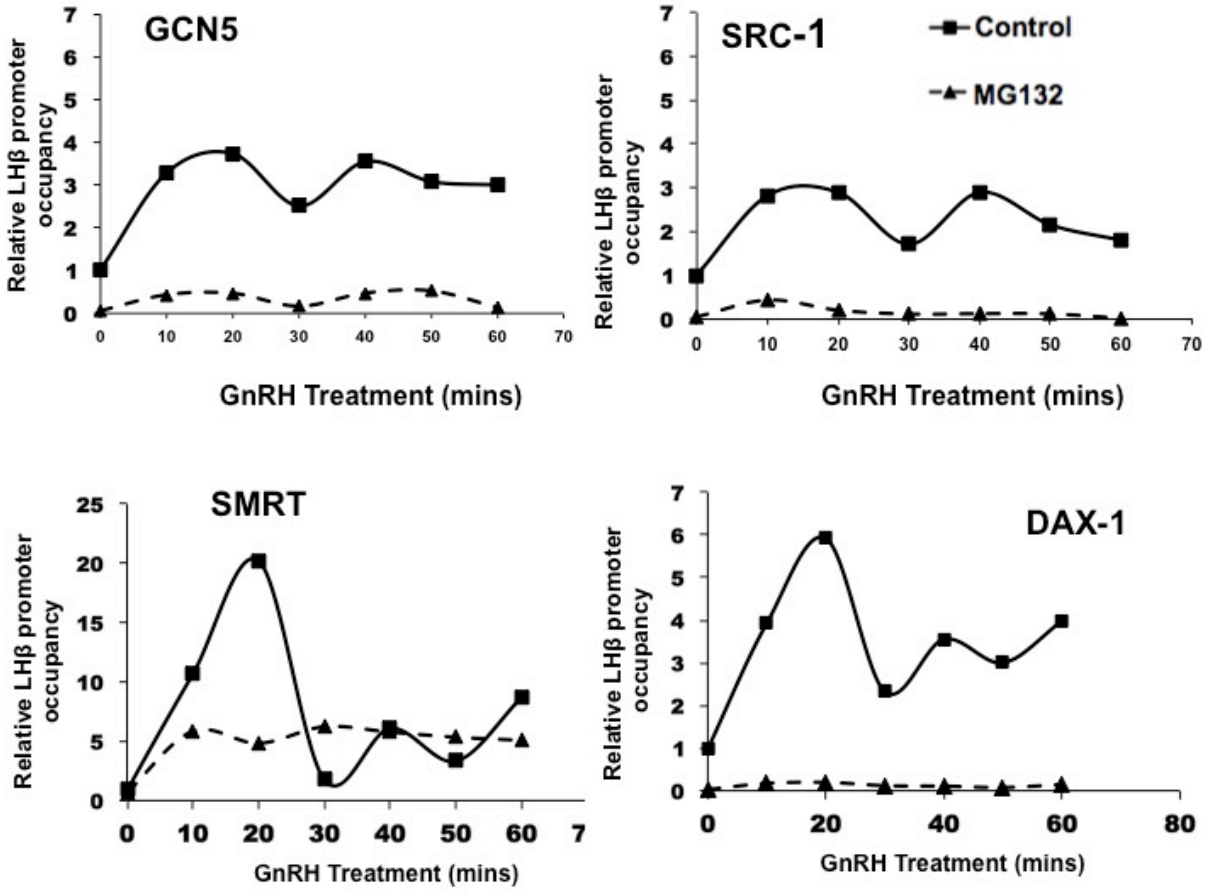

Figure 21 cyclic associations of coregulators to the LHβ promoter requires proteasomal degradation

Figure 21 Cyclic associations of coregulators to the LHβ promoter requires proteasomal degradation:

The LβT2 cells were first pre-treated with 2.5μm of α-amanitin for 1hr, then incubated with or without 50 μM MG132 (proteasome inhibitor) for 1hr. Cells were then treated with 50 nM GnRH and collected every 10min till 60min. The ChIP assay was performed using antibodies against pRNApol, SF1, GCN5, SMRT and DAX1 .LHβ promoter occupancy was measured by quantitative real time PCR using primers specific for the LHβ promoter (-102 to -1bp).

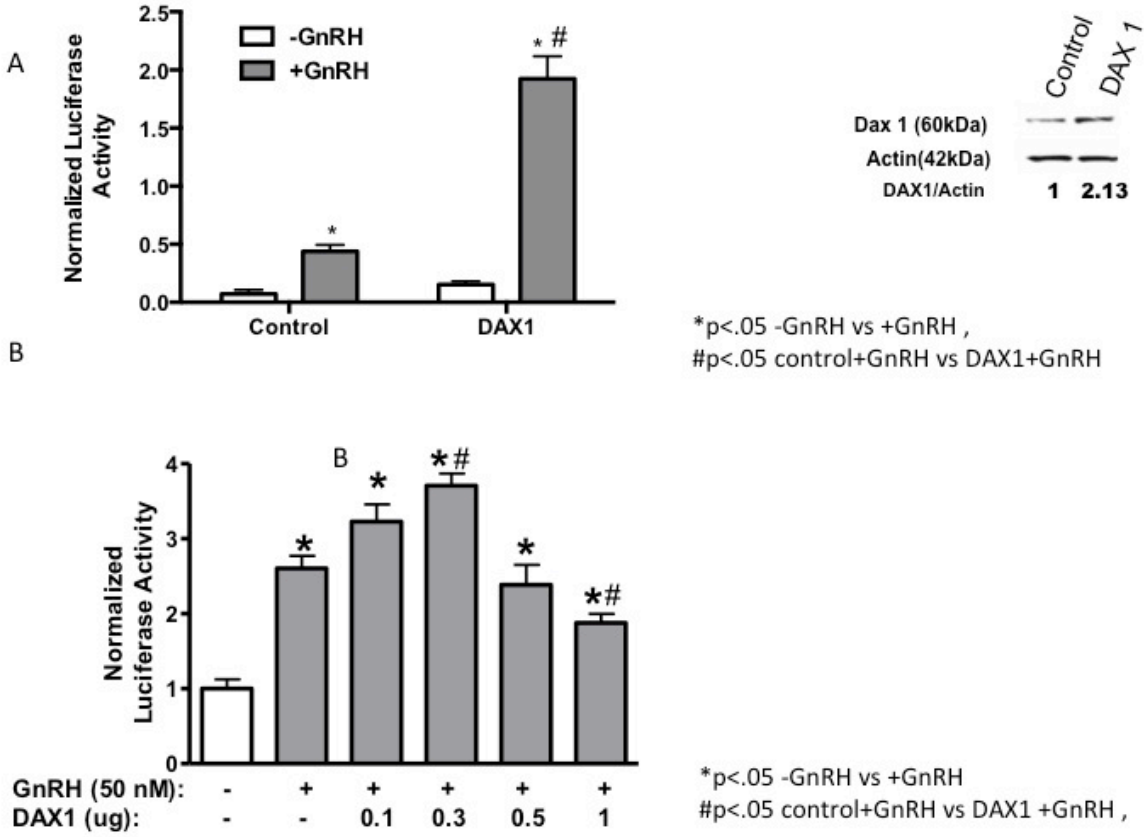

Figure 22 DAX1 modulates GnRH-stimulated LHβ transcription in a dose dependent manner

Figure 22 DAX1 modulates GnRH-stimulated LHβ transcription in a dose dependent manner: LβT2 cells were transfected with a luciferase reporter construct driven by the rat LHβ promoter (-617 to +41 bp), **A)** with or without constant dose of 0.5 mg of DAX1 or **B)** increasing concentrations of DAX1 (0.1, 0.3, 0.5, 1ug per reaction). After 48h post-transfection, cells were treated with or without 50nM GnRH for 6h and collected for luciferase assays and western blot analysis. GnRH-stimulated LHβ transcription was increased significantly with 0.3ug of DAX1 and at higher concentrations (0.5,1ug) DAX1 decreased LHβ transcription. Average and standard error were calculated for 6 samples. Statistical significance was determined using paired student's t-test. *P<0.05: –GnRH vs +GnRH, #P<0.05: Control +GnRH vs DAX1 + GnRH.

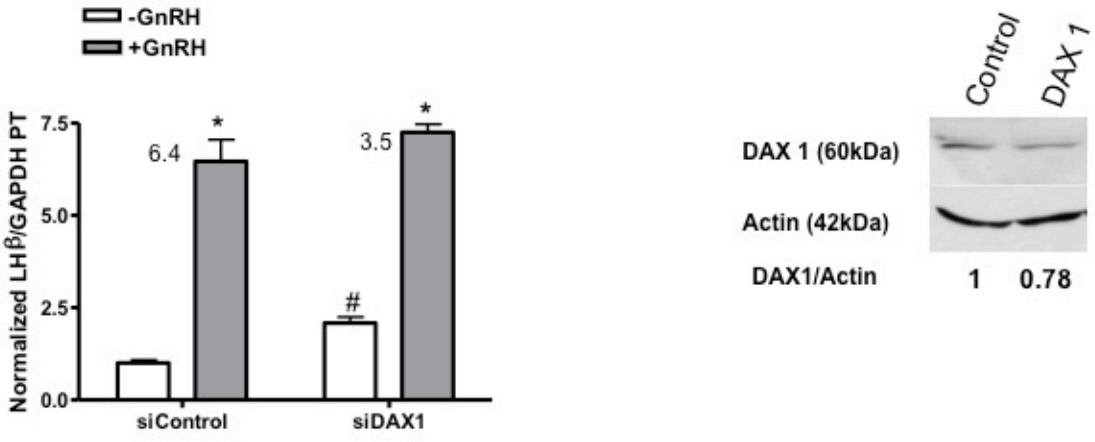

Figure 23 siDAX1 decreases the GnRH stimulation of endogenous expression of LHβ PT

Figure 23 siDAX1 decreases the GnRH stimulation of endogenous expression of LHβ PT

LβT2 cells were transfected with siRNA against DAX1 and a non-targetting siRNA as a control. After 48h cells, were treated with or without GnRH for 90mins followed by cell lysate collection, RNA extraction and western blot analysis (30 ug protein) on 10% PAGE-SDS gel. DAX1 protein was detected by immunoblotting and normalized for β-actin on the same blot. The expression of endogenous LHβ mRNA primary transcript (PT) was measured by real-time PCR. LHβPT was normalized to GAPDH mRNA levels. A representative experiment among 4 experiments has been shown. Statistical significance was determined using paired student's t-test. **P*<0.05: –GnRH vs +GnRH, #*P*<0.05: Control vs DAX1

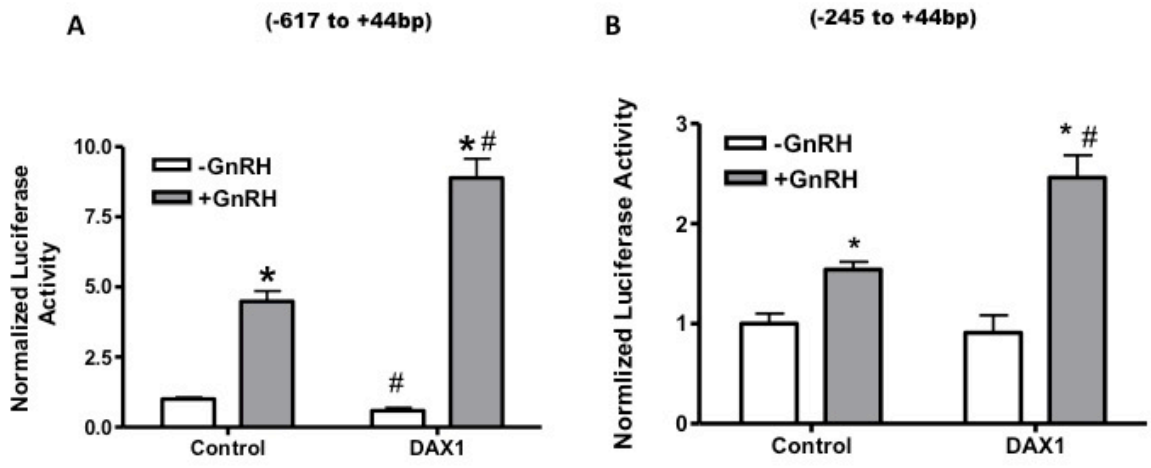

Figure 24 The proximal promoter region of LHβ is sufficient for DAX1 mediated increase of GnRH-stimulated LHβ transcription

Figure 24 the proximal promoter region of LHβ is sufficient for DAX1 mediated increase of GnRH-stimulated LHβ transcription. LβT2 cells were transfected with luciferase reporter construct driven by either **A)** the rat LHβ gene promoter (-617 to +44 bp, contains both distal and proximal GnRH responsive promoter elements), or B**)** the truncated promoter (-245 to +44 bp, contains only the proximal GnRH responsive promoter region). Constructs were co-transfected with 0.3ug of DAX1 plasmid or control plasmid to normalize total DNA. After 48 h, cells were treated with 50nM GnRH for 6hrs and collected in lysis buffer. Luciferase activity was measured, and data expressed as mean $\pm$ SE for 3 samples; the experiment was performed 3 times. Statistical significance was determined using paired student T-test. *P<0.05, –GnRH vs +GnRH, #P<0.05: Control vs DAX1

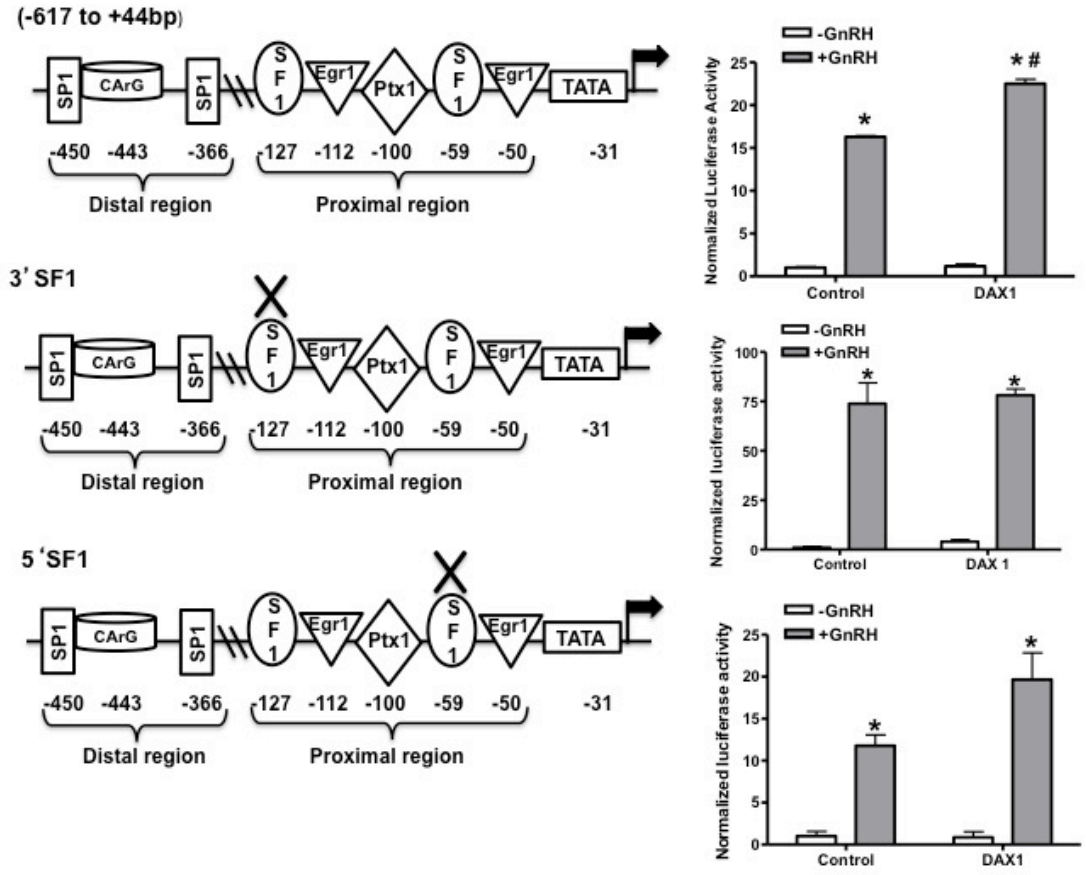

Figure 25 Potential roles of SF1 sites in DAX1 mediated GnRH stimulation of LHβ transcription.

Figure 25 Potential roles of SF1 sites in DAX1 mediated GnRH stimulation of LHβ transcription. LβT2 cells were transfected with a luciferase reporter construct driven by **A)** the rat LHβ gene promoter (-617 to +44 bp) or with similar promoter construct carrying mutation at the **B) 3'SF1** or C**)** 5'SF1 sites, with or without DAX1.Constructs were co-transfected with 0.3ug of DAX1 plasmid or control plasmid to normalize total DNA. After 48 h, cells were treated with or without 50nM GnRH for 6hrs and collected in lysis buffer. Average and standard error were calculated for 6 samples. Statistical significance was determined using paired student's t-test. **P*<0.05: –GnRH vs +GnRH, #*P*<0.05: Control vs DAX1.

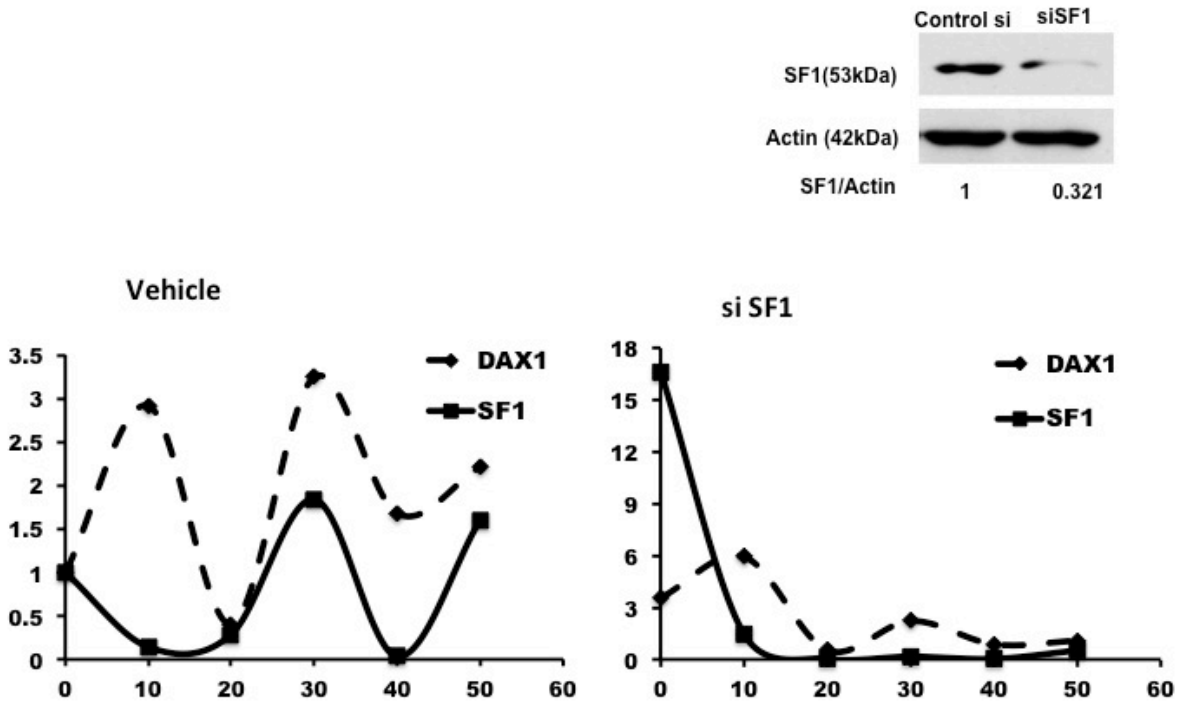

Figure 26 Cyclic association of DAX1 is independent of SF1 association on the LHβ promoter:

Figure 26 Cyclic association of DAX1 is independent of SF1 association on the LHβ promoter:

LβT2 cells were transfected with control siRNA or siRNA targeted against SF1. After 72h of post-transfection, cells were pre-treated with 2.5μm of α-amanitin for 1hr followed by washing with PBS. Cells were then treated with 50 nM GnRH and collected every 10min for 50min. ChIP assays were performed using antibodies against DAX1 and SF1. LHβ promoter occupancy was measured by quantitative real time PCR using primers specific for LHβ promoter (-102 to -1bp).

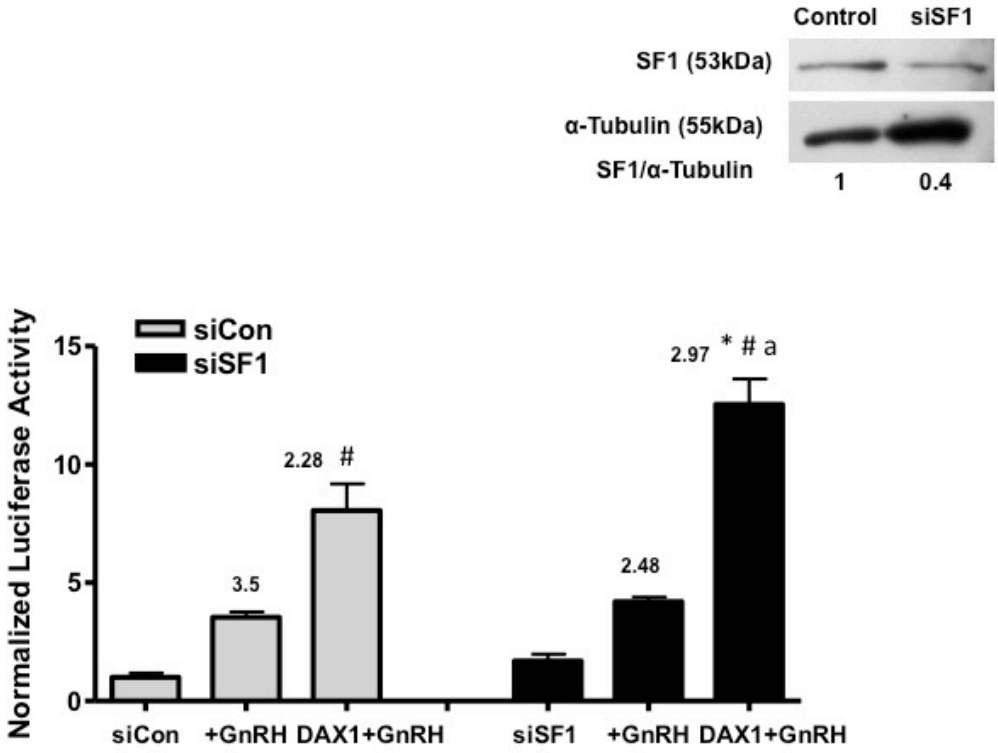

Figure 27 DAX1 mediated regulation of the LHβ promoter is not dependent on SF1

Figure 27 DAX1 mediated regulation of the LHβ promoter is not dependent on SF1

LβT2 cells were transfected with DAX1 expression plasmid (0.1ug and 0.3ug) and control siRNA or siRNA targeted against SF1. A luciferase reporter construct driven by the rat LHβ promoter (-617 to +41 bp) was transfected the following day. After 72h of post-transfection cells were treated with or without 50nM GnRH for 6hrs and collected in lysis buffer for western blot and reporter gene assays. Luciferase activity was measured, and data expressed as mean $\pm$ SE for 3-6 samples; the experiment was performed once. Statistical significance was determined using using paired student's t-test and ANOVA (with differences between treatment groups determined by Bonferroni multiple comparison test). * P<.05 siControl vs siSF1, # P<. 05 -GnRH vs +GnRH, a P<.05 +GnRH vs GnRH + DAX1.

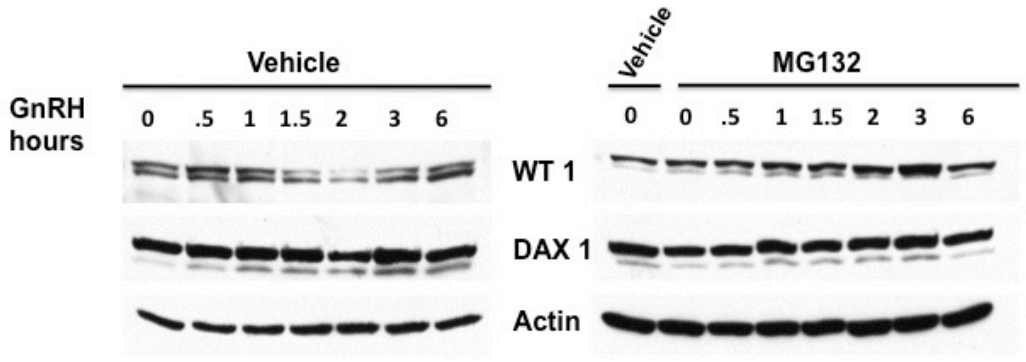

Figure 28 WT1 but not DAX1 protein levels are regulated by GnRH and proteasome activity

WT1 and DAX1 protein levels in LβT2 cells treated with or without 50nM GnRH for 0 to 6 h with (right panel) or without (left panel) proteasomal inhibitor 50 μM MG132. Cells were lysed after GnRH treatment at indicated time points and proteins (30 mg) were separated by 10% SDS-PAGE, then detected with antibodies against WT1 or DAX1. Immunoblotting for β-actin was performed on the same blots used as loading control.

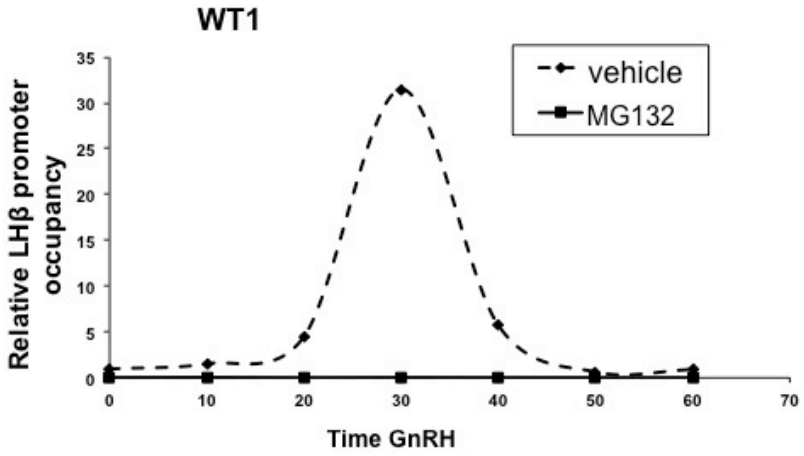

Figure 29 Proteasome degradation facilitates the cyclic association of WT1 on the LHβ promoter

The LβT2 cells were first pre-treated with 2.5μm of α-amanitin for 1hr, then incubated with or without 50 μM MG132 (proteasome inhibitor) for 1hr. Cells were treated with 50 nM GnRH and collected every 10min for 60min. A ChIP assay was performed using antibodies against WT1. LHβ promoter occupancy was measured by quantitative real time PCR using primers specific for LHβ promoter (-102 to -1bp).

Chapter 4: Discussion and Perspectives

GnRH regulation of the Gonadotropin genes: differential gene regulation

Pulsatile release of GnRH differentially regulates the synthesis and secretion of several gonadotropin genes, such as α-subunit, LHβ, and FSHβ, along with its own receptor (GnRHR) in the anterior pituitary(1). How this one hormone differentially controls several subunit genes in same location (pituitary) has been an intriguing question. The release of GnRH at various pulse frequencies is one key element which in turn differentially regulates several other processes, such as triggering selective signaling pathways, protein synthesis, protein modification and turnover to modulate relevant transcription factors and co-regulators (1).

Fast GnRH pulse frequency (1/30 mins) favors alpha-subunit, LHβ synthesis and GnRHR mRNA expression; slow GnRH pulses (1/120 min) favor FSHβ synthesis and low GnRHR expression in the rat pituitary (141). Increased expression of GnRHR was observed at faster pulses favoring LHβ synthesis. With slow pulses, GnRHR mRNA expression was low but maximal FSHβ mRNA expression occurred, which shows differential regulation of subunit genes by GnRH could occur through the variation of its own receptor levels (141). Modulation of GnRHR expression and thus effective GnRH activity might result in triggering different signaling pathways and /or expression of proteins that can differentially regulate various gonadotropin subunit genes. One study shows that acute GnRH treatment induces and long term GnRH represses LHβ

transcription by separate signaling pathways. Acute induction was mediated by PKC signaling and repression by long term GnRH was mediated by calcium signaling (142). Other studies showed GnRH utilizes specific singling pathways to induce specific subunit gene transcription; for example, ERK is important for induction of alpha-subunit, FSHβ subunit and LHβ transcription via Egr1, however, JNK is specifically involved in LHβ transcription (23). Discrepancies in studies related to GnRH action might occur due to different experimental systems; for example, primary cell culture and in-vivo systems are heterologous in nature and results might be influenced by other cell-types through paracrine signaling (142,143) . Studies using engineered GH3 cell lines derived from somatomammotropes of the pituitary might not reflect similar signaling cascades as gonadotopes (142,143). For example, GH3 cells do not express SF1, important for transcription of GnRH-R and gonadotropin subunit genes, and the cells may not contain all the requisite pathways to alter SF1 function (142,143).

There are a few investigations demonstrating alterations in GnRH signaling pathways depending on the mode of GnRH treatment. A recent study involving pulsatile and continuous GnRH treatment to LβT2 cells showed differential modulation of ERK and MAP kinase phosphatase 1 signaling. After static, continuous GnRH treatment; ERK1/2(MAPK) was significantly activated within 10 min and sustained with gradual decreases between 4-20hrs. With fast GnRH pulse treatment (1/30 mins) ERK was activated within 5 min and gradual decrease occurred by 20-30mins resulting in an oscillating pattern of activity every 30 min. At slow GnRH pulse frequency (1/120mins) the decrease took 40-50 min to come to the baseline level. Similarly, MAPK

phosphatase enzymes (MKPs), that deactivate the family of MAPK signaling molecules, showed variation at continuous and pulsatile GnRH frequencies and are maximally activated by pulses of 1/30min versus 1/120 min, confirming the differential regulation of signaling pathways by pulsatile GnRH (144). Blocking ERK-phosphorylation suppressed Egr1 mRNA expression and LHβ transcription in response to GnRH. Rapid activation of ERK might thus facilitate the translation of fast GnRH pulses (1/30mins) by rapidly inducing Egr1 and LHβ transcription (36,144). Sustained ERK activation by static GnRH treatment to LβT2 cells can stimulate LHβ transcription even without pulsatile GnRH treatment.

In this thesis we have focused on the transcriptional regulation of the LHβ subunit gene in response to constant GnRH using LβT2 cells. LβT2 cells are an immortalized murine pituitary cell line able to produce the gonadotropin proteins along with GnRH receptor, and respond to either static or pulsatile GnRH (144,145). Moreover, continuous GnRH treatment on LβT2 cells has been shown to stimulate all the gonadotropin subunit genes (α-subunit, LHβ and FSHβ) along with the GnRH receptor. In addition, presence of continuous GnRH, transcription factor association on the LHβ promoter occurred with a 30-35min interval, as expected with pulsatile GnRH treatment (29,146). LHβ primary transcript mRNA (LHβPT) was stimulated in the presence of continuous GnRH till 2 h, followed by decreasing LHβPT expression between 3-6h, correlating with GnRH-receptor desensitization (29). This suggests that LβT2 cells could be successfully used to study LHβ transcription in presence of continuous GnRH, although pulsatile GnRH treatment might be more physiological. In addition to signaling pathways, GnRH also differentially regulates the synthesis, modification and degradation of transcription

factors and their co-regulators. GnRH rapidly induces the expression of the transcription factor Egr1, which is almost undetectable in the absence of GnRH. Egr1 knockout mice are infertile due to lack of LHβ expression (59) and mutation of either Egr1 site on the LHβ promoter significantly decreased GnRH stimulated LHβ transcription (29,128). WT1 might add another layer to the Egr1-mediated LHβ transcription as it can bind to Egr1 binding sites and is regulated by GnRH. In our studies we show that WT1 protein expression is more stable compared to the transient expression of Egr1 protein, and is suppressed by GnRH. However, we could not distinguish the protein expression levels between the two WT1 splice variants, which have differential effects on LHβ transcription. WT1 could also be regulating Egr1 levels, as we have shown that Egr1 levels are reduced in presence of WT1siRNA with reduced –KTS variant mRNA. GnRH induces Egr1 and decreases –KTS mRNA expression; we speculate that –KTS is a less potent positive regulator however; it might also induce Egr1 expression to indirectly enhance LHβ transcription.

The orphan nuclear receptor SF1 is involved in the transcription of all the gonadotropin subunit genes and the GnRH receptor, and works in concert with Egr1 during LHβ transcription (33–35). WT1 has been shown to physically interact with SF1 and can also regulate SF1 gene expression (74,83). In this thesis we show that mutation of both SF1 sites abolished the repressive effect of WT1 +KTS, suggesting that the negative regulation by WT1 (+KTS) is mediated through one or both the SF1 sites on the promoter. On the contrary, mutation of 3'Egr1 site but not SF1 sites influences the WT1 (-KTS) mediated increase of LHβ transcription. Therefore it is possible that two splice variants of WT1 might form active or repressive transcriptional complexes by interacting

with different transcription factor or co-regulators to regulate LHβ transcription.

Co-regulators of SF1 such as GCN5, SRC1 and SMRT also associate to LHβ promoter in a cyclic manner. GCN5 has HAT activity and chromatin remodeling has been previously speculated to be another aspect of differential regulation by GnRH (1). For example, the HAT activity of P300 may aid in the synergy between Egr1-SF1 in response to GnRH. The co-activator activity of P300 is reinforced in presence of other transcriptional co-activators such as SRC/P160 proteins (39). In this thesis we have shown that DAX1, another co-regulator of SF1, associates with the LHβ promoter and positively or negatively regulates LHβ transcription in a dose dependent manner. DAX1 interrupted the synergy between WT1 (-KTS) and SF1 on MIS gene expression during male gonadal development (74). Although in this thesis we show that DAX1 can activate LHβ transcription independent of SF1, the mechanism of DAX1 activation was not fully explored and investigating the interaction among WT1, SF1 and DAX1 might illuminate the role of DAX1 in LHβ transcription.

GnRH also implements post-translational modifications to differentially regulate gonadotropin gene transcription. Post-translational modifications such as phosphorylation and ubiquitination have been shown to play important roles in transcription by regulating protein–protein interactions, association of co-activators and co-repressors on transcription factors, turnover of transcription factors/co-regulators, and clearing proteins from the promoter to facilitate new rounds of transcription (55,101,103,104,109). SF1 is an orphan nuclear receptor and doesn't have a known

ligand; instead, posttranslational modifications such as phosphorylation and ubiquitination regulate its activity. SF1 is phosphorylated on Serine203 by the MAPK signaling pathway in response to GnRH. Phosphorylation of SF1 regulates its interaction with other proteins such as GRIP1, SMRT and Pitx1 (44,112) . Serine203 phosphorylation also primes SF1 for ubiquitination. GnRH induced mono-ubiquitination of SF1 increases its transcriptional activity by synergizing the SF1-Pitx1 association during the LHβ transcription in LβT2 cells (112). Ubiquitination of Egr1 in response to GnRH ensures its cyclic association with the LHβ promoter to maintain several transcriptional cycles. Egr1 is post transcriptionally modified by phosphorylation, sumoylation and ubiquitination (40,64). The phosphorylated form(s) of Egr1 binds DNA more efficiently and in a dose dependent manner (65). Both WT1 splice-variants are phosphorylated by PKA and PKC which modulates transcription activity, cellular localization and DNA binding ability of WT1. In our studies we show WT1 protein levels and association to the LHβ promoter is modulated by proteasome activity in response to GnRH ,however we could not distinguish between the splice-variants in association with the promoter and their response to the proteasome. It will be interesting to test the mono and polyubiquitination levels of WT1. It might be possible that GnRH is modulating WT1 phosphorylation and thus ubiquitination and/or promoter association by PKC or PKA signaling pathways in response to GnRH (147).

Overall differential gene regulation by GnRH is a combination of different signaling pathways, protein synthesis, modification and degradation. Moreover, to maintain the periodicity of transcription cycles, GnRH regulates both activation and deactivation of signaling pathways and synthesis and degradation of the proteins (transcription factors

and co-regulators). This thesis identifies WT1 as a new-regulator for LHβ transcription that might be differentially regulated by GnRH, favoring LHβ transcription.

Another layer of regulation that remains unexplored in this study is the effect of steroid hormones that can feedback to the hypothalamus regulating GnRH pulse frequency and the pituitary to directly regulate gonadotropin gene transcription. WT1 has been shown to down regulate both AR and ER in vitro (148,149). Our preliminary data shows that DHT decreases overall WT1 protein levels in the presence of GnRH, however, it would be informative to know if the effect of DHT is splice variant specific. In PCOS patients, high androgen levels may contribute to increased LH synthesis and gene transcription. It is possible that androgen specifically down regulates WT1 (+KTS), a repressor of LHβ transcription and thereby indirectly increases LHβ transcription as observed in PCOS patients. Moreover, a study shows that DHT treatment downregulates WT1 expression in endometrial stromal cells, and significant decreases in WT1 expression was found in endometrium of PCOS patients compared to non-PCOS endometrium (150). WT1 expression is required for decidualization to facilitate successful embryo implantation. DHT has been shown to downregulate WT1 and dysregulate its target genes such as EGFR and Bcl-2, which might adversely affect embryo implantation in the endometrium (150). Investigating the effect of estrogen and androgen on WT1 mediated transcriptional regulation of LHβ transcription might give an insight into how dysregulation of steroid levels affects LH and fertility in PCOS and other infertility syndromes.

In this work we have used continuous GnRH treatment on LβT2 cells, however, to have

a deeper physiological insight, testing the effect of various GnRH pulse pattern needs to be addressed in future studies.

GnRH regulation of LHβ transcription: Proteasome Hypothesis

GnRH stimulates LHβ transcription by inducing Egr1 synthesis and stimulating the cyclic binding of the transcription factors Egr1 and SF1 on the LHβ promoter (29). A previous publication from our lab shows that blocking proteasomal degradation severely impedes the GnRH-induced cyclic association of the transcription factors Egr1 and SF1 on the LHβ promoter (29). Since proteasomal inhibition hampered the cyclic binding of these transcription factors we hypothesized there could be inhibitory proteins, either bound to the DNA or to the transcription factors Egr1 or SF1, and that those inhibitory proteins are hindering protein cycling on the promoter. These inhibitory proteins might require to be removed by proteasomal degradation in order to recruit transcriptional activators. Our candidate for the DNA-binding repressor protein was WT1 and the potential candidate for the transcription factor-bound inhibitory protein was DAX1. The zinc finger transcription factor WT1 is essential for urogenital system development. It also regulates several reproductive genes by interacting with SF1 or binding to GC-rich elements such as Egr1 binding sites (74,83,87,88) (62,63).DAX1 is expressed throughout the Hypothalamic-Pituitary-Adrenal-Gonadal axis and regulates several reproductive and steroidogenic genes via interaction with SF1; it has been suggested to have both co-activator and co-repressor properties(45,68,69,71,75)(72,135). In this thesis we investigated the potential roles for both WT1 and DAX1 in LHβ transcription using clonal mouse gonadotrope LβT2 cells. Furthermore, we have

examined the role of GnRH and the proteasome in regulating the cyclic association of the co-regulatory proteins (SRC1, GCN5, SMRT, DAX1, WT1) on the promoter.

Overall in chapter 2, we have confirmed a regulatory role of WT1 in the pituitary gonadotropes and also determined specific roles of the WT1 splice variants; -KTS, a positive regulator and +KTS, a negative regulator of LHβ transcription. Fig.30 is a simplified model that summarizes and explains the possible role of the WT1 splice variants in LHβ transcription.

Role of –KTS**:** At the basal level (in absence of GnRH) WT1–KTS could be binding to the 3'Egr1 binding site to maintain low LHβ promoter activity. This is supported by our data that shows WT1 is bound to the LHβ promoter at the basal level and WT1 (-KTS) somewhat enhances LHβ transcription at the basal level. Upon GnRH stimulation, Egr1 synthesis is induced dramatically and being a potent activator, Egr1 could replace –KTS and occupy one or both of the Egr1 binding sites to further stimulate LHβ transcription. Since Egr1 protein expression is more transient and WT1 expression is more stable, it is possible that WT1 (-KTS) continues to stimulate LHβ transcription in the presence or absence of Egr1, and a competitive and cyclic binding between Egr1 and WT1 might be occurring on the 3'Egr1 binding site. Furthermore, our WT1siRNA data suggests that WT1–KTS could be influencing Egr1 synthesis directly or indirectly, asWT1 –KTS knockdown resulted in reduced Egr1 primary transcript expression in the same experiment. WT1 (-KTS) could also interact with other proteins and/or regulate other genes to indirectly influence LHβ transcription.

Role of +KTS**:** At the basal level (in the absence of GnRH) WT1 (+KTS) may associate with one or both the SF1 sites most probably via the SF1 protein, as the SF1 binding site is not recognized by WT1. Moreover, WT1 (+KTS) could also be binding to the 3'Egr1 binding site that is adjacent to the SF1 binding site and repress LHβ promoter activity. As WT1 (+KTS) requires all 4 zinc-fingers to bind to the DNA, it is also possible that WT1 (+KTS) intrudes somewhat into the 3'SF1 binding site. Since our data shows that the mutation of the Egr1 3'site; and double mutation of 3'5' SF1sites, on the LHβ promoter compromises the repressive effect of WT1 (+KTS), these sites are vital for +KTS repressive activity. Our data shows that WT1 is bound to the LHβ promoter at the basal level. However, due to the absence of specific antibody to distinguish between the two splice variants, it could not be evaluated if one or both the WT1 splice variants bind to the LHβ promoter, or if a differential binding pattern is exhibited in presence or absence of GnRH. WT1 (+KTS) represses LHβ transcription at the basal level. Upon GnRH stimulation, Egr1 synthesis is induced dramatically and WT1 (+KTS) mRNA expression is reduced. GnRH suitably stimulates the activator, Egr1, and simultaneously represses the repressor, WT1 (+KTS), in order to stimulate LHβ transcription. GnRH possibly triggers the removal of WT1 (+KTS) from the promoter, thus facilitating Egr1 and WT (-KTS) to potentially replace WT1 (+KTS) binding to stimulate LHβ transcription. We further speculate that that WT1 (+KTS) could be interacting with or regulating Egr1 or other proteins, to directly or indirectly influence LHβ transcription. The Egr1 promoter has a GC rich region that could be a potential binding site for WT1(62,63). More importantly, the ratio of the WT1 splice variants might be crucial to balance their opposing effects in regulating LHβ transcription.

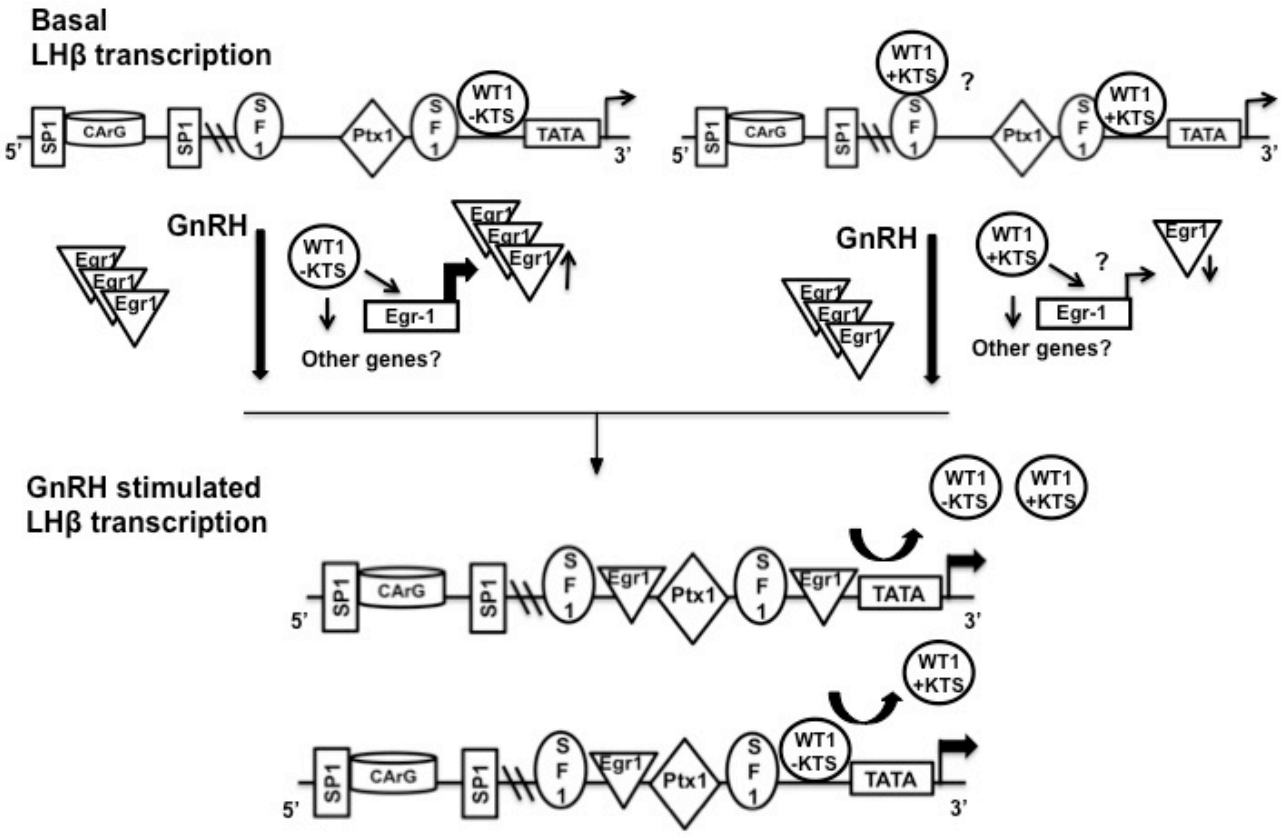

Figure 30 Role of WT1 splice variants (-KTS and +KTS) on LHβ transcription

Figure 30 Role of WT1 splice variants (-KTS and +KTS) on LHβ Transcription

WT1 (-KTS) may associate directly with the 3'Egr1 site of the LHβ promoter to stimulate basal promoter activity, but in the presence of GnRH stimulates Egr1 expression. Egr1 then binds to both the 3'- and 5'-Egr1 sites on the promoter and further increases LHβ transcription. WT1 (+KTS) may associate with the 3'-Egr1 site, but also requires the SF1 site for biological activity. Decreased expression of WT1 (+KTS) would stimulate basal LHβ expression as the suppressor is reduced. The isoforms likely compete for association to the LHβ promoter at the same sites

In Chapter 3 we have shown that the binding of the co-regulators GCN5, SRC1 (co-activators), SMRT (co-repressor) and WT1 and DAX1 (both co-activator and co-repressor activities) with the LHβ promoter is regulated by proteasome activity. We also show that whether the orphan nuclear receptor DAX1 acts as a positive or a negative regulator of LHβ transcription is dose dependent.

The model

The possible role of the proteasome in LHβ transcription is described in Fig.31 through a simplified model. At the basal level, a DNA binding repressor protein is bound to the LHβ promoter and/or the transcription factors may also be bound to repressor proteins. For example the co-repressor proteins Nab1 and Nab2 can bind to Egr1 to repress Egr1 trans activity. Similarly SMRT can act as a co-repressor of SF1 through direct physical interaction. WT1 (+KTS) may be a DNA-bound repressor protein. Upon GnRH stimulation, exchange between the co-repressor and the co-activators occur at the promoter. GnRH stimulates the ubiquitination of the repressor and co-repressor proteins to facilitate the proper recruitment of the transcription factors (Egr1, Ptx1, SF1, -KTS) along with their co-activators .In this work we have shown that GnRH stimulates the binding of the typical co-activators of SF1: GCN5, SRC1 as well as DAX1 and WT1 (-KTS). However, DAX1 can act as either co-activator co-repressor of SF1, to the LHβ promoter. The positive or negative role of DAX1 is dose dependent and context dependent in LHβ transcription. The dose dependent role of DAX1 could be due to postranslational modification modulating the protein-protein interaction or degradation of DAX1 or the protein it interacts with.

Once a transcriptionally active complex is formed, pRNApol II can bind and carry on LHβ transcription. Moreover, mono ubiquitination can assist protein-protein interactions. The proteins involved in LHβ transcription, could be modified by monoubiquitination in order to facilitate co-activator recruitment. After each round of transcription the components of the transcription complex is ubiquitinated and degraded by the proteasome machinery, to clear off the promoter and begin a new cycle of transcription.

Overall, as we speculated/suggested in our hypothesis, WT1 (+KTS) could serve as the DNA-bound inhibitory protein and DAX1 as a protein-bound regulator, positive or negative depending on its dose. In course of our research we found WT1 (–KTS) to be a positive regulator that enhances GnRH-stimulated LHβ transcription. This thesis is the first work that identifies a regulatory role of WT1 in the pituitary gonadotropes. We have also demonstrated that GnRH and proteasome activity regulates the cyclic binding of the transcription factors and their co-regulators (SRC1, GCN5, SMRT, DAX1, WT1) on the LHβ promoter. GnRH stimulation results in association and disassociation of both stimulatory and inhibitory proteins on the promoter. GnRH regulates LHβ transcription by controlling the cyclic association and dissociation of the regulatory proteins with the LHβ promoter and their degradation via the ubiquitin proteasome system after each cycle of transcription to support the dynamic nature of the transcribing chromatin.

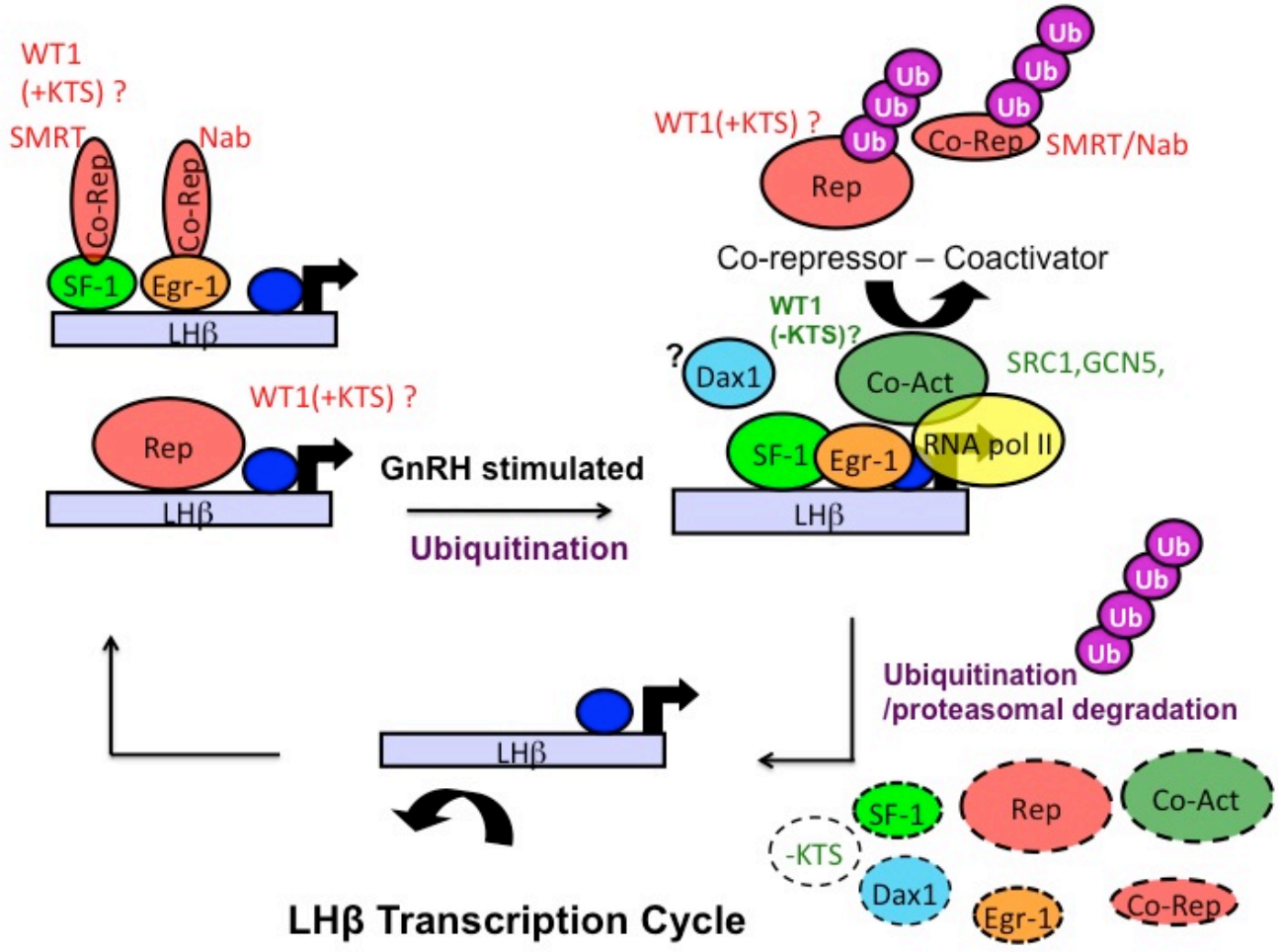

Figure 31 Role of the proteasome and regulators in GnRH stimulated LHβ transcription

Figure 31 Role of the proteasome and regulators in GnRH stimulated LHβ transcription

To stimulate the LHβ transcription GnRH facilitates the exchange of the corepressors with the transcriptional activators on the promoter. GnRH could be stimulating mono-ubiquitination, to favor protein-protein interactions between the positive regulators and poly-ubiquitination to remove the repressors via and proteasomal degradation. After each transcription cycle all the components of the transcriptional complex are degraded via proteasome activity to clear off the promoter and start a new transcriptional cycle and in this way several rounds of transcription is maintained. This overall cyclic process is regulated by GnRH at various levels; activating and deactivating several signaling molecules, transcription factors, co-regulatory proteins and regulation of post-translational modifications. GnRH mediates the co-ordination of various events in a rhythmic manner to influence LHβ transcription.

Perspective on PCOS and other diseases

PCOS is characterized by increased LH and androgen levels, therefore understanding the detailed mechanism of LH transcription was the broad goal of this study. However, the role of androgen on WT1 or DAX1 mediated effects on LHβ transcription is yet to be addressed. Preliminary data from our lab (by Carlos Santos) shows that androgen treatment down-regulates WT1 protein but not DAX1. A study on endometrial stromal cells shows cAMP-incuded WT1 expression to be down regulated in presence of DHT. The same study shows that cAMP-induced WT1 expression is significantly less in primary cells of PCOS endometrium when compared with fertile endometrium suggesting that hyperandrogenemia interferes with the required WT1 expression during embryo implantation (150). Another study showed that WT1 can bind to the AR promoter and also regulate AR target gene in presence or absence of androgen depending on the cell type (148).

In the case of LHβ transcription, the two variants play opposing roles, WT1 (-KTS) being a positive regulator and WT1 (+KTS) being a negative regulator. It will be interesting to investigate the affect of androgen on the splice variant specific regulation of LHβ transcription that can give more insight to PCOS with regard to WT1-AR interaction. The ratio between +KTS and –KTS and their interaction with AR may play a crucial role in influencing LHβ transcription. A proper ratio between the WT1 splice variants, +KTS: -KTS (2:1), is required for normal urogenital development and the reproductive system. Other human reproductive anomalies characterized by urogenital defects that occur due to WT1 mutations are WAGR, DDS and Frasier syndrome. Frasier syndrome,

characterized by sex reversal and developmental defects in the kidney and the gonads, is caused by an incorrect ratio between the WT1 splice variants. The imbalance between the splice variants in Frasier syndrome occurs due to a mutation in intron 9 of the WT1 gene, carrying the alternative splicing site that generates +KTS and –KTS proteins (94,95,126,127). Considering the significant role of WT1 on the reproductive system and the feedback mechanisms that exist between steroids in the hypothalamus and pituitary, it is difficult to evaluate the effects of WT1 mutations on the pituitary function. However, a particular WT1 mutation (IVS9+5G>A) that causes Frasier syndrome has been linked to hypergonadotropic hypogonadism and increased gonadotropin (LH and FSH) levels in the serum of the affected patients (126). In one report, the WT1 mutation (IVS9+4C>T) caused a decreased ratio of WT1 (+KTS) to WT1 (-KTS) that led to production of high levels of basal LH (127). This report is in agreement with our WT1siRNA data where the increase in basal LH transcription was observed when knockdown of both the splice variants occurred.

DAX1 is a critical protein that regulates several reproductive and steroidogenic genes in the pituitary, andrenal gland and the gonads and its interaction with SF1 and WT1 is worth exploring with regard to LHβ transcription (45,72,135).Our studies did not identify how DAX1 enhances GnRH stimulation, but it may occur through recruitment of additional co-activators, prevention of co-repressor recruitment, or stabilization of protein-protein interactions such as Egr1-LRH1/SF1. A potential interplay of DAX1 and WT1 splice variants (+KTS and –KTS) could be explored in the future, as the dosage sensitivity of DAX1, relative to WT1, has been suggested to play a role in MIS expression (74). For example, in normal males the gene dosage ratio of WT1 (-KTS):

DAX1 is 1:2, since DAX1 is an X-linked gene and WT1 is autosomal. However, in Denys Drash syndrome (WT1 mutation in human) the WT1 (-KTS): DAX1 ratio is altered. Due to the specific WT1 mutation in Denys Drash syndrome the wild-type -KTS dose is reduced and DAX1 become more abundant compared to WT1 (–KTS). This altered ratio interrupts the WT1-SF1 synergy that promotes MIS gene transcription (74). In the case of LHβ transcription, this interplay of DAX1, WT1 and SF1 proteins could be influenced by GnRH mediated posttranslational modifications or degradation, altering the DAX1: WT1 ratio and help explain the dose dependent regulation by DAX1. Moreover, the question if WT1 regulates the transcription of other gonadotropin genes such as the FSHβ or the common α-subunit or if is it specific to LHβ transcription needs to be addressed.

Finally, to investigate the importance of WT1, and our other findings in normal and PCOS animal model (PNA mice) will be of more physiological approach. Prenatally androgenized mice or PNA mice exhibits characteristics of PCOS such as disrupted estrous cycle, high LH and androgen levels (151). We speculate that WT1 (+KTS) might be repressed and /or WT1 (-KTS) might be stimulated in the pituitary of PNA mice, as androgen has stimulatory effect on LH. Our preliminary data shows DHT downregulates WT1 protein levels. Possibly the WT1 (+KTS) variant is repressed by androgen facilitating the increase in LH level. Moreover, elevated androgen levels have been shown to increase GnRH pulse frequency by interfering with the negative feedback by progesterone (13).Variation in the GnRH pulses (due to androgen) might affect WT1 expression or ratios between the WT1 splice variants and thus regulation of LHβ transcription in PNA mice.

Chapter 5: Bibliography

1. **Ferris HA, Shupnik MA.** Mechanisms for pulsatile regulation of the gonadotropin subunit genes by GNRH1. *Biol. Reprod.* 2006;74(6):993–8.

2. **McCartney CR.** Maturation of sleep-wake gonadotrophin-releasing hormone secretion across puberty in girls: potential mechanisms and relevance to the pathogenesis of polycystic ovary syndrome. *J. Neuroendocrinol.* 2010;22(7):701–9.

3. **Messinis IE.** Ovarian feedback, mechanism of action and possible clinical implications. *Hum. Reprod. Update* 2006;12(5):557–71.

4. **Griffin JE,Ojeda SR.** *Textbook of Endocrine Physiology*. 2nd ed. Oxford University Press, New York; 1992.

5. **Gharib SD, Wierman ME, Shupnik MA, Chin WW.** Molecular biology of the pituitary gonadotropins. *Endocr. Rev.* 1990;11(1):177–99.

6. **Shupnik MA.** Effects of gonadotropin-releasing hormone on rat gonadotropin gene transcription in vitro: requirement for pulsatile administration for luteinizing hormone-beta gene stimulation. *Mol. Endocrinol.* 1990;4(10):1444–50.

7. **Roberts VJ, Barth S, El-Roeiy A, Yen SS.** Expression of inhibin/activin subunits and follistatin messenger ribonucleic acids and proteins in ovarian follicles and the corpus luteum during the human menstrual cycle. *J. Clin. Endocrinol. Metab.* 1993;77(5):1402–10.

8. **Popovics P, Rekasi Z, Stewart AJ, Kovacs M.** Regulation of pituitary inhibin/activin subunits and follistatin gene expression by GnRH in female rats. *J. Endocrinol.* 2011;210(1):71–9.

9. **Welt C, Sidis Y, Keutmann H, Schneyer A.** Activins, inhibins, and follistatins: from endocrinology to signaling. A paradigm for the new millennium. *Exp. Biol. Med.* 2002;227(9):724–52.

10. **De Kretser DM, Hedger MP, Loveland KL, Phillips DJ.** Inhibins, activins and follistatin in reproduction. *Hum. Reprod. Update* 2002;8(6):529–41.

11. **Ehrmann DA.** Polycystic Ovary Syndrome. *N. Engl. J. Med.* 2005;352(12):1223–1236.

12. **Blank SK, McCartney CR, Helm KD, Marshall JC.** Neuroendocrine effects of androgens in adult polycystic ovary syndrome and female puberty. *Semin. Reprod. Med.* 2007;25(5):352–9.

13. **Pielecka J, Quaynor SD, Moenter SM.** Androgens increase gonadotropin-releasing hormone neuron firing activity in females and interfere with progesterone negative feedback. *Endocrinology* 2006;147(3):1474–9.

14. **Pastor CL.** Polycystic Ovary Syndrome: Evidence for Reduced Sensitivity of the Gonadotropin-Releasing Hormone Pulse Generator to Inhibition by Estradiol and Progesterone. *J. Clin. Endocrinol. Metab.* 1998;83(2):582–590.

15. **Eagleson CA, Gingrich MB, Pastor CL, Arora TK, Burt CM, Evans WS, Marshall JC.** Polycystic Ovarian Syndrome: Evidence that Flutamide Restores Sensitivity of the Gonadotropin-Releasing Hormone Pulse Generator to Inhibition by Estradiol and Progesterone. *J. Clin. Endocrinol. Metab.* 2000;85(11):4047–4052.

16. **Dumesic DA, Abbott DH, Padmanabhan V.** Polycystic ovary syndrome and its developmental origins. *Rev. Endocr. Metab. Disord.* 2007;8(2):127–41.

17. **Tsutsumi R, Webster NJG.** GnRH pulsatility, the pituitary response and reproductive dysfunction. *Endocr. J.* 2009;56(6):729–37.

18. **Gambineri A, Pelusi C, Vicennati V, Pagotto U, Pasquali R.** Obesity and the polycystic ovary syndrome. *Int J Obes Relat Metab Disord.* 2002;26(7):883–896.

19. **Diao F-Y, Xu M, Hu Y, Li J, Xu Z, Lin M, Wang L, Zhou Y, Zhou Z, Liu J, Sha J.** The molecular characteristics of polycystic ovary syndrome (PCOS) ovary defined by human ovary cDNA microarray. *J. Mol. Endocrinol.* 2004;33(1):59–72.

20. **Liu F, Usui I, Evans LG, Austin DA, Mellon PL, Olefsky JM, Webster NJG.** Involvement of both G(q/11) and G(s) proteins in gonadotropin-releasing hormone receptor-mediated signaling in L beta T2 cells. *J. Biol. Chem.* 2002;277(35):32099–108.

21. **Kraus S, Naor Z, Seger R.** Intracellular signaling pathways mediated by the gonadotropin-releasing hormone (GnRH) receptor. *Arch. Med. Res.* 2001;32(6):499–509.

22. **Naor Z, Benard O, Seger R.** Activation of MAPK cascades by G-protein-coupled receptors: the case of gonadotropin-releasing hormone receptor. *Trends Endocrinol. Metab.* 2000;11(3):91–9.

23. **Haisenleder DJ, Burger LL, Walsh HE, Stevens J, Aylor KW, Shupnik MA, Marshall JC.** Pulsatile gonadotropin-releasing hormone stimulation of gonadotropin subunit transcription in rat pituitaries: evidence for the involvement of Jun N-terminal kinase but not p38. *Endocrinology* 2008;149(1):139–45.

24. **Yokoi T, Ohmichi M, Tasaka K, Kimura A, Kanda Y, Hayakawa J, Tahara M, Hisamoto K, Kurachi H, Murata Y.** Activation of the luteinizing hormone beta promoter by gonadotropin-releasing hormone requires c-Jun NH2-terminal protein kinase. *J. Biol. Chem.* 2000;275(28):21639–47.

25. **Duan WR, Ito M, Park Y, Maizels ET, Hunzicker-Dunn M, Jameson JL.** GnRH regulates early growth response protein 1 transcription through multiple promoter elements. *Mol. Endocrinol.* 2002;16(2):221–33.

26. **Andrade J, Quinn J, Becker RZ, Shupnik MA.** AMP-activated protein kinase is a key intermediary in GnRH-stimulated LHβ gene transcription. *Mol. Endocrinol.* 2013;27(5):828–39.

27. **Haisenleder DJ, Ferris HA, Shupnik MA.** The calcium component of gonadotropin-releasing hormone-stimulated luteinizing hormone subunit gene transcription is mediated by calcium/calmodulin-dependent protein kinase type II. *Endocrinology* 2003;144(6):2409–16.

28. **Ferris H a, Walsh HE, Stevens J, Fallest PC, Shupnik M a.** Luteinizing hormone beta promoter stimulation by adenylyl cyclase and cooperation with gonadotropin-releasing hormone 1 in transgenic mice and LBetaT2 Cells. *Biol. Reprod.* 2007;77(6):1073–80.

29. **Walsh HE, Shupnik MA.** Proteasome regulation of dynamic transcription factor occupancy on the GnRH-stimulated luteinizing hormone beta-subunit promoter. *Mol. Endocrinol.* 2009;23(2):237–50.

30. **Weck J, Anderson AC, Jenkins S, Fallest PC, Shupnik MA.** Divergent and Composite Responsive Elements in the Rat Luteinizing Hormone Subunit Genes. 2000;(July):472–485.

31. **Curtin D, Ferris HA, Häkli M, Gibson M, Jänne O a, Palvimo JJ, Shupnik MA.** Small nuclear RING finger protein stimulates the rat luteinizing hormone-beta promoter by interacting with Sp1 and steroidogenic factor-1 and protects from androgen suppression. *Mol. Endocrinol.* 2004;18(5):1263–76.

32. **Kaiser UB, Halvorson LM, Chen MT.** Sp1, steroidogenic factor 1 (SF-1), and early growth response protein 1 (egr-1) binding sites form a tripartite gonadotropin-releasing hormone response element in the rat luteinizing hormone-beta gene promoter: an integral role for SF-1. *Mol. Endocrinol.* 2000;14(8):1235–45.

33. **Barnhart KM, Mellon PL.** The orphan nuclear receptor, steroidogenic factor-1, regulates the glycoprotein hormone alpha-subunit gene in pituitary gonadotropes. *Mol. Endocrinol.* 1994;8(7):878–85.

34. **Ingraham HA, Lala DS, Ikeda Y, Luo X, Shen WH, Nachtigal MW, Abbud R, Nilson JH, Parker KL.** The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. *Genes Dev.* 1994;8(19):2302–2312.

35. **Ngan ES, Cheng PK, Leung PC, Chow BK.** Steroidogenic factor-1 interacts with a gonadotrope-specific element within the first exon of the human gonadotropin-releasing hormone receptor gene to mediate gonadotrope-specific expression. *Endocrinology* 1999;140(6):2452–62.

36. **Burger LL, Haisenleder DJ, Aylor KW, Marshall JC.** Regulation of Lhb and Egr1 gene expression by GNRH pulses in rat pituitaries is both c-Jun N-terminal kinase (JNK)- and extracellular signal-regulated kinase (ERK)-dependent. *Biol. Reprod.* 2009;81(6):1206–15.

37. **Tremblay JJ, Drouin J.** Egr-1 is a downstream effector of GnRH and synergizes by direct interaction with Ptx1 and SF-1 to enhance luteinizing hormone beta gene transcription. *Mol. Cell. Biol.* 1999;19(4):2567–76.

38. **Salisbury TB, Binder AK, Grammer JC, Nilson JH.** Maximal activity of the luteinizing hormone beta-subunit gene requires beta-catenin. *Mol. Endocrinol.* 2007;21(4):963–71.

39. **Mouillet J-F, Sonnenberg-Hirche C, Yan X, Sadovsky Y.** P300 Regulates the Synergy of Steroidogenic Factor-1 and Early Growth Response-1 in Activating Luteinizing Hormone-Beta Subunit Gene. *J. Biol. Chem.* 2004;279(9):7832–9.

40. **Miller RS, Wolfe A, He L, Radovick S, Wondisford FE.** CREB binding protein (CBP) activation is required for luteinizing hormone beta expression and normal fertility in mice. *Mol. Cell. Biol.* 2012;32(13):2349–58.

41. **Poukka H, Aarnisalo P, Santti H, Jänne OA, Palvimo JJ.** Coregulator small nuclear RING finger protein (SNURF) enhances Sp1- and steroid receptor-mediated transcription by different mechanisms. *J. Biol. Chem.* 2000;275(1):571–9.

42. **Lawson MA, Tsutsumi R, Zhang H, Talukdar I, Butler BK, Sharon J, Mellon PL, Webster NJG, Medicine R, Neuroscience PLM, Santos SJ.** Pulse sensitivity of the luteinizing hormone beta promoter is determined by a negative feedback loop Involving early growth response-1 and Ngfi-A binding protein 1 and 2. *Mol. Endocrinol.* 2007;21(5):1175–1191.

43. **Kowase T, Walsh HE, Darling DS, Shupnik MA.** Estrogen enhances gonadotropin-releasing hormone-stimulated transcription of the luteinizing hormone subunit promoters via altered expression of stimulatory and suppressive transcription factors. *Endocrinology* 2007;148(12):6083–91.

44. **Hammer GD, Krylova I, Zhang Y, Darimont BD, Simpson K, Weigel NL, Ingraham HA.** Phosphorylation of the nuclear receptor SF-1 modulates cofactor recruitment: integration of hormone signaling in reproduction and stress. *Mol. Cell* 1999;3(4):521–6.

45. **Crawford PA, Dorn C, Sadovsky Y, Milbrandt J.** Nuclear receptor DAX-1 recruits nuclear receptor corepressor N-CoR to steroidogenic factor 1. *Mol. Cell. Biol.* 1998;18(5):2949–56.

46. **Sonoda J, Pei L, Evans RM.** Nuclear receptors: decoding metabolic disease. *FEBS Lett.* 2008;582(1):2–9.

47. **Li Y, Choi M, Cavey G, Daugherty J, Suino K, Kovach A, Bingham NC, Kliewer SA, Xu HE.** Crystallographic identification and functional characterization of phospholipids as ligands for the orphan nuclear receptor steroidogenic factor-1. *Mol. Cell* 2005;17(4):491–502.

48. **Krylova IN, Sablin EP, Moore J, Xu RX, Waitt GM, MacKay JA, Juzumiene D, Bynum JM, Madauss K, Montana V, Lebedeva L, Suzawa M, Williams JD, Williams SP, Guy RK, Thornton JW, Fletterick RJ, Willson TM, Ingraham HA.** Structural analyses reveal phosphatidyl inositols as ligands for the NR5 orphan receptors SF-1 and LRH-1. *Cell* 2005;120(3):343–55.

49. **Urs AN, Dammer E, Sewer MB.** Sphingosine regulates the transcription of CYP17 by binding to steroidogenic factor-1. *Endocrinology* 2006;147(11):5249–58.

50. **Pratt WB, Toft DO.** Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr. Rev.* 1997;18(3):306–60.

51. **Rosenfeld MG, Lunyak V V, Glass CK.** Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev.* 2006;20(11):1405–28.

52. **Cohen RN, Brzostek S, Kim B, Chorev M, Wondisford FE, Hollenberg a N.** The specificity of interactions between nuclear hormone receptors and corepressors is mediated by distinct amino acid sequences within the interacting domains. *Mol. Endocrinol.* 2001;15(7):1049–61.

53. **Freedman L.** Multimeric Coactivator Complexes for Steroid/Nuclear Receptors. *Trends Endocrinol. Metab.* 1999;10(10):403–407.

54. **Lonard DM, O'Malley BW.** The expanding cosmos of nuclear receptor coactivators. *Cell* 2006;125(3):411–4.

55. **Ismail A, Nawaz Z.** Nuclear hormone receptor degradation and gene transcription: an update. *IUBMB Life* 2005;57(7):483–90.

56. **Schimmer BP, White PC.** Minireview: steroidogenic factor 1: its roles in differentiation, development, and disease. *Mol. Endocrinol.* 2010;24(7):1322–37.

57. **Ito M, Yu RN, Jameson JL.** Steroidogenic factor-1 contains a carboxy-terminal transcriptional activation domain that interacts with steroid receptor coactivator-1. *Mol. Endocrinol.* 1998;12(2):290–301.

58. **Jacob AL, Lund J, Martinez P, Hedin L.** Acetylation of steroidogenic factor 1 protein regulates its transcriptional activity and recruits the coactivator GCN5. *J. Biol. Chem.* 2001;276(40):37659–64.

59. **Lee SL, Sadovsky Y, Swirnoff AH, Polish JA, Goda P, Gavrilina G, Milbrandt J.** Luteinizing hormone deficiency and female infertility in mice lacking the transcription factor NGFI-A (Egr-1). *Science* 1996;273(5279):1219–21.

60. **Dorn C, Ou Q, Svaren J, Crawford PA, Sadovsky Y.** Activation of luteinizing hormone beta gene by gonadotropin-releasing hormone requires the synergy of early growth response-1 and steroidogenic factor-1. *J. Biol. Chem.* 1999;274(20):13870–6.

61. **Halvorson LM, Kaiser UB, Chin WW.** The protein kinase C system acts through the early growth response protein 1 to increase LHbeta gene expression in synergy with steroidogenic factor-1. *Mol. Endocrinol.* 1999;13(1):106–16.

62. **Hamilton TB, Barilla KC, Romaniuk PJ.** High affinity binding sites for the Wilms' tumour suppressor protein WT1. *Nucleic Acids Res.* 1995;23(2):277–84.

63. **Gannon AM, Turner EC, Reid HM, Kinsella BT.** Regulated Expression of the alpha Isoform of the Human Thromboxane A2 Receptor during Megakaryocyte Differentiation: A Coordinated Role for WT1, Egr1, and Sp1. *J. Mol. Biol.* 2009;394(1):29–45.

64. **Pagel J-I, Deindl E.** Early growth response 1-A transcription factor in the crossfire of signal transduction cascades. *Indian J. Biochem. Biophys.* 2011;48(4):226–35.

65. **Huang RP, Adamson ED.** The phosphorylated forms of the transcription factor, Egr-1, bind to DNA more efficiently than non-phosphorylated. *Biochem. Biophys. Res. Commun.* 1994;200(3):1271–6.

66. **Manente AG, Pinton G, Tavian D, Lopez-Rodas G, Brunelli E, Moro L.** Coordinated sumoylation and ubiquitination modulate EGF induced EGR1 expression and stability. *PLoS One* 2011;6(10):e25676.

67. **Parker KL, Schimmer BP.** Steroidogenic factor 1: a key determinant of endocrine development and function. *Endocr. Rev.* 1997;18(3):361–77.

68. **Habiby RL, Boepple P, Nachtigall L, Sluss PM, Crowley WF, Jameson JL.** Adrenal Hypoplasia Congenita with Hypogonadotropic Hypogonadism. 1996:1055–1062.

69. **Iyer AK, McCabe ERB.** Molecular mechanisms of DAX1 action. *Mol. Genet. Metab.* 2004;83(1-2):60–73.

70. **Suzuki T, Kasahara M, Yoshioka H, Morohashi K.** LXXLL-Related Motifs in Dax-1 Have Target Specificity for the Orphan Nuclear LXXLL-Related Motifs in Dax-1 Have Target Specificity for the Orphan Nuclear Receptors Ad4BP / SF-1 and LRH-1. *Mol. Cell. Biol.* 2003;23(1):238–249.

71. **Ikeda Y, Swain A, Weber TJ, Hentges KE, Zanaria E, Lalli E, Tamai KT, Sassone-Corsi P, Lovell-Badge R, Camerino G, Parker KL.** Steroidogenic factor 1 and Dax-1 colocalize in multiple cell lineages: potential links in endocrine development. *Mol. Endocrinol.* 1996;10(10):1261–72.

72. **Babu PS.** Interaction Between Dax-1 and Steroidogenic Factor-1 in Vivo: Increased Adrenal Responsiveness to ACTH in the Absence of Dax-1. *Endocrinology* 2002;143(2):665–673.

73. **Park SY, Meeks JJ, Raverot G, Pfaff LE, Weiss J, Hammer GD, Jameson JL.** Nuclear receptors Sf1 and Dax1 function cooperatively to mediate somatic cell differentiation during testis development. *Development* 2005;132(10):2415–23.

74. **Nachtigal MW, Hirokawa Y, Enyeart-VanHouten DL, Flanagan JN, Hammer GD, Ingraham H a.** Wilms' tumor 1 and Dax-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression. *Cell* 1998;93(3):445–54.

75. **Xu B, Yang W-H, Gerin I, Hu C-D, Hammer GD, Koenig RJ.** Dax-1 and steroid receptor RNA activator (SRA) function as transcriptional coactivators for steroidogenic factor 1 in steroidogenesis. *Mol. Cell. Biol.* 2009;29(7):1719–34.

76. **Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R.** WT-1 is required for early kidney development. *Cell* 1993;74(4):679–91.

77. **Chau YY, Brownstein D, Mjoseng H, Lee WC, Buza-Vidas N, Nerlov C, Jacobsen SE, Perry P, Berry R, Thornburn A, Sexton D, Morton N, Hohenstein P, Freyer E, Samuel K, van't Hof R, Hastie N.** Acute multiple organ failure in adult mice deleted for the developmental regulator Wt1. *PLoS Genet.* 2011;7(12):e1002404.

78. **Rauscher FJ, Morris JF, Tournay OE, Cook DM, Curran T.** Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. *Science* 1990;250(4985):1259–62.

79. **Mrowka C, Schedl A.** Wilms' tumor suppressor gene WT1: from structure to renal pathophysiologic features. *J. Am. Soc. Nephrol.* 2000;11 Suppl 1:S106–S115.

80. **Gashler a L, Bonthron DT, Madden SL, Rauscher FJ, Collins T, Sukhatme VP.** Human platelet-derived growth factor A chain is transcriptionally repressed by the Wilms tumor suppressor WT1. *Proc. Natl. Acad. Sci. U. S. A.* 1992;89(22):10984–10988.

81. **Oh S, Song Y, Yim J, Kim TK.** The Wilms' tumor 1 tumor suppressor gene represses transcription of the human telomerase reverse transcriptase gene. *J. Biol. Chem.* 1999;274(52):37473–8.

82. **Hewitt SM, Hamada S, Mcdonnell TJ, Rauscher FJ, Saunders GF.** Regulation of the Proto-oncogenes bcl-2 and c- myc by the Regulation of the Proto-oncogenes bcl-2 and c-myc by the Wilms ' Tumor. 1995:5386–5389.

83. **Wilhelm D, Englert C.** The Wilms tumor suppressor WT1 regulates early gonad development by activation of Sf1. *Genes Dev.* 2002;16(14):1839–51.

84. **Kim J, Prawitt D, Bardeesy N, Torban E, Vicaner C, Goodyer P, Zabel B, Pelletier J.** The Wilms' tumor suppressor gene (wt1) product regulates Dax-1 gene expression during gonadal differentiation. *Mol. Cell. Biol.* 1999;19(3):2289–99.

85. **Dame C, Kirschner KM, Bartz K V., Wallach T, Hussels CS, Scholz H.** Wilms tumor suppressor, Wt1, is a transcriptional activator of the erythropoietin gene. *Blood* 2006;107(11):4282–90.

86. **Lee SB, Huang K, Palmer R, Truong VB, Herzlinger D, Kolquist KA, Wong J, Paulding C, Yoon SK, Gerald W, Oliner JD, Haber DA.** The Wilms tumor suppressor WT1 encodes a transcriptional activator of amphiregulin. *Cell* 1999;98(5):663–673.

87. **Hohenstein P, Hastie ND.** The many facets of the Wilms' tumour gene, WT1. *Hum. Mol. Genet.* 2006;15 Spec No(2):R196–201.

88. **Huff V.** Wilms' tumours: about tumour suppressor genes, an oncogene and a chameleon gene. *Nat. Rev. Cancer* 2011;11(2):111–121.

89. **Hastie ND.** Life , Sex , and WT1 Isoforms — Three Amino Acids Can Make All the Difference Minireview. 2001;106:391–394.

90. **Bor Y, Swartz J, Morrison A, Rekosh D, Ladomery M, Hammarskjöld M-L.** The Wilms' tumor 1 (WT1) gene (+ KTS isoform) functions with a CTE to enhance translation from an unspliced RNA with a retained intron. *Genes Dev.* 2006;20(12):1597–608.

91. **Larsson SH, Charlieu JP, Miyagawa K, Engelkamp D, Rassoulzadegan M, Ross A, Cuzin F, van Heyningen V, Hastie ND.** Subnuclear localization of WT1 in splicing or transcription factor domains is regulated by alternative splicing. *Cell* 1995;81(3):391–401.

92. **Davies RC, Calvio C, Bratt E, Larsson SH, Lamond AI, Hastie ND.** WT1 interacts with the splicing factor U2AF65 in an isoform-dependent manner and can be incorporated into spliceosomes. *Genes Dev.* 1998;12(20):3217–25.

93. **Wells J, Rivera MN, Kim WJ, Starbuck K, Haber DA.** The predominant WT1 isoform (+KTS) encodes a DNA-binding protein targeting the planar cell polarity gene Scribble in renal podocytes. *Mol. Cancer Res.* 2010;8(7):975–85.

94. **Barbaux S, Niaudet P, Gubler MC, Grünfeld JP, Jaubert F, Kuttenn F, Fékété CN, Souleyreau-Therville N, Thibaud E, Fellous M, McElreavey K.** Donor splice-site mutations in WT1 are responsible for Frasier syndrome. *Nat. Genet.* 1997;17(4):467–70.

95. **Klamt B, Koziell a, Poulat F, Wieacker P, Scambler P, Berta P, Gessler M.** Frasier syndrome is caused by defective alternative splicing of WT1 leading to an altered ratio of WT1 +/-KTS splice isoforms. *Hum. Mol. Genet.* 1998;7(4):709–14.

96. **Kohsaka T, Tagawa M, Takekoshi Y, Yanagisawa H, Tadokoro K, Yamada M.** Exon 9 mutations in the WT1 gene, without influencing KTS splice isoforms, are also responsible for Frasier syndrome. *Hum. Mutat.* 1999;14(6):466–70.

97. **Tootle TL, Rebay I.** Post-translational modifications influence transcription factor activity: a view from the ETS superfamily. *Bioessays* 2005;27(3):285–98.

98. **Nickel BE, Allis CD, Davie JR.** Ubiquitinated histone H2B is preferentially located in transcriptionally active chromatin. *Biochemistry* 1989;28(3):958–63.

99. **Herrmann J, Lerman LO, Lerman A.** Ubiquitin and ubiquitin-like proteins in protein regulation. *Circ. Res.* 2007;100(9):1276–91.

100. **Lecker SH, Goldberg AL, Mitch WE.** Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. *J. Am. Soc. Nephrol.* 2006;17(7):1807–19.

101. **Muratani M, Tansey WP.** How the ubiquitin-proteasome system controls transcription. *Nat. Rev. Mol. Cell Biol.* 2003;4(3):192–201.

102. **Wallace AD, Cidlowski JA.** Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids. *J. Biol. Chem.* 2001;276(46):42714–21.

103. **Lonard DM, Nawaz Z, Smith CL, O'Malley BW.** The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. *Mol. Cell* 2000;5(6):939–48.

104. **Nalepa G, Rolfe M, Harper JW.** Drug discovery in the ubiquitin-proteasome system. *Nat. Rev. Drug Discov.* 2006;5(7):596–613.

105. **Ruffner H, Joazeiro CA, Hemmati D, Hunter T, Verma IM.** Cancer-predisposing mutations within the RING domain of BRCA1: loss of ubiquitin protein ligase activity and protection from radiation hypersensitivity. *Proc. Natl. Acad. Sci. U. S. A.* 2001;98(9):5134–9.

106. **Martins-Branco D, Esteves AR, Santos D, Arduino DM, Swerdlow RH, Oliveira CR, Januario C, Cardoso SM.** Ubiquitin proteasome system in Parkinson's disease: a keeper or a witness? *Exp. Neurol.* 2012;238(2):89–99.

107. **McNaught KSP, Belizaire R, Isacson O, Jenner P, Olanow CW.** Altered proteasomal function in sporadic Parkinson's disease. *Exp. Neurol.* 2003;179(1):38–46.

108. **Nawaz Z, Lonard DM, Dennis AP, Smith CL, O'Malley BW.** Proteasome-dependent degradation of the human estrogen receptor. *Proc. Natl. Acad. Sci. U. S. A.* 1999;96(5):1858–62.

109. **Wu R-C, Feng Q, Lonard DM, O'Malley BW.** SRC-3 coactivator functional lifetime is regulated by a phospho-dependent ubiquitin time clock. *Cell* 2007;129(6):1125–40.

110. **David Y, Ziv T, Admon A, Navon A.** The E2 ubiquitin-conjugating enzymes direct polyubiquitination to preferred lysines. *J. Biol. Chem.* 2010;285(12):8595–604.

111. **Orford K, Crockett C, Jensen JP, Weissman AM, Byers SW.** Serine Phosphorylation-regulated Ubiquitination and Degradation of -Catenin. *J. Biol. Chem.* 1997;272(40):24735–24738.

112. **Luo Z, Wijeweera A, Oh Y, Liou Y-C, Melamed P.** Pin1 facilitates the phosphorylation-dependent ubiquitination of SF-1 to regulate gonadotropin beta-subunit gene transcription. *Mol. Cell. Biol.* 2010;30(3):745–63.

113. **Reid G, Hübner MR, Métivier R, Brand H, Denger S, Manu D, Beaudouin J, Ellenberg J, Gannon F.** Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. *Mol. Cell* 2003;11(3):695–707.

114. **Le Scolan E, Zhu Q, Wang L, Bandyopadhyay A, Javelaud D, Mauviel A, Sun L, Luo K.** Transforming growth factor-beta suppresses the ability of Ski to inhibit tumor metastasis by inducing its degradation. *Cancer Res.* 2008;68(9):3277–85.

115. **Luo M, Koh M, Feng J, Wu Q, Melamed P.** Cross talk in hormonally regulated gene transcription through induction of estrogen receptor ubiquitylation. *Mol. Cell. Biol.* 2005;25(16):7386–98.

116. **Haisenleder DJ, Dalkin AC, Ortolano GA, Marshall JC, Shupnik MA.** A pulsatile gonadotropin-releasing hormone stimulus is required to increase transcription of the gonadotropin subunit genes: evidence for differential regulation of transcription by pulse frequency in vivo. *Endocrinology* 1991;128(1):509–17.

117. **Zhao L, Bakke M, Krimkevich Y, Cushman LJ, Parlow AF, Camper SA, Parker KL.** Steroidogenic factor 1 (SF1) is essential for pituitary gonadotrope function. *Development* 2001;128(2):147–54.

118. **Dammer EB, Leon A, Sewer MB.** Coregulator exchange and sphingosine-sensitive cooperativity of steroidogenic factor-1, general control nonderepressed 5, p54, and p160 coactivators regulate cyclic adenosine 3',5'-monophosphate-dependent cytochrome P450c17 transcription rate. *Mol. Endocrinol.* 2007;21(2):415–38.

119. **Ji S-Y, Hao J-X, Li L, Zhang J, Zheng Q-S, Li X-X, Wang X-N, Han C-S, Gao F, Liu Y-X.** Expression of inhibin-alpha is regulated synergistically by Wilms' tumor gene 1 (Wt1) and steroidogenic factor-1 (Sf1) in sertoli cells. *PLoS One* 2013;8(1):e53140.

120. **Webster NJ, Kong Y, Sharma P, Haas M, Sukumar S, Seely BL.** Differential effects of Wilms tumor WT1 splice variants on the insulin receptor promoter. *Biochem. Mol. Med.* 1997;62(2):139–50.

121. **Chu Z, Andrade J, Shupnik MA, Moenter SM.** Differential regulation of gonadotropin-releasing hormone neuron activity and membrane properties by acutely applied estradiol: dependence on dose and estrogen receptor subtype. *J. Neurosci.* 2009;29(17):5616–27.

122. **Fallest PC, Trader GL, Darrow JM, Shupnik MA.** Regulation of rat luteinizing hormone beta gene expression in transgenic mice by steroids and a gonadotropin-releasing hormone antagonist. *Biol. Reprod.* 1995;53(1):103–9.

123. **Call GB, Wolfe MW.** Species differences in GnRH activation of the LHbeta promoter: role of Egr1 and Sp1. *Mol. Cell. Endocrinol.* 2002;189(1-2):85–96.

124. **Ritchie MF, Yue C, Zhou Y, Houghton PJ, Soboloff J.** Wilms tumor suppressor 1 (WT1) and early growth response 1 (EGR1) are regulators of STIM1 expression. *J. Biol. Chem.* 2010;285(14):10591–6.

125. **Sakamoto KM, Bardeleben C, Yates KE, Raines MA, Golde DW, Gasson JC.** 5' upstream sequence and genomic structure of the human primary response gene, EGR-1/TIS8. *Oncogene* 1991;6(5):867–71.

126. **Benz K, Plank C, Amann K, Mucha B, Dörr HG, Rascher W, Dötsch J.** Hypergonadotropic hypogonadism and renal failure due to WT1 mutation. *Nephrol. Dial. Transplant* 2006;21(6):1716–8.

127. **Melo KFS, Martin RM, Costa EMF, Carvalho FM, Jorge A a, Arnhold IJP, Mendonca BB.** An unusual phenotype of Frasier syndrome due to IVS9 +4C>T mutation in the WT1 gene: predominantly male ambiguous genitalia and absence of gonadal dysgenesis. *J. Clin. Endocrinol. Metab.* 2002;87(6):2500–5.

128. **Tremblay JJ, Drouin J.** Egr-1 Is a Downstream Effector of GnRH and Synergizes by Direct Interaction with Ptx1 and SF-1 To Enhance Luteinizing Hormone β Gene Transcription. Egr-1.Mol Cell Biol1999;19:2567-2576

129. **Ikeda Y, Takeda Y, Shikayama T, Mukai T, Hisano S, Morohashi KI.** Comparative localization of Dax-1 and Ad4BP/SF-1 during development of the hypothalamic-pituitary-gonadal axis suggests their closely related and distinct functions. *Dev. Dyn.* 2001;220(4):363–76.

130. **Holter E, Kotaja N, Mäkela S, Strauss L, Kietz S, Jänne O a, Gustafsson J-A, Palvimo JJ, Treuter E.** Inhibition of androgen receptor (AR) function by the reproductive orphan nuclear receptor DAX-1. *Mol. Endocrinol.* 2002;16(3):515–28.

131. **Zhang H, Thomsen JS, Johansson L, Gustafsson J-A, Treuter E.** DAX-1 Functions as an LXXLL-containing Corepressor for Activated Estrogen Receptors. *J. Biol. Chem.* 2000;275(51):39855–39859.

132. **Zhang J, Guenther MG, Carthew RW, Lazar MA.** Proteasomal regulation of nuclear receptor corepressor-mediated repression. *Genes Dev.* 1998;12(12):1775–80.

133. **Gurates B.** WT1 and DAX-1 regulate SF-1-mediated human P450arom gene expression in gonadal cells. *Mol. Cell. Endocrinol.* 2003;208(1-2):61–75.

134. **Kelly VR, Xu B, Kuick R, Koenig RJ, Hammer GD.** Dax1 up-regulates Oct4 expression in mouse embryonic stem cells via LRH-1 and SRA. *Mol. Endocrinol.* 2010;24(12):2281–91.

135. **Achermann JC, Meeks JJ, Jameson JL.** Phenotypic spectrum of mutations in DAX-1 and SF-1. *Mol. Cell. Endocrinol.* 2001;185(1-2):17–25.

136. **Fortin J, Kumar V, Zhou X, Wang Y, Auwerx J, Schoonjans K, Boehm U, Boerboom D, Bernard DJ.** NR5A2 regulates Lhb and Fshb transcription in gonadotrope-like cells in vitro, but is dispensable for gonadotropin synthesis and fertility in vivo. Lydon JP, ed. *PLoS One* 2013;8(3):e59058.

137. **Fayard E, Auwerx J, Schoonjans K.** LRH-1: an orphan nuclear receptor involved in development, metabolism and steroidogenesis. *Trends Cell Biol.* 2004;14(5):250–60.

138. **Ikeda Y, Luo X, Abbud R, Nilson JH, Parker KL.** The nuclear receptor steroidogenic factor 1 is essential for the formation of the ventromedial hypothalamic nucleus. *Mol. Endocrinol.* 1995;9(4):478–86.

139. **Fortin J, Lamba P, Wang Y, Bernard DJ.** Conservation of mechanisms mediating gonadotrophin-releasing hormone 1 stimulation of human luteinizing hormone beta subunit transcription. *Mol. Hum. Reprod.* 2009;15(2):77–87.

140. **Zheng W, Yang J, Jiang Q, He Z, Halvorson LM.** Liver receptor homologue-1 regulates gonadotrope function. *J. Mol. Endocrinol.* 2007;38(1-2):207–19.

141. **Kaiser UB, Jakubowiak A,Steinberger A and Chin WW.** Differential Effects of Gonadotropin-Releasing Hormone (GnRH) Pulse Frequency on Gonadotropin Subunit and GnRH Receptor Messenger Ribonucleic Acid Levels in Vitro. *Endocrinology* 1997:1224–1231.

142. **Vasilyev V, Lawson MA, Diapolo D, Webster NJG and Mellon PL.** Different Signaling Pathways Control Acute Induction versus Long-Term Repression of LHβ Transcription by GnRH. *Endocrinology* 2002;143(9):3414–3426.

143. **Kaiser UB, Conn PM, Chin WW.** Studies of Gonadotropin-Releasing Hormone (GnRH) Action Using GnRH Receptor-Expressing Pituitary Cell Lines1. *Endocr. Rev.* 2013;18(1):46-70

144. **Kanasaki H, Purwana I, Oride A, Mijiddorj T, Miyazaki K.** Extracellular Signal-Regulated Kinase (ERK) Activation and Mitogen-Activated Protein Kinase Phosphatase 1 Induction by Pulsatile Gonadotropin-Releasing Hormone in Pituitary Gonadotrophs. *J. Signal Transduct.* 2012;2012:198527.

145. **Alarid ET, Windle JJ, Whyte DB, Mellon PL.** Immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice. *Development* 1996;122(10):3319–29.

146. **Bédécarrats GY, Kaiser UB.** Differential regulation of gonadotropin subunit gene promoter activity by pulsatile gonadotropin-releasing hormone (GnRH) in perifused L beta T2 cells: role of GnRH receptor concentration. *Endocrinology* 2003;144(5):1802–11.

147. **Ye Y, Raychaudhuri B, Gurney A, Campbell CE, Williams BR.** Regulation of WT1 by phosphorylation: inhibition of DNA binding, alteration of transcriptional activity and cellular translocation. *EMBO J.* 1996;15(20):5606–15.

148. **Zaia A, Fraizer GC, Piantanelli L, Saunders GF.** Transcriptional regulation of the androgen signaling pathway by the Wilms' tumor suppressor gene WT1. *Anticancer Res.* 21(1A):1–10.

149. **Han Y, Yang L, Suarez-Saiz F, San-Marina S, Cui J, Minden MD.** Wilms' tumor 1 suppressor gene mediates antiestrogen resistance via down-regulation of estrogen receptor-alpha expression in breast cancer cells. *Mol. Cancer Res.* 2008;6(8):1347–55.

150. **D. Gonzalez, H. Thackeray, P. D. Lewis, A. Mantani, N. Brook, K. Ahuja, R. Margara, L. Joels, J. O. White and RSC.** Loss of WT1 Expression in the Endometrium of Infertile PCOS Patients: A Hyperandrogenic Effect? *J. Clin. Endocrinol. Metab.* 2012:957–966.

151. **Sullivan SD, Moenter SM.** Prenatal androgens alter GABAergic drive to gonadotropin-releasing hormone neurons: implications for a common fertility disorder. *Proc. Natl. Acad. Sci. U. S. A.* 2004;101(18):7129–34.