Review

Hormones in synergy: Regulation of the pituitary gonadotropin genes

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Abstract

The precise interplay of hormonal influences that governs gonadotropin hormone production by the pituitary includes endocrine, paracrine and autocrine actions of hypothalamic gonadotropin-releasing hormone (GnRH), activin and steroids. However, most studies of hormonal regulation of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the pituitary gonadotrope have been limited to analyses of the isolated actions of individual hormones. LHβ and FSHβ subunits have distinct patterns of expression during the menstrual/estrous cycle as a result of the integration of activin, GnRH, and steroid hormone action. In this review, we focus on studies that delineate the interplay among these hormones in the regulation of LHβ and FSHβ gene expression in gonadotrope cells and discuss how signaling cross-talk contributes to differential expression. We also discuss how recent technological advances will help identify additional factors involved in the differential hormonal regulation of LH and FSH.

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Abbreviations: AP-1, activator protein-1; AR, androgen receptor; ChIP, chromatin immunoprecipitation; Egr-1, early growth response-1; ER, estrogen receptor; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; HD, homeodomain element; HRE, hormone response element; LH, luteinizing hormone; HPG, hypothalamic-pituitary-gonadal; MAPK, mitogen-activated protein kinase; PCOS, polycystic ovary syndrome; PR, progesterone receptor; PKC, protein kinase C; SBE, Smad-binding element; SF-1, steroidogenic factor-1.

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1. Introduction to the gonadotropins

The hypothalamic-pituitary-gonadal (HPG) axis plays a pivotal role in every phase of mammalian reproduction including fetal development, puberty, the menstrual cycle, pregnancy, postpartum, and menopause. Fertility depends on precise hormonal regulation of this axis. Two of the most critical hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are produced exclusively in the gonadotrope cells of the anterior pituitary. They are secreted into the blood stream, primarily in response to gonadotropin-releasing hormone (GnRH) from the hypothalamus (Seeburg et al., 1987; Vale et al., 1977). Gonadal steroid hormones, such as estrogen, progesterone and testosterone, in addition to peptide hormones, such as activin, inhibin, and follistatin, modulate LH and FSH levels via feedback to the anterior pituitary, as well as to the hypothalamus. LH and FSH regulate critical aspects of reproduction in the gonads, including steroidogenesis, gametogenesis and ovulation (Burns and Matzuk, 2002).

LH and FSH are heterodimeric glycoproteins composed of a common α subunit (α-GSU) dimerized with unique β subunits (LHβ and FSHβ) (Pierce and Parsons, 1981). Since the β subunits confer the biological specificity of LH and FSH and synthesis of these subunits is the rate-limiting step for production of the mature hormones (Kaiser et al., 1997b; Papavasiliou et al., 1986), many studies have focused on the interactions of hormones within the pituitary to control LH and FSH synthesis. Comprehension of these molecular mechanisms not only provides insight into the physiology and pathology of the mammalian reproductive system, but may lead to the development of novel contraceptive methods or conversely, infertility treatments.

LH is required for reproduction in mice, since absence of the LHβ gene results in hypogonadism and infertility in both sexes (Ma et al., 2004). Humans with inactivating mutations in LHβ have normal intrauterine development due to a compensatory role of placental human chorionic gonadotropin hormone, but lack pubertal development and are infertile (Huhtaniemi, 2006; Huhtaniemi et al., 2006). FSH is essential for the fertility of female mice and plays an important, though not essential, role in male fertility. Female mice deficient in FSHβ have a block in folliculogenesis prior to the antral stage, while males are fertile but have impaired reproductive function (Kumar et al., 1997). The human FSHβ gene is critical for reproductive function in both genders. A variety of non-synonymous mutations result in absent or incomplete pubertal development in women, relatively normal pubertal development but azoospermia in men, and infertility in both women and men (Lamminen et al., 2005).

2. Differential regulation of LH and FSH synthesis

During the rodent estrous cycle, a surge of GnRH during the afternoon of proestrus triggers a surge in both LH and FSH, resulting in ovulation of the mature follicle in response to LH (Jones, 1997). Several hours later, during the morning of estrus, a secondary FSH surge occurs that does not correspond to a surge in LH (Besecke et al., 1997; Woodruff et al., 1996). In humans, the FSH bioactivity levels also increase at mid to late luteal phase through the early to mid-follicular phase of the menstrual cycle. This secondary FSH surge in rodents is essential for follicular development for the subsequent cycle (DePaolo et al., 1979; Hoak and Schwartz, 1980; Papavasiliou et al., 1986). In humans, the increase in the FSH level coincides with a critical time during folliculogenesis when the next cohort of follicles is being recruited. Transcription of the gonadotropin β subunits both precedes and correlates with LH and FSH levels in the blood. LHβ gene expression is upregulated three-fold in the afternoon of proestrus prior to the onset of the surge (Butcher et al., 1974; Zmeili et al., 1986). FSHβ mRNA levels increase four to five-fold during the afternoon of proestrus and three-fold during estrus (Halvorson et al., 1994; Ortolano et al., 1988). Thus, despite receiving identical input from autocrine, paracrine and endocrine hormonal signals, LH and FSH synthesis and secretion are differentially regulated during the estrous cycle and this regulation appears to occur primarily at the level of transcription of the β subunits.

As we discuss below, numerous studies have documented differential hormonal regulation of LHβ and FSHβ gene expression. Several mechanisms have been proposed to explain the means by which variations or alterations in gonadotropin synthesis occur. GnRH is released in a pulsatile fashion from hypothalamic neurons into the hypophyseal-portal system and differing GnRH pulse frequencies have been found to participate in differential regulation of LHβ and FSHβ [reviewed recently in Ferris and Shupnik, 2006]. Multiple studies have shown that an increase in GnRH pulse frequency favors LHβ (Burger et al., 2008; Haisenleder et al., 2008), while a decrease favors FSHβ gene expression (Dalkin et al., 1989; Haisenleder et al., 1988, 1991; Papavasiliou et al., 1986). LHβ seems to be especially sensitive to GnRH pulse frequency and amplitude, while FSHβ less so (Burger et al., 2008). In addition to GnRH, regulation of the activin/follistatin/inhibin feedback loop is thought to be important for differential LHβ and FSHβ synthesis. Follistatin synthesis in the pituitary is inversely correlated with FSHβ expression during the estrous cycle (Besecke et al., 1997; Halvorson et al., 1994). Follistatin transcription may also be regulated by differential GnRH pulse frequencies, again in the opposite manner to FSHβ (Burger et al., 2002; Kirk et al., 1994). Ovarian-derived inhibin has been shown to suppress FSH production during metestrus and diestrus (Woodruff et al., 1993), but its expression is decreased during estrus (Woodruff et al., 1996). Therefore, higher levels of bio-available activin, due to a drop in follistatin and/or inhibin levels in estrus, may selectively favor FSHβ synthesis (Besecke et al., 1997; Woodruff et al., 1996). Finally, steroid hormones, such as progesterone, testosterone, and glucocorticoids have all been shown to induce FSHβ but inhibit LHβ expression (Burger et al., 2004b; Thackray et al., 2006). In this review, we discuss the evidence that differential LHβ and FSHβ synthesis is modulated by interactions among these hormones, in concert with differential GnRH pulse control. Understanding how these signals are conveyed and integrated may be especially important in pathological conditions associated with a disturbed LH to FSH ratio such as polycystic ovary syndrome (PCOS) or obesity.

PCOS is a clinical disorder affecting ~10% of reproductive-age women. Characteristic of PCOS include increased LH pulsatility and reduced FSH levels, which lead to increased androgen production and anovulation, respectively (Chang, 2007; Hall et al., 1998; Morales et al., 1996). Hyperandrogenemia, in turn, may impair hypothalamic progesterone sensitivity and prevent slowing of GnRH pulses (Blank et al., 2006). Obesity is the most common chronic disease in the United States today, and contributes to menstrual cycle disturbances, anovulation and reduced rates of conception (Zain and Norman, 2008). Similarly to PCOS, obesity correlates with increased LH pulse frequency (Yoo et al., 2006). An increase in the frequency of LH pulses and disruption of circadian fluctuations in LH secretion have been observed in obese girls by late puberty (McCaffrey et al., 2009). Although it is still not completely understood how signals from GnRH neurons in the hypothalamus and paracrine/endocrine feedback from the anterior pituitary/gonads are interpreted by gonadotrope cells, it is clear that proper functioning of the HPG axis and maintenance of a normal LH/FSH ratio is critical for reproductive health.
3. Approaches for analyses of hormonal regulation in gonadotrope cells

Although there are distinct differences in the hormonal regulation of the HPG axis among mammalian species, there is also a great deal of conservation. For instance, FSHβ null mice, which are infertile, can have their fertility restored by replacing the mouse FSHβ gene with 10 kb of the human one containing 4 kb of the 5′ regulatory region, all exons and introns and 2 kb of the 3′ flanking region (Kumar et al., 1998). Several experimental systems have been used extensively to examine hormonal regulation of the gonadotropin genes in rodents. These include whole animals, primary pituitary cell culture and immortalized cell culture. Conclusions derived from physiological studies in rats and mice in vivo obviously benefit from the fact that the data are obtained within the endogenous hormonal setting. However, it can be cumbersome to differentiate the sites of hormone action because hormonal feedback occurs at the level of both the hypothalamus and the anterior pituitary. In rodents, separation of GnRH input from the pituitary can be accomplished biochemically with continuous GnRH agonist or antagonist administration. However, cross-talk between GnRH and other hormone signaling pathways cannot be studied effectively in this setting.

Primary pituitary cell culture is also frequently employed to study hormonal effects on gonadotropin synthesis. However, there are several caveats to these models that are worth mentioning. First, the endocrine milieu at the time of harvest can impact the results (Fallest and Schwartz, 1991), so caution must be used when interpreting data from animals in different estrous stages. Second, gonadotropes only comprise 5–15% of the cells in the anterior pituitary (Ooi et al., 2004). Four other types of secretory cells including thyrotropes, somatotropes, lactotropes and corticotropes are also present, as well as folliculostellate cells. Many hormones secreted from these cells, such as prolactin and oxytocin from lactotropes, have paracrine effects on gonadotrope cells [recently reviewed in Denef, 2008]. Folliculostellate cells have been reported to rapidly overgrow other pituitary cells in culture (Kawakami et al., 2002) and can impact the experimental outcome since they produce paracrine factors such as follistatin and PACAP [recently reviewed in Winters and Moore, 2007].

Gonadotrope cell culture is ideal for analyzing the mechanisms of individual and combined hormonal regulation of gonadotropin gene expression. Several reports describe methods to enrich gonadotropes from the mixed cell population of the pituitary. For instance, gonadotropes have been successfully enriched from rat pituitaries, though the procedure is time consuming (Childs and Unabia, 2001). More recently, gonadotropes have been purified from transgenic mice containing 4.7 kb of the ovine FSHβ promoter linked to the cell surface antigen, H-2Kβ (Wu et al., 2004). However, these gonadotrope cells are only efficiently purified from the pituitaries of male or ovariectomized female mice and the relatively low yield makes it difficult to perform detailed characterization of the molecular mechanisms involved in hormonal regulation of gonadotropin gene expression.

Two immortalized clonal cell lines were developed in our laboratory using targeted tumorigenesis with SV40 T-antigen linked to the 5′ regulatory regions of α- and β-subunits of LHβ (Alarid et al., 1996, 1998). The αT-1 cell line expresses the human gonadotropin subunit that is highly similar to the mouse homologue. The βT2 cell line expresses the human β-subunit of LHβ and secretes LH and FSH in response to LHβ/cAMP. The βT2 cell line also expresses activin, follistatin, and inhibin, as well as the receptors for activin, inhibin and steroid hormones (Lawson et al., 2001; Lewis et al., 2000; Schreiber et al., 2000; Thackray et al., 2006; Turgeon et al., 1996). Thus, the development of these immortalized cell lines set the stage for an intensive characterization of the hormonal signaling mechanisms that mediate transcription of the gonadotropin genes. Given the comprehensive experimental tools available for studying gonadotropin biology in rodents, we have focused this review on studies performed in rodents or tissues derived from them with only occasional comparisons to studies in other mammalian species.

4. Hormonal regulation of LHβ and FSHβ gene expression

4.1. GnRH

The GnRH receptor is a member of the rhodopsin family of G protein-coupled, seven-transmembrane receptors and is expressed specifically in pituitary gonadotropes (Kaiser et al., 1997a; Sealfon et al., 1997; Stojilkovic et al., 1994; Tsutsumi et al., 1992). However, the GnRH receptor has a unique structure, because it lacks an intracellular, cytoplasmic tail; a feature that allows for a much slower internalization (Hislop et al., 2001). Slower internalization may play a role in the necessity for pulsatile release of GnRH into the hypophyseal-portal system, since long-term tonic exposure adversely affects gonadotropin function (Belchetz et al., 1978; Burren and Jameson, 1989; Shupnik, 1990). Ligand binding to the GnRH receptor causes activation of G proteins, primarily Gq and G11 (Stanislaus et al., 1997), with the subsequent activation of phospholipase Cβ, release of calcium from intracellular stores, and increase in the activity of protein kinase C (PKC) and calcium/calmodulin kinase II (Haisenleder et al., 2003; Liu et al., 2002). GnRH receptor activation also leads to activation of three branches of the mitogen-activated protein kinase (MAPK) pathway: ERK1/2, JNK, and p38 (Liu et al., 2002; Roberson et al., 1995; Sundaresan et al., 1996), via PKC (Naor, 2009) or via association of GnRH receptor with Raf and calmodulin in lipid rafts (Roberson et al., 2005).

Induction of LHβ gene expression by GnRH is dependent upon PKC signaling, since the PKC inhibitor Bis-indolyl Maleimide prevents LHβ induction (Vasilyev et al., 2002a). A role for calcium has been documented (Weck et al., 1998); and may correlate with its role in the activation of conventional PKC isofoms, since calcium increase by itself after ionophore treatment is not sufficient to induce LHβ (Harris et al., 2002). The role of MAPK in LHβ induction was examined in a number of studies, but it is still inconclusive which branches of the MAPK pathway contribute to LHβ induction. The discrepancies in these studies may arise from different experimental paradigms. For instance, the length of GnRH treatment may be important, since ERK1/2 are activated rapidly after 2–5 min, while p38 and JNK are maximally activated after 30–60 min (Yokoi et al., 2000; Harris et al., 2002). Most studies agree that the ERK1/2 and JNK branches of the MAPK pathway are required for LHβ induction by GnRH, while a role of p38 is controversial (Yokoi et al., 2000; Harris et al., 2002; Yamada et al., 2004). These results correlate with the fact that ERK1/2 and JNK are involved in the growth factor induction of early growth response-1 (Egr-1) which is the only known factor induced by GnRH that regulates transcription of LHβ.

GnRH induces mammalian LHβ gene expression through rapid induction of the immediate-early gene, Egr-1 (Kaiser et al., 2000; Weck et al., 2000). Upon induction by GnRH, Egr-1 interacts with both tissue-specific, and ubiquitous, basal factors to regulate the LHβ promoter [reviewed in Jorgensen et al., 2004]. Female Egr-1 null mice are infertile and lack expression of the LHβ gene, indicating that other Egr family members (Egr-2,3,4) are unable to compensate (Lee et al., 1996; Topilko et al., 1998). The proximal promoter region contains two Egr-1 sites in tandem with two sites for steroidogenic factor-1 (SF-1) located on either side of a homeodomain element (HD) (Halvorson et al., 1996; Quirk et al., 2001) (Fig. 1). The pituitary-specific HD protein, Ptx1, has been shown to
bind the homeodomain element in heterologous cells (Tremblay and Drouin, 1999), while in LβT2 cells, the protein binding to the site is related to Otx1, rather than Ptx1 (Rosenberg and Mellon, 2002). Synergistic interactions among SF-1, Egr-1 and Ptx1 are essential for GnRH induction of LHβ gene expression (Dorn et al., 1999; Tremblay and Drouin, 1999). The distal region of the rodent promoter contains several Sp1 binding sites (Kaiser et al., 1998) and an overlapping CARG element, important for pulsatile GnRH induction in LβT2 cells (Weck et al., 2000) (Fig. 1). Sp1 interacts with the Egr-1/Sp1/HD complex in a spatially dependent manner (Kaiser et al., 2000; Weck et al., 2000). The co-activator, SNURF (Curtin et al., 2004) and the scaffold protein, p300 (Mouillet et al., 2004) bridge distal and proximal promoter regions and may potentiate Egr-1 action, and therefore, the GnRH effect. β-catenin has also been reported to play a role in GnRH induction of LHβ, since it accumulates in the nucleus following GnRH treatment and increases SF-1 and Egr-1-mediated LHβ transcription through an interaction with SF-1 (Gardner et al., 2007; Salisbury et al., 2007).

Recent studies have also analyzed mechanisms involved in the responsiveness to pulsatile GnRH treatment and frequency-dependent expression of the LHβ gene. For instance, proteasome function was reported to be critical for LHβ responsiveness and GnRH treatment was shown to mediate ubiquitination of SF-1 and Egr-1 which results in the constant cycling of these factors on and off of the LHβ promoter (Walsh and Shupnik, 2009). This may allow the LHβ gene to respond to each pulse of GnRH. Sustained induction of Egr repressor proteins from the Nab family at low GnRH frequency may also be critical in turning off the signal and/or adjusting the LHβ response to high frequency of GnRH (Lawson et al., 2007).

GnRH regulates FSHβ at the transcriptional level, since serum FSH levels are reduced 60–90% in mice lacking GnRH compared to wild-type mice (Mason et al., 1986) and one pulse of GnRH administered to castrated, testosterone-replaced rats increased FSHβ gene expression four-fold (Dalkin et al., 2001). GnRH regulation of FSHβ gene expression is mediated by the PKC and MAPK signaling pathways (Coss et al., 2004; Liu et al., 2005; Vasilyev et al., 2002b; Bonfil et al., 2004).

GnRH regulation of the FSHβ gene also occurs through the induction of intermediate, immediate-early genes, which constitute activator protein-1 (AP-1). AP-1 consists of a variety of dimers of Fos isoforms (c-Fos, FosB, Fra-1 and Fra-2) and Jun isoforms (c-Jun, JunB and JunD), most of which are induced by GnRH (Karak et al., 2003; Wurmbach et al., 2001). While Fos proteins can only heterodimerize with members of the Jun family to form transcriptionally active complexes, Jun proteins can also make homodimers. In the ovine FSHβ promoter, two putative AP-1 binding sites were identified using JAR placental cells (Strahl et al., 1997, 1998). More recently, we demonstrated that AP-1 plays a role in GnRH induction of the murine FSHβ gene (Fig. 1). Specifically, we showed that the role of MAPK, in the GnRH induction of FSHβ gene, is via the induction of c-Fos and JunB (Coss et al., 2004; Liu et al., 2002), which, with c-Jun and FosB, bind and induce FSHβ promoter. The human FSHβ promoter has also been shown to be regulated by AP-1 proteins through two AP-1 response elements (Wang et al., 2008). AP-1, like Egr-1 on the LHβ gene, interacts with factors involved in basal expression of FSHβ, such as NF-Y, in the mouse and possibly USF1, in the rat promoter (Ciccone et al., 2008; Coss et al., 2004) to integrate GnRH responsiveness. A study by Johnson et al. (1992) indicated that c-Fos may play an important role in reproduction, since c-Fos knockout animals were reported to have small ovaries with atretic follicles (Johnson et al., 1992). Although the levels of gonadotropin hormones were not measured in these animals, the phenotype is reminiscent of FSHβ knockout mice (Kumar et al., 1997). GnRH regulation of the rat FSHβ gene also involves the CREB transcription factor (Ciccone et al., 2008), which binds the homologous CRE/AP-1 half-site. Although CREB deficient mice do not exhibit changes in FSH levels, other family members, such as CREM or ATF, may compensate for CREB deficiency (Hummler et al., 1994), or, alternatively, CREB may play a role only in rat FSHβ expression in a species-specific manner.

### 4.2. Activin

Activin was originally identified as a gonadal peptide that stimulates FSH production (Ling et al., 1986). It is a dimer of two β subunits, of which there are two critical isoforms, A and B. Activin binds the type II receptor, which leads to heterodimerization and phosphorylation of the type I receptor (Attisano and Wrana, 2002). Activin has also been shown to be critical for embryonic development. Knock-in of activin B into the activin A locus rescued neonatal lethality in mice, but revealed that activin A is required for ovarian and testicular growth (Chang et al., 2002). Mice deficient in the activin type II receptor survive to adulthood, as opposed to mice lacking the type I receptor, but have lower FSH levels and the females are infertile (Matzuk et al., 1995). Following receptor binding and phosphorylation of type I receptors, the type I receptor then phosphorylates Smad2 and Smad3. The phosphorylated Smads bind to Smad4 and translocate into the nucleus to activate transcription of target genes (Attisano and Wrana, 2002; Massague, 1998). Smad3 and 4 can bind DNA directly at Smad-binding elements (SBE), although their binding affinity is rather weak, while Smad2 does not bind directly to DNA (Massague, 1998; Shi et al., 1998). Inhibin (a heterodimer of one of the activin subunits and a unique α subunit) (Nakamura et al., 1990) and follistatin (a potent activin-binding protein) (Shimasaki et al., 1988) antagonize...
activin action. Interestingly, activin, follistatin and inhibin are all expressed in the mature pituitary gonadotrope and can function in an autocrine manner (Kaiser et al., 1992; Kagawa et al., 1991; Roberts et al., 1989). Follistatin is also synthesized by folliculostellate cells in the pituitary and can regulate activin availability in a paracrine manner (Gospodarowicz and Lau, 1989; Kawakami et al., 2002).

**LH**

**gene expression and secretion** has been shown to be regulated by activin in rodents (Attardi and Miklos, 1990; Burger et al., 2002), monkeys (McLachlan et al., 1989; Stouffer et al., 1993), human fetal cells (Blumenfeld and Ritter, 2001), and mouse primary pituitary cultures (Coss et al., 2005), although far less potently than FSHβ. Our studies demonstrated that activin induction of LHβ occurs through three adjacent activin-response elements (ARE, Fig. 1), which are juxtaposed to the SF-1, Egr-1 and homeodomain elements in the proximal region of the LHβ promoter (Coss et al., 2005). Upon activation, Smad3 and 4 bind the middle ARE element (Fig. 1) to induce the LHβ gene. Similarly to other Smad-regulated genes, the interaction of Smads with the LHβ promoter is probably strengthened by their interaction with a homeodomain protein such as Ptx1 or Otx1 (Datta et al., 2000; Germain et al., 2000). Alternatively, multiple activin-response elements in close proximity, such as the three in the LHβ promoter, permit cooperative binding of Smad proteins and allow them to overcome their low binding affinity (Lopez-Rovira et al., 2002). Smad3-deficient male mice were analyzed for gonadotropin gene expression, since females have an ovarian phenotype that disregulates the feedback (Tomic et al., 2002). We demonstrated that Smad3-null mice express lower levels of both LHβ and FSHβ (Coss et al., 2005), substantiating a role of activin in LHβ expression.

Activin is a potent inducer of the rodent FSHβ gene. Activin increases the release of FSH from the pituitary (Ling et al., 1986) and induces FSHβ expression in gonadotrope cells (Weiss et al., 1995). For activin induction of FSHβ, Smad3 seems to be a limiting factor, since its overexpression can induce a FSHβ luciferase reporter (Gregory et al., 2005; Lamba et al., 2006). The FSHβ promoter contains three activin-response elements, identified to date. One of them, located at −267 in the mouse FSHβ promoter, is a classical consensus Smad-binding site, comprised of a palindromic sequence GTCTAGAC (Bernard, 2004; Gregory et al., 2005; Suszko et al., 2005) (Fig. 1), but it does not appear to play a major role in the overall activin induction (Coss et al., 2007; McGillivray et al., 2007) and is not present in the human promoter. Two other AREs, containing a half-site AGAC motif, were identified in the FSHβ proximal promoter and are critical for activin induction in all species examined (Bailey et al., 2004) (Fig. 1). These elements are likely bound by other proteins, such as Pbx and Prep, which can interact with Smads and tether them to the promoter following their activation (Bailey et al., 2004; Suszko et al., 2003). These proteins may not only provide higher affinity binding, but may also afford signal specificity, if they are tissue-restricted binding partners. In addition to the Smad signaling pathway, the TAK1 pathway has been identified as another potential player in activin induction of FSHβ transcription (Safwat et al., 2005). Various BMPs can also stimulate FSHβ expression (although not as potently as activin) or can potentiate activin stimulation of FSHβ expression (Lee et al., 2007; Nicol et al., 2008; Otsuka and Shimasaki, 2002). However, the mechanism of their action is not known, nor is it understood whether Smads 1/5/8 when activated by BMP signaling, function through the same sites as Smads 2/3 activated by activin.

### 4.3. Steroids

The ovarian steroid hormones, estrogen and progesterone, regulate gonadotropin gene expression, as well as secretion, by negative and positive feedback to the hypothalamus and the anterior pituitary. Removal of ovarian hormones via ovariectomy in rodents results in increased levels of LH and FSH. For LH, these levels return to baseline after exogenous estrogen treatment (Shupnik et al., 1988). This negative feedback appears to occur via estrogen receptor (ER) α since knockout of the receptor in mice results in increased LH levels (Couse et al., 2003; Glidewell-Kenney et al., 2008). LH suppression by estrogen is also abolished by treatment with a GnRH antagonist, indicating the hypothalamic nature of the estrogen negative feedback (Shupnik and Fallest, 1994). In contrast to its action in the hypothalamus, estrogen has been reported to directly induce LHβ gene expression in gonadotropes (Shupnik et al., 1989a, b). The mechanism of estrogen action on the LHβ promoter is still unclear. ER was shown to bind an imperfect estrogen response element at −1189 in the distal rat LHβ promoter (Shupnik and Rosenzweig, 1991; Shupnik et al., 1989b), but it is not conserved in other mammalian species. Alternatively, ER can be recruited to the LHβ promoter through interactions with SF-1 and Ptx1 (Luo et al., 2005). Finally, estrogen has been shown to potentiate GnRH induction of LHβ indirectly by enhancing the expression of Egr-1 and suppressing the expression of ZEB, a transcriptional repressor that binds at −381 and −182 on the rat LHβ promoter (Kowase et al., 2007).

Estrogen appears to play only a partial role in suppressing FSH levels since its addition after ovariectomy does not result in full suppression (Shupnik et al., 1988). Studies provide evidence that another ovarian hormone such as inhibin is important for suppression of FSH levels (Dalkin et al., 1990, 1993; Gharib et al., 1987). There is very little evidence that estrogen directly modulates FSHβ gene expression in rodents. Estrogen did not alter the expression of FSHβ mRNA in ovariectomized rats treated with a GnRH antagonist (Dalkin et al., 1993; Shupnik et al., 1989a) or in female rat pituitary fragments (Shupnik and Fallest, 1994). Our experiments with the murine FSHβ promoter in LBT2 cells agree with these studies because estrogen did not stimulate FSHβ gene expression even in the presence of transfected ERO or ERβ (Thackray et al., 2006). Thus, instead of a direct effect, estrogen may regulate FSH indirectly by modulating GnRH or activin action. Interestingly, activin B expression was elevated in pituitary tissues from ERαKO mice (Couse et al., 2003).

Progesterone exerts both inhibitory and facilitating feedback effects on gonadotropin synthesis and secretion, through indirect regulation of GnRH production in the hypothalamus and direct regulation in the anterior pituitary [reviewed in Levine et al., 2001]. Studies on the role of progesterone in reproductive tissues, including the gonadotrope cell, are complicated by the fact that one of the functions of estrogen is to stimulate the expression of the progesterone receptor (PR) (Romano et al., 1989). PR is essential for female reproduction. Female mice carrying a null mutation of PR are infertile; they cannot respond to GnRH surge conditions and do not experience an elevation of LH and FSH levels prior to ovulation (Chappell et al., 1997).

Since LHβ mRNA levels decrease during estrus when progesterone levels are at their highest, progesterone is an excellent candidate for countering the stimulatory effects of GnRH and activin on LHβ gene expression. Contrary to studies measuring LHβ mRNA levels in ovariectomized rats or in primary pituitary cells treated with estrogen and progesterone (Kerrigan et al., 1993; Park et al., 1996), we recently demonstrated that progesterone can suppress both basal transcription and GnRH- or activin-stimulated LHβ gene expression in gonadotrope cells (Thackray et al., 2006; Thackray and Mellon, 2008). It is possible that earlier studies did not observe progesterone suppression because of the stimulatory effect of estrogen on LHβ gene expression. In our studies, we enhanced PR levels directly by overexpressing the receptor in LBT2 cells instead of using estrogen to induce PR, thus avoiding the effects of estrogen on LHβ gene expression. The suppressive effects of pro-
gesterone mapped to a region between −300 to −150 of the LHβ promoter (Thackray et al., 2009) (Fig. 1). This region was shown to be both necessary and sufficient for the suppression, since deletion of this region abrogated the progesterone suppression and fusing this region to a heterologous promoter recapitulated the suppression. Additionally, chromatin immunoprecipitation (ChiP) analysis revealed that PR was recruited to the LHβ promoter, though gel-shift assays did not detect direct binding of PR to the necessary regions of the promoter. Since the progesterin suppression of LHβ depended on the DNA binding domain of PR, these results suggest that suppression is mediated by indirect binding of PR or “tethering” to the LHβ promoter.

In contrast to the LHβ promoter, many studies support the hypothesis that progestins induce FSHβ gene expression in the anterior pituitary [reviewed in Burger et al., 2004b]. Both basal and GnRH-induced FSHβ mRNA levels increased in rats treated with estrogen and progesterone (Attardi and Fitzgerald, 1990; Kerrigan et al., 1993). Progesterone antagonists also blocked FSH secretion and mRNA expression during the pre-ovulatory FSH surge (Ringstrom et al., 1997) and the secondary FSH surge (Knox and Schwartz, 1992; Szabo et al., 1998). Reporter genes containing either the proximal rat or ovine FSHβ promoter responded to progesterin treatment in primary rat or ovine pituitary cultures, respectively (O’Connor et al., 1997; Webster et al., 1995). Our studies also demonstrated that progesterone induced the murine FSHβ promoter in LT2 cells (Thackray et al., 2006). In contrast to LHβ, progesterone induction of the mouse FSHβ promoter required direct binding and transactivation of PR. ChiP and gel-shift analysis demonstrated that PR bound to the proximal mouse FSHβ promoter in vivo and in vitro (Thackray et al., 2006). The progesterone responsiveness on the FSHβ promoter mapped to a region between −500 and −95 of the proximal mouse promoter; this region contains six putative hormone response elements (HREs) previously described in the rat and ovine promoters. Five of these HREs appear to play a functional role, including a consensus direct repeat element at −381 (Fig. 1). Collectively, our studies provide strong evidence that progesterone can directly modulate LHβ and FSHβ synthesis, as opposed to indirect means such as altering the balance of the activin/Inhibin/follistatin feedback loop. These results also support the hypothesis that progesterone differentially regulates gonadotropin gene expression by inhibiting LHβ and inducing FSHβ gene expression in the pituitary, and, thus, progesterone may modulate the secondary FSH surge.

In addition to progesterins, there is considerable evidence that androgens directly modulate the levels of LHβ and FSHβ mRNA in the anterior pituitary. Androgens have been reported to repress LHβ gene expression in both castrated, GnRH antagonist-treated rats (Burger et al., 2004a; Wierman and Wang, 1990) and primary pituitary cell culture (Winters et al., 1992). Additionally, androgens have been shown to suppress transcription of LHβ in immortalized gonadotrope cells. Androgen treatment suppressed GnRH-induced transcription of the rat LHβ promoter, likely through a direct protein–protein interaction between the androgen receptor (AR) and Sp1 (Curtin et al., 2001), while androgens were reported to suppress the bovine LHβ promoter through a protein–protein interaction between the androgen receptor (AR) and Sp1 (Curtin et al., 2001). Like progestins, androgens upregulate FSHβ mRNA levels. For example, castrated GnRH antagonist-treated rats exhibited a selective increase in FSHβ mRNA upon treatment with testosterone (Burger et al., 2004a; Dalkin et al., 1992; Paul et al., 1990; Wierman and Wang, 1990). Studies in primary pituitary cells confirmed that the activation of FSHβ expression by testosterone occurs at the level of the pituitary (Charib et al., 1990; Leal et al., 2003; Winters et al., 1992). Our studies using LT2 cells also demonstrated that the gonadotrope cell was sufficient for this response and that activation of both the murine and ovine FSHβ promoter by androgens was due to direct androgen receptor binding to HREs in the proximal promoter (Spady et al., 2004; Thackray et al., 2006) (Fig. 1).

Similarly to progestins and androgens, glucocorticoids can regulate LHβ and FSHβ synthesis in the anterior pituitary. A suppressive effect of glucocorticoids on GnRH-induced LHβ gene expression, as well as LH secretion, was observed in castrated rats (Rosen et al., 1991). Our recent studies showed that LHβ mRNA levels were inhibited by glucocorticoids in LT2 cells (Thackray et al., 2006). In contrast to LHβ, a selective increase in FSHβ gene expression in response to glucocorticoids has been demonstrated in rats (McAndrews et al., 1994; Ringstrom et al., 1991), as well as in primary pituitary cells (Bohnack et al., 2000; Kilen et al., 1996; Leal et al., 2003). We also demonstrated glucocorticoid induction of the murine FSHβ promoter in LT2 cells (Thackray et al., 2006).

5. Synergy between hormonal signaling pathways modulates FSHβ gene expression

Levels of bio-available activin increase during estrus, presumably due to decreases in follistatin and inhibin levels. GnRH and 3-keto steroid hormones also convey signals to the pituitary

![Fig. 2. Activin synergizes with GnRH (A and B) or progesterone (C and D) to induce FSHβ gene expression. A luciferase reporter containing 1 kb of the mouse FSHβ promoter was transiently transfected into LT2 cells. After overnight starvation in serum-free media, the cells were treated with vehicle control (C), 10 ng/ml activin (A), 10 nM GnRH (G), 100 nM R5020 (a synthetic progesterin; P), activin and GnRH co-treatment (AG) or activin and progesterin (AP), as indicated. The asterisks indicate significant differences from the vehicle-treated control, while pound signs indicate a synergistic interaction as defined by a two-way ANOVA (p < 0.05). The dashed line in A and C represents the level at which the hormone co-treatment is additive rather than synergistic. In B and D, synergy is demonstrated graphically as described in Slinker (1998). If the lines diverge, there is synergy, whereas if the lines remain parallel, there is no interaction. The circles represent control and GnRH (B) or progesterin (D) treatment alone. The squares represent activin treatment, both alone and with GnRH (B) or progestin (D).]
gonadotrope during this time. Thus, we propose that interactions between activin and these other hormonal signaling pathways are responsible for differential LHβ and FSHβ synthesis and the secondary FSH surge. In particular, our recent studies have demonstrated that GnRH and steroid hormones, such as progestins, androgens and glucocorticoids, can synergistically cooperate with activin to specifically induce FSHβ mRNA levels (Fig. 2). Synergy is defined as an interaction or cooperation of two hormones resulting in a combined effect greater than the sum of their individual effects. In Fig. 2A and B, activin and GnRH synergize while in C and D, activin and progesterone synergize. See Slinker (1998) for a succinct and useful discussion about synergy and the use of two-way ANOVA to identify an interaction between two treatments (Slinker, 1998). Confusion in the literature about interactions between hormonal signaling pathways mostly results from incorrect statistical analysis of the data. For instance, data is sometimes incompletely analyzed by one-way ANOVA despite the inherent two-way factorial design of the experiment. One-way ANOVA analysis can demonstrate that the combined effect of the two treatments is statistically different from either treatment alone, but not whether the interaction is additive or synergistic. We have used a dashed line in Fig. 2A and C to represent the level at which the hormone co-treatment would be additive rather than synergistic, which can be determined using two-way ANOVA. Fig. 2B and D illustrate this synergistic interaction with non-parallel lines.

5.1. Synergy between activin and GnRH

Activin and GnRH have been shown to synergistically induce rodent FSHβ gene expression (Coss et al., 2007; Gregory et al., 2005) and this synergism is specific for FSHβ. Follistatin diminishes the level of FSHβ mRNA levels in rat primary pituitary cells (Besecke et al., 1996) and reduces FSHβ promoter activity (Coss et al., 2007; Pernasetti et al., 2001). Follistatin also reduces the GnRH induction of FSHβ. Since gonadotrope cells secrete activin, these results support the concept that autocrine activin can synergize with GnRH and potentiate its effect. As we discussed previously, both GnRH and activin induce LHβ. However, co-treatment of LβT2 cells with activin and GnRH results in an additive LHβ induction (Coss et al., 2007; Yamada et al., 2004), suggesting independent and separate molecular mechanisms for induction, as opposed to their synergistic interaction on FSHβ. Therefore, synergistic induction of the FSHβ gene by activin and GnRH is not replicated on the LHβ gene, indicating that synergy is not due to an induction of the GnRH receptor and is likely an effect of direct action of these two hormones on the FSHβ promoter. The mechanism of the synergy between activin and GnRH on FSHβ entails cross-talk between signaling pathways that impinge on p38 MAPK and result in an increased amount of c-Fos protein and Smad3 phosphorylation (Coss et al., 2007). The human FSHβ gene was also reported to be synergistically induced by activin and GnRH (Wang et al., 2008), although the mechanism of this synergistic induction has yet to be elucidated. In the murine promoter, mutation of the −267 consensus SBE did not affect the level of synergy, while mutation of the AP-1 sites, both in the mouse (Coss et al., 2007) and human promoters (Wang et al., 2008) reduced the synergism. Only when the Smad site was mutated together with the AP-1 sites, in the mouse FSHβ promoter, was the synergy completely abrogated. On monomerized AP-1 elements, which lack activin responsiveness, there was also an increase in expression with activin and GnRH co-treatment, compared to GnRH treatment alone (Coss et al., 2007). The minor contribution of the −267 Smad site is not that surprising, since it has been shown that Smads and AP-1 can interact and induce the AP-1 response element of the human collagenase promoter (Qing et al., 2000). It is likely that Smads can be tethered to the FSHβ promoter through interaction with other transcription factors such as AP-1. On the other hand, Smad–DNA binding may still play a role, since there was a significant increase in synergy when both AP-1 and Smad elements were linked together on a heterologous promoter (Coss et al., 2007). Thus, during estrus and the secondary FSHβ surge, which is characterized by a slower GnRH pulse frequency, synergy between GnRH and activin may specifically induce FSHβ gene expression to promote folliculogenesis (Fig. 3).

Fig. 3. A model of hormonal regulation of differential LH and FSH synthesis in pituitary gonadotrope cells. There are multiple ways that peptide and steroid hormones can influence gonadotropin transcription. On the morning of proestrus, activin signaling is minimized due to high levels of the inhibitory molecules, follistatin (FS) and inhibin (1). GnRH signaling is also muted, resulting in low levels of both LHβ and FSHβ mRNA (2). On the afternoon of proestrus, GnRH pulsatility and amplitude increase dramatically prior to ovulation leading to increased transcription of LHβ and FSHβ (3). Estrogen may also play a direct role in inducing LHβ gene expression (4). During estrus, the levels of bio-available activin increase due to a reduction in follistatin and inhibin levels, resulting in increased levels of FSHβ mRNA (5). Concurrently, GnRH pulses become slower, which may favor FSHβ transcription directly and indirectly via reduced induction of follistatin (6). Moreover, progesterone action leads to induction of FSHβ but suppression of LHβ gene expression (7). Finally, synergy between activin and GnRH or progesterone signaling pathways enhances the positive action of these hormones on FSHβ transcription (8) and, thus, may help regulate the secondary FSH surge.
5.2. Synergy between activin and steroid hormones

Gonadal steroids and glucocorticoids differentially modulate LH and FSH synthesis by suppressing LHβ and inducing FSHβ gene expression. It has also been suggested that activin action in the pituitary gonadotrope contributes to steroid induction of the FSHβ promoter. For example, Miyake et al. (1993) observed increased levels of FSH in primary pituitary cells after co-treatment with activin and gonadal steroid hormones (Miyake et al., 1993). Furthermore, steroid enhancement of FSH secretion was blunted by inhibin (Dahl et al., 1992) and follistatin (Bohsnack et al., 2000). Several studies also indicated that increased secretion of FSH resulted from a combinatorial effect of activin and steroids on FSHβ gene expression. The anti-progestin and anti-glucocorticoid, RU-486, suppressed activin induction of FSHβ gene expression in primary pituitary cell culture (Szabo et al., 1998). In addition, co-treatment of primary pituitary cells with activin and testosterone or glucocorticoids enhanced FSHβ mRNA levels above those observed with activin alone (Leal et al., 2003).

Regulation of FSH transcription by interactions between activin and steroid hormones could occur through direct or indirect means. Steroids may modify the activin/follistatin/inhibin feedback loop such that activin action on the FSHβ promoter is enhanced. Alternatively, components of the activin and steroid hormone signaling pathways could directly interact on the FSHβ promoter. In support of an indirect mechanism, testosterone has been shown to suppress follistatin mRNA and primary transcripts in rat primary cell cultures (Bilezikjian et al., 1996; Burger et al., 2004a). Smad mRNA and Smad phosphorylation have also been reported to change after testosterone treatment in primary pituitary cells (Burger et al., 2007). However, if steroid action were simply indirect, we would expect that all regulatory regions responsive to activin would be affected and this is not what is observed. Both the LHβ and FSHβ promoters are active, even if FSHβ is induced in LβT2 cells after activin and steroid hormone co-treatment, while LHβ is suppressed (McGillivray et al., 2007; Thackray and Mellon, 2008). These experiments demonstrated that the gonadotrope itself is sufficient for synergy between activin and progestins, androgens or glucocorticoids. Smad transcription factors can interact directly with steroid receptors (Chipuk et al., 2002; Hayes et al., 2001; Kang et al., 2002; Li et al., 2003; Song et al., 1999; Thackray and Mellon, 2008). Moreover, we showed that synergy between activin and 3-keto steroid hormones occurs directly on the FSHβ promoter and requires Smad and steroid receptor signaling, as well as DNA binding to the FSHβ promoter (Thackray and Mellon, 2008). This synergistic interaction between activin and 3-keto steroid hormones may play an important physiological role by participating in the generation of the secondary FSH surge that occurs during the early morning of estrus in rodents (Fig. 3).

6. Conclusions and future directions

In the female, normal reproductive function involves a pattern of regular ovulatory cycles that is achieved through precise integration of stimulatory and inhibitory signals between the hypothalamus, pituitary and ovary. Insight into the molecular mechanisms of hormonal regulation of LH and FSH synthesis may be critical for understanding not only the ovulatory cycle, but also fetal development, puberty, pregnancy, postpartum, and menopause. In this review, we have highlighted recent studies demonstrating that synergy between activin and GnRH, as well as activin and steroid hormones, in concert with variations in GnRH amplitude and frequency, may be responsible for the differential regulation of LH and FSH synthesis, particularly during the secondary FSH surge.

As illustrated in Fig. 1, hormonal regulation of the LHβ and FSHβ promoters is extremely complex. Although numerous molecular mechanisms of hormone action on these promoters have been elucidated over the past decade, there are potentially many more factors that may play important roles. For instance, factors important in pituitary gland development such as LIM homeodomain proteins have been shown to regulate FSHβ gene expression (West et al., 2004), but whether they also convey hormone responsiveness or interact with hormone-induced factors has not been examined. It is also true that we are just beginning to understand the interactions between transcription factors involved in hormonal regulation of gonadotropin synthesis and cofactors involved in chromatin remodeling or the activation of basal transcriptional machinery.

The use of more physiologically relevant model systems is also required to confirm results obtained in immortalized gonadotrope cells. The generation of a mouse co-expressing the GnRH receptor and Cre recombinase (GRIC) (Wen et al., 2008) that can be crossed to a ROSA 26-yellow fluorescent protein mouse to generate purified gonadotropes by fluorescence-activated cell sorting may be particularly useful in this regard. Gonadotropes purified from transgenic mice containing 4.7 kb of the ovine FSHβ promoter linked to the cell surface antigen, H-2Kb (Wu et al., 2004) may be an alternative. Moreover, genetically modified mice can be used to test the necessity of specific regulatory elements or transcription factors in hormonal regulation of the LHβ and FSHβ promoters (Ferris et al., 2007; Huang et al., 2001; Su et al., 2007).

Just as the gonadotropin β subunits are regulated by cross-talk among GnRH, activin and steroid hormone signaling pathways, it is highly likely that other proteins important for gonadotrope function are regulated as well. Several microarray studies have defined transcriptional targets of GnRH in LβT2 cells (Kakar et al., 2003; Lawson et al., 2007; Wurmbach et al., 2001). We and others have also recently demonstrated that activin substantially modifies the transcriptional response to GnRH (Mazhawidza et al., 2006; Zhang et al., 2006). Defining the complete transcriptome for these hormones, both alone and in combination, will not be trivial. However, the application of ChIP-chip (Ren et al., 2000) and ChIP-sequencing techniques [reviewed in Shendure and Ji, 2008] will expedite the process. The use of tissue-targeted and conditional knockout mice may also help identify additional hormonally regulated genes. In addition to the GRIC mouse described above, several transgenic mice have been developed that express Cre recombinase in a pituitary and gonadotrope-specific manner (Charles et al., 2008; Cushman et al., 2000; Naik et al., 2006), and can be used to analyze the role of specific transcription factors or signaling molecules exclusively in gonadotropes.

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diagram


