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Gonadotropin-Releasing Hormone Pulse Sensitivity of Follicle-Stimulating Hormone-β Gene Is Mediated by Differential Expression of Positive Regulatory Activator Protein 1 Factors and Corepressors SKIL and TGIF1

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Gonadotropin synthesis and release is dependent on pulsatile stimulation by the hypothalamic neuropeptide GnRH. Generally, slow GnRH pulses promote FSH production, whereas rapid pulses favor LH, but the molecular mechanism underlying this pulse sensitivity is poorly understood. In this study, we developed and tested a model for FSHβ regulation in mouse LβT2 gonadotropes. By mining a previous microarray data set, we found that mRNA for positive regulators of *Fshb* expression, such as *Fos* and *Jun*, were up-regulated at slower pulse frequencies than a number of potential negative regulators, such as the corepressors *Skil*, *Crem*, and *Tgif1*. These latter corepressors reduced *Fshb* promoter activity whether driven by transfection of individual transcription factors or by treatment with GnRH and activin. Overexpression of binding or phosphorylation-defective ski-oncogene-like protein (SKIL) and TG interacting factor (TGIF1) mutants, however, failed to repress *Fshb* promoter activity. Knockdown of the endogenous repressors SKIL and TGIF1, but not cAMP response element-modulator, increased *Fshb* promoter activity driven by constant GnRH or activin. Chromatin immunoprecipitation analysis showed that FOS, SKIL, and TGIF1 occupy the FSHβ promoter in a cyclical manner after GnRH stimulation. Overexpression of corepressors SKIL or TGIF1 repressed induction of the *Fshb* promoter at the slow GnRH pulse frequency but had little effect at the fast pulse frequency. In contrast, knockdown of endogenous SKIL or TGIF1 selectively increased *Fshb* mRNA at the fast GnRH pulse frequency. Therefore, we propose a potential mechanism by which production of gonadotropin *Fshb* is modulated by positive transcription factors and negative corepressors with different pulse sensitivities. **(*Molecular Endocrinology* 25: 0000–0000, 2011)**

The hypothalamic neuropeptide GnRH is essential for normal mammalian reproductive function. GnRH acts on gonadotrope cells in the anterior pituitary to increase the production and secretion of the gonadotropin hormones, LH and FSH (1, 2). These hormones are heterodimeric proteins of the glycoprotein hormone family, sharing a common α-subunit but having different β-subunits (*Lhb* and *Fshb* in the mouse). LH and FSH are crucial for the develop-

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Abbreviations: AP-1, Activator protein 1; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus; CRE, cAMP response element; CREB, cAMP response element-binding protein; CREM, cAMP response element-modulator; DCREB, DIEDML-CREB; EGR, early growth response; HDAC, histone deacetylase complex; *hpg*, hypogonadal; hSKIL, human SKIL; hTGIF, human TGIF; ICER, inducible cAMP early repressor; JNK, Jun-N-terminal kinases; SF1, steroidogenic factor 1; siRNA, small interfering RNA; SKIL, ski-oncogene-like protein; SMAD, mothers against decapentaplegic homolog; TGIF, TG interacting factor.

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ment and function of male and female gonads (3, 4). GnRH is also important for proliferation and development of gonadotropes. *Hpg* mice, harboring a deletion in the GnRH gene, have fewer gonadotropes and reduced gondotropin levels and are infertile (5). Clinically, hypogonadotropic hypogonadism is associated with mutations in the human GnRH receptor gene, underscoring the significance of GnRH signaling for normal reproductive function (6–8).

GnRH is secreted from the hypothalamus in a pulsatile fashion and causes gonadotropes to release LH and FSH in an episodic manner (9). This GnRH pulsatility is important for proper gonadotropin production because chronic exposure to GnRH or its analogs leads to a reversible suppression of gonadotropin release. It has been established *in vivo* that LH and FSH synthesis and secretion are differentially sensitive to GnRH pulse frequency (10). Generally, slow GnRH pulse frequencies favor FSH production and secretion, whereas rapid frequencies favor LH production and secretion (10–16). Such differential production and secretion is important for the female menstrual cycle in which a decrease in the GnRH pulse frequency increases FSH production at the late luteal phase to promote follicle selection and maturation, whereas an increase in the GnRH pulse frequency triggers the LH surge before ovulation (17). Part of the pulse frequency dependence for LH and FSH occurs at the transcriptional level because continuous and pulsatile GnRH leads to differential gonadotropin gene regulation (13, 15). Nonetheless, the mechanistic basis for the ability of gonadotropes to distinguish pulse frequency is not well understood because the majority of studies of GnRH signaling are performed in static culture using continuous GnRH treatment.

Studies using the *Lhb* promoter in cultured cells and animal models have suggested that steroidogenic factor 1 (SF1) and early growth response (EGR) 1 are involved in the regulation of the *Lhb* promoter (18–20). Using a pulse perifusion system, we demonstrated that the rat *Lhb* promoter is positively regulated by transcription factors EGR1 and EGR2, which are expressed at high pulse frequencies and by SF1, which is expressed under basal conditions and low pulse frequencies. Corepressors dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1, NAB1, and NAB2 are induced at low pulse frequencies and are able to repress *Lhb* promoter induction mediated by SF1, EGR1, and EGR2 (21). These observations allowed us to propose a model in which *Lhb* mRNA production is repressed at low GnRH pulse frequencies due to expression of NAB and dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome corepressors but increased the expression of positive transcription factors EGR1/2 at high GnRH pulse frequencies can overcome NAB repression, leading to the increased production of *Lhb* mRNA.

The *Fshb* promoter is more complicated because both GnRH and activin signaling are involved its regulation. It has been reported that activator protein 1 (AP-1) complexes including FOS, JUN, FOSB, and JUNB are induced by GnRH and can bind to the *Fshb* promoter region to stimulate transcription (22–24). The AP-1 half-site between positions −72 and −69 in the proximal promoter region that is conserved in mouse, rat, and human is crucial for GnRH-mediated *Fshb* promoter induction (24). Additionally, activin receptor-mediated activation of phosphorylated mothers against decapentaplegic homolog (SMAD)-2/4 and SMAD3/4 heterodimers is essential for *Fshb* expression because they interact with activin-responsive element sites on the *Fshb* promoter region (25–29). A recent study using the proximal rat *Fshb* promoter reported that the corepressor-inducible cAMP early repressor (ICER) is involved in the pulse regulation of *Fshb* promoter via competing with cAMP response element-binding protein (CREB) for the cAMP-responsive element (CRE) site (30). SMAD-mediated transcription is also subject to negative regulation. SKI and ski-oncogene-like protein (SKIL) (also known as SnoN) are members of the SKI family of nuclear protooncogenes that interfere with TGF-β-mediated SMAD signaling in various cell lines; however, their role in pituitary gonadotropes has not been investigated (31–34). Both corepressors are known to associate with histone deacetylase complex 1 (HDAC)-1 through binding to nuclear receptor corepressor-1 and SIN3A and repress their target genes (35, 36). Aside from SKIL and SKI, corepressor TG-interacting factor (TGIF) 1 can also associate with SMAD (37). Repression of SMAD-dependent transcription by TGIF involves the recruitment of HDAC instead of the coactivator EP300 into the SMAD complex (38). Association of JUN is also important for TGIF-mediated repression of SMAD signaling (39). This makes the differential pulse sensitivity of SKIL, SKI, and TGIF of particular relevance to GnRH and activin regulation of *Fshb*.

Based on our previous microarray data, we predicted that the corepressors SKIL and TGIF1 might be involved in negative feedback regulation of *Fshb* subunit expression. We hypothesized that pulse sensing by the *Fshb* promoter may be related to the relative abundance of stimulatory verses inhibitory transcription factors on the promoter under different pulse frequencies. We demonstrate that the transcriptional activators and corepressors are differentially expressed at fast GnRH pulse frequencies in mouse pituitary gonadotrope-derived cell line

LβT2 cells. Using overexpression, knockdown, and transfection of mutants, we demonstrate that SKIL, cAMP response element-modulator (CREM), and TGIF1 suppress *Fshb* promoter induction mediated by GnRH and/or activin and that SKIL and TGIF1 selectively repress *Fshb* promoter at slow pulse frequency. We also show that FOS, SKIL, and TGIF1 occupy the *Fshb* promoter in a cyclical manner.

Results

The AP-1 factors FOS and JUN and the corepressor proteins SKIL, TGIF1, and CREM are induced with continuous and pulsatile GnRH treatment

We initially mined our pulsatile GnRH microarray data set for factors that could regulate the *Fshb* promoter (21). We found that the mRNA for the AP-1 factors *Fos* and *Jun* and corepressors *Skil*, *Tgif1*, *Tgif2*, and *Crem* were induced with increasing pulse frequency at both 100 and 10 nM GnRH (Supplemental Fig. 1, A and B, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). We confirmed these observations using quantitative PCR and showed that *Fos*, *Jun*, *Skil*, *Tgif1*, *Icer*, and *Crem*, but not *Ski*, were induced after a single pulse of GnRH (Supplemental Fig. 1, C and D). We also verified that *Skil* and *Tgif1* are induced in primary rat pituitary cultures (Supplemental Fig. 1E). We then investigated induction at the protein level. Cells were stimulated with continuous 100 nM GnRH and protein expression assessed over 6 h. c-*fos* and c-*jun* were induced maximally at 2 h of GnRH treatment (Fig. 1A and Supplemental Fig. 2), which is consistent with the maximal mRNA induction at 30–60 min via quantitative PCR (Supplemental Fig. 1C), and protein expression was still detectable at 6 h. Multiple bands were detected for both FOS and JUN. The upper form of JUN corresponds to a band detected by an antibody to phospho-JUN (Ser73) (Supplemental Fig. 3A), so we believe that the upper bands represent the phosphorylated versions of FOS and JUN, phosphorylated at the carboxyl and amino termini, respectively, as has been shown previously (42–45). The lower unphosphorylated form of FOS is induced initially, but then the upper phosphorylated form predominates at later times. The converse is true for JUN: the early-induced form corresponds to the phosphorylated form, but at later times the unphosphorylated form predominates. The corepressors SKIL and TGIF1 were induced maximally at 4 h, and expression was maintained for 6 h (Fig. 1A), which was consistent with the maximal mRNA induction observed at 90 min (Supplemental Fig. 1C). The TGIF1 antibody detected two bands that are likely to be unphosphorylated (lower band) and phosphorylated (upper band) forms of TGIF1 based on prior published data (Supplemental Fig. 3B) (46). Unlike FOS and JUN, GnRH increased both forms of TGIF1. In contrast to the other proteins, only a slight induction of CREM was detected at 6 h (Fig. 1A).

We then checked whether a single 100-nM GnRH pulse would induce these transcription factors. Both FOS and JUN were induced maximally at 2 h, similar to the continual treatment, but dropped rapidly thereafter (Fig. 1A). Maximal induction of the corepressors TGIF1 and SKIL occurred at 2–4 h after a single pulse of GnRH (Fig. 1A), but CREM did not respond to a single GnRH pulse. The level of the SKI protein remained unchanged upon both single-pulse and continuous GnRH treatment in agreement with the mRNA data (Fig. 1A and Supplemental Fig. 1). A representative β-tubulin blot is shown to demonstrate equal loading. Finally, we tested the response of these proteins to multiple pulses of GnRH. LβT2 cells were treated with 5 min pulses of 1 and 10 nM GnRH every 30 or 120 min for 6 h. The lower doses of GnRH were used for the multiple pulse experiments to prevent desensitization. FOS and JUN were induced at both pulse frequencies but showed greater induction at the faster frequency (Fig. 1A). In contrast, SKIL and TGIF1 were induced only at the faster GnRH pulse frequency and were not induced at the slower pulse frequency (Fig. 1A). Again we did not see an induction of the CREM protein (Fig. 1A). As expected, SKI protein levels did not change with GnRH treatment. In most cases, treatment with 10 nM GnRH led to a higher induction FOS, JUN, TGIF1, and SKIL protein expression than 1 nM GnRH treatment (Fig. 1A).

Repression of *Fshb* promoter activity by SKIL, SKI, TGIF1, and CREM

To test whether these corepressors could repress the *Fshb* gene, we initially used a standard promoter transfection/overexpression model with continuous GnRH treatment because this paradigm has been used to investigate both *Lhb* and *Fshb* promoter regulation. A mouse −1.5-kb *Fshb* promoter-luciferase reporter (*Fshb*-luc) was cotransfected with TGIF1, SKIL, or SKI expression vectors into LβT2 cells. None of the corepressors reduced basal *Fshb* promoter activity (Fig. 1B). Because GnRH induces the *Fshb* promoter via the AP-1 site, we stimulated promoter activity by cotransfecting expression vectors for FOS, or JUN individually or in combination. Transfection of individual vectors led to a 1.8-fold increase in *Fshb* promoter activity, and cotransfection of mouse TGIF1, SKIL, or SKI reduced *Fshb* promoter activity to basal levels (Fig. 1B). When transfected in combination, FOS and JUN led to a 3.8-fold increase in *Fshb*

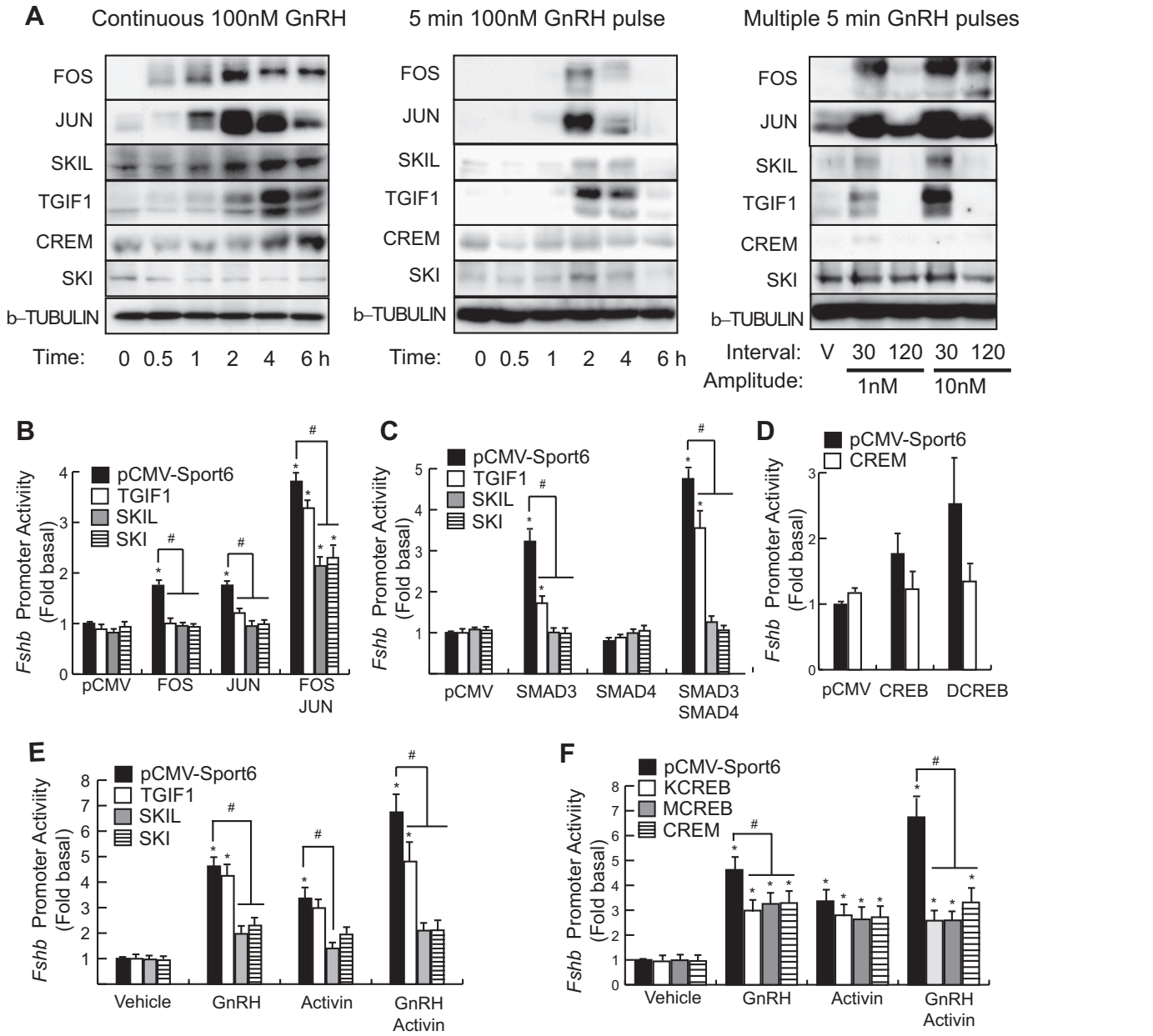

FIG. 1. Induction of AP-1 factors and corepressor proteins by GnRH and the repression of *Fshb* promoter activity by SKIL, SKI, TGIF1, and CREM in LβT2 cells. A, Induction of protein expression by continuous 100 nM GnRH (*left panel*), after a single pulse of 100 nM GnRH (*middle panel*), or after multiple 5-min pulses of 1 or 10 nM GnRH every 30 or 120 min for 6 h in static culture (*right panel*). V, Vehicle control. Cell lysates were blotted for FOS, JUN, SKIL, TGIF1, CREM, or β-tubulin as a loading control. Each experiment was repeated at least three times. Quantification of protein induction is given in Supplemental Fig. 2. B–F, Cells were cotransfected with the −1.5-kb *Fshb*-luc reporter gene and the indicated expression vectors. B, Cotransfection of *Fos*, *Jun*, *Skil*, *Ski*, and *Tgif1* or pCMVSPORT6. C, Cotransfection of *Smad3*, *Smad4*, *Smad3/4*, *Skil*, *Ski*, and *Tgif1* or pCMVSPORT6. D, Cotransfection of *Creb*, *DCreb*, *Crem*, or pCMVSPORT6. E, Cotransfection of *Skil*, *Ski*, and *Tgif1* or pCMVSPORT6 in presence of activin A and GnRH. Transfected cells were treated for 16 h with 25 ng/ml activin A, and then 100 nM GnRH was added for a further 6 h. F, Cotransfection of *MCreb*, *KCreb*, *Crem*, or pCMVSPORT6. Transfected cells were treated for 16 h with 25 ng/ml activin A, and then 100 nM GnRH was added for a further 6 h. Data represent the mean and SE of at least three independent experiments. *, Significant difference ($P < 0.05$) compared with vehicle-treated control group; #, significant difference ($P < 0.05$) between designated groups.

promoter activity. Cotransfection of SKIL or SKI reduced *Fshb* promoter activity, but TGIF1 was less effective under these conditions (Fig. 1B).

Because SKIL, TGIF1, and SKI are known to form complexes with SMAD, we then tested whether these corepressors can repress *Fshb* promoter activity stimulated by SMAD proteins. LβT2 cells were transfected with expression vectors for SMAD3 or SMAD4 or both along with the *Fshb*-luc reporter gene. SMAD3 transfection increased *Fshb* promoter activity 3-fold (Fig. 1C). SMAD4 transfection alone did not increase promoter activity, but SMAD3 and SMAD4 cotransfection increased promoter activity 5-fold. Cotransfection of SKIL or SKI reduced SMAD3- or SMAD3/4-stimulated promoter ac-

tivity to basal levels (Fig. 1C). TGIF1 also repressed *Fshb* promoter activity but not as strongly as SKI or SKIL. Similar experiments were attempted with SMAD2, but it had no effect in our hands (Supplemental Fig. 3C). Thus, the corepressors SKIL, SKI, and TGIF1 repress both AP-1- and SMAD-stimulated *Fshb* promoter activity.

Mutation of the conserved CRE reduces GnRH-stimulated activation of the rat *Fshb* gene promoter (30); therefore, we wanted to investigate whether CREB and its corepressor CREM were important for GnRH regulation of the mouse *Fshb* promoter (47). LβT2 gonadotropes were transfected with the − 1.5-kb *Fshb*-luc reporter construct along with expression vectors for CREB or a constitutively active mutant DIEDML-CREB (DCREB), with or without CREM. DCREB is known to stimulate via the CRE more strongly than CREB due to its increased interaction with CREB binding protein (48). Transfection of CREB and DCREB induced *Fshb* promoter activity 2- to 3-fold, whereas the cotransfection of CREM with either reduced the activity to basal levels (Fig. 1D).

Finally, we tested the effect of these corepressors on GnRH- and/or activin-stimulated *Fshb* promoter activity. LβT2 cells were transfected with or without expression vectors for the corepressors along with the −1.5-kb *Fshb*-luc reporter construct (Fig. 1E). In the absence of the corepressors, both activin treatment (25 ng/ml, 16 h) and GnRH treatment (100 nM, 6 h) increased in *Fshb* promoter activity 3- to 4-fold (Fig. 1E). Activin and GnRH cotreatment induced *Fshb* promoter activity 7-fold. Cotransfection of either SKIL or SKI reduced *Fshb* promoter activity induced by GnRH, activin, or both (Fig. 1E). In contrast, TGIF1 reduced promoter activity only after activin and GnRH cotreatment but did not have a significant repressive effect on cells stimulated with GnRH or activin individually (Fig. 1E). Human TGIF2 also repressed GnRH and activin-stimulated FSHβ promoter activity in a similar manner to mouse TGIF1 (Supplemental Fig. 3D). We performed similar experiments to test whether CREM or dominant-negative forms of CREB could repress FSHβ promoter activity mediated by GnRH, activin, or both. Expression vectors for CREM, KCREB, or MCREB were transfected with the −1.5-kb *Fshb*-luc reporter construct. KCREB and MCREB are dominant-negative mutants of CREB, KCREB having a point mutation in its DNA binding motif such that it heterodimerizes with endogenous CREB and reduces its binding to CRE, and MCREB having a point mutation in its phosphorylation site (S133A) such that it can bind to the CRE site but can not bind to coactivator CREB binding protein (49). Treatment with GnRH (100 nM, 6 h) and/or activin (25 ng/ml, 16 h) stimulated the *Fshb* promoter as before (Fig. 1F). Transfection of CREM or the dominant-negative mutants KCREB and MCREB reduced promoter activity stimulated by GnRH alone and both GnRH and activin, but not by activin alone (Fig. 1F), consistent with the known requirement of the CRE solely for GnRH induction of *Fshb*.

Knockdown of SKIL or TGIF1 increases *Fshb* promoter activity

The experiments above demonstrated that overexpression of the corepressors SKIL, SKI, CREM, and TGIF1 represses *Fshb* promoter activity. To test whether the endogenous corepressors regulate the *Fshb* promoter, SKIL, TGIF1, and CREM were knocked down using small interfering RNA (siRNA). We tested the functional effect of TGIF1 knockdown on FOS/JUN-stimulated FSHβ promoter activity because TGIF1 binds to JUN (39). TGIF1 knockdown increased basal *Fshb* promoter activity at 48 and 72 h and also increased FOS/JUN-stimulated promoter activity at 48 and 72 h (Fig. 2A). We tested the functional effect of SKIL knockdown on SMAD3-stimulated *Fshb* promoter activity because SKIL binds to SMAD3 (32). There was no increase in basal *Fshb* promoter activity at 24 or 48 h, but we observed a significant increase in basal activity at 72 h of SKIL knockdown and in SMAD3-stimulated *Fshb* promoter activity at both 48 and 72 h, resulting in a 6-fold stimulation at 72 h (Fig. 2B). Finally, we tested the functional effect of the CREM knockdown on DCREB-stimulated *Fshb* promoter activity. Although the CREM knockdown did not increase basal promoter activity, it significantly increased DCREB-stimulated promoter activity at both 42 and 72 h (Fig. 2C).

Having established the functional effect of the knockdowns using AP-1, SMAD, and DCREB-stimulation, we tested the effect of the knockdowns on GnRH- and activin-stimulated promoter activity. Treatment with GnRH (100 nM, 6 h), activin (25 ng/ml, 16 h), or both led to increase in *Fshb* promoter activity as before. Knockdown of SKIL resulted in further stimulation of *Fshb* promoter activity by GnRH, activin, or both (Fig. 2, D and E). Interestingly, TGIF1 knockdown increased promoter activity to a noticeable degree only when cells were cotreated with both GnRH and activin (Fig. 2, D and E). This last observation is consistent with the earlier finding that TGIF1 overexpression reduced promoter activity only when stimulated by both GnRH and activin. Unexpectedly, CREM knockdown did not increase promoter activity stimulated by GnRH or activin (Fig. 2, D and E), indicating that endogenous CREM does not regulate the induction of *Fshb* by GnRH or activin. Because endogenous CREM did not appear to modulate *Fshb* promoter activity, we excluded CREM from further study.

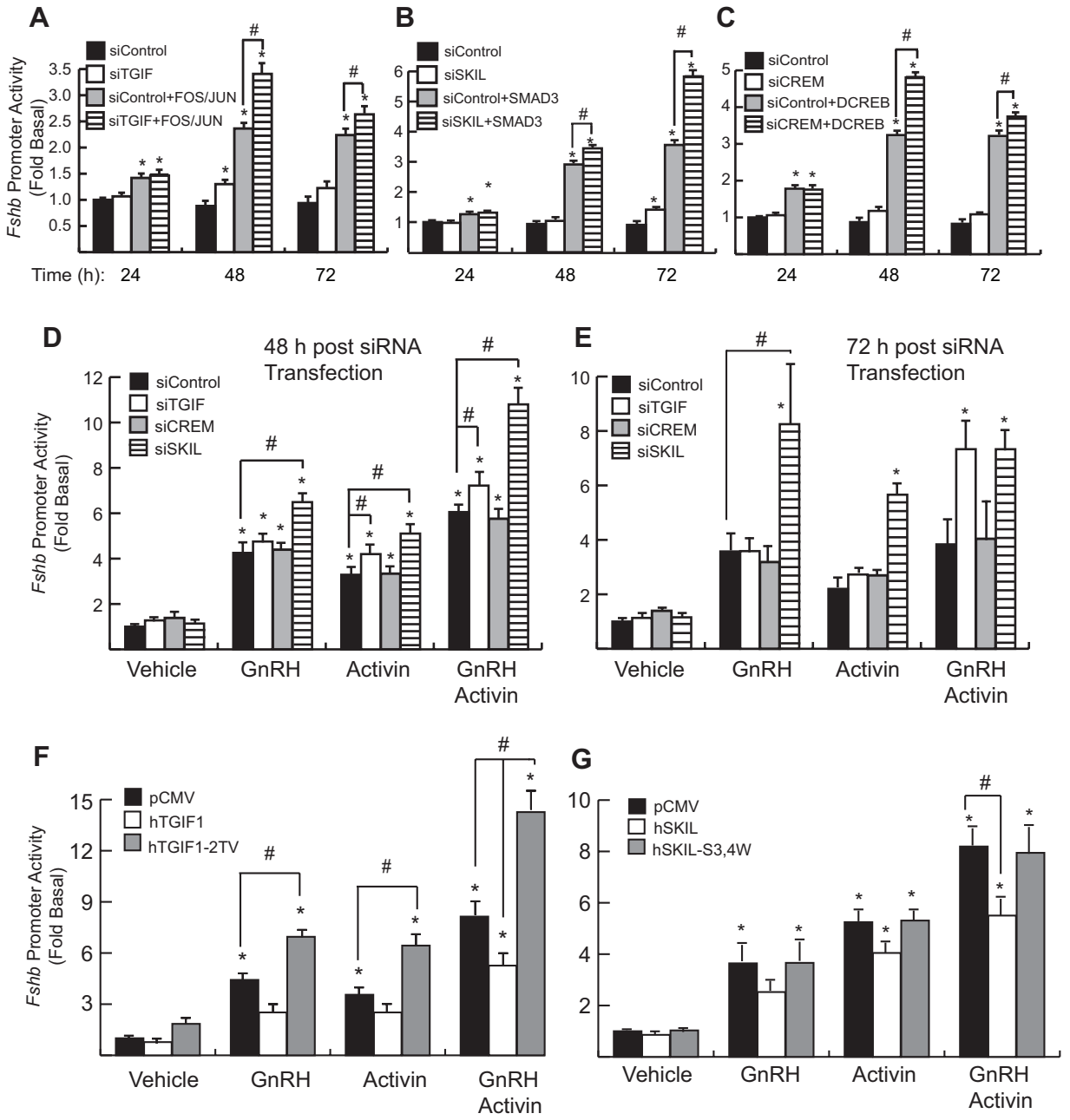

FIG. 2. Stimulation of *Fshb* promoter activity by corepressor knockdown or expression of mutant corepressors. In all panels, cells were cotransfected with the −1.5-kb *Fshb*-luc reporter gene and the indicated siRNA and/or expression vectors. A, Cotransfection of *Fos*/*Jun* and siRNA against *Tgif1* or control siRNA. B, Cotransfection of *Smad3* and siRNA against *Skil* or control siRNA. C, Cotransfection of *DCreb* and siRNA against *Crem* or control siRNA. D, Cotransfection of siRNA against the corepressors *Tgif1*, *Skil*, *Crem*, or control. Transfected cells were treated for 16 h with 25 ng/ml activin A, and then 100 nM GnRH was added for a further 6 h and harvested 48 h after siRNA transfection. E, Similar experiment to D but cells harvested 72 h after transfection. F, Cotransfection of wild-type human *TGIF1* or mutant human *TGIF1*-2TV, which contains valine substitutions at the ERK phosphorylation site threonines 235 and 239. G, Cotransfection of human wild-type *Skil* or mutant *Skil*-S3,4W, which contains alanine substitutions at amino acids 85–88 to eliminate SMAD2 and SMAD3 binding and tryptophan 318 to eliminate SMAD4 binding. Data represent the mean and SE of at least three independent experiments. *, Significant difference ($P < 0.05$) compared with vehicle-treated control group; #, significant difference ($P < 0.05$) between designated groups.

Loss-of-function TGIF1 and SKIL mutants do not repress the *Fshb* promoter

As an alternative strategy to confirm that TGIF1 and SKIL are involved in the repression of the *Fshb* promoter, LβT2 cells were transfected with the −1.5-kb *Fshb*-luc reporter and expression vectors for mutant corepressors. For TGIF, the mutant (hTGIF-2TV) contains threonine to valine substitutions at positions 235 and 239 in human TGIF (hTGIF), residues that are sites for ERK-dependent phosphorylation and regulation of protein stability (46, 50). The SKIL mutant (hSKIL-S3,4W) contains alanine substitutions at amino acids 85–88 and 318 in human

SKIL (hSKIL). This mutant is deficient in SMAD2 and SMAD3 binding due to mutation of residues 85–88 and deficient in SMAD4 binding due to mutation of tryptophan 318 (32). Overexpression of human TGIF1 reduced *Fshb* promoter activity in response to GnRH, activin, or both, similar to that seen by mouse TGIF1, reaching significance for both GnRH and activin (Fig. 2F). Unexpectedly, transfection of cells with the hTGIF-2TV mutant increased *Fshb* promoter activity in GnRH, activin, or cotreated cells compared with respective control groups, suggesting that the hTGIF-2TV mutant acts as a dominant negative to inhibit endogenous mouse TGIF1. Transfection of wild-type TGIF1 led to a large increase in a slower migrating, phosphorylated form of TGIF1, but transfection of the 2TV mutant led to expression of low levels of a faster migrating band, consistent with the known protein-stabilizing effect of phosphorylation (Supplemental Fig. 3B). Wild-type hSKIL reduced *Fshb* promoter activity significantly when cells were stimulated with both GnRH and activin, similar to mouse SKIL. As expected, the mutant hSKIL-S3,4W did not repress promoter activity compared with the respective treated control, suggesting that binding to the SMAD is required for repression (Fig. 2G).

Modulation of SKIL and TGIF1 expression alters the sensitivity of the *Fshb* subunit gene to GnRH pulses

The next series of experiments were designed to test whether the corepressors alter the response to pulsatile GnRH. We verified that the LβT2 cells respond appropriately to GnRH pulse frequency by treating cells with 5-min pulses of 1 and 10 nM GnRH at 30- and 120-min intervals for 6 h (Fig. 3A). At the lower pulse amplitude (1 nM GnRH), the slower GnRH pulse frequency led to a higher induction of *Fshb* mRNA, in agreement with the differential expression observed in other cells *in vitro* and in intact animals *in vivo* (16, 51). Interestingly, a higher amplitude GnRH pulse (10 nM GnRH) led to similar differential *Fshb* mRNA expression, but the

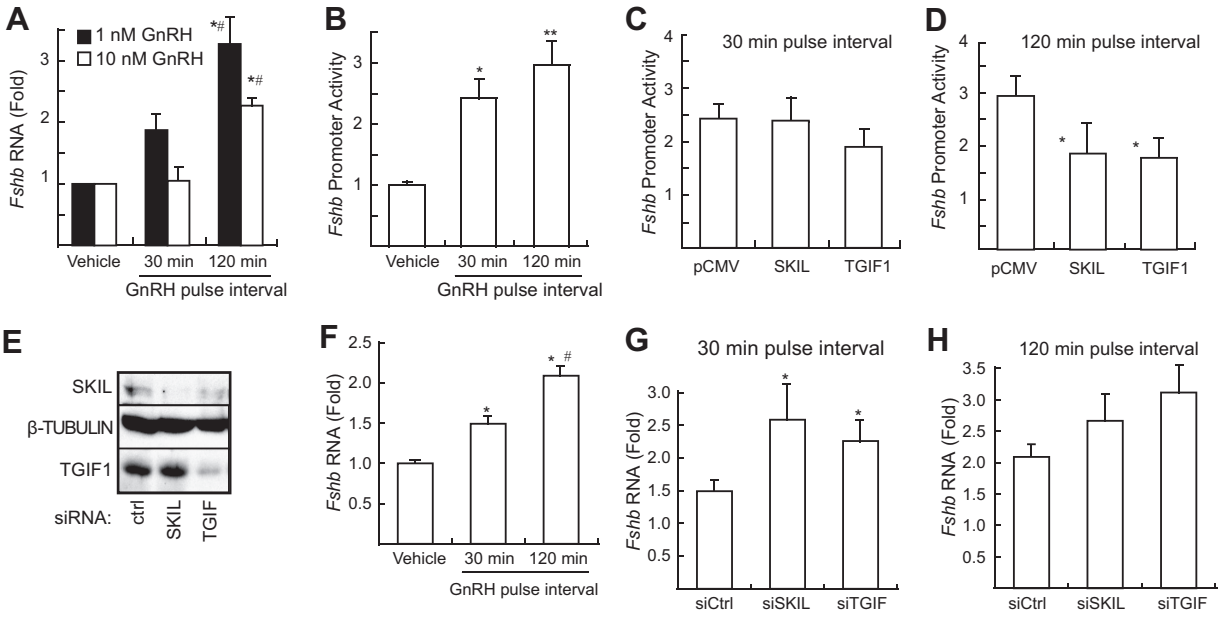

FIG. 3. Corepressors SKIL and TGIF1 reduce *Fshb* promoter activity in response to pulsatile GnRH. A, Measurement of *Fshb* mRNA in LβT2 cells treated with 5-min pulses of 1 and 10 nM GnRH at intervals of 30 and 120 min over 6 h. Data represent the means and SE of three independent experiments. *, Significant difference ($P < 0.05$) compared with vehicle-treated group; #, significant difference ($P < 0.05$) compared with respective 30-min GnRH pulse frequency-treated group. B–D, LβT2 cells were cotransfected with −1.5-kb *Fshb*-luc, tklacZ, and expression vectors for *Tgif1*, *Skil*, or control pCMVSPORT6 vector as indicated. Cells were treated with 5-min pulses of 10 nM GnRH in a pulse perifusion system at intervals of either 30 or 120 min for 6 h. Two nanograms per milliliter activin A were added to all of the groups at an interval of 120 min to maintain basal promoter activity. B, Effect of different pulse frequencies in the control-transfected cells. *, Significant ($P < 0.05$) increase relative to vehicle control; **, significant ($P < 0.01$) increase relative to vehicle control. C, Effect of TGIF1 or SKIL overexpression on *Fshb* induction by GnRH at 30-min pulse intervals. D, Effect of TGIF1 or SKIL overexpression on *Fshb* induction by GnRH at 120-pulse intervals. *, Significant decrease ($P < 0.05$) relative to pCMVSPORT6-transfected cells. E, Verification of siRNA knockdown in LβT2 cells electroporated with the indicated siRNA. Forty-eight hours later, cells were treated with 100 nM GnRH for 4 h to induce TGIF1 and SKIL. Protein expression was measured by immunoblotting using the indicated antibodies. Data were quantified using densitometry (Supplemental Fig. 2F). F–H, LβT2 cells were electroporated with the siRNA against *Tgif1* or *Skil* for 48 h before being treated with 5-min pulses of 10 nM GnRH at intervals of either 30 or 120 min for 6 h in static culture. F, Induction of *Fshb* mRNA by GnRH pulses in the presence of the control siRNA. *, Significant difference from vehicle-treated control ($P < 0.05$); #, significant difference from 30 min pulse frequency treatment ($P < 0.05$). G, Effect of *Tgif1* or *Skil* knockdown on *Fshb* induction by GnRH at 30-min pulse intervals. *, Significant difference from control siRNA-transfected group ($P < 0.05$). H, Effect of *Tgif1* or *Skil* knockdown on *Fshb* induction by GnRH at 120-min pulse intervals.

overall induction was less at both fast- and slow-pulse frequencies (Fig. 3A).

We then tested whether SKIL and TGIF1 are involved in GnRH pulse regulation of *Fshb*. The *Fshb*-luc reporter was transfected into LβT2 cells on cytodex beads along with expression vectors for SKIL or TGIF1 or pCMV-SPORT6 control. Cells were treated with 5-min pulses of 10 nM GnRH in perifusion culture at 30- or 120-min pulse intervals for 6 h. A perifusion cultures was used for the promoter transfections because we have previously shown that the response of the promoter to GnRH pulses is better in perifusion than static culture. Because endogenous activin is important for basal FSHβ promoter expression and is removed by the perifusion system, 2 ng/ml activin was added to pulse-treated and control groups every 120 min to support basal promoter activity. To ensure that addition of activin did not alter the differential induction of FOS, JUN, SKIL, and TGIF1, we assessed transcription factor induction in these cells. Although activin slightly increases the basal levels of these transcription factors, it did not alter the differential induction of SKIL and TGIF1 by fast and slow GnRH pulse frequencies (Supplemental Fig. 4A). Both 30- and 120-min GnRH pulse frequencies increased FSHβ promoter activity in control cells transfected with pCMVSPORT6 (Fig. 3B). Like the endogenous FSHβ gene, the 120-min pulse frequency tended to give higher induction than the 30-min frequency, but the difference was not significant (Fig. 3B). No repression of FSHβ promoter activity by TGIF1 or SKIL was observed at the faster pulse frequency (Fig. 3C), but both TGIF1 and SKIL significantly reduced the *Fshb* promoter activity at the slower frequency (Fig. 3D), which is consistent with our earlier observation that endogenous SKIL and TGIF1 proteins are induced at the high GnRH pulse frequency, so overexpression would have less effect.

To confirm the role of the endogenous corepressors in GnRH pulse regulation of *Fshb*, SKIL or TGIF1 expression was knocked down in LβT2 cells. We verified initially that the siRNA decreased protein expression (Fig. 3E and Supplemental Fig. 4B) and then assessed *Fshb* gene induction. Cells were transfected with scrambled control, TGIF1, or SKIL siRNA and were treated with fast and slow GnRH pulse frequencies in static culture for total of 6 h. Pulses in static culture, rather than perifusion culture, were used because we have found that the response of the endogenous gene is more robust. As before, 2 ng/ml activin was added to media to maintain basal *Fshb* gene expression. For cells transfected with control siRNA, slow GnRH pulses caused a significantly higher induction of *Fshb* mRNA compared with cells exposed to fast pulses, as expected from previous experiments (Fig. 3F). When cells transfected with SKIL or TGIF1 siRNA were treated with fast GnRH pulses, the SKIL and TGIF1 knockdown groups significantly increased *Fshb* mRNA induction (Fig. 3G). In contrast, when these cells were treated with slow GnRH pulses, no significant difference was observed compared with control siRNA-transfected cells (Fig. 3H). The knockdown would be expected to be functional only at the high GnRH pulse frequency when endogenous TGIF1 and SKIL are induced.

Changes in FOS, JUN, SKIL, and TGIF1 occupancy of the *Fshb* promoter in response to GnRH treatment

The overexpression and knockdown experiments implicated FOS, JUN, SKIL, and TGIF1 in the regulation of the *Fshb* promoter. To investigate whether these are direct effects on the *Fshb* promoter, we measured promoter occupancy by chromatin immunoprecipitation (ChIP). Initially, LβT2 cells were stimulated with 100 nM GnRH over 6 h, and then chromatin was precipitated using antibodies to FOS, JUN, TGIF1, or SKIL. The ChIP data were averaged using three independent PCR products to different regions of the *Fshb* promoter from multiple experiments and were normalized to the basal, unstimulated samples to determine the dynamic changes over time. All four factors bound to the *Fshb* promoter, but their occupancy changed over the 6 h of GnRH stimulation (Fig. 4A). Binding of FOS and TGIF1 appears to increase after GnRH treatment, but SKIL binding appears high initially and then decreases. All three factors appear to cycle on and off the promoter with a periodicity of approximately 2–3 h (Fig. 4B). In contrast, JUN occupancy increases gradually over time, being significant at 5 and 6 h.

We then studied occupancy of the *Fshb* promoter in response to a single 100-nM GnRH pulse (Fig. 4C). The results were noticeably different from the continuous treatment (Fig. 4D). FOS occupancy on the *Fshb* promoter increased at 2 h and then declined to basal levels. JUN occupancy peaked at 4 h and then fell to basal, in contrast to the results with constant GnRH. TGIF1 occupancy also differed in that occupancy initially fell at 2 h from the basal level and then rebounded in parallel with the increase in JUN binding at 4 h before falling again. SKIL occupancy did not oscillate but rather declined gradually over 6 h.

The kinetics of FOS, JUN, SKIL, and TGIF1 protein induction, occupancy of the *Fshb* promoter, and induction of the *Fshb* mRNA in response to multiple pulses of GnRH

Once we had established that these factors occupy the *Fshb* promoter in response to GnRH, the important ques-

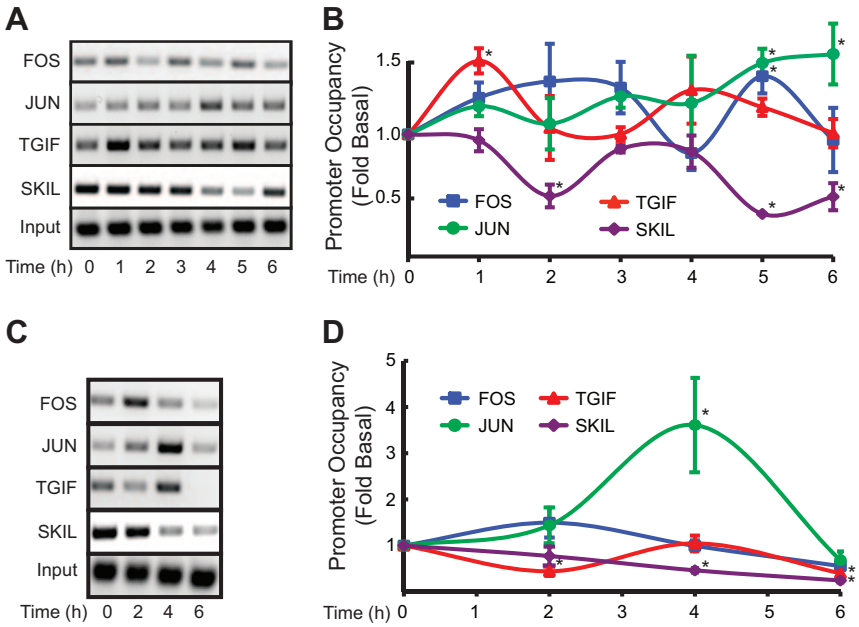

FIG. 4. *Fshb* promoter occupancy by FOS, JUN, SKIL, and TGIF1 in response to GnRH. A, ChIP assay was performed with LβT2 cells treated with continuous 100 nM GnRH for the indicated times. Chromatin was immunoprecipitated using antibodies to FOS, JUN, TGIF1, and SKIL. Precipitated chromatin was amplified using three independent sets of primers to the proximal *Fshb* promoter. Representative PCR are shown. Amplification of input DNA is shown as a control for chromatin recovery. B, Quantification of the ChIP assay. Results are the mean of at least three experiments with three independent primer pairs. Data are normalized to respective inputs and then to their respective zero time points. *, Significant difference from respective 0 h ($P < 0.05$). C, ChIP assay was performed with LβT2 cells treated with a single 5-min pulse of 100 nM GnRH for the indicated times using antibodies to FOS, JUN, TGIF1, and SKIL as before. The panels show representative PCR. D, Quantification of ChIP after a single pulse of GnRH. Data were normalized and analyzed as before. *, Significant difference from respective 0 h ($P < 0.05$). Data represent the mean and SE of at least two independent experiments.

tion was whether occupancy would be altered in the context of multiple GnRH pulses. Because the protein expression and ChIP data with continuous or single-pulse GnRH was dynamic, it was important to include a time course in the multiple pulses experiment. To allow the assessment of the effect of preconditioning with multiple GnRH pulses on protein expression, *Fshb* promoter occupancy, and *Fshb* mRNA induction, the experimental paradigm was designed so that the LβT2 cells would receive 5-min GnRH pulses (1 or 10 nM) every 30 or 120 min for 6 h followed by a final 5-min pulse of GnRH to synchronize timing relative to the last pulse. Cells were then harvested immediately or at 30, 60, or 120 min after that last pulse. In all cases, culture medium was changed every 30 min to control for effects due to autocrine stimulation by secreted factors. We initially assessed protein induction under this paradigm. For both 1- and 10-nM GnRH pulses, FOS and JUN proteins were induced at 30–60 min in agreement with data from static cultures. Immediately after the last pulse, cells that had been preconditioned at the GnRH high pulse frequency showed higher basal levels of FOS and JUN, consistent with the earlier data, but also showed prolonged expression at 120 min after the final pulse compared with the cells preconditioned with slow GnRH pulses (Fig. 5A and Supplemental Fig. 5). The corepressors TGIF1 and SKIL were strongly induced only in cells preconditioned by fast 10-nM GnRH pulses (Fig. 5A and Supplemental Fig. 4). Furthermore, basal TGIF1 levels were elevated in cells receiving the fast GnRH pulses, similar to FOS and JUN.

Fshb promoter occupancy by these transcription factors was then studied in cells exposed to multiple 10-nM GnRH pulses using the same experimental design (Fig. 5B). We used only 10 nM GnRH because it gave more robust changes in protein expression. All four proteins showed changes in promoter occupancy over the 120-min time course after the final pulse (Fig. 5C). For cells preconditioned with fast GnRH pulses, promoter occupancy by TGIF1 or FOS was closely correlated with promoter occupancy elevated compared with vehicle-pulsed cells immediately after the pulse and then decreasing to basal levels at 60 min before increasing again at 120 min (Fig. 5C). In cells preconditioned with slow GnRH pulses, the correlation between FOS and TGIF1 occupancy was less apparent. Basal occupancy by both FOS and TGIF1 is again elevated relative to vehicle-pulsed cells, but occupancy by FOS transiently decreased at 30 min and rebounded at 60 and 120 min, whereas occupancy by TGIF1 decreased gradually over time. Occupancy by JUN and SKIL was also correlated under both conditions but did not change significantly (Fig. 5C).

Because our pulse-sensing model is that *Fshb* promoter activity is determined by the balance of the positive and negative factors, we normalized the promoter occupancy of FOS and JUN to either TGIF1 or SKIL. Relative promoter binding of FOS to either TGIF1 or SKIL increases significantly over time in cells that have been preconditioned with slow GnRH pulses but does not change in cells preconditioned with fast GnRH pulses (Fig. 6A). The relative binding of JUN to TGIF1 or SKIL shows a similar pattern (Fig. 6A). These results indicate that cells exposed to a slow GnRH pulse frequency tend to have a higher abundance of stimulatory transcription factors bound to the *Fshb* promoter.

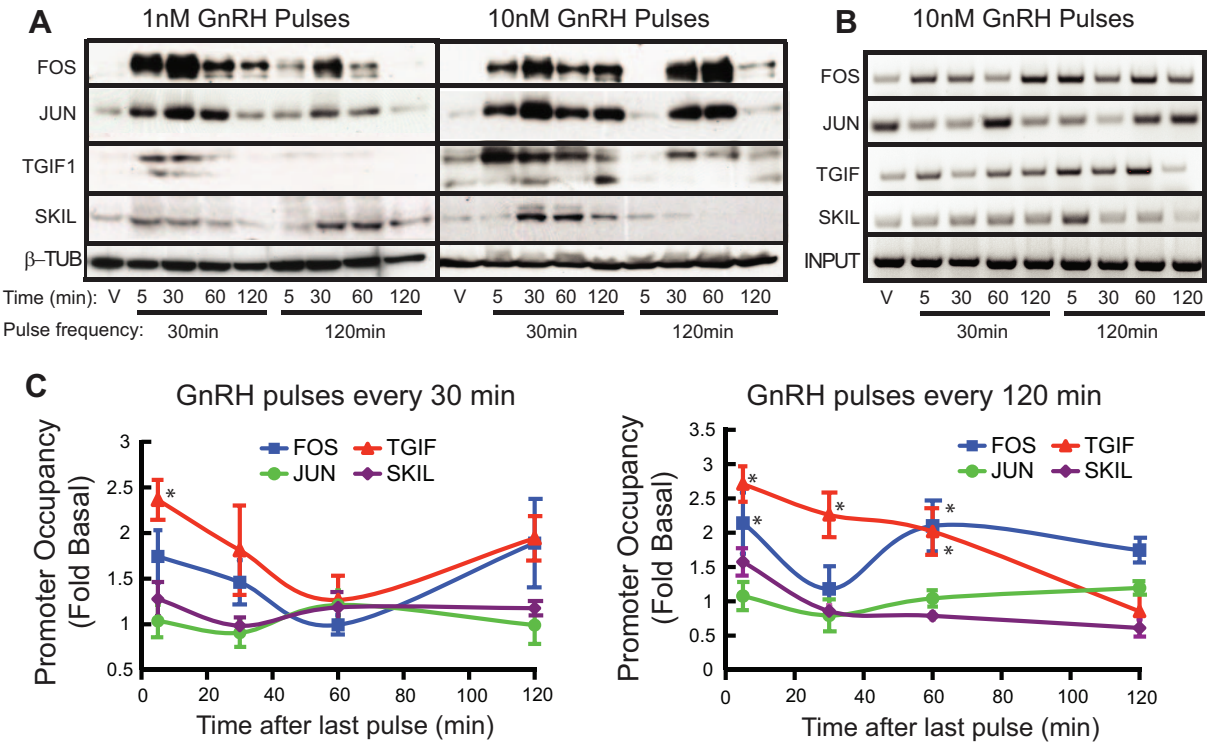

FIG. 5. Kinetics of transcription factor expression and *Fshb* promoter occupancy in cells exposed to fast and slow GnRH pulses. LβT2 cells were serum starved for 24 h and then treated with 5-min pulses of 1 or 10 nM GnRH every 30 or 120 min for 6 h. After 6 h, cells were treated with one final synchronizing pulse of GnRH and then harvested immediately or 30, 60, or 120 min after the last pulse. A, Time course of induction of FOS, JUN, TGIF1, and SKIL protein expression. Representative blots are shown. Blots were reblotted for β-tubulin for loading control and were quantified by densitometry (Supplemental Fig. 5). B, Time course of promoter occupancy by ChIP after the last GnRH pulse. Chromatin was immunoprecipitated with antibodies to FOS, JUN, TGIF1, and SKIL. The panels show representative PCR. C, Quantification of the ChIP data after a multiple pulse of GnRH. Data were normalized to respective inputs and then to vehicle-treated cells as before. *, Significant difference from respective 0 time point ($P < 0.05$). Data represent the mean and SE of at least two independent experiments.

Finally, if the balance of stimulatory to inhibitory transcription factors increases over time in cells exposed to slow GnRH prepulses, then a similar increase in *Fshb* mRNA should be seen. Therefore, the induction of *Fshb* mRNA after the final GnRH pulse in cells preconditioned with multiple pulses was also measured to test whether there is a correlation between transcription factor induction, promoter binding, and mRNA synthesis. *Fshb* mRNA was elevated immediately after the final pulse in cells preconditioned with slow GnRH pulses, which is in agreement with our earlier results (Fig. 6B). Additionally, *Fshb* mRNA was further elevated over 120 min after the pulse in cells previously exposed to slow 1- or 10-nM GnRH pulses (Fig. 6B). Although a slight increase in *Fshb* mRNA was also detected in cells exposed to the high GnRH pulse frequency, the increase was less dramatic than in the cells exposed to the slow pulse frequency.

Discussion

Based on the results presented here, we propose a model for GnRH pulse regulation of *Fshb* in which induction of stimulatory AP-1 factors FOS and JUN at a slow GnRH pulse frequency leads to increased transcription of *Fshb*, but as the GnRH pulse frequency increases, the corepressors SKIL and TGIF1 are induced to dampen further *Fshb* induction (Fig. 7). We observed further that FOS, TGIF1, and SKIL bind to the proximal *Fshb* promoter region in a cyclical manner with a periodicity of approximately 2 h, similar to the optimal pulse frequency for *Fshb* gene induction. Cyclical promoter occupancy has been demonstrated for EGR1 and SF1 on the *Lhb* gene with a periodicity of 30 min, which again correlates with the optimal pulse frequency for *Lhb* gene induction (52). It is possible that these periodicities of binding underlie the optimum pulse sensitivities of the two genes because the cycles of binding due to each pulse create positive reinforcement of the signal.

Our data also point to a disconnection between protein expression and promoter binding. For example, in cells exposed to fast GnRH pulses, FOS, JUN, TGIF1, and SKIL are all induced after the final pulse, but promoter binding of both FOS and TGIF1 decreases over the same period before rebounding, and occupancy by JUN and SKIL do not change. It is possible that the elevated basal protein levels due to the prior pulses are sufficient to saturate binding to the *Fshb* promoter, so the further in-

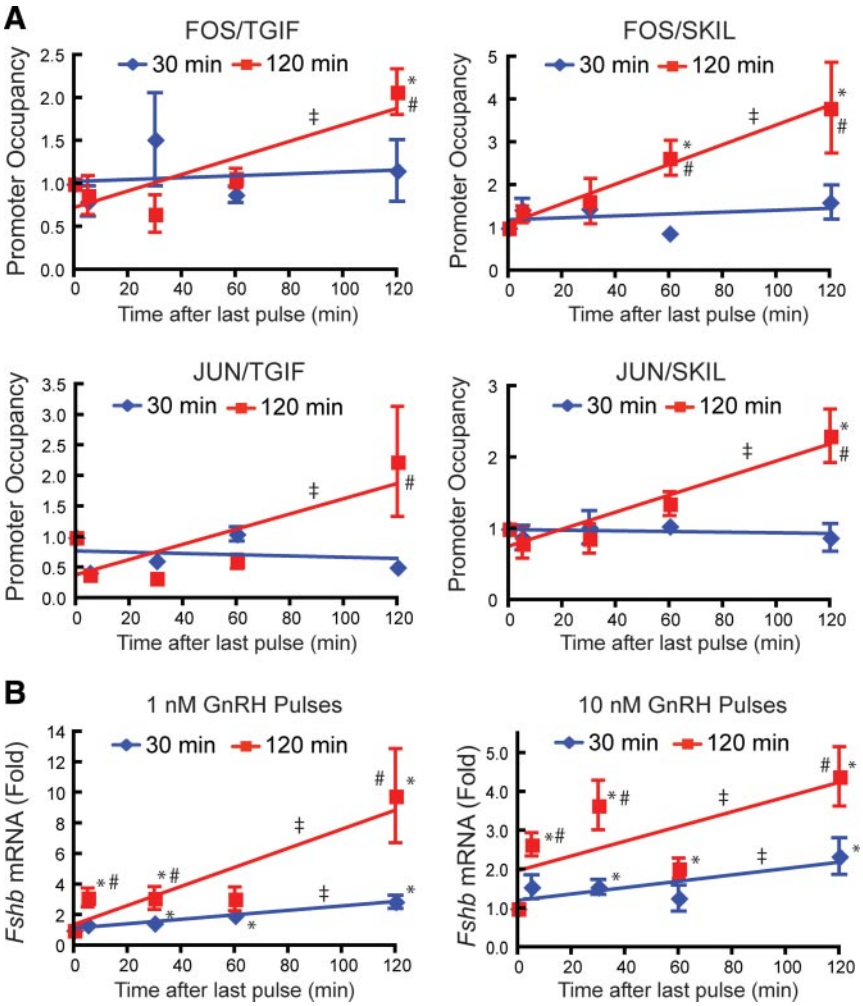

FIG. 6. Ratio of transcription factor to corepressor occupancy after the final pulse and kinetics of *Fshb* mRNA induction in cells exposed to fast and slow GnRH pulses. A, Quantification of the ChIP data from Fig. 5 plotted as FOS/TGIF1, FOS/SKIL, JUN/TGIF1, or JUN/SKIL promoter occupancy ratios. B, *Fshb* mRNA induction after final pulse using the experimental paradigm from Fig. 5. #, Significant difference compared with the respective 30-min pulse interval group ($P < 0.05$); *, significant difference compared with the vehicle-treated, zero time point ($P < 0.05$). The promoter occupancy and mRNA induction data were fit using linear regression; ‡, the linear regression line has a significantly nonzero slope ($P < 0.01$). Refer to Supplemental Table 2 for specific r^2 and P values. The time-course data represent the mean and SE of three independent experiments.

crease in protein has no effect. The observed decrease in promoter occupancy by FOS and TGIF1 after the last pulse may reflect a signaling event rather than a change in expression. Indeed, phosphorylation of JUN on Ser63/73 or Thr91/93 by the Jun-N-terminal kinases (JNK) triggers degradation of JUN and FOS (53–55). JNK are robustly activated by GnRH, which may serve to trigger promoter clearance followed by renewed binding as the JNK signal wanes. The situation in cells exposed to slow GnRH pulses is quite different. Basal expression of the four factors due to the prior pulses is low and only FOS and JUN are strongly induced after the last pulse. Promoter binding of FOS shows a transient decrease and then recovers quickly, again likely reflecting JNK mediate promoter clearance, but TGIF1 binding decreases continually over time, perhaps reflecting that TGIF1 protein is not induced under these conditions, so there is less protein to allow repopulation of the promoter. Comparing promoter binding of the positive transcription factors to the corepressors provides perhaps the best representation of the data. The relative binding of FOS/JUN to TGIF1/SKIL reveals that the ratio of positive to negative factors increases over time in cells exposed to slow GnRH pulses but does not change in cells exposed to fast pulses. This change in relative occupancy correlates closely with the increase in FSHβ mRNA after the final pulse as would be predicted by our model.

How these transcription factors cycle on the *Fshb* promoter is an important question. Ubiquitin-mediated proteosomal degradation of EGR1 and SF1 was shown to be important for cycling on the *Lhb* promoter. The degradation of FOS is more complicated because it is subject to both ubiquitin-dependent and ubiquitin-independent degradation by the proteosome (53). A major C-terminal destabilization element in monomeric FOS is an unstructured domain that directs ubiquitin-independent degradation by the 20S proteosome core. Phosphorylation of this domain on Ser362/Ser374 by ERK/ribosomal protein S6 kinase polypeptide 1/2, or formation of a JUN/FOS heterodimer prevents degradation by the ubiquitin-independent pathway (53). When the C-terminal destabilization element is removed, ubiquitin-dependent degradation via the 26S proteosome can be observed due to an N-terminal destabilization element. As mentioned earlier, JUN and FOS degradation is triggered by phosphorylation of JUN, but the ubiquitinated residues have not been identified (54, 55).

Although we have shown that the corepressors SKIL and SKI repress *Fshb* expression, the mechanisms underlying their action are not fully understood. Both interact with SMAD proteins (35, 36) and repress target genes by binding to nuclear receptor corepressor-1 and mSin3A recruiting HDAC1 (35, 36, 56, 57). Interestingly, we found that SKIL and SKI can also repress

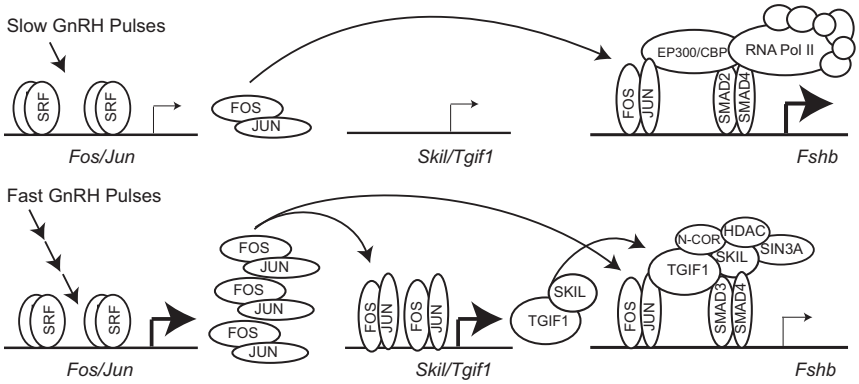

FIG. 7. Model for pulse regulation by the *Fshb* gene. The transcription factors FOS and JUN are induced at slow GnRH pulse frequencies, but the corepressors SKIL and TGIF1 do not change. This leads to an increased ratio of promoter occupancy by stimulatory transcription factors to inhibitory corepressors causing increased *Fshb* mRNA induction. Because the GnRH pulse frequency increases, there is additional induction of the corepressors SKIL and TGIF1 so the ratio of promoter occupancy by stimulatory transcription factors to inhibitory corepressors does not change. The presence of the corepressors on the *Fshb* promoter dampens induction of the *Fshb* mRNA at the faster GnRH pulse frequency, preventing further induction.

AP-1- or GnRH-stimulated *Fshb* promoter activity, although neither of these proteins has been shown to bind AP-1 directly (58, 59). Repression by SKIL and SKI may also depend on posttranscriptional modification of these proteins. For example, SKIL can be modified by small ubiquitin-like modifier-ylation on residues 50 and 383 via a specific small ubiquitin-like modifier E3 ligase, which leads to repression of TGF-β signaling in a promoter-specific manner (60, 61). SKI and SKIL are also subject to ubiquitination and proteosomal degradation that limits their repressive effect. Whether such mechanisms are involved in repression of GnRH and activin-mediated induction of the *Fshb* promoter remains unknown. Although SKI was not induced by GnRH, it may still be important for GnRH pulse frequency-mediated repression of *Fshb* because SKI preferentially forms a heterodimer with SKIL and such heterodimers are more active than individual SKI or SKIL homodimers (62, 63).

Unlike SKIL and SKI, TGIF1 was able to modulate *Fshb* promoter activity only when both GnRH and activin were added. TGIF1 can interact with both JUN and the SMAD (39); therefore, it is possible that TGIF1 requires activation of both GnRH and activin signaling and occupancy of the AP-1 sites and SMAD binding element. Like SKIL, TGIF1 is known to associate with HDAC (38, 64) and repression is under posttranslational control. TGF-β activation of the Ras-MAPK kinase-ERK pathway in HaCaT and Cos cells phosphorylates TGIF1 (46, 50). Two C-terminal threonines are phosphorylated by ERK and increase protein stability, possibly by interfering with ubiquitination and proteosomal degradation (46). GnRH stimulation of LβT2 gonadotropes induces two TGIF1 protein bands that we believe represent the phosphorylated and unphosphorylated form of TGIF1 based on previous publications. The phosphorylation of TGIF1 is important for its transcriptional repression because the TGIF-2TV mutant was unable to repress promoter activity but rather had a dominant-negative effect on the endogenous TGIF. The importance of ERK phosphorylation of TGIF raises the interesting question of whether GnRH alters the phosphorylation state of TGIF1 and hence its ability to repress *Fshb* transcription.

Contrary to our expectations, although overexpression of CREM reduced *Fshb* promoter activity, knockdown had no effect. A recent study has demonstrated that ICER, an alternatively spliced product of the *Crem* gene, regulates *Fshb* promoter pulse sensitivity through binding to the CRE site in the proximal *Fshb* promoter (30). The ICER splice variant is transcribed from the internal P2 promoter of the *Crem* gene (65). The probes and primers we used to detect *Crem* expression target a conserved 3′ region and measure all *Crem* isoforms including ICER, and we verified that both *Crem* and ICER mRNA are induced similarly. We do not see a robust induction of CREM protein in contrast to the published strong induction of ICER (30). It is possible that the longer period of perfusion for 20 h used by Ciccone *et al.* (30) allows for a more robust increase in protein. Both studies demonstrated that CREM or ICER overexpression represses *Fshb* promoter activity in transfected LβT2 cells, and Ciccone *et al.* (30) further demonstrated that transfected ICER binds to the *Fshb* promoter by ChIP. We show that CREM, and dominant-negative CREB mutants, specifically block GnRH stimulation of *Fshb*, whereas Ciccone *et al.* used overexpression of CREB coupled with GnRH stimulation to show the repression by ICER in static culture.

Where the two studies differ is that we are not able to see an effect of CREM/ICER knockdown on GnRH-stimulated *Fshb* promoter activity. Our knockdown approach used a mixture of three synthetic siRNA, and we verified a functional knockdown by measuring the DCREB stimulation of the *Fshb* promoter, whereas Ciccone *et al.* (30) used a short hairpin RNA targeted against a conserved region of ICER. In addition, the two studies also used different *Fshb* promoters; we used 1.5 kb of the mouse promoter, whereas the earlier study used the proximal −140-bp rat promoter, which might account for some of the differences in the results.

In summary, we have shown that AP-1 family members FOS and JUN positively regulate and the SKIL and TGIF1 corepressors negatively regulate the *Fshb* promoter at different GnRH pulse frequencies and amplitudes. The findings are consistent with a model in which positive factors are induced at low GnRH pulse frequency and amplitude to induce *Fshb* expression, but the corepressors are induced at higher GnRH pulse frequencies and amplitudes to dampen further induction of *Fshb*. Further studies are planned to determine whether such a model explains the differential regulation of FSH production observed during the female reproductive cycle.

Materials and Methods

Materials

GnRH was purchased from Sigma Chemical Co. (St. Louis, MO) or the National Hormone and Peptide Program (Torrance, CA). Activin A was purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal horseradish peroxidase-linked antirabbit antibodies, anti-SKIL (sc-9141), anti-SKI (sc-9140), anti-CREM (sc-440), anti-TGIF1 (sc-9084), anti-FOS (sc-52), anti-JUN (sc-1694), and anti-β-tubulin (sc-9104) as well as ChIP-grade versions of FOS, JUN, TGIF, and SKIL antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). *Skil* (sc-36519), *Tgif1* (sc-36660), *Crem* (sc-37701), and control (sc-37007) siRNA were also purchased from Santa Cruz Biotechnology. Protein G-Dynabeads immunoprecipitation kit (Invitrogen 100.07D), DMEM, and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA) and Cellgro (Mediatech, Inc., Manassas, VA). The human wild-type hemagglutinin-tagged TGIF1 expression vector was obtained from addgene (addgene.org). The human TGIF1 mutant (threonine 235/239 to valine) and control vector were obtained from Dr. Joan Massagué (Sloan Kettering Cancer Institute, New York, NY). The human TGIF2 expression vector was obtained from Dr. David Wotton (University of Virginia, Charlottesville, VA). Human wild-type and SMAD-binding deficient mutant SKIL and control vector were obtained from Dr. Kunxin Luo (University of California, Berkeley, Berkeley, CA). CREB, DCREB, KCREB, and MCREB expression vectors were obtained from Dr. Jane Reusch (University of Colorado Health Sciences Center, Aurora, CO). The −1.5-kb mouse *Fshb* promoter-luciferase construct (−1.5 kb *mFshb*-luc) was obtained from Dr. Pamela Mellon (University of California, San Diego, San Diego, CA). Expression vectors for mouse FOS, JUN, SKIL, TGIF1, SKI, CREM, and pCMVSPORT6 were purchased from American Type Culture Collection (ATCC.org). All other reagents were purchased from either Sigma or Fisher Scientific (Pittsburgh, PA).

Single pulse, multiple pulses, and continuous GnRH treatment in static culture

LβT2 cells were maintained as described in a previous publication (21). For single-pulse experiments, LβT2 cells were grown to confluence in six-well or 12-well plates (BD Biosciences, San Jose, CA), washed once with serum free medium, and incubated in fresh serum-free medium for 24 h. Cells were stimulated with either 10 or 100 nM GnRH for 5 min, and then GnRH-containing medium was removed and fresh 37 C serum-free medium was added. At the indicated times, the medium was removed and cells were washed once with PBS. Cells were harvested for RNA or protein extraction at various times thereafter. For continuous GnRH treatment, the same protocol was followed except that the culture medium was not changed after GnRH addition.

For multiple pulses in static culture, LβT2 cells were grown to confluence in six-well plates, washed once with serum-free medium, and incubated in fresh serum-free medium for 24 h. Cells were stimulated with either 1 or 10 nM GnRH, or with vehicle, for 5 min at 30- or 120-min intervals for 6 h. Every 30 min, serum-free medium was removed, and fresh warmed serum-free medium was added to all three groups, irrespective of GnRH stimulation to ensure all groups were treated equally and to correct for autocrine stimulation by factors secreted by the LβT2 cells. After 6 h, medium was removed and cells were washed once with PBS before harvesting for RNA or protein extraction. For pulse kinetic experiments, cells were treated with pulses as above, given one last 5-min pulse at 6 h and then harvested at the indicated times after the last synchronized pulse.

Quantitative RT-PCR

RNA was extracted from LβT2 cells using RNA-Bee (Tel-Test, Friendswood, TX). First-strand cDNA was synthesized using a high-capacity cDNA synthesis kit (Roche Applied Science, Indianapolis, IN). Samples were run in 20-μl reactions on an MJ Research Chromo4 instrument using iSYBR Green (Bio-Rad, Hercules, CA) and sequence-specific primers for *Fos*, *Jun*, *Tgif1*, *Skil*, *Crem*, *Ski*, and *Fshb* (Supplemental Table 1). Cycle threshold values were extracted by manually setting the threshold midway between basal and maximum fluorescence on a log10 scale. Gene expression levels were calculated after normalization to the housekeeping gene, *Gapdh* or *Rplp0* (also known as *M36B4*), using the ΔΔcycle threshold method and expressed as relative RNA levels compared with the control.

Western blotting

LβT2 cells were lysed on ice in the radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40, 1 mM EDTA, 0.05% protease inhibitor, 0.04% β-mercaptoethanol, 1× NuPAGE lithium dodecyl sulfate sample buffer), boiled for 5 min to denature proteins, and sonicated for 5 min to shear the chromosomal DNA. Equal volumes (10–20 μl) of these lysate were separated by SDS-PAGE on 10 or 12% gels and electrotransferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA). The membranes were blocked with 5% nonfat dried milk in Tris-buffered saline-Tween 20 (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.1% Tween 20). Blots were incubated with primary antibodies in blocking buffer for 60 min at room temperature and then incubated with horseradish peroxidase-linked secondary antibodies followed by chemiluminescent detection using Pico luminescent substrate (Thermo Scientific, Rockford, IL). Films were scanned and data were quantified by densitometry (Carestream Molecular Imaging Software, Rochester, NY). Intensity of each sample band was normalized to the

respective β-tubulin band. Data were plotted as fold basal change.

ChIP assay

ChIP was performed as previously described (24). Briefly, LβT2 cells were grown on 150-mm tissue culture plates to confluency. They were treated with a single pulse, multiple pulses, or continuous GnRH as indicated. Cells were cross-linked using 1% formaldehyde, lysed, and sonicated to produce the chromatin lysate. Protein G-coated dynabeads were prepared and conjugated with indicated antibodies according to the manufacturer's protocol. The chromatin lysate was incubated with conjugated dynabeads overnight. The beads containing the antigen-antibody complex were washed successively with low-salt buffer, high-salt buffer, LiCl wash buffer, and then finally three times with dynabead buffer. Chromatin was eluted using the elution buffer provided with the dynabeads and then Tris/EDTA buffer at 65 C and constant agitation. The cross-linking was reversed by heating the samples at 65 C overnight. The samples were then treated with Proteinase K, and DNA was purified using a PCR purification kit (QIAGEN, Valencia, CA). PCR was performed on immunoprecipitated samples as well as input samples using three different sets of primers targeting the proximal *Fshb* promoter region (Supplemental Table 1). Data were quantified by densitometry (Carestream Molecular Imaging Software), and intensities of bands produced by three different sets of primers were averaged. Sample band intensities were normalized to respective input DNA and data were plotted as the fold change relative to the basal, unstimulated sample. As a negative control in the initial experiments, unconjugated beads were used to immunoprecipitate the *Fshb* promoter region. No detectable bands were observed upon PCR of these samples (data not shown).

Perifusion and promoter activity assays

LβT2 cells were routinely maintained in 175-cm^2 flasks in DMEM-supplemented with 10% fetal bovine serum at 37 C with 5% CO_2. In the perifusion experiments, 12–15 $\times$ 10^7 cells were plated on a 1-ml bed volume of cytodex 3 microcarrier beads in a 10-cm petri dish with DMEM supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin and grown for 5 d in an atmosphere of 5% CO_2. Subsequently, cells were washed once in fresh serum-free DMEM and transfected with 4 μg of a 2:1:1 ratio of mouse −1.5 kb *Fshb*-luc, corepressor expression vector or pCMVSPORT6 control, and cytomegalovirus (CMV)-βgal. Transfections were performed using Fugene 6 (Roche Applied Science) according to the manufacturer's recommendations. After 16 h, cells were loaded into the perifusion chambers as described in previous publication (21) and pulsed with 10 nM GnRH at given pulse frequencies. Two nanograms per milliliter activin were supplemented every 120 min to maintain basal FSHβ promoter activity. After the perifusion, cells were lysed in 100 mM PBS containing 0.1% Triton X-100, vortexed for 30 sec, and clarified by centrifugation at 14,000 $\times$ *g*. Cell lysates were assayed for luciferase (luciferase assay system; Promega Corp., Madison, WI) and β-galactosidase (Galacto-Light Tropix, Bedford, MA) activity according to manufacturer's instructions in a 96-well plate using a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA). Luciferase activity was normalized for cotransfected β-galactosidase activity and then expressed relative to basal unstimulated cells.

For monolayer transfections, LβT2 cells were plated in six- or 12-well plates. Transfection was performed using Fugene 6 reagent or Transfast (Promega) following the manufacturer's protocol. For most experiments, each well was transfected with 500 ng of −1.5 kb *Fshb*-luc, 50 ng of tk-lacZ, and 50 ng of each expression vector for 12-well plates or 1.5 μg of −1.5 kb *Fshb*-luc, 150 ng of tk-lacZ, and 150 ng of each expression vector for six-well plates. For human wild-type and mutant SKIL and TGIF1, 215 ng of expression vectors was used per well in six-well plates. Empty vectors were used to balance DNA mass as necessary. Control experiments contained the empty pGL3 reporter plasmid. The following day, the cells were switched to serum-free DMEM supplemented with 0.1% BSA. Twenty-five nanograms per milliliter activin were added to specified wells in various experiments. After overnight incubation, the cells were treated with vehicle or a single pulse of 100 nM GnRH for 6 h. For experiments in which no GnRH or activin was used, cells were harvested after a specified time with vehicle treatment. Cell lysates were assayed for luciferase and β-galactosidase activity as above.

siRNA knockdowns

LβT2 cells were seeded into 12-well culture plates 24 h before transfection. The cells (60–70% confluence) were transfected with 100 nM siRNA as well as −1.5 kb *Fshb*-luc, tk-lacz, *Smad3*, and *Fos* and/or *Jun* expression vectors using 2 μl Fugene HD (Roche) using similar ratios of plasmids as above. The cells were cultured for the given time and harvested. For GnRH-activin experiments, the medium was replaced with fresh serum-free medium after the given siRNA treatment. Twenty-five nanograms per milliliter activin A were added when applicable. The next day 100 nM GnRH was added to the indicated groups. Six hours after GnRH treatment, cells were washed once with PBS and assayed for luciferase-β-galactosidase activity or immunoblotted for protein expression as described above. For multiple pulse experiments, siRNA was electroporated into cells using a Microporator at 1300 V pulse amplitude, 20 msec pulse width, and two pulses (BTX/Harvard Apparatus, Holliston, MA).

Primary pituitary cultures

Primary pituitary cells were obtained as described with minor modifications (40, 41). Briefly, anterior pituitaries were rapidly removed from 18-d-old male rats, placed in freshly prepared DMEM supplemented with glutamax, cut into small pieces, and incubated in 0.25% trypsin for 30 min. After addition of deoxyribonuclease and fetal bovine serum, the fragments were dispersed into individual cells in freshly prepared Krebs-Ringer bicarbonate buffer without Ca^{2+} or Mg^{2+} and filtered through a 70-μm cell strainer (BD Biosciences, Bedford, MA). Pituitary cells (400,000 per well) were plated in 12-well plates coated with poly-lysine in DMEM (low glucose) supplemented with 10% horse serum, 2.5% fetal calf serum, 1% minimum essential medium Eagle nonessential amino acids, Fungizone, and gentamicin [GIBCO (Invitrogen), Carlsbad, CA]. The following day, cells were washed with serum-free medium DMEM with high glucose-F12 supplemented with 0.1% BSA and stimulated with 100 nM GnRH for the indicated times. RNA was extracted from

cells and quantitative PCR performed as described above using appropriate primers for rat SnoN and TGIF.

Statistical analysis

Data were analyzed using Prism software (GraphPad, La Jolla, CA) using ANOVA for multiple group comparisons with the significance threshold set at $P < 0.05$. The significance of differences between individual conditions was determined using a Tukey *post hoc* test again with $P < 0.05$. Student's *t* test was used for specific pair-wise comparisons with significance being $P < 0.05$. Linear regression analysis was performed using Prism. The r^2 value for the fit was determined and the line was tested for significant nonzero slope at a significance value of $P < 0.05$. All the experiments were repeated at least three times unless otherwise indicated.

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