Forkhead Box O1 Is a Repressor of Basal and GnRH-Induced Fshb Transcription in Gonadotropes

Danalea V. Skarra, David J. Arriola, Courtney A. Benson, and Varykina G. Thackray

Department of Reproductive Medicine and the Center for Reproductive Science and Medicine, University of California, San Diego, La Jolla, CA 92093

Synthesis of the gonadotropin beta subunits is tightly controlled by a complex network of hormonal signaling pathways that may be modulated by metabolic cues. Recently, we reported that insulin regulates FOXO1 phosphorylation and cellular localization in pituitary gonadotropes and that FOXO1 overexpression inhibits Lhb transcription. In the current study, we investigated whether FOXO1 modulates Fshb synthesis. Here, we demonstrate that FOXO1 represses basal and GnRH-induced Fshb transcription in L/H9252T2 cells. Additionally, we show that PI3K inhibition, which increases FOXO1 nuclear localization, results in decreased Fshb mRNA levels in murine primary pituitary cells. FOXO1 also decreases transcription from the human FSHB promoter, suggesting that FOXO1 regulation of FSHB transcription may be conserved between rodents and humans.

Although the FOXO1 DNA binding domain is necessary for suppression of Fshb, we do not observe direct binding of FOXO1 to the Fshb promoter, suggesting that FOXO1 exerts its effect through protein-protein interactions with transcription factors required for Fshb synthesis. FOXO1 suppression of basal Fshb transcription may involve PITX1 since PITX1 interacts with FOXO1, FOXO1 repression maps to the proximal Fshb promoter containing a PITX1 binding site, PITX1 induction of Fshb or a PITX1 binding element in CV-1 cells is decreased by FOXO1, and FOXO1 suppresses Pitx1 mRNA and protein levels. GnRH induction of an Fshb promoter containing a deletion at -50/-41 or -30/-21 is not repressed by FOXO1, suggesting that these two regions may be involved in FOXO1 suppression of GnRH-induced Fshb synthesis. In summary, our data demonstrate that FOXO1 can negatively regulate Fshb transcription, and suggest that FOXO1 may relay metabolic hormonal signals to modulate gonadotropin production.

FSH is produced by gonadotrope cells in the anterior pituitary and plays a key role in mammalian fertility. In females, it is required for ovarian folliculogenesis, while in males it promotes spermatogenesis in conjunction with testosterone (1, 2). Female Fshb knockout mice exhibit an arrest in ovarian folliculogenesis prior to the antral stage, while males are fertile but have impaired reproductive function (3). In contrast, the human FSHB gene appears to be critical for reproductive function in both genders. Nonsynonymous mutations result in absent or incomplete pubertal development and infertility in women, as well as azoospermia and infertility in men (4).

FSH is a heterodimeric glycoprotein composed of a common alpha subunit, shared with LH and TSH, as well as a unique beta subunit that confers biological specificity. Transcription of the Fshb gene is dynamically regulated during the estrous cycle. Alterations in Fshb mRNA levels are observed prior to changes in serum FSH levels, indicating that transcription of Fshb is a rate-limiting step for production of the mature hormone (5, 6). Proper regulation of FSH levels is important for fertility. Low FSH levels are associated with defective follicular growth, while high levels are associated with premature ovarian failure (7).

Abbreviations: AP1 activator protein 1; AP1BE AP1 binding element; β-gal β-galactosidase; DBD DNA binding domain; DMEM Dulbecco’s Modification of Eagle’s Medium; EGR1 early growth response protein 1; FBE forkhead binding element; FBS fetal bovine serum; GFP green fluorescent protein; HD homeodomain; HDDBE HD binding element; HSD Honestly Significant Difference; LH LH receptor binding domain; LIM homeobox gene; luc luciferase; NFY nuclear transcription factor Y; PBS phosphate buffered saline; PITX paired-like homeodomain transcription factor; PITXBE PITX binding element; SF1 steroidogenic factor 1

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Basal Fshb transcription involves multiple transcription factors, including LIM homeobox gene (LHX), nuclear transcription factor Y (NFY) and paired-like homeodomain transcription factor (PITX) (8). LH3 is reported to bind the porcine Fshb promoter at several binding sites, including one element which is highly conserved among mammals (9). Additionally, NFY was shown to bind to a site at –76/-72 in the murine Fshb promoter and mutation of this site decreased basal Fshb synthesis by 30% (10). Furthermore, Lamba et al. showed that PITX1/2 binds to a conserved element at –54/-49 of the murine Fshb promoter and that mutation of this binding element reduced basal Fshb gene expression (11).

Hormonal regulation of Fshb gene expression has been shown to occur via multiple signaling pathways (12). GnRH increases Fshb transcription via PKC and MAPK signaling pathways through the induction of the activator protein 1 (AP1) transcription factors, c-FOS and JUNB, which bind and induce the Fshb promoter, along with c-JUN and FOSB (13–18). AP1 also interacts with factors involved in basal expression of Fshb, such as NFY, in the mouse and USF1, in the rat to integrate GnRH responsiveness (16, 19). Activin is also an inducer of the Fshb gene (20). Activin signaling through SMAD and FOXL2 proteins also appears to be critical for transcriptional regulation of the Fshb gene (8, 21).

In addition to reproductive hormones, metabolic hormones may regulate FSH production at the level of the gonadotrope. While insulin, IGF1 and leptin treatment of rat primary pituitary cells have been reported to increase FSH, as well as LH levels (22–25), it is not clear whether this is due to an effect of these hormones on Fshb transcription vs production and/or secretion of the mature hormone. One possibility is that metabolic hormones regulate Fshb transcription in gonadotropes through the activity of downstream effectors such as the FOXO subfamily of forkhead transcription factors. This family is composed of FOXO1, 3, 4 and 6. The transcriptional activity of FOXOs is controlled by posttranslational modifications in multiple cell and tissue types (26–28). FOXO phosphorylation has been shown to be regulated by insulin, IGF1 and leptin (29–31). Insulin stimulation induces PI3K/AKT activation, triggering AKT phosphorylation of FOXO1 on residues Thr24, Ser256, and Ser319 (32). This results in FOXO1 export from the nucleus to the cytoplasm, where it is sequestered by 14–3–3 proteins, preventing FOXO1 transcriptional activity. While considerable progress has been made in understanding the role of FOXOs in reproductive tissues such as the ovary and uterus (33, 34), FOXO regulation of gonadotropin hormone production in pituitary gonadotrope cells remains largely unexplored.

Recently, we reported that insulin signaling regulated FOXO1 phosphorylation in a PI3K-dependent manner in immortalized gonadotropes, similarly to what has been observed in other cells (26, 35, 36). Since we also discovered that overexpression of FOXO1 suppressed basal and GnRH-induced Lhb transcription, the purpose of this study was to determine whether FOXO1 can regulate Fshb synthesis. We show that overexpression of FOXO1 in LβT2 cells results in decreased basal and GnRH-induced transcription from murine and human FSHB-luc reporters as well as endogenous Fshb mRNA. In addition, we show that inhibition of PI3K, which increases FOXO1 nuclear localization (35), results in decreased basal and GnRH-induced Fshb mRNA in murine primary pituitary cells. We also demonstrate that the FOXO1 suppression maps to the proximal Fshb promoter. Moreover, our data suggest that the FOXO1 suppression occurs through protein–protein interactions with factors necessary for Fshb transcription such as PITX1, since the FOXO1 DNA binding domain (DBD) is required for the suppression but FOXO1 does not appear to bind directly to the proximal Fshb promoter. This idea is further supported by the fact that PITX1 induction of Fshb or a PITX1 binding element in CV-1 cells is suppressed by FOXO1, that PITX1 interacts with FOXO1, and that two regions of the proximal Fshb promoter (-50/-41 and -30/-21) appear to be necessary for FOXO1 suppression of GnRH-induced Fshb transcription.

Materials and Methods

Murine Primary Pituitary Cell Culture

Seven-week old, male C57 Black 6 mice (Harlan Laboratories, Indianapolis, IN) were housed in the UCSD vivarium for one week under standard conditions. All animal procedures were conducted in accordance with the UCSD Institutional Animal Care and Use Committee requirements. Eight mice were sacrificed and their pituitaries were collected in ice-cold Dulbecco’s A Phosphate-buffered saline (PBS). After a PBS rinse, the pituitaries were minced on ice with fine scissors and then placed in dissociation media containing phosphate buffered 0.25% collagenase (Life Technologies). The pituitaries were minced into small pieces on ice and then placed in dissociation media containing phosphate buffered 0.25% collagenase (Life Technologies). The pituitaries were minced into small pieces on ice and then placed in dissociation media containing phosphate buffered 0.25% collagenase (Life Technologies). The pituitaries were minced into small pieces on ice and then placed in dissociation media containing phosphate buffered 0.25% collagenase (Life Technologies). The pituitaries were minced into small pieces on ice and then placed in dissociation media containing phosphate buffered 0.25% collagenase (Life Technologies). The pituitaries were minced into small pieces on ice and then placed in dissociation media containing phosphate buffered 0.25% collagenase (Life Technologies). The pituitaries were minced into small pieces on ice and then placed in dissociation media containing phosphate buffered 0.25% collagenase (Life Technologies). The pituitaries were minced into small pieces on ice and then placed in dissociation media containing phosphate buffered 0.25% collagenase (Life Technologies). The pituitaries were minced into small pieces on ice and then placed in dissociation media containing phosphate buffered 0.25% collagenase (Life Technologies). The pituitaries were minced into small pieces on ice and then placed in dissociation media containing phosphate buffered 0.25% collagenase (Life Technologies). The pituitaries were minced into small pieces on ice and then placed in dissociation media containing phosphate buffered 0.25% collagenase (Life Technologies). The pituitaries were minced into small pieces on ice and then placed in dissociation media containing phosphate buffered 0.25% collagenase (Life Technologies).
treated with DMSO or 50 μM LY294002 (EMD Biosciences, San Diego, CA) for 1 hour, then treated with 0.1% BSA vehicle or 30 nM GnRH (Sigma-Aldrich Co., St. Louis, MO) along with DMSO or LY294002 for an additional 5 hours, after which the cells were lysed to obtain total RNA.

Immortalized Cell Culture

Cell culture was performed using the LβT2 cell line which has many characteristics of a mature, differentiated gonadotrope (37, 38). CV-1 cells lacking PITX1 were also used (39, 40). The cells were maintained in 10 cm plates in DMEM with 10% FBS and penicillin/streptomycin antibiotics (Gibco/Invitrogen, Grand Island, NY) at 37°C and 5% CO₂.

Adenoviral Infection

Adenoviral vectors containing cDNA of green fluorescent protein (Ad-GFP) and constitutively active FOXO1 (T24A/S256A/S319A) (Ad-FOXO1-CA) were generously provided by Dr. Domenico Accili. LβT2 cells were seeded at 2 × 10⁶ cells/well on 6-well plates. The next morning, cells were transduced with a multiplicity of infection of 200 of Ad-GFP or Ad-FOXO1-CA for 6 hours, then switched to serum-free DMEM. 24 hours after adenoviral infection, cells were treated with vehicle (0.1% BSA) or 10 nM GnRH for 1 or 6 hours as noted.

Quantitative RT-PCR

Total RNA was extracted from LβT2 cells with TRIzol Reagent (Life Technologies, Carlsbad, CA) following the manufacturer’s protocol. Contaminating DNA was removed with DNA-free reagent (Life Technologies). 2 μg of RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturer’s protocol. Quantitative real-time PCR was performed in an iQ5 iCycler using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA) at 37°C and 5% CO₂. The cells were maintained in 10 cm plates in DMEM with 10% FBS and penicillin/streptomycin antibiotics (Gibco/Invitrogen, Grand Island, NY) at 37°C and 5% CO₂.

Statistical Analyses

Quantitative RT-PCR and transient transfection experiments were performed in triplicate and each experiment was performed using the LβT2 cell line which has many characteristics of a mature, differentiated gonadotrope (37, 38). CV-1 cells lacking PITX1 were also used (39, 40). The cells were maintained in 10 cm plates in DMEM with 10% FBS and penicillin/streptomycin antibiotics (Gibco/Invitrogen, Grand Island, NY) at 37°C and 5% CO₂.

Plasmid Constructs

The pcDNA3 Flag human FOXO1 and pcDNA3 Flag FOXO1-CA expression vectors were previously described (32). We obtained the pcDNA3 FOXO1ΔDBD (∆Δ208–220) from Dr. William Sellers (Addgene plasmid 10694). The pcDNA3 murine PITX1 expression vector was described previously (41). The construction of the −1000 murine Fshb luciferase (luc) reporter plasmid and 5’ truncations were described previously (16, 42). The 10 bp deletions of the −398 Fshb promoter from −70/−11 were generously provided by Dr. Djurdjica Coss. The −1028/+7 human FSHB-luc reporter plasmid was provided by Dr. Daniel Bernard. The 4xFBF-luc was constructed by inserting four repeats of a consensus forhead binding element (FBE) (CCGTAAAACA) upstream of a minimal −81 Herpes simplex virus thymidine kinase promoter in pGL3 using KpnI and Nhel restriction enzyme sites while the 4xHDBE-luc contains four repeats of a consensus bico/end-related homeodomain binding element (HDBE) (ACTAATCCCT) (39). The elements are underlined. Sequences were confirmed by dideoxyribonucleotide sequencing.

Mutagenesis

The QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to generate mutations in a plasmid containing the murine Fshb promoter. Mutagenesis was performed with the following mutations in the −54/−49 PITX1 and −72/−69 AP1 binding elements, respectively: 5’-GGTGAATCCGAC-3’ and 5’-ATTGGTCCCG-3’ (mutated bases are indicated in bold). The sequences of the promoters were confirmed by dideoxyribonucleotide sequencing.

Transient Transfection

LβT2 cells were seeded at 4.5 × 10⁵ cells/well on 12-well plates and transfected 18 hours later, using Polyjet (SignaGen Laboratories, Rockville, MD) following the manufacturer’s instructions. CV-1 cells were seeded at 1.5 × 10⁵ cells/well. For all experiments, the cells were transfected with 400 ng of the indicated luc reporter plasmid and 200 ng of a β-galactosidase (β-gal) reporter plasmid driven by the Herpes Virus thymidine kinase promoter to control for transfection efficiency. The cells were switched to serum-free DMEM containing 0.1% BSA, 5 mg/L transferrin, and 50 mM sodium selenite 6 hours after transfection. After overnight incubation in serum-free media, the cells were treated with vehicle (0.1% BSA) or 10 nM GnRH for 6 hours, as indicated.

Luciferase and β-galactosidase Assays

To harvest the cells, they were washed with 1xPBS and lysed with 0.1 M K-phosphate buffer pH 7.8 containing 0.2% Triton X-100. Lysed cells were assayed for luc activity using a buffer containing 100 mM Tris-HCl pH 7.8, 15 mM MgSO₄, 10 mM ATP, and 65 mM luciferin. β-Gal activity was assayed using the Tropix Galacto-light assay (Applied Biosystems, Foster City, CA), according to the manufacturer’s protocol. Both assays were measured using a Veritas Microplate Luminometer (Promega, Madison, WI).

Statistical Analyses

Quantitative RT-PCR and transient transfection experiments were performed in triplicate and each experiment was performed using the LβT2 cell line which has many characteristics of a mature, differentiated gonadotrope (37, 38). CV-1 cells lacking PITX1 were also used (39, 40). The cells were maintained in 10 cm plates in DMEM with 10% FBS and penicillin/streptomycin antibiotics (Gibco/Invitrogen, Grand Island, NY) at 37°C and 5% CO₂.
repeated at least three times. For the transient transfections, the data were normalized for transfection efficiency by expressing luc activity relative to β-gal and relative to the empty reporter plasmid to control for hormone effects on the vector DNA. The data were analyzed by Student’s t test for independent samples or one-way analysis of variance (ANOVA) followed by post hoc comparisons with the Tukey-Kramer Honestly Significant Difference (HSD) test using the statistical package JMP 10.0 (SAS, Cary, NC). Significant differences were designated as P < .05.

**Immunofluorescence**

LBT2 cells were seeded onto poly L-lysine coverslips (BD Biosciences, Franklin Lakes, NJ) at 4.5 × 10^5 cells/well in 12-well plates and then transfected 18 hours later with pcDNA3 Flag human FOXO1 or pcDNA3 Flag FOXO1-CA expression vectors. Six h after transfection, the cells were switched to serum-free DMEM containing 0.1% BSA, 5 ml/L transferrin, and 50 mM sodium selenite for overnight incubation. The next day, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10 minutes. Cells were then washed in PBS twice and permeabilized with Nonidet P-40 solution (PBS containing 0.2% Nonidet P-40, 20% goat serum, 1% BSA) for 1 hour at room temperature. Cells were washed twice in PBS and then incubated with mouse anti-Flag (Sigma, 1:100, F3165) primary antibody in blocking buffer (PBS containing 20% goat serum, 1% BSA) for 24 hours at 4°C. Cells were washed 3 times with PBS for 5 minutes and incubated with Alexa488-conjugated goat antimouse secondary antibody (Invitrogen, 1:300, A11001) in blocking buffer for 1 hour at room temperature. Cells were washed twice in PBS and then incubated with mouse anti-Flag (Sigma, 1:100, F3165) primary antibody in blocking buffer (PBS containing 20% goat serum, 1% BSA) for 24 hours at 4°C. Cells were washed 3 times with PBS for 5 minutes and incubated with Alexa488-conjugated goat antimouse secondary antibody (Invitrogen, 1:300, A11001) in blocking buffer for 1 hour at room temperature. Cells were washed twice with PBS for 5 minutes and incubated with 300 mM DAPI (Invitrogen) for 4 minutes, then washed 3 times with PBS for 5 minutes each. Coverslips were mounted using Prolong Gold (Invitrogen) and cells were viewed using a Nikon Eclipse TE 2000-U inverted fluorescence microscope. Digital images were collected using a CoolSNAP EZ cooled CCD camera (Roper Scientific, Trenton, NJ) and analyzed with the Version 2.3 NIS-elements image analysis system.

**Electrophoretic Mobility Shift Assay (EMSA)**

Flag-FOXO1-CA was transcribed and translated using a TnT Coupled Reticulolysate System (Promega Corp., Madison, WI). The oligonucleotides were end-labeled with T4 polynucleotide kinase and [γ-^32P] ATP. 4 μL of TnT lysate was incubated with 1 fmol of ^32P-labeled oligo at 4°C for 30 minutes in a DNA-binding buffer [10 mM Hepes pH 7.8, 50 mM KCl, 5 mM MgCl2, 0.1% Nonidet P-40, 1 mM dithiothreitol, 2 μg poly(dI-dC), and 10% glycerol]. After 30 minutes, the DNA binding reactions were run on a 5% polyacrylamide gel (30:1 acrylamide: bisacrylamide) containing 2.5% glycerol in a 0.25x TBE buffer. One fourth of the ^35S-labeled in vitro transcribed-translated product was electrophoresed on a 10% SDS-polyacrylamide gel. One fourth of the ^35S-labeled in vitro transcribed-translated product was loaded onto the gel as input.

**Results**

**Overexpression of Constitutively Active FOXO1 and PI3K Inhibition Decreases Basal and GnRH-Induced Fshb mRNA Levels**

To ascertain whether the FOXO1 transcription factor can suppress Fshb gene expression, we transduced LBT2...
cells with adenovirus (Ad) expressing GFP or constitutively active FOXO1 (FOXO1-CA) and measured Fshb mRNA levels using quantitative RT-PCR. FOXO1-CA is localized in the nucleus due to the inability of insulin/growth factor signaling to phosphorylate the mutated residues (Figure 1A). As observed previously (45), GnRH induced Fshb transcription by 3 fold (Figure 1B). We also demonstrated that Fshb mRNA synthesis was significantly decreased in cells infected with Ad-FOXO1-CA vs Ad-GFP (Figure 1B). Basal Fshb mRNA levels were reduced by 67% while GnRH-induced Fshb transcription was decreased by 84%. These results indicate that FOXO1 can suppress endogenous Fshb synthesis in the context of the native chromatin.

We then determined whether repression of Fshb transcription could be observed in murine primary pituitary cells. Since there is no pharmacological activator of FOXO1, we used a PI3K inhibitor to mimic constitutively active FOXO1 since inhibition of PI3K results in FOXO1 nuclear localization in gonadotropes (35). As shown in Figure 1C, treatment of dispersed pituitary cells with 30 nM GnRH resulted in a significant 1.7-fold induction of Fshb. Inhibition of PI3K with 50 μM LY294002 resulted in a substantial decrease in basal and GnRH-induced Fshb mRNA levels (79% and 95%, respectively). Although pharmacological inhibition of PI3K may affect multiple PI3K/AKT targets (46), these results demonstrate, for the first time, that inhibition of PI3K results in

**FIGURE 1.** Constitutively Active FOXO1 and Inhibition of PI3K Reduces Basal Transcription and GnRH Induction of Fshb mRNA in Gonadotropes. A. Diagram illustrating wild-type FOXO1 and constitutively active FOXO1-CA (T24A/S256A/S319A). DBD, DNA binding domain; NLS, nuclear localization signal; NES, nuclear export signal. B. LβT2 cells were transduced with Ad-GFP or Ad-FOXO1-CA for 6 hours, then switched to serum-free media. 24 hours after adenoviral infection, cells were treated with 0.1% BSA vehicle (Veh) or 10 nM GnRH for 6 hours, as indicated. The results represent the mean ± SEM of three experiments performed in triplicate and are presented as amount of Fshb mRNA relative to Gapdh. * indicates that Fshb transcription is significantly repressed by FOXO1-CA compared to GFP using Student’s t test. C. After overnight incubation in serum-free media, dispersed murine primary pituitary cells were pretreated with DMSO or 50 μM LY294002 for 1 hour, then treated with Veh or 30 nM GnRH along with DMSO or LY294002 for an additional 5 hours. The results represent the mean ± SEM of four experiments performed in triplicate and are presented as amount of Fshb mRNA relative to Gapdh. # indicates that GnRH significantly increased Fshb mRNA levels compared to Veh using Student’s t test. * indicates that Fshb transcription is significantly repressed by LY294002 compared to DMSO using Student’s t test.
decreased basal and GnRH-induced Fshb transcription in murine primary pituitary cells, potentially through increased nuclear localization of FOXO1.

FOXO1 Suppresses Basal Transcription and GnRH Induction of Murine Fshb-luc

Since FOXO1-CA suppressed endogenous Fshb mRNA levels, FOXO1 or FOXO1-CA were overexpressed in LβT2 cells to determine whether FOXO1 regulates the Fshb promoter. Initially, we determined the cellular localization of pcDNA3 Flag human FOXO1 and pcDNA3 Flag human FOXO1-CA transiently transfected in LβT2 cells and incubated in serum-free media. As shown in Figure 2A, FOXO1 is predominantly localized in the cytoplasm with some nuclear localization while FOXO1-CA is localized in the nucleus. We then tested whether FOXO1 transfected into LβT2 cells could induce transcription from a minimal promoter containing a characterized forkhead binding element (FBE). FOXO1 increased transcription of four copies of a consensus FBE linked to a luc reporter (4xFBE-luc) (Figure 2B). In contrast, when 200 ng of FOXO1 or FOXO1-CA were transiently transfected with the –1000 murine Fshb promoter linked to a luc reporter (mFshb-luc), we observed repression of Fshb synthesis (Figure 2C). More specifically, basal Fshb transcription was decreased by 50% and 99%, respectively (Figure 2D) while GnRH-induced Fshb was reduced by 63% and 29%, respectively (Figure 2E).

FOXO1 Suppresses Transcription of Human FSHB-luc

Given that the proximal murine and human FSHB promoters are highly conserved (8), we hypothesized that FOXO1 would have a similar repressive effect on human FSHB gene expression as on murine Fshb. When 200 ng of FOXO1 was transiently transfected with the –1028/+7 human FSHB promoter linked to a luciferase reporter (hFSHB-luc), we observed repression of basal and GnRH-induced FSHB synthesis (Figure 3A-C). Basal FSHB transcription was reduced 38% by FOXO1 while GnRH-induced FSHB gene expression was reduced by 52%.

FOXO1 Suppression Maps to the Proximal Fshb Promoter

Since the proximal Fshb promoter contains PITX1, NFY and AP-1 binding elements necessary for basal and GnRH induction of Fshb (Figure 4A), we determined which regions of the Fshb promoter were required for FOXO1 suppression using 5’ truncation analysis of the promoter. Basal transcription and GnRH induction were measured using –1000, –500, –304, –95, and –64 Fshb-luc reporter plasmids (Figure 4B). FOXO1 suppression of
basal Fshb gene expression and GnRH induction still occurred with the −64 Fshb-luc (Figure 4C-D), suggesting that elements present in the 64 base pairs upstream of the transcription start site are sufficient for FOXO1 repression of basal and GnRH-induced Fshb transcription.

The DNA-Binding Domain of FOXO1 Is Required for Suppression of Fshb Synthesis

To determine whether FOXO1 repression requires the FOXO1 DBD, we tested whether a DNA-binding deficient FOXO1, termed FOXO1-ΔDBD (Δ208–220) (Figure 5A), could elicit a repressive response. As a control for the level of protein expression, we showed that FOXO1 and FOXO1-ΔDBD are stably expressed when transfected into LβT2 cells (Figure 5B). We then demonstrated that while overexpression of FOXO1 reduced Fshb gene expression, the FOXO1-ΔDBD did not alter basal Fshb synthesis (Figure 5C) or suppress GnRH-induced Fshb expression (Figure 5D). These results indicate that
the DBD of FOXO1 is necessary for FOXO1 repression of Fshb basal transcription and GnRH induction on the Fshb promoter and suggest that FOXO1 exerts an effect by either binding to the Fshb promoter or indirectly through FOXO1 DBD interaction with other factors.

**FIGURE 5.** DNA Binding Domain of FOXO1 Is Required to Suppress Basal and GnRH-induced Fshb Gene Expression. A. Diagram illustrating FOXO1-ΔDBD (Δ208-220). B. LβT2 cells were transfected with pcDNA3 empty vector (EV), pcDNA3-FOXO1 (WT) or pcDNA3-FOXO1-ΔDBD for 6 hours, then switched to serum-free media. 24 hours after transfection, the cells were harvested. Western blot analysis was performed on whole cell extracts using FOXO1 and β-Tubulin primary antibodies and a horseradish peroxidase-linked secondary antibody. A representative image is shown. C-D. The –1000 murine Fshb-luc reporter was transfected into LβT2 cells along with EV, FOXO1 or FOXO1-ΔDBD, as indicated. After overnight incubation in serum-free media, cells were treated for 6 hours with 0.1% BSA or 10 nM GnRH. The results represent the mean ± SEM of three experiments performed in triplicate and are presented as basal transcription relative to empty vector (C) or fold GnRH induction relative to the vehicle control (D). * indicates that Fshb-luc transcription is significantly repressed by FOXO1 compared to EV or FOXO1-ΔDBD using one-way ANOVA followed by Tukey’s HSD post hoc test.

**FIGURE 6.** FOXO1 Does Not Bind Directly to the Proximal Fshb Promoter. TnT Flag-FOXO1-CA was incubated with a consensus FBE, –95/-61, –65/31, or –35/-1 Fshb probes and tested for complex formation in EMSA. A. FOXO1-CA-DNA complex on the FBE is shown in lane 1, while anti-Flag supershift is shown in lane 3 and IgG control in lane 2. The FOXO1-CA-DNA complex (arrow), antibody supershift (ss) and nonspecific binding of proteins (ns) are indicated on the left of the gel. B. Self-competition with excess cold FBE is shown in lane 14, lack of competition with mutated consensus FBE (Figure 6B, lane 15). Incubation with oligos encompassing the –95/-1 region of the Fshb promoter did not result in competition (Figure 6B, lanes 16–18). These results suggest that, in contrast to the consensus FBE, FOXO1 does not bind to the proximal Fshb promoter via a high affinity binding site.

FOXO1 Does Not Bind to the Proximal Fshb Promoter via a High Affinity Binding Site

Since the FOXO1 repression mapped to the proximal Fshb promoter and required the FOXO1 DBD, we performed EMSA to determine whether FOXO1 could bind to the proximal promoter in vitro. Three 35-mer oligonucleotide probes were designed to span the –95/-1 region relative to the transcription start site. Flag-FOXO1-CA, synthesized with TnT rabbit reticulocyte lysate, bound to an oligonucleotide probe containing a consensus FBE (Figure 6A, lane 1) but not to probes encompassing the –95/-1 region of the Fshb promoter (Figure 6A, lanes 4, 7, and 10). TnT Flag-FOXO1 also bound to a consensus FBE (data not shown). To identify which complex contained the Flag-FOXO1-CA bound to the FBE, we supershifted the complex with an anti-Flag antibody (Figure 6A, lane 3) but not with control IgG (Figure 6A, lane 2). This complex was not present when rabbit reticulocyte lysate containing the pcDNA3 empty vector was used (data not shown). This complex also showed evidence of self-competition (Figure 6B, lane 14) but did not compete with a mutated consensus FBE (Figure 6B, lane 15). Incubation with oligos encompassing the –95/-1 region of the Fshb promoter did not result in competition (Figure 6B, lanes 16–18). These results suggest that, in contrast to the consensus FBE, FOXO1 does not bind to the proximal Fshb promoter via a high affinity binding site.
FOXO1 Suppression of Basal Fshb Transcription May Involve PITX1

Since the −64 Fshb-luc containing the PITX1 binding site was sufficient to elicit FOXO1 suppression, we tested whether Fshb transcription due to PITX1 overexpression was reduced by FOXO1. We performed these experiments in CV-1 cells lacking PITX1 because LβT2 cells contain PITX1 (39, 40). We first tested whether a multimer of a bicoid-related homeodomain binding element (HDBE) was sufficient to elicit the FOXO1 suppressive effect. As shown in Figure 7A, PITX1 induction of the 4xHDBE was significantly decreased by FOXO1. We then showed that overexpression of PITX1 resulted in a 14-fold induction of Fshb gene expression which was reduced 73% by FOXO1 (Figure 7C). In addition, we tested whether the PITX1 binding element in the proximal Fshb promoter shown in Figure 7B was necessary for the PITX1 induction. Our results demonstrated that the PITX1 binding element was absolutely required for PITX1 induction of Fshb gene expression in CV-1 cells (Figure 7C).

![Graph](image)

FIGURE 7. FOXO1 Suppression of Basal Fshb Transcription May Involve PITX1. A. The 4xHDBE-luc reporter was transiently transfected into CV-1 cells along with pcDNA3 empty vector (EV), FOXO1 and PITX1, as indicated. The results represent the mean ± SEM of three experiments performed in triplicate and are presented as fold PITX1 induction relative to pcDNA3 empty vector. * indicates that transcription is significantly repressed by FOXO1 compared to EV using Student’s t test. B. Diagram illustrating the mutations in the PITX1 binding element (PITXBE) in the murine Fshb promoter. C-D. The −1000 Fshb-luc reporter and the Fshb PITX1 mutant (mut) were transiently transfected into CV-1 (C) or LβT2 (D) cells along with EV, FOXO1 and PITX1, as indicated. The results represent the mean ± SEM of three experiments performed in triplicate and are presented as fold PITX1 induction relative to pcDNA empty vector (C) or luc/β-gal (D). * indicates that Fshb-luc transcription is significantly repressed by FOXO1 compared to EV using Student’s t test while # indicates that the PITX1 mut was significantly repressed compared to the wild-type Fshb promoter. E. GST interaction assays were performed using bacterially expressed GST-fusion proteins (indicated above each lane) and 35S-labeled in vitro transcribed and translated FOXO1, FOXO1-ΔDBD and GFP (indicated on the left of the panels). GFP was used as a negative control. The GST-fusion proteins included GST alone and GST-PITX1. One quarter of the protein used in the interaction assay was loaded in the lane marked input. The experiment was repeated several times with the same results and a representative experiment is shown.

FOXO1 Interacts with PITX1

Since FOXO1 can suppress PITX1 induction of Fshb and the 4xHDBE in CV-1 cells, we investigated whether FOXO1 can physically interact with PITX1. We tested whether FOXO1 or DNA-binding deficient FOXO1 interacts with PITX1 by incubating a GST-PITX1 fusion protein with in vitro transcribed and translated 35S-labeled FOXO1 or FOXO1-ΔDBD in pull-down experiments. As shown in Fig-
ure 7E, there was minimal interaction between the GST-PITX1 fusion protein and the negative control (35S-GFP) or with GST alone incubated with FOXO1 or FOXO1-ΔDBD. In addition, FOXO1 bound to PITX1 while there was no detectable interaction between FOXO1-ΔDBD and PITX1, indicating that the interaction between FOXO1 and PITX1 requires the FOXO1 DBD.

**FOXO1 Reduces Pitx1 mRNA and Protein Levels**

Since the FOXO1 DBD is required for suppression of Fshb but FOXO1 does not appear to bind to the proximal Fshb promoter, we hypothesized that FOXO1 may modulate Fshb transcription indirectly by regulating the amount of PITX1 in the cells. In these experiments, LβT2 cells were transduced with Ad-GFP or Ad-FOXO1-CA and Pitx1 mRNA levels were measured using quantitative RT-PCR (Figure 8A). FOXO1-CA significantly decreased Pitx1 mRNA levels by 57%. We also tested whether the decrease in Pitx1 mRNA levels translated into lower PITX1 protein levels. As shown in Figure 8B-C, FOXO1-CA transduction of LβT2 cells resulted in a 40% reduction in PITX1 protein levels. These results suggest that FOXO1 may suppress Fshb transcription indirectly through regulation of Pitx1 mRNA and protein levels.

**FOXO1 Does Not Regulate c-Fos or c-Jun mRNA and Protein Levels**

Since GnRH induction of the murine Fshb promoter involves the intermediate early genes, c-FOS and c-JUN that comprise the transcriptional AP1 complex, we determined whether FOXO1-CA overexpression alters production of these two genes. LβT2 cells were transduced with Ad-GFP or Ad-FOXO1-CA and c-fos and c-jun mRNA levels were measured using quantitative RT-PCR (Figure 9A and C). Similar to previous reports (47, 48), 1 hour of GnRH treatment substantially increased c-fos and c-jun mRNA levels (Figure 9B and D). However, FOXO1-CA overexpression had little or no effect on c-fos and c-jun mRNA. We also demonstrated that there was no effect of FOXO1-CA overexpression on c-FOS and c-JUN protein levels (Figure 9E).

**FOXO1 Suppression of GnRH-Induced Fshb Transcription Involves Two Regions of the Proximal Fshb Promoter at –50/-41 and –30/-21**

Since GnRH induction involves an AP1 binding element in the proximal Fshb promoter, we tested whether this site was necessary for FOXO1 suppression of Fshb transcription. Mutation of the AP1 binding element shown in Figure 10A reduced GnRH induction of Fshb transcription (Figure 10B), as previously reported (16). However, overexpression of FOXO1 reduced transcription of Fshb further, indicating that FOXO1 may act to suppress Fshb transcription indirectly through the AP1 binding element.
Fsbb from the mutated promoter by 50% compared to 59% for the wild-type promoter (Figure 10B), indicating that the API1 binding element is not required for FOXO1 suppression. Since FOXO1 suppression of GnRH-induced Fsbb transcription occurred within the −64 Fsbb promoter lacking the API1 binding element, we sought to map which regions of the proximal promoter were required for the suppression. Ten bp deletions of the Fsbb promoter ranging from −70/-61 to −20/-11 within the −398 Fsbb-luc were compared to wild-type −398 Fsbb-luc. Basal transcription of Fsbb from either wild-type or mutated promoters was suppressed by FOXO1 (data not shown), suggesting that none of the 10 bp regions individually were required for FOXO1 suppression of basal Fsbb synthesis. Interestingly, GnRH induction of Fsbb was reduced with the mutated promoters compared to the wild-type promoter (Figure 10C), indicating that GnRH induction involves other regions of the Fsbb promoter in addition to the API1 binding element. Moreover, FOXO1 was unable to repress GnRH induction of Fsbb from promoters containing deletions at −50/-41 or −30/-21 (Figure 10C), indicating that these regions may play a role in FOXO1 suppression of Fsbb gene expression induced by GnRH.

Discussion

Our studies suggest that the FOXO1 transcription factor may regulate fertility through modulation of gonadotropin beta subunit gene expression in pituitary gonadotrope cells. Overexpression of wild-type or constitutively active FOXO1 greatly diminished basal and GnRH-induced Fsbb mRNA levels and Fsbb-luc expression in LβT2 cells (Figures 1 and 2), similarly to the repressive effect of FOXO1 on Lhb gene expression (35). Although wild-type FOXO1 is predominantly localized in the cytoplasm under serum-free media conditions, the level of nuclear FOXO1 appears to be sufficient to suppress Fsbb transcription (Figure 2) (35). Our results demonstrating that inhibition of PI3K results in increased nuclear localization of FOXO1 (35) and decreased basal and GnRH induction of Fsbb mRNA in primary pituitary cells (Figure 1) provides support for the idea that FOXO1 is a repressor of Fsbb transcription in a more physiological context. The suppres-

![FIGURE 9. FOXO1 Suppression of GnRH-Induced Fsbb Transcription Does Not Involve Regulation of c-Fos or c-Jun mRNA or Protein Levels. LβT2 cells were transduced with Ad-GFP or Ad-FOXO1-CA for 6 hours, then switched to serum-free media. 24 hours after adenoviral infection, cells were treated with 0.1% BSA vehicle (Veh) or 10 nM GnRH for 1 hour, as indicated. Cells were harvested for total mRNA (A-D) or protein (E). A-D. The results represent the mean ± SEM of three experiments performed in triplicate and are presented as amount of c-Fos mRNA relative to Gapdh (A), fold GnRH induction of c-Fos relative to Veh (B), c-Jun mRNA relative to Gapdh (C), fold GnRH induction of c-Jun relative to Veh (D). * indicates that transcription is significantly repressed by FOXO1-CA compared to GFP using Student's t test. E. Western blot analysis was performed on whole cell extracts using c-FOS, c-JUN and FOXO1 primary antibodies and a horseradish peroxidase-linked secondary antibody. A representative image is shown. doi: 10.1210/me.2013-1185 mend.endojournals.org 11
sion of Fshb and Lhb transcription by FOXO1 is not due to a global repressive mechanism since reporter plasmids containing the insulin response element from the IGFBP1 gene (35) or a consensus FBE (Figure 2) are induced by FOXO1 in LβT2 cells while endogenous c-fos and c-jun mRNA levels are not altered by adenoviral transduction of LβT2 cells with FOXO1-CA (Figure 9). In addition, FOXO1 repression of transcription is not an artifact of the reporter plasmids since substantial repression of endogenous Fshb and Lhb mRNA was observed in LβT2 cells transduced with constitutively active FOXO1 (Figure 1) (35).

At this time, it remains to be determined whether FOXO1 regulates transcription of the gonadotropin beta subunits in human gonadotropes. There is one report that FOXO1 colocalized with Lhb-staining gonadotropes in a human male pituitary section (49). Our results also suggest that FOXO1 regulation of gonadotropin levels may occur in humans since FOXO1 suppressed transcription of a reporter plasmid containing either the human FSHB or LHB promoter in LβT2 cells (Figure 3) (35).

So, how does FOXO1 regulate gonadotropin gene expression via transcriptional repression? Although there are several similarities between FOXO1 repressive effects on the Fshb and Lhb promoters, there are promoter-specific mechanisms that are also required for FOXO1 suppression of the individual promoters. First, the DBD of FOXO1 is required for the repression of both genes (Figure 5) (35), although the specific residues involved have not yet been mapped and it is unknown whether residues required for the repression differ from those necessary for binding to DNA. Second, there is no evidence that FOXO1 binds directly to either the Fshb or Lhb proximal promoters (Figure 6) (35). It is possible that FOXO1 binds to these promoters with such low affinity that it cannot be detected in a gel-shift assay, although FOXO1 binding to a consensus FBE is not altered by 100-fold excess of cold oligonucleotides from the proximal Fshb or Lhb promoters. Thus, our data support the idea that FOXO1 elicits a repressive effect on the gonadotropin beta subunit promoters through indirect mechanisms involving the FOXO1 DBD. This idea is not altogether unexpected since FOXO1 has been shown to interact with other proteins such as SMADS through its DBD (50).

Given that FOXO1 has been reported to physically interact with other proteins to regulate transcription of specific target genes (51), it is plausible that FOXO1 represses basal transcription and GnRH induction of the Fshb and Lhb promoters through specific protein-protein interactions with transcription factor(s) or cofactors necessary for gonadotropin synthesis. Although basal transcription of Fshb involves several factors, including LHX3 and NFY, FOXO1 suppression of basal Fshb gene expression mapped to the –64/-1 region of the Fshb promoter (Figure 4), suggesting that FOXO1 interaction with factors binding to this region eg, PITX1 may play a role in the repression of transcription. In comparison, FOXO1 sup-

**FIGURE 10.** FOXO1 Suppression of GnRH-Induced Fshb Transcription Involves Two Elements in the Proximal Fshb Promoter. A. Diagram illustrating the mutations in the AP1 binding element (AP1BE) in the murine Fshb promoter. B. The –398 Fshb-luc reporter and the Fshb AP1 mutation (mut) were transiently transfected into LβT2 cells along with pcDNA3 empty vector (EV) or FOXO1, as indicated. After overnight incubation in serum-free media, cells were treated for 6 hours with 0.1% BSA vehicle or 10 nM GnRH. The results represent the mean ± SEM of three experiments performed in triplicate and are presented as fold GnRH induction relative to the vehicle control. * indicates that Fshb-luc transcription is significantly repressed by FOXO1 compared to EV using Student’s t test while # indicates that the AP1 mut was significantly repressed compared to the wild-type Fshb promoter. C. The –398 Fshb-luc reporter and 10 bp deletions ranging from –70/-61 to –20/-11 were transiently transfected into LβT2 cells along with EV or FOXO1, as indicated. After overnight incubation in serum-free media, cells were treated for 6 hours with 0.1% BSA or 10 nM GnRH. The results represent the mean ± SEM of three experiments performed in triplicate and are presented as fold GnRH induction relative to the vehicle control. * indicates that Fshb-luc transcription is significantly repressed by FOXO1 compared to EV using one-way ANOVA followed by Tukey’s HSD post hoc test.
pression of basal Lhb transcription mapped to the −150/−87 region of the Lhb promoter, which contains PITX1 and SF1 binding sites (35). Since the PITX1 binding site in the proximal Fshb promoter is conserved among mammals and mutation of this site in the murine and human promoters reduced FSHB transcription (11), it is interesting to speculate that FOXO1 suppression of basal FSHB transcription via PITX1 may also be conserved.

The hypothesis that PITX1 plays a role in FOXO1 suppression of the Fshb promoter is supported by our results demonstrating that PITX1 induction of the Fshb promoter in CV-1 cells is repressed by FOXO1, that PITX1 induction of a consensus HDBE is also decreased by FOXO1 and that PITX1 physically interacts with FOXO1 (Figure 7). The fact that the FOXO1 DBD is requires for the interaction between FOXO1 and PITX1 is also in agreement with our data showing that the FOXO1 DBD is necessary for FOXO1 repression of Fshb. Although this is the first report of a physical interaction between PITX1 and FOXO1, PITX2 interacts with another member of the forkhead transcription factor family, FOXC1 via the C-terminal activation domain of FOXC1 and the homeodomain of PITX2 (52). Interestingly, the PITX1 binding element in the proximal Fshb promoter was not required for FOXO1 suppression in LBT2 cells, suggesting that the repression can occur without PITX1 binding to DNA. It is noteworthy that PITX1 was reported to activate the rat or human Fshb promoter even when the PITX1 binding element was mutated, suggesting that PITX1 activation can occur through protein-protein interactions with factors such as SF1, in addition to direct DNA binding (11, 53–55). Altogether, our studies imply that FOXO1 interaction with PITX1 may be important for suppression of basal Fshb and possibly Lhb transcription.

In addition to the protein-protein interaction between FOXO1 and PITX1 which may regulate Fshb transcription, it is possible that FOXO1 modulates Fshb mRNA levels through regulation of Pitx1 mRNA and protein levels. Both PITX1/2 are expressed in Rathke’s pouch at embryonic day 10.5 and in the developing anterior lobe at e11.5 (56, 57). PITX1/2 are also highly expressed in adult gonadotrope cells and have been reported to regulate transcription of the alpha subunit, Fshb, Lhb and the GnRH receptor (39, 57). Although knockout of PITX1 had little effect on pituitary gland development, lack of PITX1/2 resulted in a more severe defect in pituitary growth and differentiation than the PITX2 knockout alone (58). These studies indicate that PITX1/2 may have distinct and overlapping functions in pituitary development and gonadotropin hormone production in the adult. Interestingly, little is known about the factors that regulate PITX1/2 expression during pituitary development and in the adult (59). Since overexpression of FOXO1 in LBT2 cells resulted in a significant reduction of Pitx1 mRNA and PITX1 protein levels (Figure 8), it is possible that FOXO1 may repress Pitx1 synthesis through regulation of the Pitx1 promoter.

GnRH responsiveness of the Fshb and Lhb promoters occurs due to the induction and binding of gene-specific transcription factors such as AP1 to the Fshb promoter and early growth response protein 1 (EGR1) to the Lhb promoter. Our results suggest that FOXO1 suppression of GnRH-induced Fshb gene expression does not occur through regulation of AP1. Transduction of LBT2 cells with FOXO1-CA did not alter c-Fos and c-Jun mRNA or protein levels (Figure 9). In addition, FOXO1 suppression of the GnRH induction of Fshb still occurred on the −64 Fshb promoter lacking the characterized AP1 binding element (Figure 4). We also demonstrated that the AP1 binding element was not necessary for FOXO1 to elicit a repressive effect on Fshb transcription (Figure 10). These results are not that surprising, given that GnRH responsiveness on the murine Fshb promoter appears to involve additional regions outside the AP1 binding element (16). This is also illustrated by the fact that the −64 Fshb promoter was still responsive to GnRH, that mutation of the AP1 element reduced, but did not abolish, the GnRH induction, and that 10 bp deletions of the proximal Fshb promoter from −70/−61 to −30/−21 all resulted in reduction of GnRH responsiveness. Although other factors involved in the GnRH induction of murine Fshb have not yet been identified, our studies highlight two regions between −50/−41 and −30/−21 which are required for FOXO1 suppression (Figure 10) and suggest that factors other than AP1 are targeted by FOXO1. It should be noted that the −50/−41 region partially overlaps the PITX1 binding element while the −30/−21 region overlaps the TATA box.

In contrast to the Fshb promoter, FOXO1 suppression of GnRH responsiveness on the Lhb promoter mapped to the −87/−1 region which contains an EGR1 binding element (35). Our previous studies also demonstrated that FOXO1 suppressed Lhb transcription induced by EGR1 overexpression in LBT2 cells. Overexpression of FOXO1 with PITX1 or SF1 also suppressed EGR1 induction of Lhb in CV-1 cells. These results provide support for the idea that an interaction between FOXO1 and EGR1 may be responsible for FOXO1 suppression of GnRH-induced Lhb transcription while FOXO1 interactions with as yet unknown factors are responsible for FOXO1 suppression of GnRH-induced Fshb gene expression.

In summary, we demonstrate that FOXO1 suppresses
basal transcription and GnRH induction of the Fshb gene, potentially through protein-protein interactions between FOXO1 and transcription factors recruited to the proximal Fshb promoter such as PITX1 as well as FOXO1 regulation of Pitx1 mRNA and protein levels. If FOXO1 is regulated by metabolic hormone signaling in gonadotropes and represses Fshb and Lhb transcription, as our studies suggest, then it may prove to be an important factor in the regulation of gonadotropin production in situations of caloric insufficiency or excess. Further studies are necessary to comprehend how interactions among FOXO1, PITX1, EGR1, and other factors contribute to the regulation of Fshb and Lhb transcription in pituitary gonadotropes. Investigation of the regulation of PITX1 synthesis may also provide insight into the mechanisms of FOXO1 repression. Additional studies will also help elucidate how FOXO1 integrates input from multiple hormonal signaling pathways to regulate reproduction under favorable or adverse environmental conditions.

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Address all correspondence and requests for reprints to: Varykina G. Thackray, Ph.D., Department of Reproductive Medicine, University of California, San Diego, 9500 Gilman Drive, MC 0674, LA Jolla, CA 92093, USA, Tel.: (858) 822–7693; Email:vthackray@ucsd.edu

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