Androgen Regulates Follicle-Stimulating Hormone \(\beta\) Gene Expression in an Activin-Dependent Manner in Immortalized Gonadotropes

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Little is known about the molecular mechanisms of androgen regulation of the FSH\(\beta\) gene; however, studies suggest that it consists of a complex feedback loop that involves multiple mechanisms acting at both the level of the hypothalamus and the pituitary. In the present study, we address androgen regulation of the FSH\(\beta\) gene in immortalized gonadotrope cells and investigate the roles of activin and GnRH in androgen action. Using transient transfection assays in the FSH\(\beta\)-expressing mouse gonadotrope cell line, L\(\beta\)T2, we demonstrate that androgens stimulate expression of an ovine FSH\(\beta\) reporter gene in a dose-dependent manner. Mutation of either of two conserved androgen response elements at \(-245/-231\) and \(-153/-139\) within the proximal region of the ovine FSH\(\beta\) gene promoter abolishes this stimulation, and androgen receptor binds directly to the \(-244\) ARE in vitro. Androgen induction of the FSH\(\beta\) reporter gene is also dependent upon the activin autocrine loop present in the L\(\beta\)T2 cells, as well as an activin-response element at \(-138/-124\) of the FSH\(\beta\) gene. However, activin regulation of other genes remains unaffected by androgens. In addition, androgens stimulate expression of a mouse GnRH receptor reporter gene, and thus may indirectly augment the response of the FSH\(\beta\) gene to GnRH.

Taken together, these data demonstrate that, in mouse gonadotropes, androgens act directly on the ovine FSH\(\beta\) gene to stimulate expression by a mechanism that is dependent upon activin, as well as acting indirectly, potentially through a second mechanism that may be dependent upon induction of GnRH receptor. (Molecular Endocrinology 18: 925–940, 2004)

**Androgens** are a class of sex steroids that play important roles in sexual development and reproduction in both males and females. Androgen action at both the hypothalamus and the anterior pituitary regulates synthesis and secretion of the heterodimeric gonadotropin hormones LH and FSH. At the level of the hypothalamus, androgens reduce steady-state levels of GnRH mRNA (1, 2) and GnRH peptide (3, 4) and modulate processing of the GnRH precursor (5) in rats, all resulting in dampened GnRH stimulation of LH and FSH. At the level of the pituitary gland, androgen action differs in a species-and gonadotropin subunit gene-specific manner. Androgens repress both \(\alpha\)-subunit of the glycoprotein hormones (\(\alpha\)-GSU) and LH\(\beta\)-subunit gene expression in GnRH antagonist-treated rats (6) and rat primary pituitary cell cultures (7, 8).

Recently, two different molecular mechanisms by which androgen receptor (AR) represses LH\(\beta\) gene expression have been elucidated using the immortalized mouse gonadotrope cell line, L\(\beta\)T2 (9, 10). Both studies show that repression is dependent upon GnRH stimulation and that the mechanisms are indirect, requiring protein-protein interactions with Sp1 (9) or steroidogenic factor 1 (10), rather than through AR binding to the LH\(\beta\) gene.

In contrast to LH\(\beta\) regulation, the mechanisms through which androgens modulate FSH\(\beta\) expression at the level of the pituitary are unknown. A growing body of evidence indicates that androgens act at the level of the pituitary to stimulate, rather than repress, FSH\(\beta\) gene transcription in rodents. Previous studies demonstrated that testosterone (T) increases FSH\(\beta\) mRNA levels by 2-fold in GnRH antagonist-treated rats, whereas \(\alpha\)-GSU and LH\(\beta\)-subunit mRNA levels are decreased (11, 12). Furthermore, T selectively induces FSH\(\beta\) mRNA in both male and female primary rat pituitary cell cultures (7, 8, 13). In addition to these actions of androgen on FSH\(\beta\) transcription, androgens have been shown to modulate levels of follistatin (FS) in the rat pituitary in vivo and both activin and FS in cultured rat pituitary cells (8, 14, 15), indicating that the mechanism of androgen action on FSH\(\beta\) might be indirect, through modulation of the activin/FS system in vivo.

Abbreviations: AR, Androgen receptor; ARE, androgen response element; CMV, cytomegalovirus; DHT, dihydrotestosterone; FBS, fetal bovine serum; FS, follistatin; GnRH-R, GnRH receptor; GRAS, GnRH receptor-activating sequence; GRE, glucocorticoid response element; \(\alpha\)-GSU, \(\alpha\)-subunit of the glycoprotein hormones; HPG, hypothalamic-pituitary-gonadal; MMTV, mouse mammary tumor virus; PRE, progestosterone response element; RSV, Rous sarcoma virus; SBE, Smad binding element; Smad, Sma- and Mad-related protein; T, testosterone; TK, thymidine kinase.

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the pituitary. Furthermore, GnRH receptor (GnRH-R) within the pituitary of rodents is modulated by androgens (16–20), which in turn may alter responsiveness of FSHβ to GnRH, and thus act as another indirect mechanism through which androgens regulate FSHβ expression at the level of the pituitary. Because androgens stimulate GnRH-R expression in mouse pituitary (9, 21), in contrast to the inhibitory effect most commonly observed in rats (18–20), androgens may stimulate mouse FSHβ gene expression through multiple mechanisms, including via enhancing responsiveness of FSHβ to GnRH.

Historically, the lack of an appropriate gonadotrope cell culture model has impeded efforts to characterize the molecular mechanisms of steroid regulation of FSHβ gene expression. In this study, the LβT2 mouse gonadotrope cell line serves as a model system to discriminate androgen action at the level of the isolated gonadotrope from that of the whole pituitary gland by eliminating the variables associated with whole-animal studies or complex primary pituitary cultures. The LβT2 cells express GnRH, androgen, and activin receptors, and produce activin and FS, providing a model that allows investigation of the interactions of these hormones on the expression of gonadotropin genes (22, 23). We find that androgens act directly within gonadotropes to stimulate FSHβ gene transcription independent of GnRH, and that this androgen stimulation occurs through two conserved androgen response elements (AREs) within the FSHβ gene, at least one of which specifically binds to AR in vitro. Furthermore, we demonstrate that androgen regulation is dependent upon the activin autocrine loop in the gonadotrope and specifically on an element in the FSHβ gene that is also required for activin responsiveness. However, androgens do not alter the activin responsiveness of an activin response element from the GnRH-R gene, indicating that the mechanism of androgen action is not through global modulation of the activin/FS system. Furthermore, activin and androgen act synergistically to induce FSHβ gene expression, as do androgen and GnRH. Finally, androgens stimulate mouse GnRH-R gene expression in gonadotropes, revealing a potential mechanism by which androgens might enhance the response of the ovine FSHβ gene to GnRH. Thus, we demonstrate that androgen stimulation of FSHβ gene transcription at the level of the gonadotrope is dependent upon activin action and requires both AREs and elements conferring activin response, providing insight into the mechanisms of androgen regulation of FSH.

RESULTS

Androgens Stimulate FSHβ Transcription in a Dose-Dependent Manner

The ovine FSHβ gene regulatory region drives gene expression and is hormonally regulated in the LβT2 mouse gonadotrope cell line (22). Furthermore, androgen regulation of the rat and bovine LHβ subunit genes has been studied in this model (9, 10). Here, we have used this cell line model system to address the question of whether androgens stimulate FSHβ gene expression by acting directly and solely within gonadotropes. We chose to utilize the ovine (o) FSHβ promoter for three reasons. First, it is the longest mammalian FSHβ promoter clone available (~4.7 kb). Second, the accuracy of the targeting and hormonal responses of the ovine gene has been demonstrated in transgenic mice (24). Finally, the ovine gene is regulated by activin and GnRH in LβT2 cells (22), and the signal transduction of the GnRH response has been elucidated (25).

LβT2 cells were transiently transfected with a luciferase reporter gene in which expression was driven by approximately 5.5 kb of the ovine FSHβ gene regulatory region with the first exon and the first intron (ofFSHβ-Luc), deprived of steroids [10% charcoal-stripped fetal bovine serum (FBS)] for 24 h and then treated with 0.1% ethanol vehicle or T at a range of concentrations for 24 h. T treatment stimulates FSHβ promoter activity in a dose-dependent manner, with statistically significant induction starting at 100 pm T.

Fig. 1. Androgen Stimulates ofFSHβ Transcription in LβT2 Cells

LβT2 cells were transfected with 4.7 kb ofFSHβ-luciferase reporter plasmid, deprived of steroids for 24 h, and then treated with either ethanol vehicle or various concentrations of T (top panel) or DHT (bottom panel) for 24 h. Data were pooled from three independent experiments (n = 3 replicates per treatment group for each experiment) and expressed as the mean ± SEM of the ratio of luciferase/CMV-β-galactosidase activity, normalized to the vehicle-treated mean. * Indicates a statistically significant difference (P < 0.05) from vehicle-treated mean. ** Indicates a highly significant difference (P < 0.0001) from the vehicle-treated mean.
(P = 0.006) and a maximal 2-fold stimulation (P < 0.0001) at 100 nM (Fig. 1). The nonaromatizable androgen, dihydrotestosterone (DHT), similarly stimulates ofSHβ-Luc transcriptional activity when administered for 24 h, with a significant induction at 1 nM (P < 0.05), and peak induction at 10 nM DHT (P < 0.0001; Fig. 1). These data demonstrate that androgens are capable of stimulating FSHβ gene transcription in a dose-dependent manner by acting solely and directly upon gonadotropes. Furthermore, the stimulation of FSHβ is likely due to androgenic rather than estrogenic activity, because both the nonaromatizable (DHT) and the more physiological, aromatizable (T) androgens have identical stimulatory effects on FSHβ promoter activity. Taken together, these data indicate that physiologically relevant concentrations of androgens stimulate FSHβ gene expression by acting directly within gonadotropes. This androgen regulation of FSHβ occurs in a specific and saturable, dose-dependent manner typical of classic steroid hormone action.

Two AREs in the Proximal Promoter Are Required for Androgen Response

To further elucidate the mechanism(s) through which androgens stimulate the FSHβ gene, we mapped regions of the oFSHβ gene promoter that confer androgen response using truncation-deletion analysis. LβT2 cells were transiently transfected with a series of truncations of the oFSHβ gene regulatory region ranging in length from 982–105 bp upstream of the transcription start site, and the ability of 100 nM DHT, administered for 24 h, to induce FSHβ gene transcription was tested (Fig. 2). DHT significantly induced reporter gene activity by 1.9–(P < 0.0001), 1.7–(P < 0.0001), and 1.8-fold (P < 0.0001), respectively, in transfections of reporter genes containing 982, 561, or 401 bp upstream of the oFSHβ gene mRNA start site. The degree of DHT stimulation of these three promoters was identical to that of the 4.7-kb flanking region of the oFSHβ gene (see Fig. 1). Further truncation to −105 resulted in a loss of the ability to respond to DHT (P = 1.0). These data demonstrate that androgen response is conferred by the 296 bp region of the oFSHβ gene-proximal promoter between −401 and −105 bp.

In examining the −401 to −105 region of the oFSHβ gene promoter for candidate AREs, homology searches revealed three candidate sites, located at −245/−231, −212/−198, and −153/−139 (Fig. 3A). These three elements have homology to known AREs (Fig. 3A), including a shared consensus ARE/glucocorticoid response element (GRE)/progestosterone response element (PRE) from the mouse mammary tumor virus (MMTV) long terminal repeat (26, 27), consensus AREs from various androgen responsive genes (reviewed in Ref. 28), and an experimentally deduced sequence proven to be highly selective and specific for AR (hasp-ARE) (29). Of the three candidate AREs, only the one at −245/−231 contains a perfect consensus half-site (TGTTCT). Overall, the −245/−231 and −153/−139 putative AREs exhibit the highest homology to previously characterized AREs (Fig. 3A). In contrast, the putative ARE at −212/−198 has higher homology to the shared ARE/GRE/PRE of MMTV (71%) than do either the −245/−231 (47%) or the −153/−139 (47%) sites, and a lower homology (66%) than either the −245/−231 (73%) or −153/−139 (80%) sites to the hasp-ARE. These data implicate these three sites as potential targets for AR action and suggest the −245/−231 and −153/−139 sites may have higher specificity for AR and thus are most likely to be AREs.

This entire 211-bp proximal region of the oFSHβ gene (−248 to −137), encompassing all three putative ARE motifs, is remarkably well conserved among nu-

**Fig. 2.** Localization of the Androgen-Responsive Region in the oFSHβ Regulatory Region

LβT2 cells were transfected with a series of truncated oFSHβ luciferase reporter plasmids (−982, −561, −401, and −105), deprived of steroids for 24 h, and then treated with ethanol vehicle or 100 nM DHT for 24 h. Data were pooled from five independent experiments (n = 4–6 replicates per treatment group for each experiment) and expressed as the mean ± SEM of the ratio of luciferase to CMV-β-galactosidase activity, normalized to the vehicle-treated mean within the same plasmid group. * Indicates a statistically significant difference (P < 0.0001) from the vehicle-treated mean within the same plasmid group.
Numerous mammalian species (Fig. 3B). The −245 and −153 AREs are the most highly conserved sites, and the C/G bases known to be critical for function of the consensus ARE/GRE/PRE are perfectly conserved in all species examined. The less than perfect overall conservation homology of the middle ARE (−212/−198) site is due to insertions of a single guanidine base in FSHβ of rodent species and of a CAT duplication in the mouse, rat, and human FSHβ genes compared with the sheep, cow, and pig FSHβ genes (Fig. 3B). Previously, Webster et al. (30) identified these three same sites within the oFSH proximal promoter to be PREs that bind PR in vitro and confer progesterone response when present as multimers on a heterologous promoter. We hypothesized that one or more of these three PREs may also act as AREs, because both AR and PR are known to bind the same DNA elements within certain genes, including the long-terminal repeat of the MMTV gene promoter (26). To determine the role of these putative regulatory elements in androgen stimulation of FSHβ, we examined the ability of DHT treatment (24 h) to stimulate promoter activity when the sequences from the −245/−231, −212/−198, or −153/−139 candidate ARE motifs were disrupted by site-directed mutagenesis (mutations shown in Fig. 4A). As shown in Fig. 2, expression of the wild-type −982 FSHβ reporter was stimulated 1.9-fold by DHT (P < 0.0001). A 2-bp mutation (GT to CC) within the conserved half-site of the putative ARE at −245/−231 completely abolished DHT stimulation of reporter gene expression in the context of the −985 FSHβ promoter (P = 1.0). Similarly, a 2-bp mutation (GA to CC) of the half-site of the putative ARE at −153/−139 also completely abolished the ability of DHT to stimulate the FSHβ promoter (P = 1.0). In contrast, mutation of 2 critical C/G base pairs within the putative ARE at −212/−198, one in each half-site, had no deleterious effect on the ability of DHT to induce FSHβ promoter activity (1.65-fold compared with 1.9-fold induction of −985 FSHβ; P = 0.453). Similarly, a CA to CC mutation of the −202/−198 half-site of the puta-
AR Binds to the −245 ARE

To determine whether AR can bind directly to an ARE in the oFSHβ subunit gene, we obtained full-length human AR protein by overexpressing it in baculovirus-infected Sf9 insect cells using a Flag-tagged AR baculovirus (kindly provided by E. Wilson). Cells were treated with androgens (1 mM DHT final concentration) for 24 h before harvest. Soluble whole-cell extract was incubated with a 29-bp probe to the conserved −245 oFSHβ ARE and analyzed by EMSA (Fig. 5). The resulting complex was supershifted by anti-AR antibody but not by IgG, showed self-competition, failed to compete with a mutant probe containing the same mutations noted in Fig. 4, and was competed by an ARE consensus sequence (Materials and Methods). We conclude that AR can bind directly and specifically to the −245 ARE element in the oFSHβ subunit gene. However, under these conditions, we were unable to demonstrate AR binding to the −153/−139 ARE in EMSA. This might be due to the fact noted above, that only the −245/−231 ARE contains a perfect consensus half-site (TGTTCT) for AR binding.
Androgen Stimulation is Dependent on the Activin Autoregulatory Loop

In addition to elucidating the mechanism through which androgens act within gonadotropes to directly stimulate the oFSHβ gene, we also examined whether androgens act within the gonadotrope through additional mechanisms to indirectly regulate FSHβ gene expression. Previous studies in rats in vivo and in cultured rat pituitary cells have shown that androgens can modulate the levels of activin and FS in the pituitary (8, 14, 15, 31, 32). To determine whether the activin autocrine loop, the key regulatory system of FSH synthesis and secretion, plays a role in androgen stimulation of FSHβ transcription, we examined the effects of coadministration of FS or activin on androgen stimulation of FSHβ expression. LβT2 cells were transiently transfected with the 4.7 kb oFSHβ-Luc reporter, incubated overnight in DMEM containing 10% charcoal-treated FBS and 250 ng/ml FS to block endogenous activin action, followed by treatment with vehicle or 100 nM DHT ± 250 ng/ml FS or 10 ng/ml activin-A for 24 h (Fig. 6). As expected, treatment with either DHT or activin alone induced FSHβ promoter activity by 1.5-fold (P = 0.0015) and 11-fold (P = 0.000003), respectively. Interestingly, addition of FS abolished the ability of DHT to stimulate FSHβ promoter activity, whereas addition of activin with DHT resulted in induction of FSHβ promoter activity by 19-fold, significantly greater than either DHT (P = 0.000007) or activin treatment alone (P = 0.0039). Using the methodology as described by Slinker (33), analysis of the data by two-way ANOVA reveals a synergistic increase upon activin and androgen cotreatment that is significantly (P < 0.0001) different than an additive effect. Therefore, these data indicate that there is a synergistic interaction between androgen and the activin/FS system in the regulation of the FSHβ promoter as well as a complete dependence upon activin as a permissive agent for androgen action. Thus, the endogenous activin secretion by the gonadotrope plays a crucial role in androgen regulation of FSHβ gene expression.

The Activin Autoregulatory Loop Does Not Affect Androgen Action Globally, nor Vice Versa

To determine whether the interaction between androgen and activin is specific to the FSHβ gene or involves a general effect of the activin system on AR function or vice versa, we examined both the effect of FS on androgen induction of the highly androgen-sensitive MMTV promoter and the effect of androgen on FS or activin regulation of the highly activin-sensitive GnRH receptor-activating sequence (GRAS) element of the mouse GnRH-R gene promoter (34). In the first series of experiments, LβT2 cells were transiently transfected with the MMTV-Luc reporter plasmid, pretreated overnight with DMEM containing 10% charcoal-treated FBS and 250 ng/ml FS, and treated with vehicle or 100 nM DHT ± 250 ng/ml FS for 24 h (Fig. 7A). DHT stimulated MMTV promoter activity by 2.56-fold (P = 0.024). FS treatment did not inhibit MMTV promoter activity, nor did FS diminish the ability of DHT to stimulate the MMTV promoter. These data indicate that FS blockade of androgen action is gene specific, rather than due to a global perturbation of AR function in the gonadotrope.

A route for androgen regulation of FSHβ gene expression in pituitary that has been previously suggested is androgen regulation of activin system components such as FS (14, 15). To determine whether androgens were globally affecting activin action, LβT2 cells were transiently transfected with the activin-sensitive GRAS-Luc reporter plasmid containing the activin response element from the GnRH-R gene (34, 35), pretreated overnight with DMEM containing 10% charcoal-treated FBS and 250 ng/ml FS, and treated with vehicle or 100 nM DHT ± 10 ng/ml activin-A ± 250 ng/ml FS for 24 h (Fig. 7B). FS treatment inhibited GRAS-Luc reporter activity by 70% (P < 0.00000001), whereas activin treatment stimulated reporter activity by 6-fold (P = 0.0000000001). DHT alone had no effect and DHT in combination with FS or activin had
no effect on either FS repression or activin stimulation of GRAS promoter activity. These data suggest that the stimulatory effect of androgens on FSHβ gene expression is not due to modulating bioavailable levels or activity of activin, its receptors, FS, or other activin-regulatory system components present in gonadotropes. Taken together, these data show that androgen regulation of FSHβ gene expression is not due to an indirect action on the activin/FS system, but likely involves interaction with the activin system specifically within the context of the FSHβ gene promoter.

### Androgen Regulation Is Dependent upon an Activin-Responsive Element in the FSHβ Gene

To investigate potential interactions of the androgen- and activin-regulatory systems on the FSHβ gene, we addressed the potential role of an activin response element in the FSHβ gene in androgen action. Recent data from our laboratory indicate that a putative Smad and Mad-related protein (Smad) binding element (SBE) located at −138/−124 of the oFSHβ gene promoter is crucial for activin regulation of transcription (35a). In the current study, we used an activin-insensitive −138/−124 mutant of oFSHβ-Luc reporter to further elucidate the molecular mechanism of interaction between androgen and the activin loop in the regulation of FSHβ transcription. LβT2 cells were transiently transfected with either the −982 truncation of oFSHβ-Luc, the −982 truncation of oFSHβ-Luc with the mutation in the −245/−231 ARE, or the −982 truncation of oFSHβ-Luc with a 2-bp mutation in the putative SBE half-site (Fig. 8A), pretreated overnight with 10% charcoal-treated FBS and treated with vehicle or 100 nM DHT ± 10 ng/ml activin-A for 24 h. These experiments were performed in serum-free conditions, and we did not include a FS pretreatment to lower the endogenous activin; hence, the magnitude of the activin induction is smaller than in Fig. 6. This is not due to differences in the activin responsiveness of the −982 vs. the −4.7-kb oFSHβ promoter (35a). As shown in Fig. 8B, DHT or activin treatment alone each stimulated activity of the −982 oFSHβ-Luc reporter by approximately 2-fold (P = 0.001 and P < 0.000001, respectively), and combined DHT plus activin treatment stimulated reporter activity by 4-fold (P < 0.000001). The oFSHβ-Luc reporter with the ARE mutation was not affected by DHT alone (P < 0.64) and was induced 1.7-fold by activin alone (P = 0.00004), and the cotreatment of DHT with activin did not significantly increase reporter activity above that of activin treatment alone (P = 0.28; 1.9-fold vs. 1.7-fold).

As expected, the oFSHβ-Luc reporter in which the −138/−124 putative SBE half-site has been disrupted by a double-point mutation was not significantly induced by activin treatment alone (1.2-fold; P = 0.213). Interestingly, the −138/−124 activin nonresponsive mutant of the oFSHβ-Luc reporter was completely insensitive to DHT treatment alone (0.98-fold vs. vehicle; P = 0.11) or DHT enhancement of activin induc-
tion (0.90-fold vs. vehicle and activin; \( P = 0.446 \)) of FSH\( \beta \) reporter gene activity. Therefore, androgen induction of FSH\( \beta \) requires both the \(-245/-231\) ARE and \(-138/-124\) SBE sites, whereas activin response requires the \(-138/-124\) SBE site, but may be slightly dampened by loss of the \(-245/-231\) ARE. These data provide strong evidence in support of the activin dependence of androgen stimulation demonstrated in Fig. 7. Moreover, they suggest that an interaction between AR and the activin-regulatory system occurs through at least two distinct cis elements in the proximal oFSH\( \beta \) promoter.

Androgen Enhances GnRH Stimulation of FSH\( \beta \)

Gene Expression

In addition to modulating the activin/FS system in the pituitary, androgens have been shown to regulate GnRH sensitivity via alterations in the level of GnRH-Rs (16–20). In fact, in the case of the LH\( \beta \) gene, GnRH stimulation was required to observe androgen repression in the L\( \beta \)T2 cell model (9, 10). We hypothesized that androgens may act upon the gonadotrope to alter the responsiveness of the FSH\( \beta \) gene to GnRH and thereby indirectly regulate its expression. To test this hypothesis, L\( \beta \)T2 cells were transfected with the 4.7-kb oFSH\( \beta \)-luciferase reporter and the ability of \( 10^{-12} \) to \( 10^{-8} \) M DHT administered for 24 h to affect GnRH (10 nM for 6 h) induction of oFSH\( \beta \) promoter activity was examined (Fig. 9). Treatment for 24 h with 10 nM DHT induced FSH\( \beta \) promoter activity by 1.6-fold (\( P = 0.032 \)), and 6 h treatment with 10 nM GnRH induced promoter activity by 1.7-fold (\( P < 0.015 \)). These results were expected because both androgens (Fig. 1) and GnRH (22, 25) are each able to independently stimulate FSH\( \beta \) expression in L\( \beta \)T2 cells. Interestingly, when increasing concentrations of DHT were coadministered with 10 nM GnRH, the oFSH\( \beta \) promoter was increasingly stimulated in a DHT dose-dependent manner. The 1 nM DHT concentration, when coadministered with GnRH, resulted in a 2.75-fold induction of FSH\( \beta \) promoter activity, which was significantly greater than either 10 nM DHT (\( P < 0.001 \)) or 10 nM GnRH (\( P < 0.001 \)) treatment alone. The maximal stimulation of 3-fold was observed in cells cotreated with 10 nM DHT and GnRH. FSH\( \beta \) promoter activity of this treatment group was also significantly greater than that of cells treated with either DHT (\( P < 0.001 \)) or GnRH (\( P < 0.001 \)) alone.

Analysis of the data by two-way ANOVA reveals a
synergistic increase upon cotreatment with GnRH and androgen that is significantly \( (P < 0.001) \) different than an additive effect (33). These data suggest that androgens, in addition to directly stimulating FSH\(\beta\) expression through novel AREs, may also be enhancing the ability of GnRH to stimulate FSH\(\beta\) expression. To test this hypothesis, we examined whether androgens are able to modulate responsiveness of gonadotropes to GnRH by inducing the GnRH-R gene.

**Androgen Stimulates GnRH-R Gene Expression in a Time- and Dose-Dependent Manner**

One mechanism through which androgens could enhance response of gonadotropes to GnRH is by stimulating expression of the GnRH-R. We examined the effects of androgen treatment on expression of the mouse GnRH-R gene in L\(\beta\)T2 cells transfected with a luciferase reporter plasmid the expression of which was driven by 1.2 kb of the mouse GnRH-R gene promoter. L\(\beta\)T2 cells were deprived of steroids for 24 h, and then incubated for 24 h with media containing either 0.1% ethanol vehicle alone or increasing concentrations of DHT. Treatment for 24 h with DHT resulted in a gradual dose-dependent increase in mouse GnRH-R promoter activity (Fig. 10A). Statistically significant induction of GnRH-R gene expression was observed at 1 \( (P = 0.002) \), 10 \( (P < 0.0001) \), and 100 nM \( (P < 0.0001) \) DHT concentrations, with maximal stimulation at 10 nM. These data indicate that at
doses physiologically relevant to adult male mice, androgen stimulates expression of the mouse GnRH-R gene in a dose-dependent manner.

To further characterize the mechanism of androgen stimulation of GnRH-R gene expression, we treated transfected LβT2 cells with either ethanol vehicle or 10 nM DHT for 6, 12, 24, or 48 h (Fig. 10B). DHT (10 nM) treatment for 6 h had no effect on GnRH-R promoter activity. As the length of exposure to DHT was increased, the induction of promoter activity increased. DHT treatment for 12 h resulted in a modest 1.2-fold induction of promoter activity but was not statistically significant \( (P = 0.15) \). As expected from the results of Fig. 10A, 24 h of 10 nM DHT resulted in a statistically significant 1.4-fold \( (P < 0.0001) \) induction of GnRH-R promoter activity. DHT treatment for 48 h resulted in an even greater 1.9-fold \( (P < 0.0001) \) induction of GnRH-R reporter gene expression. These data indicate that a physiologically relevant dose of androgen stimulates GnRH-R gene expression in a time-dependent manner.

**Androgen Enhancement of GnRH Stimulation Persists after Abrogation of Direct Androgen Stimulation of FSHβ Expression**

We have demonstrated that androgen stimulates GnRH-R gene expression in the mouse gonadotrope; however, these data do not discount that the effect of DHT and GnRH treatment in the stimulation of FSHβ promoter activity might still be due to cross-talk between the androgen and GnRH signaling pathways rather than due to androgen causing sensitization of the gonadotrope to GnRH. To test this alternate hypothesis, we examined the ability of DHT and GnRH to stimulate promoter activity of a 4.7-kb oFSHβ-luciferase expression plasmid that had been rendered insensitive to direct androgen stimulation. The −245/−231 ARE was mutated in the same manner as in Fig. 4, but in the context of the 4.7-kb oFSHβ 5′-flanking region. As described in Fig. 4, this same mutation completely abolished androgen stimulation in the context of the −982 oFSHβ promoter. As expected, 24 h of 10 nM DHT failed to result in stimulation of reporter gene activity in LβT2 cells transiently transfected with the 4.7-kb oFSHβ-luciferase plasmid containing the ARE mutation \( (P = 0.85; \) Fig. 11). This indicates that mutation of this ARE alone is sufficient to block androgen induction even in the context of the −4.7 kb promoter. GnRH (10 nM) for 6 h induced mutant FSHβ promoter activity by 2.2-fold compared with vehicle-treated controls \( (P < 0.0001) \). Interestingly, when 10 nM DHT was coadministered with GnRH, the activity of the ARE mutant oFSHβ promoter was still significantly increased above that of GnRH treatment alone by 1.78-fold \( (P < 0.0001) \). These data indicate that androgens act indirectly through GnRH to induce FSHβ promoter activity, even when androgens are no longer able to directly induce FSHβ expression. Taken together with the data from Figs. 9 and 10, these results support the hypothesis that androgens also act in the gonadotrope to indirectly promote transcription of the FSHβ gene by stimulating GnRH-R expression and thereby enhancing responsiveness of the gonadotrope and FSHβ gene to GnRH. Alternatively, it is also possible that androgen could be affecting the sensitivity of the LβT2 cell to GnRH by augmenting the signal transduction pathways through which GnRH is acting on the FSHβ gene.

**DISCUSSION**

Accumulating evidence indicates that the steroid hormone feedback regulation of gonadotropin synthesis involves multiple mechanisms, with distinct actions at the levels of both the hypothalamus and pituitary gland. Very little is known about how androgenic steroids regulate the FSHβ gonadotropin subunit gene at the level of the pituitary gland, the molecular mechanisms involved, or even whether the gonadotrope itself is the direct target of androgen. The key impediment to such investigations has been the lack of an appropriate FSHβ-expressing gonadotrope model system. Recent studies using highly sensitive RT-PCR (22), immunohistochemistry (36), and RNase protection assays (37) have demonstrated that the LβT2 mouse
gonadotrope cell line expresses endogenous FSHβ, and that expression of FSHβ in LβT2 cells is regulated by the same factors known to regulate FSHβ in vivo, such as GnRH, activin, and FS (22). Previous studies had reported that LβT2 cells, like normal gonadotropes, express ER (38) and AR (23). The data presented in the current study confirm that endogenous AR is functional and active in LβT2 cells, demonstrating the relevance of the LβT2 cell line as a model system to study the action of androgenic steroid hormones.

Previous literature has demonstrated that androgen regulation of FSHβ gene expression appears to be species specific and involves actions at both the hypothalamus and pituitary gland. In rats, for example, androgens act through at least two opposing mechanisms to regulate FSH synthesis, i.e. an inhibitory action at the hypothalamic level that is GnRH dependent and a stimulatory action at the pituitary level that is independent of GnRH (7, 11–13). In primates, as in rats, androgens act both at the hypothalamic level in a GnRH-dependent manner and at the pituitary level in a GnRH-independent manner. However, unlike the rat, both mechanisms of androgens appear to be inhibitory to FSHβ expression in primates (39, 40). In the current study, we elucidate the activin-dependent, GnRH-independent, molecular mechanism through which androgens stimulate expression of the oFSHβ gene at the level of the pituitary gonadotrope. Furthermore, we identify a GnRH-dependent mechanism of androgen stimulation that potentially functions through the induction of GnRH-R gene expression.

Several investigators have demonstrated, using either GnRH antagonist-treated rats or primary cultures of rat pituitary cells, that at the level of the pituitary gland, androgens increase steady-state levels of FSHβ mRNA (7, 8, 11–13) and primary transcript (41) and enhance stability of FSHβ transcripts (11) through unknown mechanisms independent of GnRH. These studies, however, were not able to address whether androgen regulation of FSHβ expression occurs by direct actions on the FSH-producing gonadotrope or is indirectly mediated by one of the other many distinct populations of the anterior pituitary. This is an important distinction because gonadotropes comprise only a small minority (5–15%) of the total secretory cell types within the anterior pituitary (42), and several anterior pituitary cell types express AR in vivo (43–45). Moreover, the different pituitary cell populations communicate with each other through paracrine, juxtacrine, and endocrine mechanisms (46). The data presented herein specifically address whether the androgen regulation of FSHβ expression observed at the level of the whole pituitary gland also occurs at the level of the gonadotrope. Taken together, these data indicate that physiologically relevant concentrations of androgens stimulate FSHβ gene expression by acting directly within gonadotropes. This androgen regulation of FSHβ occurs in a specific and saturable, dose-dependent manner typical of classic steroid hormone action. Furthermore, androgen induction of FSHβ transcription occurs within 24 h, a physiologically relevant time frame when compared with the diurnal changes in androgen levels that occur in many different animal genera, including rodents (47, 48).

Our finding that two of the three sites within the proximal oFSHβ promoter shown to act as PREs by Webster et al. (30) also act as AREs, demonstrates that PR and AR likely use distinct mechanisms with shared components. Because disruption of the −212/-198 PRE does not interrupt androgen stimulation of FSHβ, it must not be crucial to the mechanism of androgen regulation. Although the necessity of the three PREs for progesterone stimulation was not examined in the studies by Webster et al. (30), all three PREs were shown to specifically bind PR in gel shift assays and confer progesterone response when multimerized on a heterologous promoter, demonstrating their sufficiency as PREs. In contrast, we have shown that two of these elements (−245/−231 and −153/−139) are individually required for androgen induction of the oFSHβ gene, i.e. that neither is sufficient when the other is mutated. Notably, only one of these elements could be demonstrated to bind to AR protein in vitro (−245/−231). This may indicate that the −153/−139 element is low affinity despite the fact that its mutation ablates androgen responsiveness. Perhaps the −153/−139 element is required to coordinate accessory or interacting proteins that provide context for the action of the AR binding at the −245/−231 element or perhaps other binding proteins present in the LβT2 cell nuclei are required to allow AR to bind to the −153/−139 sequence.

Interestingly, two of the three PRE/ARE sequences within the oFSHβ gene proximal promoter are highly conserved among mammalian species, including pigs, cows, rats, mice, and humans (Fig. 4). The high degree of conservation of these sequences would suggest that they play important roles in androgen and/or progesterone regulation of FSHβ in a wide variety of species. However, the significance of this high degree of sequence conservation in mammals is complicated by observations of androgen inhibition of FSH synthesis in primate model systems. In castrated rhesus macaques treated with GnRH antagonist, administration of T significantly reduced the levels of serum FSH, suggesting an inhibitory role of androgen in FSH synthesis at the level of the pituitary gland in nonhuman primates (39). Similarly, in primary pituitary cell cultures from hypogonadal (hpg), GnRH-deficient, human FSHβ promoter-containing transgenic mice, administration of testosterone propionate or DHT for 24 h reduced steady-state levels of human FSHβ transgene mRNA (40). The authors postulated that differences in the FSHβ gene between humans and rats were a possible reason for the contradiction of their data to that observed in rats. Our finding that both sequences responsible for androgen action within the oFSHβ gene are extremely well conserved among mammals does not support such a hypothesis, and therefore, the
relative differences in the responses of the rat and sheep gene to androgen (stimulatory) compared with that of the human gene (inhibitory) are probably not due to sequence differences. We cannot, however, disregard the possibility that subtle sequence differences in regions flanking the highly conserved 110-bp region of the proximal FSHβ promoter examined could affect sensitivity to androgens. Alternatively, the two ARE sequences identified in the current study may be crucial to the actions of AR in FSHβ expression for both sheep and humans, but interaction of AR with different combinations of coactivators or corepressors on the FSHβ gene may ultimately determine whether androgens will stimulate or repress expression in a given species.

The dependence of androgen activation of the oFSHβ gene on the activin autocrine loop indicates a potential mechanism for species differences. For example, both Smad 3 and Smad 4 are known to bind AR in vitro, and overexpression of AR affects expression of Smad 3/4-sensitive genes and vice versa (49–51). This is of physiological importance because Smads are downstream mediators of the activin receptor. Activin is a key physiological regulator of FSH synthesis and secretion and is believed to be a principal mechanism through which FSH is regulated differentially from LH (52). Thus, the dependence of androgen induction on the presence of endogenous activin indicates that activin acts as a permissive agent for the regulation of this gene by androgen.

Androgens have been hypothesized to act in the anterior pituitary through modulation of the components of the activin and FS system (8, 14, 15, 31, 32). Our finding that FS blocks the action of androgens on the FSHβ promoter might seem to support this hypothesis. However, the further demonstration that androgens have no effect on activin induction of an activin-response element from the GnRH-R gene indicates that the role of activin/FS system plays in androgen action is gene specific, not a global alteration in the bioavailability of activin or FS or in the level or responsiveness of the activin receptors. Furthermore, the removal of activin by FS tends to increase, rather than decrease, the response of MMTV to androgens, indicating that activin is not necessary for the action of AR on expression of other genes. Finally, mutation of only one ARE in the FSHβ promoter prevents androgen regulation despite the presence of the intact activin-response elements and activin responsiveness indicating that androgen is not regulating this promoter by altering the levels of activin or FS. This finding is supported by a recent study by Burger et al. (41) in which androgen was shown to induce FSHβ primary transcript independent of pituitary FS mRNA regulation. The mechanism of the activin dependence is further elucidated by the demonstration that mutation of an element required for activin action on the oFSHβ gene abrogates androgen induction despite the presence of both intact AREs. This indicates the potential for an interaction between these elements perhaps through AR/SMAD protein-protein interactions, as has been demonstrated in other systems (49–51).

In addition to species-specific and activin-dependent effects of direct androgen action on FSHβ, the current study also supports a species-specific effect of indirect androgen action on FSHβ. Studies using rats have most commonly demonstrated that the levels of GnRH-R and GnRH-R gene expression are reduced by androgen treatment (18–20). However, this does not appear to be the case in the mouse. Naik et al. (21) determined by radioligand binding studies that castration reduced the number of GnRH binding sites (GnRH-R-Rs) within the pituitary gland of male mice by approximately 50%, and that T replacement at the time of castration completely prevented the castration-mediated decline in the number of GnRH-R-Rs. More recently, Curtin et al. (9) demonstrated that the levels of GnRHR mRNA are increased approximately 1.7-fold by 24 h of 1 nu DHT in LβT2 cells. Our data confirm the findings of these earlier studies that androgens stimulate GnRH-R gene expression in the mouse gonadotrope. Furthermore, our demonstration of the dose and time dependency of androgen stimulation of a mouse GnRH-R reporter gene in mouse gonadotropes indicates the specificity of this apparent species-specific androgen action. Although our data demonstrate that androgens synergistically enhance GnRH stimulation of FSHβ gene expression, and that this same regimen of androgen treatment induces GnRH-R gene expression, these data do not prove that the mechanism through which androgens act synergistically with GnRH to stimulate FSHβ gene expression depends on an increase in GnRH-R number. It remains possible that this synergism and the remaining effects of androgen on the ARE-mutated FSHβ gene are due to androgen effects on the GnRH signaling cascade. Gonadotropes possess a reservoir of spare GnRH-Rs (53), and increasing the number of cell-surface GnRH-Rs does not necessarily increase the responsiveness to GnRH (54, 55). Furthermore, studies have shown that GnRH can regulate the level of FS (56, 57) in rat pituitary, providing another possible mechanism for the interaction of GnRH and androgen on the FSHβ gene. However because GnRH was shown to induce FS levels (56, 57), this would be counter to our finding of GnRH induction of the FSHβ gene. Nevertheless, our data are strongly coincidental, and the hypothesis that the two androgen-regulated events are linked is an alluring one.

The physiological significance of the opposing mechanisms of androgen-positive and -negative feedback regulation of FSHβ at the pituitary and hypothalamic levels, respectively, may seem paradoxical or counterproductive. However, these two opposing mechanisms of androgen feedback regulation of FSHβ have potential benefits. First, the contrary effects of androgen at the pituitary and hypothalamic levels provide a means through which a single hormone, androgen, may differentially regulate synthesis
of the two gonadotropin hormones, LH and FSH, by acting differently at only one hypothalamic-pituitary-gonadal (HPG) axis site (gonadotrope) without requiring different actions on the other axis sites (hypothalamus and gonads). Thus, expression of the two gonadotropin genes could be altered differentially in a subtle yet complex manner using relatively simple mechanisms. This is further supported by our suggestion that androgens may stimulate responsiveness of the gonadotrope to GnRH and, in turn, further enhance FSHβ transcription. In this manner, androgens could selectively stimulate FSHβ gene transcription by two mechanisms occurring at the level of the gonadotrope. Additionally, the dependence on activin of androgen regulation of the FSHβ further distinguishes the responses of the two gonadotropin genes. Another advantage of opposing mechanisms of androgen regulation of FSH is that in males, which have sufficient concentrations of androgen to stimulate FSH expression, the opposing mechanisms of androgen action in the context of the entire HPG axis would result in relatively stable expression of FSH at a time of dramatic and precipitous decline in LH. Androgen acts at the hypothalamus to reduce GnRH availability, which by itself results in reduction of both LH and FSH expression (due to loss of GnRH stimulation). Simultaneously at the level of the gonadotrope, androgen stimulates the FSHβ gene directly and may also increase the number of GnRH-Rs, which would result in each gonadotrope being more sensitive to the GnRH that is still available. When the results of each mechanism within the context of the entire HPG axis are added together, they balance each other out so that net FSHβ expression is unchanged or perhaps even modestly increased. The increased expression of GnRH-R would not, however, enhance GnRH induction of LH, because androgen interferes with the binding of downstream GnRH-R-induced signal transducers to the LHβ gene itself (9, 10). Because AR repression of LHβ expression at the level of the gonadotrope appears to be downstream of the GnRH-R, increasing GnRH-R does not alter the inhibitory effect of androgen on LH, resulting in inhibition of GnRH stimulation of LH by androgen. The physiological relevance of these combined opposing mechanisms is applicable both to nonseasonal animals like rats, which would require steady FSH levels to maintain spermatogenesis throughout the year, and to seasonal animals like sheep, in which seasonally increasing androgen levels could result in seasonal net increases in FSH and thereby dramatically increase spermatogenesis during the breeding season. Interestingly, FSH levels in male rats do not fluctuate substantially once maturity is reached, whereas both androgen and LH levels surge diurnally (48, 58, 59). In male sheep, androgen and FSH levels rise simultaneously at the beginning of the breeding season (60). The physiological significance of the findings of the current study, that androgens act directly through AR binding to ARE(s) on FSHβ in an activin-dependent manner, and may act indirectly through stimulation of GnRH-R expression at the level of the gonadotrope to stimulate FSHβ gene expression, is likely relevant to the underlying physiological mechanisms of steroid hormone feedback regulation of reproduction in a variety of both seasonal and nonseasonal breeding animal species.

**MATERIALS AND METHODS**

**Hormones**

T and DHT were purchased from Sigma Chemical Co. (St. Louis, MO). All steroid stock solutions were prepared by dissolving crystalline hormone in 100% ethanol at a 10 μM concentration and stored in lightproof borosilicate glass vials at 4°C. Before each experiment, fresh steroid treatment preparations were made by diluting the 10 μM stock in 100% ethanol to 10³-fold higher concentration than the final target concentration, and subsequently, 1 μl of diluted steroid was added to 1 ml total volume of media to achieve the final target concentration of steroid in 0.1% ethanol vehicle. The human recombinant activin A was obtained from Calbiochem (San Diego, CA). Recombinant mouse FS 288 was kindly provided by Shunichi Shimasaki. Both were resuspended in PBS with 0.1% BSA. GnRH was obtained from Sigma.

**Hormone Treatments**

For all transient transfection experiments, 8 h after transfection, the Ljβ2 cells were preincubated in steroid-free DMEM supplemented with 10% charcoal/dextran-treated FBS for 20 h, followed by the appropriate treatment protocol. In the steroid experiments, fresh DMEM with 10% charcoal/dextran-treated FBS containing T or DHT was added, and the cells were incubated for 6–48 h as indicated in the figure legends. For the activin/FS experiments in Figs. 6 and 7, the preincubation media was DMEM with 10% charcoal/dextran-treated FBS containing 100 nm DHT and/or 200 ng/ml activin A was added, and the cells were incubated for 24 h. In the activin/DHT experiments in Fig. 8, the transfection and treatment procedure was the same as that of Figs. 6 and 7, except that DMEM supplemented with 10% charcoal/dextran-treated FBS without FS was used for the 20-h preincubation before treatment for 24 h with 100 nm DHT and/or 10 ng/ml activin A. In the GnRH/DHT experiments, steroid-free DMEM with 10% charcoal/dextran-treated FBS containing 100 nm DHT and/or 200 ng/ml recombinant human activin-A was added, and the cells were incubated for 24 h. In the GnRH/DHT experiments in Fig. 9, the transfection and treatment procedure was the same as that of Figs. 6 and 7, except that DMEM supplemented with 10% charcoal/dextran-treated FBS without DHT was used for the 20-h preincubation before treatment for 24 h with 100 nm DHT and/or 10 ng/ml activin A. In the GnRH/DHT experiments, the media were changed to serum-free DMEM supplemented with 0.1% BSA and 5 ng/ml transferrin containing DHT, and the cells were incubated for 24 h. Six hours before harvest, additional serum-free DMEM (with BSA/transferrin supplement) containing DHT and/or GnRH was added, and the cells were incubated for the remaining 6 h until harvest. The cells were then harvested, and luciferase and β-galactosidase assays were performed.

**Construction of Plasmids for Transfection**

A 5.5-kb region of the oFSHβ gene (ofSHβ) encompassing 4741 bp of the promoter and 759 bp downstream from the +1 transcription start site was subcloned into the pGL3-Basic luciferase promoter plasmid (Promega Life Science, Madison, WI) as described previously (22). The cloning of the –982 truncation was described previously (25). Cloning of
EMSA

Full-length, human AR containing a Flag epitope tag was overexpressed in Sf9 cells via a baculovirus expression system (64). The Sf9 cells were inoculated with virus at a multiplicity of infection of 1.0 and allowed to grow for an additional 48 h at 27 C. They were treated for the last 24 h before harvest with 1 mM DHT (final concentration). Cells were harvested by centrifugation at 1500 rpm for 15 min, washed once in TG buffer (10 mM Tris-HCl, pH 8.0; and 10% glycerol) and frozen as a pellet at −80 C. The Sf9 cells were lysed in a homogenization buffer (20 mM Tris-HCl, pH 7.5; 350 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 0.5 µg/ml leupeptin, 10 µg/ml bacitracin, 2 µg/ml aprotinin, 1 µg/ml pepstatin). All procedures were done at 0–4 C. The cell lysate was centrifuged at 40,000 rpm for 30 min, and the supernatant was taken as a soluble whole-cell extract. The ability of AR to bind an oligonucleotide from the oFSHβ promoter was determined by EMSA. AR was incubated with 1 fmol of 32P-labeled oligonucleotide at 4 C 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(dl-dC), and 10% glycerol) in the absence or presence of the C-19x polyclonal antibody to AR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit IgG control, or 1000-fold excess of the −245, −245 mutant or ARE consensus oligonucleotides. The 245 oligo is 5’-CAAG-GTGAAAGGAGTGGGGTGCTTCTACTATAA-3’; the 245 mutant is 5’-CAAGGTAAGAAAGGAGGTTTGCTTCTACTATA-3’; and the ARE consensus is 5’-ACGGGTGGAAAGCGCGTGCTTCTTGGC-3’. The oligonucleotide was end-labeled with T4 DNA polymerase and [γ-32P]ATP. After 30 min, the DNA binding reactions were electrophoresed on a 5% polyacrylamide gel (40:1 acrylamide:bisacrylamide) containing 2.5% glycerol in a 0.25× TBE buffer (20 mM Tris-borate, pH 8; 20 mM boric acid; 0.5 mM EDTA).

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Probes

Address for T.J.S.: Center for Reproduction of Endangered Species, Zoological Society of San Diego, PO Box 120551, San Diego, California 92112-0551.

Cell Culture and Transient Transfection

LßT2 cells were maintained in 100-mm diameter dishes in DMEM (Celligro, Mediatech, Inc., Herndon, VA) supplemented with 10% FBS (Omega Scientific, Inc., Tarzana, CA) at 5°C with 5% CO2. Charcoal-treated FBS was also obtained from DMEM (Cellgro, Mediatech, Inc., Herndon, VA) supplemented with 5% CO2. Luminometer (PerkinElmer Corp., Norwalk, CT) as described previously (22).

 Luciferase and β-Galactosidase Assays

LßT2 cells were washed once with 1× PBS and then 40 µl of lysis solution (GALAC-to-light assay system, Tropix, Bedford, MA) was added to each well of the 24-well plates. Cells were then incubated at room temperature on a plate shaker for 5 min to detach and lyse cells. The contents of the wells were then transferred to microcentrifuge tubes on ice and centrifuged at 12,300 rpm for 8 min at 4 C. Lysed sample (20 µl) was assayed for luciferase activity, and 10 µl were aliquoted, incubated at 48 C for 1 h to heat inactivate endogenous β-galactosidase, and then assayed for β-galactosidase activity from the reporter gene. Luciferase and β-galactosidase activity were measured using an EGG Berthold Monoplate Luminometer (PerkinElmer Corp., Norwalk, CT) as described previously (22).
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