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Stimulation of FSHβ Transcription by Blockade of Endogenous Pituitary Follistatin Production

Efficacy of Adenoviral-Delivered Antisense RNA in the Rat

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This study investigated FSHβ transcriptional responses to the suppression of endogenous follistatin (FST) production using FST antisense RNA (FST-AS) expressing adenovirus constructs in female rat pituitary cells in vitro. Adenoviral delivery systems were characterized and optimized using an adenovirus–green fluorescent protein construct, and maximal infection (85–90% of cells) was seen 48 h post adenovirus treatment. A 424 bp fragment, which included the translational start site and exons 1–3 of the rat FST gene, was subcloned in the reverse orientation into an adenovirus vector. Construct efficacy was tested using cultured rat pituitary cells infected with the adenovirus–FST-AS construct. Infection with adenovirus–FST-AS increased FST-AS mRNA expression in a dose-dependent manner, reduced FST protein expression to undetectable levels, and stimulated increases in FSHβ primary transcript and FSH secretion. Treatment with testosterone alone stimulated FSHβ primary transcript and FSH release, and responses were doubled in the presence of adenovirus–FST-AS. These results demonstrate the effectiveness of adenovirus FST-AS in suppressing pituitary FST protein expression and enhancing FSH biological responses at the transcriptional level. Thus, the FST-deficient rat gonadotrope cell is a model that allows for the investigation of factors regulating FSHβ expression, which might otherwise involve the autocrine/paracrine actions of FST.

Key Words: Follistatin antisense; adenovirus-delivered RNA; FSHβ.

Introduction

Synthesis and secretion of the pituitary gonadotropin FSH is primarily regulated by GnRH from the hypothalamus and by steroids and peptides from the gonads and/or pituitary. Within the pituitary, gonadal steroids (estradiol, progesterone, and testosterone) regulate the FSHβ gene directly (via steroid response elements) *(1,2)*. They also have indirect effects via activation of specific signal transduction pathways *(3,4)*, and by actions on expression of specific peptides (i.e., activin B and follistatin) *(5–8)* that are released from gonadotrope and other pituitary cell types. Gonadal steroids also regulate FSH secretion/gene expression through stimulatory and inhibitory actions on GnRH pulsatile secretory activity *(9)*. Together these factors coordinate the FSH secretory patterns necessary to induce/maintain cyclical follicular development in females of mammalian species.

Follistatin (FST) is produced by pituitary gonadotrope and folliculostellate cells, and regulates FSH secretion/expression in an autocrine/paracrine manner by binding activin with high affinity *(10,11)*. Follistatin-bound activin is prevented from binding to activin plasma membrane receptors, and is thus unable to activate the activin receptor–mediated intracellular signal transduction pathway within the gonadotrope *(12)*. Follistatin is found in two major isoforms, which are generated by alternate splicing of a single gene product. The full length 315 amino acid isoform is generated from translation of all six FST exons, whereas the shorter 288 amino acid FST isoform is derived from exons 1–5. Both isoforms have similar binding affinities for activin *(13)*.

Pituitary FST expression varies during the rat estrous cycle and following ovariectomy *(14,15)*, and is regulated by pulsatile GnRH (i.e., selectively stimulated by rapid frequency pulses), gonadal steroids, and gonadal peptides *(16, 17)*. Studies have shown that FST suppresses FSH, at both the secretory and transcriptional level *(16,18)*. However, the physiological role of FST in the regulation of FSHβ subunit transcription/secretion remains to be fully characterized, because a FST-deficient cell model has yet to be developed. Follistatin knockout mice have several embryonic defects and die soon after birth *(19)*. Also, attempts at using anti-

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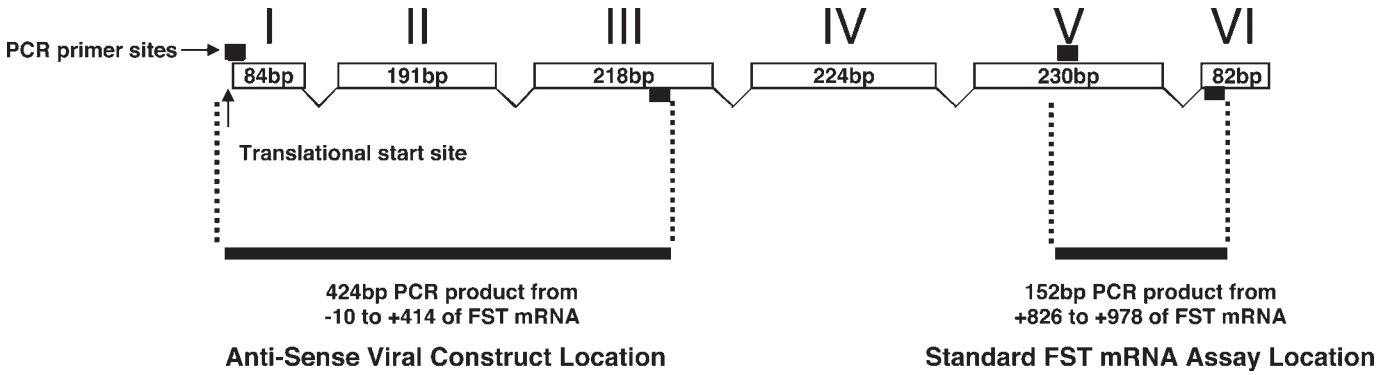

Fig. 1. Rat follistatin (FST) gene map of FST antisense [FST-AS; directed to the translational start site (TSS) and exons 1–3] and our FST mRNA PCR assay (exons 5–6). Solid blocks indicate positions of primers used.

FST serum to neutralize endogenous FST have had limited success *(20)*. The use of antisense (AS) oligodeoxynucleotides (ODNs) or RNAs to block the synthesis/actions of specific gene products has previously been shown to be an effective method to investigate the role of those products in regulating biological systems *(21,22)*. Thus, the present study was conducted to develop a FST-deficient rat pituitary cell model using adenovirus (Ad) vector FST-AS constructs, in order to investigate the role of endogenous FST in the regulation of FSHβ transcription.

Results

Preliminary studies were conducted using follistatin AS-ODNs delivered by lipofection. The administration of AS-ODN directed to the translational start site (TSS; see *Materials and Methods* for details) increased FSHβ primary transcript (PT) levels (twofold; $p < 0.05$) and FSH secretion (twofold; $p < 0.05$) after 33 h of treatment, whereas the intron 3-splice site AS-ODN was ineffective (data not shown). These studies provided useful initial information regarding the efficacy of administering an antisense construct directed to the TSS, and support the notion that loss of FST played a role in the increase in FSH synthesis and secretion. However, in repeat experiments we observed a marked variation in the magnitude of cellular responses. As a result, this strategy was abandoned in favor of an adenovirus (Ad) vector delivery system, which would provide higher levels of RNA expression in a more reliable manner.

To assess infectivity, dose and timecourse studies were conducted using an adenovirus vector containing a cytomegalovirus–green fluorescent protein construct (Ad-CMV-GFP) given to cultured rat pituitary cells. Results showed that GFP expression was evenly distributed over all cells and maximal expression (85–90% of cells at both 24 and 48 h post-infection) were seen using a MOI (multiplicity of infection or plaque forming units) of 10 (data not shown). To confirm that adenovirus vectors were the optimal delivery system for rat pituitary cells, we compared GFP expression in Ad-CMV-GFP–treated cells vs cells exposed to a lentivirus-CMV-GFP construct. Approximately 90% of Ad-CMV-GFP–treated cells expressed GFP at 48 h, but, in contrast, only 5–10% of lentiviral-CMV-GFP–treated cells expressed GFP at 48 h (data not shown). Owing to these findings, adenoviral constructs served as the primary model for further gene transfer studies.

Based on preliminary ODN–lipofection studies, the AS sequence selected included the TSS, and the location of the 424 bp AS construct inserted into the viral shuttle vector (exons 1–3) is shown in Fig. 1. The gene map also shows the location of the primers for our FST mRNA quantitative PCR assay (exons 5–6, 152 bp product) *(16)*. Figure 2 (left panel), RT-PCR using primers to exons 1–3 (which measures both antisense and native-sense mRNAs), revealed that treatment with Ad-CMV-FST-AS (5 MOI) generated fivefold-higher PCR product vs Ad-CMV-(no insert) controls ($p < 0.05$). In contrast, FST mRNA (exons 5–6) expression was similar in both groups. Thus, any effects of the AS would likely be attributable to perturbation of translation, not mRNA stability. Infection with Ad-CMV-FST-AS increased FST antisense expression in a dose-dependent manner (Fig. 2, right panel), with maximal (12- to 13-fold) increases seen with the 10 and 20 MOI doses.

Follistatin protein expression was assessed in Ad-CMV-FST-AS (10 MOI)–treated pituitary cells, 48 h post-infection. Figure 3 shows that in vitro expression of cellular FST protein after Ad-CMV-(no insert) treatment was somewhat reduced compared to levels seen in vivo (Con group). In contrast, FST protein was undetectable in Ad-CMV-FST-AS–treated cells. Also of note, the fact that protein expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was not altered suggest that the cellular response to AS-FST was FST-specific.

Adenoviral dose response studies revealed that both FSHβ PT and FSH secretion were consistently increased in Ad-CMV-FST-AS–treated cells, and the 10 MOI dose was optimal (data not shown). FST-AS stimulated a twofold increase in FSHβ PT and a 60% rise in FSH secretion vs Ad CMV-(no insert) controls ($p < 0.05$; Fig. 4, Veh-treated groups). Also of note, LHβ PT concentrations were not sig-

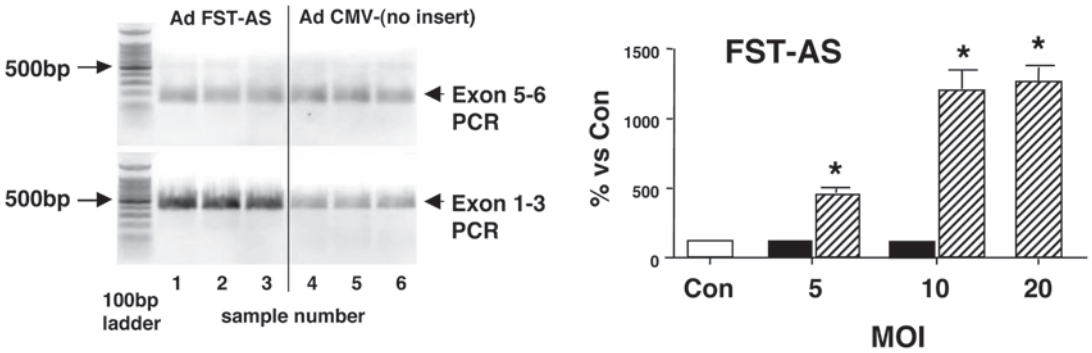

Fig. 2. Expression of FST-AS (exons 1–3, 424 bp; left panel/lower gel autoradiograph shown) and native FST mRNA (exons 5–6, 152 bp; left panel/upper gel autoradiograph shown) in Ad-CMV-FST-AS (adenovirus vector containing a cytomegalovirus-FST-AS construct) and Ad-CMV-(no insert)–treated cells at 48 h post-infection (see gene map in Fig. 1). Pituitary cells were infected with Ad-CMV-FST-AS using MOIs of 5–20. Controls either received no virus (Con) or Ad-CMV-(no insert) (n = 8–11 per group). Cell lysates were collected and FST-AS and native FST determined by PCR. Left panel shows a representative autoradiograph (5 MOI); right panel shows FST-AS concentrations in Con (open bar), Ad CMV-(no insert) (5 and 10 MOI; black bars) and FST-AS (5-20 MOI; hatched bars) groups. *$p < 0.05$ vs CMV-(no insert) controls (5 and 10 MOI).

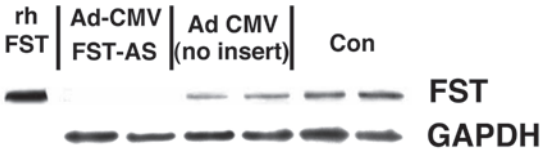

Fig. 3. FST protein expression in response to Ad FST-AS. Pituitary cells were infected with Ad FST-AS (10 MOI) or Ad CMV-no insert (10 MOI) and cellular FST protein determined 48 h later by immunoblot. Non-treated lysates of pituitaries collected in vivo were included as controls (Con). Antibody binding to recombinant human (rh) FST is also shown. Blots were stripped and re-blotted for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control.

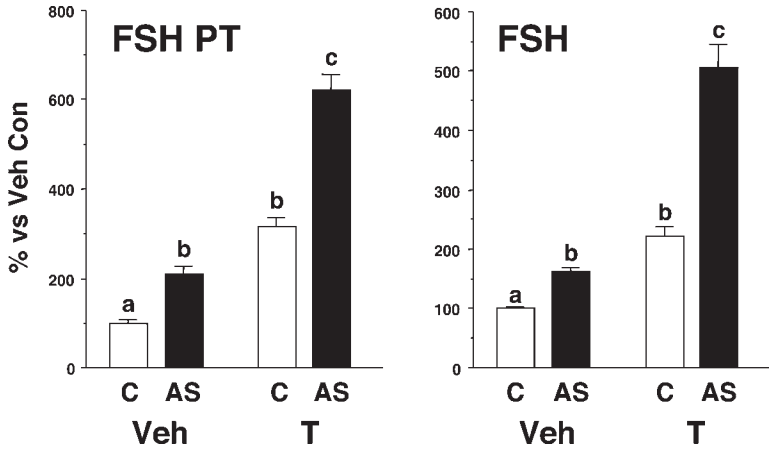

Fig. 4. FSHβ primary transcript (PT) and FSH secretory responses to treatment with Ad-CMV-FST-AS. Studies were performed in the presence of vehicle (Veh) or testosterone (T) treatment. Cultured rat pituitary cells were infected with Ad-CMV-FST-AS (AS; 10 MOI) or Ad-CMV-(no insert) (10 MOI) as control (C) and 48 h later cell lysates and culture media collected (n = 5–6 per group representing pooled data from three separate experiments). Bars with different letters (a–c) are significantly ($p < 0.05$) different.

nificantly different between Ad-CMV-FST-AS–treated and Ad CMV-(no insert)–treated groups (data not shown), supporting the specificity of the effect on FSHβ transcription.

Further studies examined the effectiveness of testosterone (T) to stimulate FSHβ transcription in FST-deficient pituitary cells (Fig. 4). Testosterone alone stimulated a threefold increase in FSHβ PT [vs vehicle-treated Ad-CMV-(no insert) controls; $p < 0.05$], which increased a further twofold after exposure to Ad-CMV-FST-AS ($p < 0.05$). Thus, the magnitude of FSHβ PT responses to FST-AS (twofold) was similar in both vehicle and T-treated groups, and the combination of FST-AS plus T was additive. Testosterone also stimulated a twofold increase in FSH secretion into the media [$p < 0.05$ vs vehicle-treated Ad-CMV-(no insert) controls], with a further (twofold) increase seen after FST-AS treatment ($p < 0.05$). LH release was not affected by either FST-AS or T treatment [media LH in Veh plus Ad-CMV-(no insert) controls = 2.9 ± 0.5 ng/mL; Veh plus Ad-

CMV-FST-AS = 3.2 ± 0.2 ng/mL; T plus Ad-CMV-(no insert) controls = 2.7 ± 0.4 ng/mL; T plus Ad-CMV-FST-AS = 3.0 ± 0.2 ng/mL].

Discussion

The present report describes the characterization of a FST-deficient rat pituitary model using an adenoviral construct to deliver FST-AS in vitro. The data clearly demonstrate that 48 h post-infection the cells express FST-AS and endogenous FST protein expression is effectively suppressed, resulting in increased FSH transcription and secretion. As FST gene knockout is lethal in mice *(19)* and previous efforts to immunoneutralize FST using antibodies have had limited effectiveness *(20)*, investigation of endogenous FST on FSH transcriptional regulation has been complicated. A previous report by Weiss et al. *(23)* characterized the use of a perifusion system to remove endogenous FST for rat pituitary in vitro studies. However, the current model will allow study of the roles and mechanisms of action of GnRH and activin on the gonadotrope in the future, without the complication of interfering FST action, and will not require the implementation of a labor-intensive perifusion in vitro model system. Preliminary studies used AS-ODNs delivered by lipofection, and provided insights into a potentially effective AS construct region (i.e., TSS), but were found to lack consistency in FSH biological responses. This likely relates to reproducible sequestering of sufficient concentrations of AS-ODNs per cell from experiment to experiment. For that reason, we changed our focus to characterize an Ad delivery system.

The use of AS ODNs, RNAs, or dominant/negatives (D/Ns) to block the synthesis/actions of specific gene products are effective methods to investigate the role of those products in regulating biological systems *(21,22)*. Antisense ODNs are generally targeted to the transcriptional start site, to translational start site, or to downstream regions, such as exon/intron splice sites. Data obtained in vivo reveal that neurons readily take up ODNs, and their use is an effective method to examine central nervous system (CNS) regulatory pathways *(24)*. Lipofection is an efficient delivery method for short-term studies in some cell systems, whereas for longer durations (days to weeks), the intracellular production of ODNs, RNAs (AS or expression vectors), or D/Ns usually requires transfection with Ad vectors *(25,26)*. Indeed, the results of a recent report showed that rats given ip injections of an Ad construct, containing a constituitively active FST-288 coding sequence, expressed high levels of FST for up to 12 d in vivo, and attenuated FSH secretion in infected rat pituitary cells in vitro *(27)*.

Pituitary FST gene expression changes dynamically during the rat estrous cycle *(14)* and is regulated by GnRH at the transcriptional level *(28)*, with rapid frequency GnRH pulses optimally stimulating FST mRNA levels *(17)*. Follistatin transcription and mRNA levels increase in response to ovariectomy and are regulated by gonadal peptides, with both inhibin and activin suppressing FST gene expression *(5,7,15,28)*. Thus, changes in pituitary FST production limit the ability to interpret results of treatment-induced changes in FSHβ transcription and FSH secretion in most current in vivo and in vitro experimental paradigms.

In both the female and male rat pituitary, physiological concentrations of androgens stimulate FSHβ gene expression at the transcriptional and post-transcriptional levels *(2,16,29)*, but the mechanisms of action are not fully understood. We have recently shown that T, but not estradiol, selectively stimulates pituitary extracellular signal regulated kinase (ERK) activation and that ERK plays a role in androgen stimulation of FSHβ expression in the rat *(4)*. Other investigators have shown that the ovine FSHβ promoter contains two androgen responsive elements (conserved in the rat promoter), which mediate responses to androgen, but are also dependent on the presence of an adjacent activin (Smad) responsive element *(2)*.

The role that FST plays in the regulation of basal FSHβ expression and in the stimulatory actions of androgens on FSHβ remains to be fully characterized. Our data indicate that pituitary FST inhibits FSHβ transcription and secretion in the basal state. Androgens suppress FST mRNA and protein expression *(8,30)* in parallel to the androgen-induced increase in FSHβ PT *(16)*. Thus, androgen stimulation of FSHβ expression could involve a reduction in intracellular FST, leading to increases in bioavailable activin *(5)*. However, we demonstrated that T stimulates FSHβ PT in vitro in the presence of excess exogenous FST *(16)*, suggesting that T stimulation of FSHβ transcription occurs independently of modulation by FST. The present results support those findings. As shown in Fig. 4, the magnitude of responses to FST-AS [vs Ad-CMV-(no insert) controls] was similar in both vehicle and T-treated groups, suggesting that T stimulates FSHβ transcription independently of actions on pituitary FST expression.

In conclusion, the present study demonstrates the efficacy of Ad FST-AS in specifically suppressing pituitary FST protein expression and enhancing FSH transcriptional and secretory responses. This model provides a useful FST-deficient cell system for delineation of the intracellular mechanisms involved in GnRH and activin action on FSHβ transcriptional activation.

Materials and Methods

In Vitro Experimental Models

Female rats (225–250 g; 55–70 d of age) were euthanized under anesthesia in accordance with the National Research Council *Guide for the Care and Use of Laboratory Animals* and approved by the University of Virginia Animal Care and Use Committee. To control for alterations in pituitary physiology across the estrous cycle, pituitaries from 36 random

cyclic female rats were pooled and dissociated for each experiment in media containing 0.35% collagenase, 0.1% hylauronidase, and 0.01% DNase. Following dissociation, the cell suspension was aliquoted into culture wells [three pituitary equivalents (3–4 million cells) per well] containing 22 mm plastic coverslips coated with polylysine (Sigma Chemical; St Louis, MO). The cells were cultured for 48 h before initiating ODN or viral vector treatment. The in vitro procedure and culture medium constituents have previously been described *(4,31,32)*. Each experiment was repeated a minimum of three times to confirm results.

Testing Ad FST-AS Construct Efficacy

Rat pituitary cells were dissociated and pre-cultured for 48 h on polylysine-coated coverslips, then infected with the Ad-CMV-FST-AS construct (5, 10, 20 MOI) for 24 h. Controls received Ad-CMV-(no insert) (5, 10 MOI), no Ad, or Ad-CMV-GFP (to assess infectivity). Viral vector–containing media were removed from the cells after 24 h and replaced with fresh media (without viral vector). Culture media and cells were collected 48 h post-infection, and media FSH and cell FSHβ PT measured by RIA and RT-PCR, respectively.

Testosterone (T) Studies

For the T treatment study (Fig. 4), 48 h after dissociation, cultured pituitary cells were transferred to wells containing charcoal-stripped fetal bovine serum (10%) and horse serum (5%) plus Ad-CMV-FST-AS or Ad-CMV-(no insert) (10 MOI) in the culture media for 24 h. The next day, coverslips were transferred to wells containing media without virus plus T (500 pg/mL), or vehicle (to controls) and incubated for an additional 24 h. At the completion of each experiment, culture media and cells were collected.

Lipofection Delivery Studies

Preliminary studies examined the utility of blocking FST production in vitro, by delivering AS-ODNs via lipofection. Because AS-ODNs directed to different parts of the gene can have markedly different efficacy, we used two FST AS-ODNs strategies. The first, two 15-mer AS-ODNs back-to-back across the translational start site (TSS) designed to reduce protein production (see FST gene map, Fig. 1). The second was directed to the intron-3 splice site of the FST gene to reduce mRNA stability. The ODNs were modified (i.e., phosphorothioate-conjugated bases) to inhibit degradation in cell culture. Control ODNs were scrambled versions with similar ATCG content. To test AS-ODN effectiveness, female rat pituitaries were dissociated, plated on polylysine-coated coverslips, and cultured. Forty-eight hours later, cells were transferred to low-sera transfection media [1 μg of each AS-ODN in 50 μL Opti-MEM containing 3 μL lipofectAMINE 2000 reagent (Invitrogen, Carlsburg, CA)], and incubated for 20 min. Six hours after transfection, 1 mL of media was removed for FSH assay, and cells were transferred to fresh media. Media was also collected at 24 and 33 h for FSH assay. Also at 33 h, cells were harvested, RNA extracted, and FSH primary transcript (PT) measured by RT-PCR (see below).

Delivery of Viral Vector Constructs

Green Fluorescent Protein Viral Vector Constructs

To assess infectivity, we used an Ad-CMV green fluorescent protein construct (Ad-CMV-GFP, provided by the University of Iowa Gene Transfer Vector Core Services Facility). Female rat pituitary cells were plated onto polylysine-coated coverslips and cultured for 48 h. The Ad-CMV-GFP construct was added at 5, 10, and 25 MOI per rat pituitary cell and GFP expression examined under UV light 24 and 48 h later. To confirm that Ad vectors are the optimal delivery system for rat pituitary cells, we compared GFP expression in Ad-CMV-GFP–treated cells vs cells given a lentivirus–CMV-GFP construct (lentivirus–CMV-GFP vector kindly provided by Dr. Tom Lanigan, University of Michigan Vector Core Laboratory).

Characterization of the Ad-CMV-FST-AS Construct

We prepared an Ad-CMV construct to express FST-AS. A 424 bp fragment that included the TSS of the rat FST gene was amplified from rat pituitary RNA by RT-PCR (see Fig. 1) and cloned into pGEMT Easy. The fragment was excised (ECO RI), subcloned into shuttle vector pAd 5' CMVK-NpolyA and screened to determine antisense orientation (by sequencing using the primer to the CMV promoter) and 40 μg sent to the University of Iowa Gene Transfer Vector Core Services Facility for amplification and purification. Preparations were titered and checked for wild-type adenovirus using biological and PCR methods.

Assays

FST Immunoblot Assay

Studies assessed FST protein expression in Ad FST-AS (10 MOI)–treated pituitary cells. Cultured cells were lysed 48 h after administration of Ad FST-AS or Ad CMV-empty (to controls). Cell lysate protein samples from cultured cells (100 μg), in vivo rat pituitaries (100 μg) and recombinant human (rh) FS (500 μg) were resolved by electrophoresis (4–20% SDS-PAGE). Proteins were transferred to nitrocellulose filters and immunoblotted using a primary monoclonal antibody that binds rat FST (FS274-287; kindly provided by Drs. W. Crowley and P. Sluss, Harvard School of Medicine), as previously described *(31,32)*. The secondary antibody was horseradish peroxidase (HRP)–conjugated goat anti-mouse (Santa Cruz Biotech; Santa Cruz, CA). Bands were detected using the Super Signal Pico West chemiluminescent system (Pierce, Rockville, IL), followed by autoradiography. To assess loading, blots were stripped (Restore Buffer, Pierce) and re-blotted with a primary antibody for GAPDH (rabbit anti-GAPDH, Santa Cruz Biotech) and goat anti-rabbit-HRP as secondary antibody (Upstate Cell Signaling Solutions, Lake Placid, NY).

Quantitative RT-PCR

FSHβ primary transcript (PT) concentrations were determined by quantitative RT-PCR assay, as previously described *(16)*. In brief, regions of intron/exon were amplified using specific oligonucleotide primers and a size-altered competitive template RNA (CT). Primary transcript concentrations were expressed as femtomoles/100 μg pituitary RNA. The FSHβ PT intraassay coefficient of variation (%CVs) was 5%. In order to reduce the effect of interassay variation, all samples from each experiment were run within a single PCR assay.

Radioimmunoassays (RIAs)

FSH and LH secretory responses were measured in culture medium by RIA, using reagents provided by the National Hormone and Pituitary Program. The assays were performed by the University of Virginia Center for Research in Reproduction, Ligand Assay and Analysis Core Facility. The RIA standards were NIDDK RP-2 (for FSH) and RP-3 (for LH). The assay sensitivities were 1 ng/tube for FSH and 0.07 ng/tube for LH. The intraassay %CVs were 6.3% and 8.3% for FSH and LH assay, respectively. All samples from each experiment were run in a single RIA assay.

Statistical Analysis

The data were analyzed by one-way analysis of variance, with differences ($p < 0.05$) between treatment groups determined by Duncan's multiple range test.

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