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LIM Homeodomain Transcription Factor Isl-1 Enhances Follicle Stimulating Hormone-β and Luteinizing Hormone-β Gene Expression and Mediates the Activation of Leptin on Gonadotropin Synthesis

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The Lin-11, Isl-1, and Mec-3 (LIM) homeodomain transcription factor Isl-1 has been reported to be involved in pituitary development in the early stages of mouse embryogenesis. Our recent studies have shown that Isl-1 is mainly located in the pituitary gonadotropes throughout pituitary development and persists to adulthood. We still do not know the physiological functions of Isl-1 expression and its related mechanisms in the pituitary gland. The aim of the present study was to examine the hypothesis that Isl-1 is involved in regulating pituitary gonadotropin hormone (FSH/LH) production by activating *FSHβ* and *LHβ* gene expressions. We have shown that Isl-1 activates FSHβ and LHβ subunit promoters and endogenous gene transcription in LβT2 cells. In addition, Isl-1 overexpression significantly increased FSH synthesis and secretion but not LH. The actions of Isl-1 were not observed when the homeodomain or LIM1 domains are mutated. This demonstrates that Isl-1 induction of FSHβ and LHβ is by both direct and indirect binding of Isl-1 to DNA sequences. Furthermore, Isl-1 expressional level was up-regulated in LβT2 cells after exposure to GnRH, activin, and leptin. However, RNA interference-induced knockdown of Isl-1 significantly reduced the effect of leptin but did not obviously influence the stimulating effects of GnRH and activin on LH and FSH production. In conclusion, the results demonstrate that the LIM-homeodomain transcription factor Isl-1 functions to increase *FSHβ/LHβ* gene transcription, and mediates the effects of leptin on gonadotropin synthesis. **(*Endocrinology* 151: 4787–4800, 2010)**

The pituitary gonadotropins, FSH and LH, are heterodimeric glycoproteins composed of a common α-subunit (α-glycoprotein hormone α-subunit), and specific β-subunits (FSHβ or LHβ). In males and females, both these hormones are essential for gonadal function including folliculogenesis, spermatogenesis, and gonadal steroids and protein production in both sexes (1–3). Although the structures of these hormones are known, the mechanisms that regulate the synthesis and differential secretion of FSH and LH are not fully understood (4).

It is generally thought that the secretion of GnRH from the hypothalamus is key in regulating the gene expression of the gonadotropin β-subunits (5–7). GnRH increases hormonal production by binding to its transmembrane receptor. This binding increases intracellular calcium, which stimulates protein kinase C to activate nuclear proteins, including MAPK family members (8, 9). In addition to GnRH, the synthesis and secretion of gonadotropins are also regulated by the stimulating and inhibiting actions of estrogen and androgen (10) and peptides such as activin,

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Abbreviations: aa, Amino acids; ChIP, chromatin immunoprecipitation; D, postnatal day; E, embryonic day; Egr-1, early growth response protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HD, homeodomain; HEK, human embryonic kidney; HOMO, homeodomain; HPG, hypothalamus-pituitary-gonadal; ICC, immunocytochemistry; IHC, immunohistochemistry; JAK, Janus tyrosine kinase; Lhx3, LIM class of homeodomain protein-3; LIM, Lin-11, Isl-1, and Mec-3; OB-Ra, short form of the leptin receptor; OB-Rb, long form of the leptin receptor; p, phosphorylated; Pitx, paired-like homeodomain transcription factor; SF-1, steroidogenic factor 1; STAT, signal transducer and activator of transcription.

inhibin (11, 12), leptin, and bone morphogenetic proteins (13, 14).

Leptin is a protein hormone synthesized and secreted mainly by adipocytes (14). In addition to its well-known regulating effects on appetite and metabolism, leptin is also vital in reproduction. Mutations in either the leptin (14) or the receptor (15) genes results in infertility. Leptin administration reverses reproductive abnormalities in leptin-deficient (*ob/ob*) mice (16) and accelerates sexual maturation and puberty in normal female (17) and transgenic mice (18). Although it is generally thought that the hypothalamus is a key site of action, the mechanism of leptin action in the reproductive system remains elusive. Leptin acts within the hypothalamus to stimulate GnRH release, which then triggers the subsequent release of FSH and LH, and stimulates the development and function of gonads (19). However, it was shown that leptin's action on the hypothalamus-pituitary-gonadal (HPG) axis appears at different levels of this axis (20, 21). The pituitary is a direct target of leptin action because leptin receptors are expressed in the pituitaries of many animals (22–26), and the cell types expressing leptin receptors include gonadotropes. In addition, leptin induces a bell-shaped dose-response curve of LH and FSH release from incubated anterior pituitaries in rats, pigs, and cattle (27–29), with almost the same effect on release of LH as GnRH (28, 29). These results suggest that leptin plays an additional role in regulating fertility by directly modulating pituitary LH and FSH synthesis and secretion. However, the pituitary intracellular pathways responsible for this direct action are still unknown. Studies have shown that leptin functions through Janus tyrosine kinase (JAK)-signal transducer and activator of transcription (STAT) signals in brain and other tissues (30–32).

The extrapituitary regulators, such as GnRH and activin, modulate LHβ and FSHβ subunit gene expression through a number of nuclear transcription factors. The factors involved in *LHβ* transcription include steroidogenic factor 1 (SF-1), early growth response protein 1 (Egr-1), paired-like homeodomain transcription factor (Pitx)-1, p8, Sp1, and nuclear factor Y (33, 34). *FSHβ* gene expression is mediated by SF-1, nuclear factor Y, activator protein-1, LIM class of homeodomain protein-3 (Lhx3), and Pitx class factors (34). The interplay among the different factors and genes are not fully understood.

The Lin-11, Isl-1, and Mec-3 (LIM)-homeodomain (HD) transcription factor Isl-1 is comprised of two tandem LIM domains and a HD. The homeodomain with a helix-turn-helix structure binds to regulatory DNA sequences of its target genes. The LIM domains are mainly involved in protein-protein interactions that regulate the activity of LIM-HD (35). Isl-1 was originally shown to function as an insulin gene enhancer binding protein (36). It plays critical roles in cell determination, proliferation and differentiation in the nervous system (37, 38), heart (39), and pituitary gland (40). In mice, it is specifically expressed in the pituitary pouch rudiment at early development stages (41). *Isl-1*$^{-/-}$ mice fetuses die at approximately embryonic day (E) 10 and the analysis at E9.5 demonstrates that the oral pouch is small and primitive with a conspicuously thinner wall (42). The exact function of Isl-1 on the fetal pituitary development is still unclear because *Isl-1* gene knockout causes developmental anomalies in the whole embryo (38), and pituitary cell differentiation happens mainly after E12.5 in the mouse (41). Our previous studies have shown that Isl-1 is mainly localized in gonadotropes, and Isl-1 expression parallels gonadotropic differentiation throughout the development in the sheep fetus and chicken embryo (43, 44). Additional studies should determine whether Isl-1 functions to promote pituitary gonadotrope differentiation and/or hormone production by activating *FSHβ* and *LHβ* genes.

The aims of the present study were to determine the effect of Isl-1 on *LHβ* and *FSHβ* gene expression and hormone production in gonadotropes and to identify whether Isl-1 is involved in mediating the signal pathways of GnRH, activin, and leptin in regulating LH and FSH production. The results show that Isl-1 enhances *FSHβ*/*LHβ* gene transcription and FSH production. It is out of our expectation that Isl-1 is involved in mediating the signal pathway of leptin but probably not GnRH and activin on gonadotropin production in LβT2 cells.

Materials and Methods

Animals and tissue collections

Kunming male mice were raised in a controlled temperature of 25 ± 1 C on a 12-h light, 12-h dark cycle. The animal experiments were approved by the Chinese Association for Laboratory Animal Sciences. For immunohistochemistry, pituitary glands of three postnatal day (D) 40 mice were separated and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 4 h at room temperature, embedded in paraffin, and cut into 5-μm sections. In dietary manipulation experiments, 30 male mice at 21 D were randomly divided into three groups. Mice in the high-fat diet group were fed a diet containing 20% fat for 2 wk. The control and fasted groups were fed a regular grain diet, but the fasted group was starved for 48 h but with *ad libitum* drinking water. For determining serum leptin concentrations, the mice with dietary manipulation and normal mice aged 21 D (17 ± 2 g), 40 D (24 ± 2 g), and 60 D (37 ± 3 g) were anesthetized, and blood samples were collected from the orbital sinus (n ≥ 6 for each group). Serum was harvested and frozen at −20 C until RIA was conducted. After blood was taken, the mice were killed by cervical dislocation. Pituitaries were separated and divided into three factions per group for RT-PCR.

Immunohistochemistry (IHC) and immunocytochemistry (ICC)

Pituitary sections were dewaxed, rehydrated, and treated with 10% normal goat serum in PBS and incubated with rabbit antirat LHβ (1:50; National Hormone and Peptide Program, Torrance, CA) and rabbit antihuman FSHβ (1:50; Zymed, San Francisco, CA) at 4 C for 12 h. The section was then incubated with fluorescein isothiocyanate-labeled goat antirabbit IgG (1:50; Scottish Antibody Product Unit, Carluke, UK) at room temperature for 3 h. After washing three times with PBS, the slides were observed under a fluorescence microscope (Leica Microsystems, Cambridge, UK) and photographed. Immunohistochemistry for Isl-1 detection was performed as previously described (43). The percentage of LHβ or FSHβ costaining for Isl-1 was counted.

For ICC, LβT2 cells were fixed in 4% paraformaldehyde for 10 min and permeabilized with 95% methanol at −20 C for 10 min and then blocked in 10% normal goat serum for 1 h. Subsequently, Isl-1 in the LβT2 cells was stained using the same protocol as IHC.

Plasmid construction

The −226 to +7 bp (−226LHβ) and −712 to +7 bp (−712LHβ) regions of the *LHβ* gene and −1836 to +56 bp (−1836FSHβ) region of *FSHβ* gene were amplified from mouse genomic DNA by the PCR method using specific primers: LH-β, −226 bp, 5′-ACCTTGTTTCCCGTGCTT-3′ and 5′-TCTTGATACCTTCCCTACCTT-3′; LH-β, −712 bp, 5′-GACCGAATTTGCCCAGTA-3′ and 5′-TCTTGATACCTTCCCTACCTT-3′; FSH-β, −1836 bp, 5′-ATATAAACATCCCATCTCCA-3′ and 5′-ATCAAGTGCTGCTACTCACC-3′. The −620 FSHβ was obtained from the −1836 FSHβ by *Mfl*I restriction enzyme digestion. These fragments were respectively inserted into the pGL3.0-basic luciferase reporter vector (Promega, Madison, WI). The expression vector for murine Isl-1 pXJ40-myc-Isl-1 vector was kindly provided by Dr. Xinmin Cao (Institute of Molecular and Cell Biology, Singapore). The truncated mutants of Isl-1 were generated by restriction enzyme digestion and PCR methods. The LIM1 deletion (ΔLim1) lacks amino acids (aa) 18–70, whereas the LIM2 deletion (ΔLim2) lacks aa 95–109 in the Isl-1 peptide, and ΔLim1 and -2 combines both deletions. The homeodomain mutated deletion (ΔHomo) lacks aa 193–213 in the Isl-1 homeodomain.

Cell culture and transient transfections

The mouse pituitary LβT2 and human embryonic kidney (HEK)-293T cells were cultured in 10% fetal bovine serum-DMEM (Life Technologies, Inc.-Invitrogen, Carlsbad, CA) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin and incubated at 37 C with 5% CO_2. Transient transfections or cotransfections were performed using Fugene HD or 6 transfection reagents (Roche Applied Science, Basel, Switzerland) according to the manufacturer's recommendations. All transfection experiments were performed at least three times.

Luciferase assays

LβT2 cells plated in 24-well plates were transfected with the Isl-1 expression vector or control vector, LHβ or FSHβ luciferase reporter vectors, and pTK-Ranilla vector (Promega) at a ratio of 10:4:1. Cells were harvested 24 h after transfection. Luciferase activity was measured using a dual-luc assay kit (Vigorous, Beijing, China) on a Modulus microplate luminometer (Turner Biosystems, Sunnyvale, CA). The values shown by the fluc to rluc ratio were normalized to an empty luciferase reporter control.

HEK293T cells were cotransfected with pSilencer3.0-H1-Isl1ix (x indicates the targeted site) and pSilencer3.0-H1-shRNA control vector, respectively, with psiCHECK-Isl-1 (Promega) to estimate their interferential effects. Luciferase activity was measured 48 h later using a dual-luciferase reporter assay. Interference efficiency of each pSilencer3.0-H1-Isl1ix was shown by its rluc to fluc ratio to that of pSilencer3.0-H1-shRNA.

Radioimmunoassays

Transfected LβT2 cells (6×10^5 per 60 mm flask) were cultured in 10% fetal calf serum-DMEM for 24 h. Cell media were then replaced by DMEM (3 ml/flask). Cells were further incubated for 48 h before harvesting. In Isl-1 expression-interference experiments, cells were subjected to 10 ng/ml rat recombined leptin (Abcam, Cambridge, UK), 10 nM GnRH analog (Sigma-Aldrich, St. Louis, MO), 25 ng/ml activin A (Peprotech, London, UK) or an equal volume DMEM for another 24 h. The media were then collected and the cells were lysed in 400 μl lysis buffer for LH and FSH determinations. Cellular protein concentrations were determined by bicinchoninic assay. Experiments were performed four to six times. The hormone concentrations were normalized to the protein concentration.

LH, FSH, and leptin were analyzed using RIA reagents provided by the Beijing North Institute Biological Technology (Beijing, China). The minimum detectable concentrations were 1 mIU/ml for FSH and LH and 0.45 ng/ml for leptin. For each RIA the intra- and interassay coefficients of variation were less than 15% and less than 10%, respectively.

PCR and RT-PCR

Total RNA was extracted using Trizol, and cDNA was generated from 2 μg RNA in a 25-μl reaction mixture using Moloney murine leukemia virus (Promega) according to the manufacturer's protocols. *LHβ*, *FSHβ*, and internal control glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression were assayed by quantitative RT-PCR using primers as described previously (45–47). *Isl-1* primers were designed by Primer Express 3.0 (Applied Biosystems, Foster City, CA) and confirmed to amplify a single product of the expected size via dissociation analysis and gel electrophoresis. Its sequences are: sense, 5′-AGTCATCCGAGTGTGGTTTC-3′, antisense, 5′-CATGCTGTTGGGTGTATCTG-3′. Amplification was carried out on the ABI PRISM 7500 sequence detection system (Applied Biosystems), and each sample was assayed three times in duplicate. The relative abundance of the genes was determined using the ABI PRISM's software.

Leptin receptors, the short form (OB-Ra) and the long form (OB-Rb), were detected by PCR using primers as previous described (48). Amplifications were carried out on PCR instrument (Bio-Rad, Hercules, CA) using the following protocol: 94 C for 5 min (one time); 94 C for 50 sec; 54 C for 30 sec; 72 C for 30 sec (35 times); 72 C for 10 min; and 4 C holding.

Western blot analysis

Western blots were performed as described previously (49). Goat polyclonal Isl-1 antibody and mouse monoclonal GAPDH antibody were purchased, respectively, from R&D (Minneapolis, MN) and Ambion (Austin, TX). All experiments were performed three times. For quantification, the membranes were

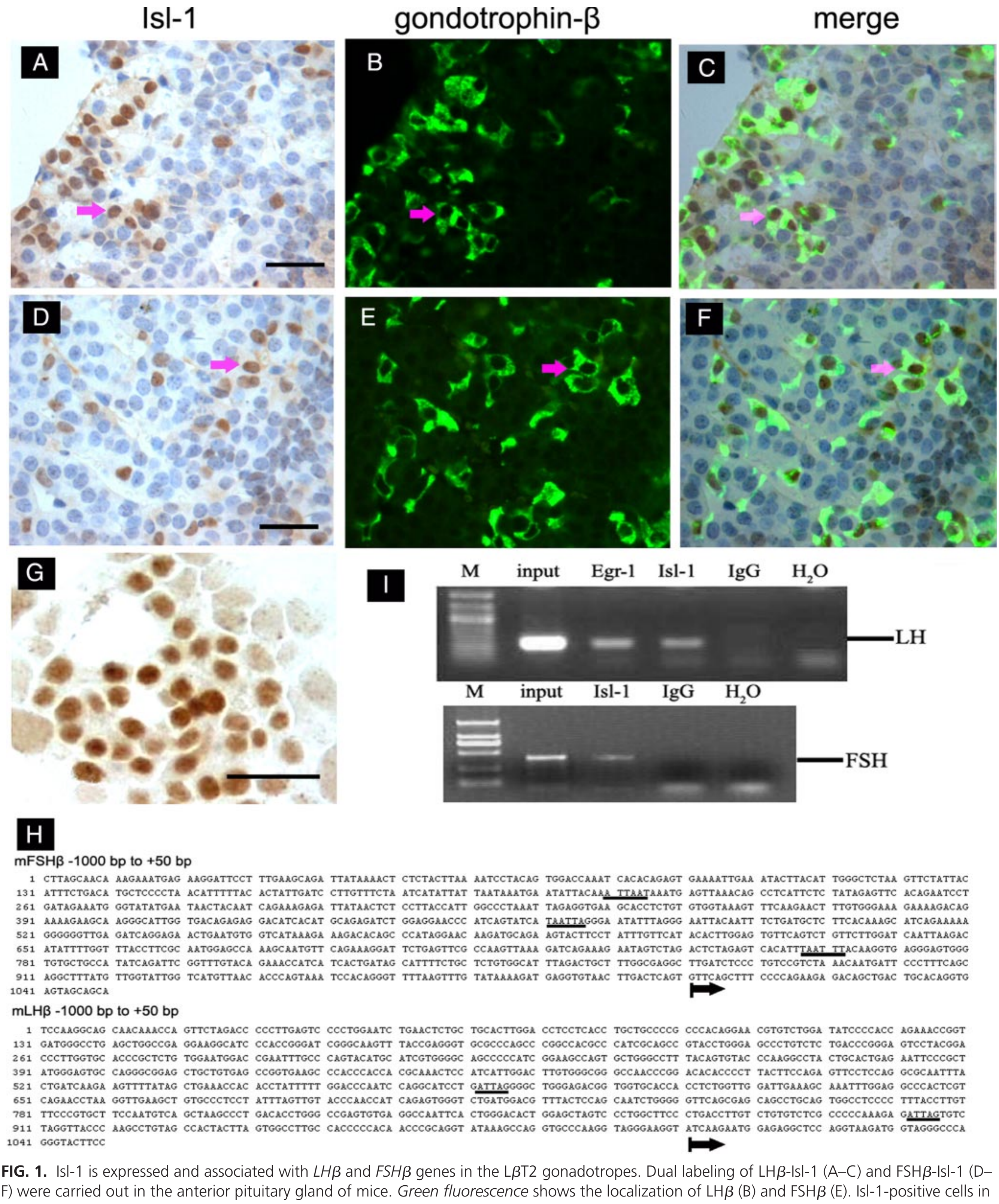

FIG. 1. Isl-1 is expressed and associated with *LHβ* and *FSHβ* genes in the LβT2 gonadotropes. Dual labeling of LHβ-Isl-1 (A–C) and FSHβ-Isl-1 (D–F) were carried out in the anterior pituitary gland of mice. *Green fluorescence* shows the localization of LHβ (B) and FSHβ (E). Isl-1-positive cells in the same field of the same slide were stained *brown* (A and D). On their respective *right* are merged images (C and F). *Purple arrows* denote representative double-stained cells. *Bar*, 30 μm. Three mice were examined in this experiment. A representative result is shown. G, ICC of Isl-1 in the LβT2 cells. Nuclear localizations of Isl-1 are stained *dark brown*. *Bar*, 30 μm. H, Sequence analysis of mouse FSHβ and LHβ promoter regions of −1000 to +50 bp from the transcriptional sites. The putative Isl-1 binding sites are *underlined*. *Arrows* indicate the start site and the direction of transcription. *Box sequences* are primer binding sites of ChIP analysis. I, ChIP analysis. A cell aliquot before precipitation was designated as the input sample. PCR amplified 234-bp (LHβ) or 401-bp (FSHβ) regions in the promoters, respectively. Egr-1 was used as a positive control in precipitating the LHβ promoter. IgG is a negative control provided by the kit.

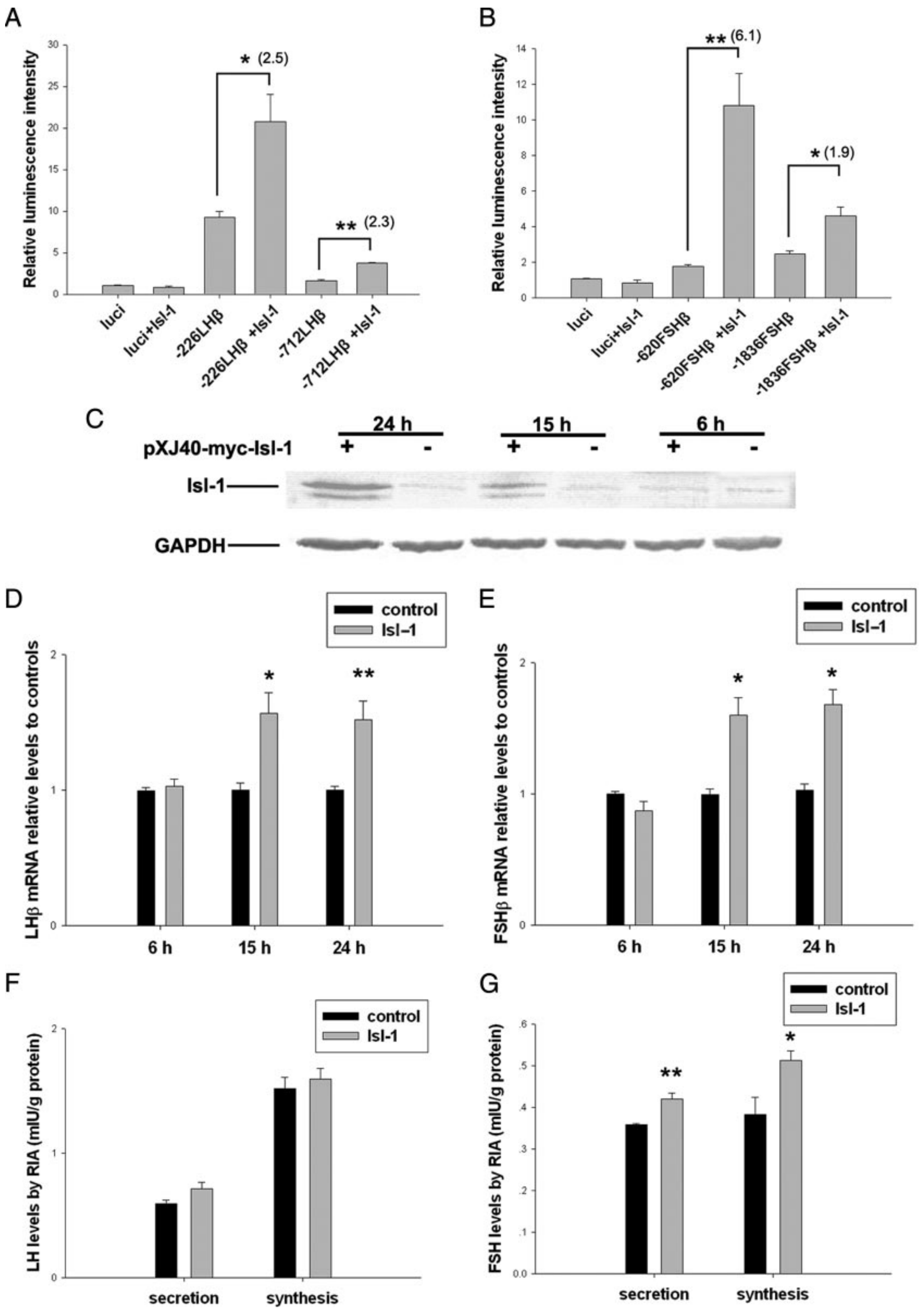

FIG. 2. Isl-1 induces the expressions of *LHβ* and *FSHβ* genes and production of FSH. A and B, Effects of Isl-1 expression vectors on the transiently transfected *LHβ* (A) and *FSHβ* (B) gene promoters fused to luciferase reporter genes. The empty luciferase vector (luci) was also transfected as a control. The stimulating amount of Isl-1-induced luciferase activity is denoted in *parentheses*. The experiments were repeated three times. C–E, Isl-1 increased LHβ and FSHβ mRNA levels. The pXJ40-myc-Isl-1 and control vectors were transfected into LβT2 cells, respectively. Isl-1 protein (C), LHβ mRNA (D), and FSHβ mRNA (E) levels were analyzed 6, 15, and 24 h later by Western blot and RT-PCR, respectively. The present data of each assay were normalized to respective 6-h control (control vector transfected 6 h). Each sample was assayed three times in duplicate. E and F, Changes of LH (E) and FSH (F) levels after transfection of Isl-1 expression vectors. The pXJ40-myc-Isl-1 and control vectors were transfected into LβT2 cells, and concentration of LH and FSH in the media (secretion) and in the cellular extracts (synthesis) were determined 72 h later by RIA. In the data shown, hormone concentration is normalized to the corresponded protein concentration. Values are all means ± SEM from four to six separate experiments. *, $P < 0.05$; **, $P < 0.01$.

scanned on AlphaImager 2200 (Alpha Innotech Corp., San Leandro, CA). The results were analyzed by densitometry.

For ERK and STAT3 analysis, membranes were blocked and then incubated in 1:1000 diluted primary antibodies overnight at 4 C. The phosphorylated (p)-ERK1/2, p-STAT3, and total ERK1/2 antibodies were from Cell Signaling Technology (Beverly, MA). The STAT3 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). After incubation for 1–2 h with secondary antibodies (horseradish peroxidase conjugated), immunoreactive bands were visualized by chemiluminescence (Millipore, Bedford, MA). In each case, the membrane was initially probed with the antibody against the phosphorylated protein and then stripped using a buffer containing 2% sodium dodecyl sulfate, 62.5 mM Tris (pH 6.8), and 0.1 M 2-mercaptoethanol for 30 min at 50 C. After washing, immunodetection was repeated using an antibody against the total protein.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using a ChIP assay kit (Active Motif, Carlsbad, CA) as previously described (50). Isl-1 antibody was from R&D. Antibodies of nonspecific IgG and Egr-1 (Santa Cruz Biotechnology) were used as negative and positive (only for LHβ) controls, respectively. Some of the protein-DNA was not precipitated but set aside for total chromatin examination (termed input). After DNA was purified, the aimed sequence, from −226 to +7 of the mouse *LHβ* gene and −687 to −286 bp of the *FSHβ* gene, was amplified by PCR using the primer sequences of −226 LHβ see *Plasmid construction*) and FSH-β: 5′-TACCATTGGCCCTA-AAT-3′ and 5′-TTGGCGAACTCAGAATCCTT-3′.

Statistical analysis

All data were analyzed using one-way ANOVA, followed by Student's *t* test. All values are expressed as means ± SEM. A $P < 0.05$ was considered significant.

Results

Isl-1 is expressed in the pituitary and gonadotrope-derived LβT2 cells

IHC results showed Isl-1 expression in the mouse pituitary gland. Dual-staining and cell-counting results showed that 64 ± 5% (mean ± SEM) LHβ immunopositive cells and 74 ± 6% (mean ± SEM) of FSHβ-immunopositive cells expressed Isl-1 in the mouse pituitary (Fig. 1, A–F). These data demonstrate that most gonadotropes express Isl-1 in the mouse pituitary. ICC results confirmed that Isl-1 was expressed in mouse gonadotrope-derived LβT2 cells (Fig. 1G).

Isl-1 interacts with *LHβ* and *FSHβ* genes in LβT2 cells

Sequence analysis showed that mouse *FSHβ* and *LHβ* genes contain two putative Isl-1 response elements (ATTAG) between −1000 and +50 bp upstream from the transcription sites (Fig. 1H). ChIP experiments were conducted to determine whether Isl-1 binds to the regulatory regions of endogenous *LHβ* and *FSHβ* genes in LβT2 cells. The primers encompassing the ATTAG box were used to detect LHβ and FSHβ promoter sequences in genomic DNA precipitation (Fig. 1H). A 234-bp region of mouse LHβ promoter was amplified from Isl-1-immunoprecipitated DNA as well as input chromatin and the DNA recruited by the LHβ transcription factor Egr-1 (Fig. 1G, *top panel*). A 401-bp FSHβ promoter sequence was also generated from Isl-1 immunoprecipitate (Fig. 1G, *bottom panel*). No PCR-amplified product was detected when the Isl-1 antibody was replaced by a nonspecific control antibody or in the water-template control (Fig. 1G). Taken together, these results confirm that Isl-1 interacts with the promoters of both FSHβ and LHβ in gonadotropes.

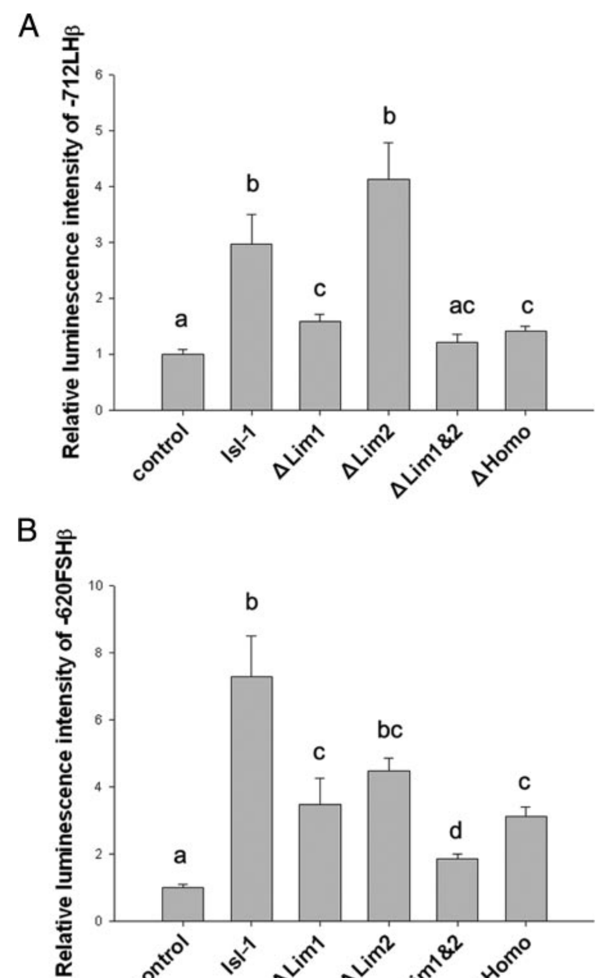

FIG. 3. Isl-1 mutants regulate −712LHβ (A) and −620FSHβ (B) promoter activities in LβT2 cells. The same quantity of wild and mutated Isl-1 expression vectors were cotransfected into cells with −712*LHβ* or −620*FSHβ* promoter reporter genes. Results are expressed as a ratio of firefly luciferase to renilla luciferase and then normalized to that of control. ΔLim1, LIM1 domain mutated; ΔLIM2, LIM2 domain mutated; ΔLim1 and 2, both LIM1 and LIM2 domain mutated; ΔHomo, homeodomain mutated. The experiments were repeated three times. Data are shown as means ± SEM. Significant differences ($P < 0.05$) are shown by *different letters*.

Isl-1 increases FSHβ and LHβ promoter activity and affects hormone production

To determine whether Isl-1 is involved in regulating *LHβ* and *FSHβ* gene transcription, pXJ40-myc-Isl-1 was transfected into LβT2 cells with either *LHβ* or *FSHβ* promoter-luciferase genes. A dual-luciferase reporter assay showed that Isl-1 enhanced the promoter activities of −226LHβ and −712LHβ about 2.5-fold ($P < 0.05$) and 2.3-fold ($P < 0.01$), respectively (Fig. 2A). −620FSHβ and −1836FSHβ were also stimulated by Isl-1 about 6.1-fold ($P < 0.01$) and 1.9-fold ($P < 0.05$), respectively (Fig. 2B). Meanwhile, Isl-1 overexpression did not change the luciferase activity of pGL3.0-basic ($P > 0.05$).

To determine whether the overexpression of Isl-1 affects endogenous transcriptional activity of gonadotropin β-subunits, we examined variations of their mRNA levels in pXJ40-myc-Isl-1- or control vector-transfected LβT2 cells. Western blot results indicated that Isl-1 expression did not increase until 15 and 24 h after transfection of pXJ40-myc-Isl-1 (Fig. 2C). Correspondingly, both LHβ and FSHβ transcripts were significantly elevated to about 1.5-fold at 15 and 24 h ($P < 0.01$) but were not changed at 6 h ($P > 0.05$, Fig. 2, D and E). In contrast, there was no change in LHβ or FSHβ transcripts in control vector-transfected cells throughout ($P > 0.05$, Fig. 2, D and E).

Although Isl-1 significantly increased both LHβ and FSHβ mRNA levels, RIA results showed that Isl-1 resulted in a significant increase only in intracellular ($P < 0.01$) and released ($P < 0.05$) FSH (Fig. 2G) but not LH ($P > 0.05$, Fig. 2F).

Isl-1 HOMO and LIM domains regulate FSHβ and LHβ promoters' activities

LIM-HD Isl-1 has one homeodomain and two separated LIM domains, interacting with target DNAs and proteins, respectively. To determine which domains are involved in the observed regulation of *LHβ* and *FSHβ* gene transcription in LβT2 cells, four truncated forms of Isl-1 protein were constructed: ΔLim1, ΔLim2, ΔLim1 and 2, and ΔHomo. Activation of Isl-1 on both −712 LHβ and −620FSHβ was decreased ($P < 0.05$, Fig. 3) when LIM1, LIM1 and 2, or the homeodomain was mutated. In contrast, deletion of LIM2 alone did not significantly change the stimulating effects of Isl-1, whether on −712LHβ or −620FSHβ ($P > 0.05$, Fig. 3).

GnRH, activin, and leptin all increase the expression levels of LIM-HD Isl-1

To determine the potential for Isl-1 to mediate the effects of GnRH, activin, and leptin on gonadotropins, we determined mRNA levels of Isl-1 in LβT2 cells exposed to GnRH, activin, and leptin using RT-PCR. After GnRH treatment, there was a significant increase in the Isl-1 mRNA level at 1 h ($P < 0.01$), which peaked at 3 h ($P < 0.01$) and decreased thereafter to the control level by 24 h ($P > 0.05$, Fig. 4A). In response to activin and leptin, Isl-1 mRNA level increased by 3 h ($P < 0.05$), peaked at 12 h, and stayed for at least 24 h ($P < 0.01$, Fig. 4, B and C). These results suggest that the LIM-HD transcription factor Isl-1 is potentially involved in gonadotropin-regulating effects of GnRH, activin, and leptin in gonadotropes.

Knockdown of Isl-1 expression in the LβT2 cells

To directly test the role of Isl-1, five short hairpin RNA constructs targeting mouse *Isl-1* gene (Fig. 5A) were designed to inhibit Isl-1 expression in LβT2 cells. Each construct was screened individually for inhibiting the activation of the psiCHECK-Isl-1 luciferase in transient transfection assays. pSilencer-H1-Isli891 was the best inhibitor, reducing Isl-1 expression by about 75%, whereas Isli511 resulted in a 64% reduction. Isli521, Isli1138, and Isli1288 resulted in a 49, 35, and 37% reduction, respectively (Fig. 5B).

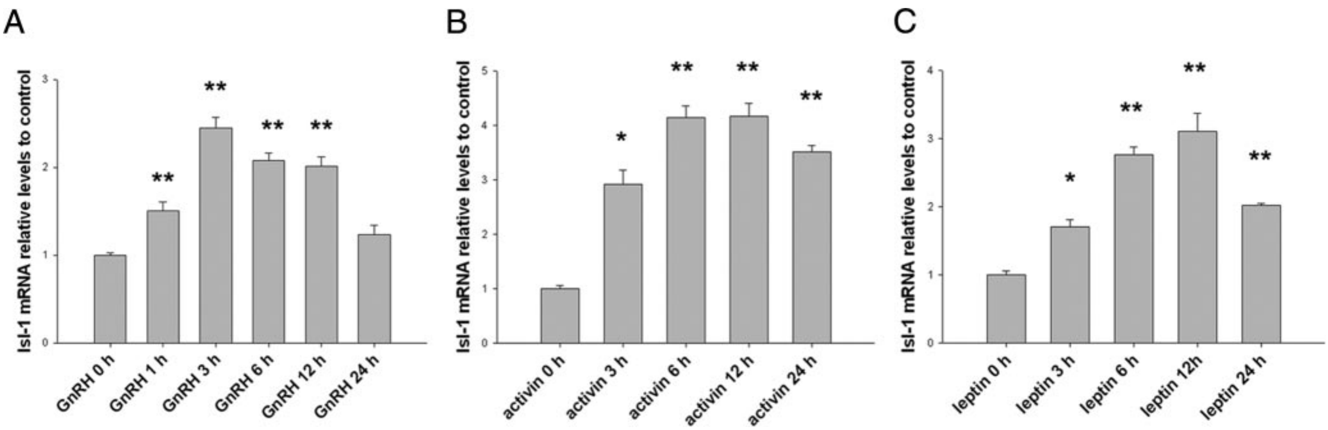

FIG. 4. The relative levels of Isl-1 mRNA levels in LβT2 cells treated by GnRH (A), activin (B), and leptin (C) for different times. Cells were treated with 10 nM GnRH, 25 ng/ml activin, or 10ng/ml leptin for 3, 6, 12, and 24 h. mRNA levels of Isl-1 and reference gene, *GAPDH*, were determined by RT-PCR. Data are shown as means ± SEM from three experiments in duplicate and normalized to their respective control. *, $P < 0.05$; **, $P < 0.01$.

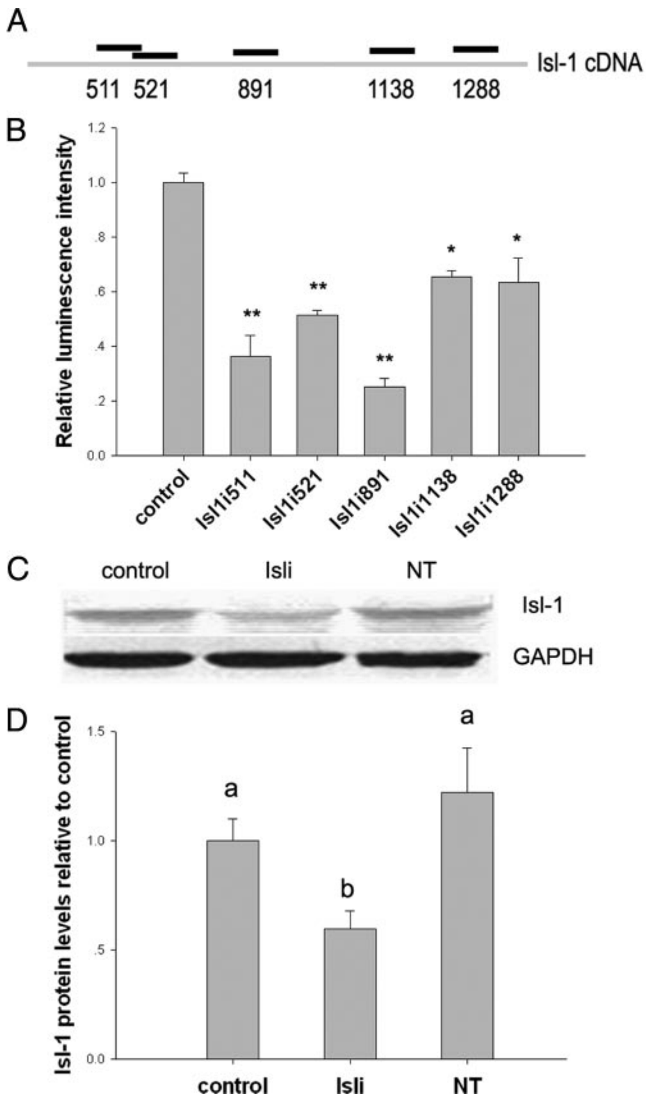

FIG. 5. Silencing effects of Isl-1 interference vector. A, Sketch map indicates the targeted sites of several Isl-1 interference vectors (named pSilencer-H1-Islix) in the mouse Isl-1 cDNA. B, psiCHECK-Isl-1 was transfected into HEK293T cells together with pSilencer-H1-Islix. Dual-luciferase ratios were expressed as ratios of renilla luciferase to firefly luciferase, and they were normalized to pSilencer-H1-shRNA control. Data are shown as means ± SEM from three repeated experiments. *, $P < 0.05$; **, $P < 0.01$. C and D, Western blot analysis of Isl-1 protein levels in LβT2 cells transfected with pSilencer-H1-shRNA, pSilencer-H1-Isli891, and nontreated cells (NT). GAPDH is blotted as an internal control. The integrated density value (IDV) of Isl-1 and GAPDH products were determined using the AlphaImager 2200 software package (Alpha Innotech), and Isl-1 expressional levels were expressed according to IDV. Data from three experiments were all normalized to the control and are shown as means ± SEM. Significant differences ($P < 0.05$) are shown by *different letters*.

To assess the pSilencer-H1-Isli891-inhibiting effects in gonadotropes, protein levels of Isl-1 were determined in LβT2 cells. Isl-1 protein levels in empty vectors and non-treated cells did not change significantly ($P > 0.05$) but decreased about 40.2% ($P < 0.05$, Fig. 5, C and D) in cells transfected with pSilencer-H1-Isli891, confirming its efficacy of reducing Isl-1 expression.

Isl-1 is involved in leptin's regulation of LH and FSH synthesis

The intracellular and medium concentrations of LH and FSH in Isl-1 knockdown and control cells were determined after treatment with GnRH, activin, and leptin to assess the role of Isl-1 in their functions.

In Isl-1 knockdown cells in the absence of GnRH, activin, or leptin (Fig. 6, NT group), the levels of secreted FSH decreased ($P < 0.05$, Fig. 6B). There was no effect on intracellular or secreted LH levels ($P > 0.05$).

GnRH treatment caused a small increase of LH secretion in LβT2 cells ($P < 0.05$), and Isl-1 knockdown did not influence this change ($P > 0.05$, Fig. 6A). Activin did not change the levels of LH ($P > 0.05$, Fig. 6A); however, it significantly promoted both synthesized ($P < 0.05$) and secreted ($P < 0.01$) FSH levels in wild-type cells, which also were not changed by Isl-1 interference ($P > 0.05$, Fig. 6B). In contrast, leptin increased intracellular LH ($P < 0.05$, Fig. 6A) and FSH ($P < 0.05$, Fig. 6B) concentrations in control vector-transfected cells. Both were significantly ($P < 0.01$ for LH, Fig. 6A; $P < 0.05$ for FSH, Fig. 6B) decreased in Isl-1 knockdown cells, indicating that Isl-1 mediates leptin regulation of LH and FSH synthesis.

Leptin induces Isl-1 expression by JAK-STAT

PCR results showed that both the OB-Ra and OB-Rb forms of the leptin receptor are expressed in LβT2 cells as well as in mouse pituitary, hypothalamus, and ovary (Fig. 7A). We next examined leptin-stimulated signals in LβT2 cells. Cultured cells were exposed to leptin for up to 60 min. Cell lysates were immunoprecipitated with antiphospho-STAT3 or antiphospho-ERK antibodies. The leptin-dependent increases in STAT3 and ERK activity were both detectable at 5 min (Fig. 7B), followed by a sustained increase from 5 to 60 min (Fig. 7B). These results indicate that leptin activates both STAT3 and ERK signals in LβT2 cells. In addition, leptin-induced expression of Isl-1 mRNA was blocked by both the JAK-selective tyrosine kinase inhibitor AG490 and STATs' phosphorylation suppressor parthenolide ($P < 0.05$, Fig. 7D). In contrast, PD98059, a specific antagonist of ERK1/2, did not modify the transcription level of Isl-1 induced by leptin ($P > 0.05$). Interestingly, PD98059 alone also increased basal Isl-1 expressions ($P < 0.05$, Fig. 7D).

The relation of pituitary Isl-1 mRNA levels to serum leptin concentration

To further identify the potential relationship of Isl-1 and leptin *in vivo*, we measured the pituitary Isl-1 mRNA by RT-PCR and serum leptin levels by RIA in mice at different ages and with different feeding patterns. The results showed that Isl-1 mRNA increased significantly from

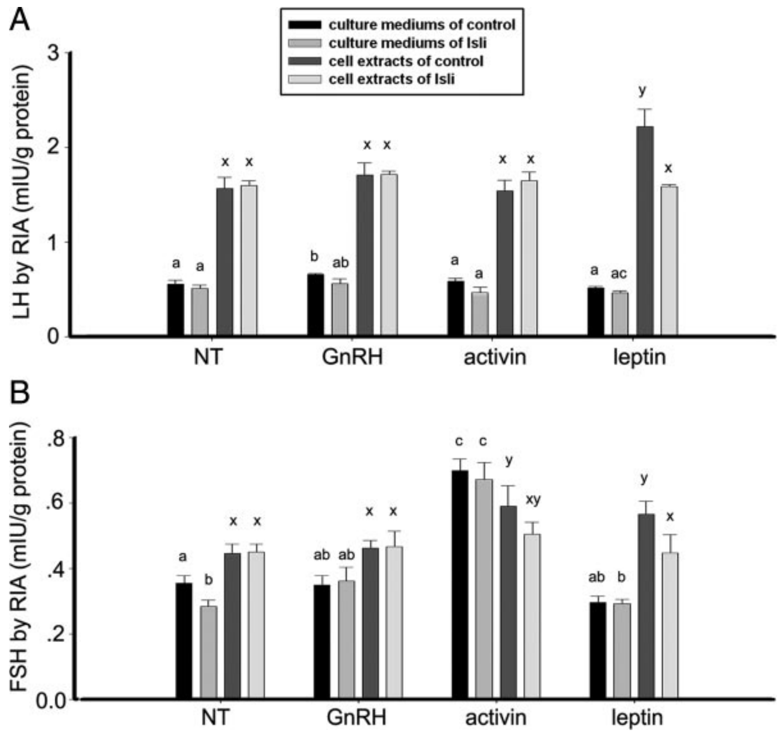

FIG. 6. Isl-1 is involved in leptin regulation of gonadotropin synthesis. pSilencer-H1-Isli891 and control vectors were transfected into LβT2 cells, and after 48 h, 10 nM GnRH, 25 ng/ml activin and 10 ng/ml leptin were respectively added 24 h before harvest. The LH (A) and FSH (B) hormone concentration in media and cellular extracts were determined by RIA, and the data are shown by hormone concentration normalized to the corresponding cellular protein concentration. Values are means ± SEM from four to six separate experiments. Significant differences are indicated by *different letters* ($P < 0.05$). NT, Nontreated cells.

21 D to 60 D ($P < 0.05$, Fig. 8A), whereas serum leptin levels increased significantly only at 60 D ($P < 0.05$, Fig. 8B). However, the changes of pituitary Isl-1 mRNA level did parallel global leptin concentrations in dietary manipulated mice, with both increasing significantly ($P < 0.01$) after feeding with the high-fat diet but were not significantly ($P > 0.05$) changed by starvation for 48 h (Fig. 8, C and D).

Discussion

Isl-1 expression in the developing pituitary gland has been reported in mice (41, 51), chicks (44, 52), and sheep (43). The dominant cell types expressing Isl-1 in the pituitary gland are the FSHβ- and LHβ-expressing gonadotropes. Our present results agree with these findings and show that Isl-1 is mainly expressed in pituitary gonadotropes of prepubertal to adult mice and in gonadotrope-derived cell line, LβT2. The discovery that LβT2 cells express Isl-1 provides an *in vitro* tool for studying the transcriptional effect of Isl-1 on *LH/FSHβ* gene expression and hormone production.

Previous studies on the function of Isl-1 in the pituitary have focused on the early stage of embryo development. *Isl-1* null mice embryos show developmental anomalies at E9.5 and die at E10 (38, 42), 2.5 d before pituitary cell differentiation and hormone production (41). Therefore, this *Isl-1* gene-knockout animal model cannot be used to study the function of Isl-1 in pituitary development and hormone secretion. Upon that premise, the present study switches to *in vitro* study, to demonstrate the relationship of Isl-1 and gonadotropin.

First, we have shown that Isl-1 not only increases both LHβ and FSHβ promoter activities but also elevates their mRNA levels about 1.5-fold. These changes in mRNA agree with luciferase assay results, which indicated that Isl-1 causes a similar stimulation on long-form *LHβ* and *FSHβ* promoter genes, which are, respectively, increased 2.3- and 1.9-fold. The elevation of mRNA strongly indicates that Isl-1 probably affects synthesis and secretion of gonadotropins by altering *LHβ* and *FSHβ* gene expression, which should result in increased hormone production. However, the results have shown that both synthesis and secretion levels of FSH are increased by Isl-1 in LβT2 cells but not that of LH.

Differential responses of LH and FSH may relate to their different synthesis and secretion patterns. Indeed, the LH and FSHβ subunits have quite distinct amino acid sequences and variable oligosaccharide chains (53). Glycosylation of polypeptides is a posttranslational event, which influences the rate of hormone dimmer assembly (54) and thus synthesis. Furthermore, LH and FSH also exhibit different secretion patterns. Previous studies in sheep have confirmed that LH is stored in electron-dense granules within the gonadotrope (55) associated with *SgII* (56–58), and FSH appears in electron light granules, possibly associated with chromogranin A (57–59). The release and subsequent plasma concentrations of FSH appear more closely related to the amount of FSH being produced within the gonadotrope, and also to the levels of transcription of FSHβ mRNA (4). In contrast the release of LH occurs via a regulated pathway with little relationship to the levels of transcription of LHβ mRNA. This is probably why LH was not changed by Isl-1, although the expression level of LHβ mRNA was up-regulated.

Similar to other LIM-HD transcription factors, Isl-1 contains two LIM domains and a HD. To study the roles of these domains in regulating *LHβ* and *FSHβ* gene expression, we constructed four Isl-1 mutants. The results showed that the HD is required for full Isl-1 transcrip-

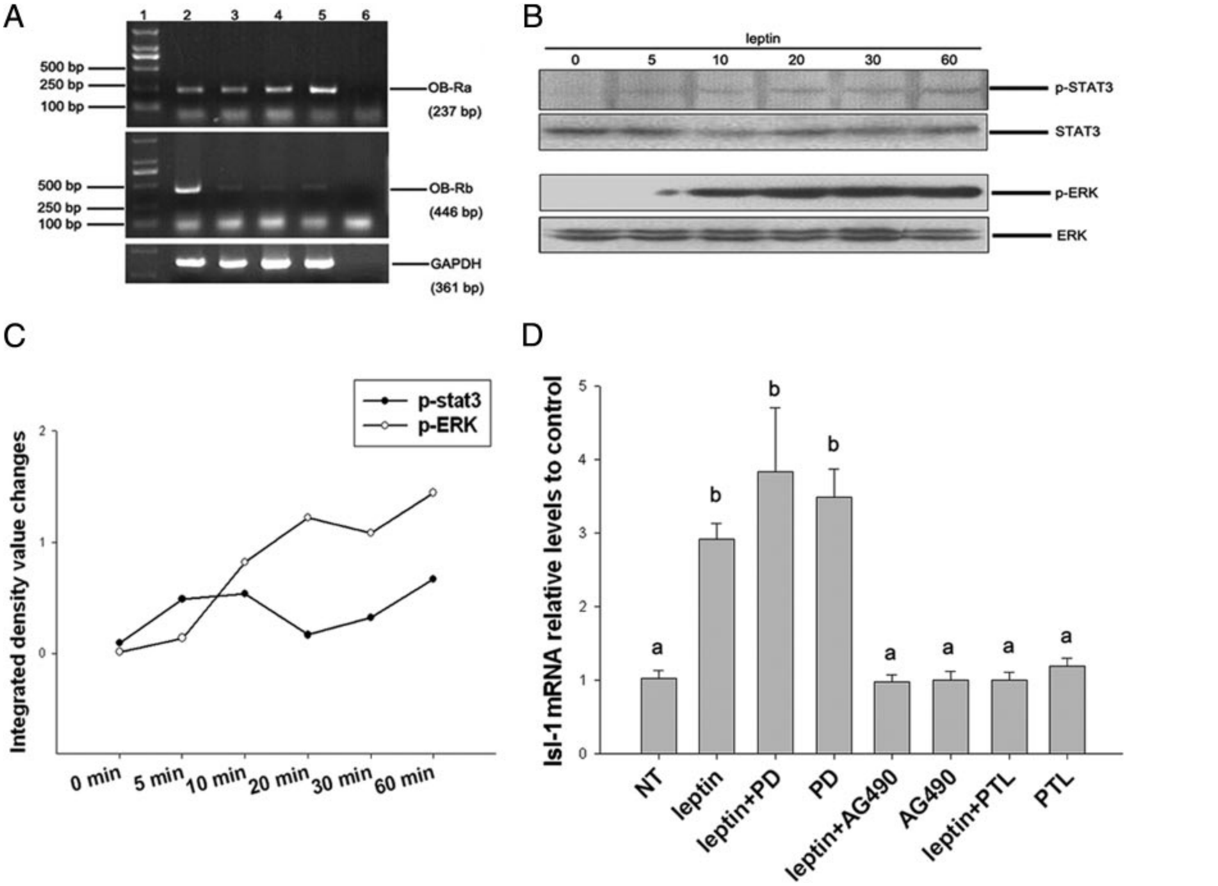

FIG. 7. Intracellular signals of leptin in up-regulating Isl-1 in the LβT2 cells. A, Expressions of leptin receptor mRNAs in LβT2 cells and several tissues of the adult mouse. Lane 1, DNA ladder; lane 2, mouse hypothalamus; lane 3, mouse pituitary; lane 4, LβT2 cell; lane 5, mouse ovary; lane 6, reverse transcriptase blank without Moloney murine leukemia virus. OB-Ra and OB-Rb products are, respectively, 237 and 446 bp. GAPDH is used as a positive control. Experiments were repeated three times, and one representative is shown. B, Leptin activates ERK and STAT3 signals in the LβT2 cells. The LβT2 cells were cultured with 10 ng/ml leptin for 0 (no treatment control), 5, 10, 20, 30, and 60 min, respectively, and then total protein extracts were prepared. Levels of phosphorylated ERK, phosphorylated STAT3, total ERK, and total STAT3 proteins were detected by Western blot. C, Changes of p-STAT3/STAT3 and p-ERK/ERK Levels. Data were calculated using integrated density value ratio of bands in B, which were determined by AlphaImager 2200 (Alpha Innotech). D, The relative levels of Isl-1 mRNA in LβT2 cells treated by leptin and several inhibitors. Inhibitors were added 1 h before leptin. All inhibitors had a final concentration of 20 μM. After 12 h exposure to leptin, Isl-1 expressions in each group were determined by RT-PCR and then normalized to that of control. Data are shown as means ± SEM from three repeated experiments. Significance ($P < 0.05$) is shown by *different letters*. NT, Nontreated cells; PTL, parthenolide.

tional activity on *LHβ* and *FSHβ* genes, suggesting that Isl-1 directly interacts with *LHβ* and *FSHβ* DNA. ChIP results confirmed that the binding site of Isl-1 probably lies within the regions of −226 to +7 bp on the *LHβ* genes and −687 to −286 bp on the *FSHβ* gene, although the exact binding motifs requires further work. In addition, the full transcriptional activity of Isl-1 does not occur when the LIM1 domain is absent. This indicates that Isl-1 also influences LHβ and FSHβ transcription by interacting with other proteins through LIM1. Isl-1 is capable of interacting with Lhx3 and the LIM-interacting partners, Ldb1, to form a trimer using its ligand-binding domain and LIM1 domains (60). Isl-1 also functions in synergy with other transcription factors, such as SF-1 (51), hepatocyte nuclear factor-4α (61), and class B bHLH transcription factor, BETA2 (62). The specific associating proteins were not identified in this study. Possibly, one of the reasons of Isl-1's different magnitude of the effect on the LHβ and FSHβ promoter activities is related to different specific transcription factor interactions, *e.g.* Lhx3 has been shown to regulate *FSHβ* but not *LHβ* promoters (63). Collectively, it is likely that the Isl-1-mediated regulation of the *LHβ* and *FSHβ* promoters incorporates several mechanisms involving both direct and indirect interactions.

More importantly, the regulatory effect on FSH synthesis and secretion potentially extends the role of Isl-1 to the HPG axis. GnRH, steroids, and cytokines produced by the hypothalamus and gonads possibly regulate the expression or activation of Isl-1 in the pituitary, thus increasing FSH hormone production. Hormone measurements demonstrate that GnRH induces LH but not FSH secretion, and activin stimulates FSH but not LH secretion, which is in agreement with previous studies (7, 45, 64). However, Isl-1 does not appear to have a close rela-

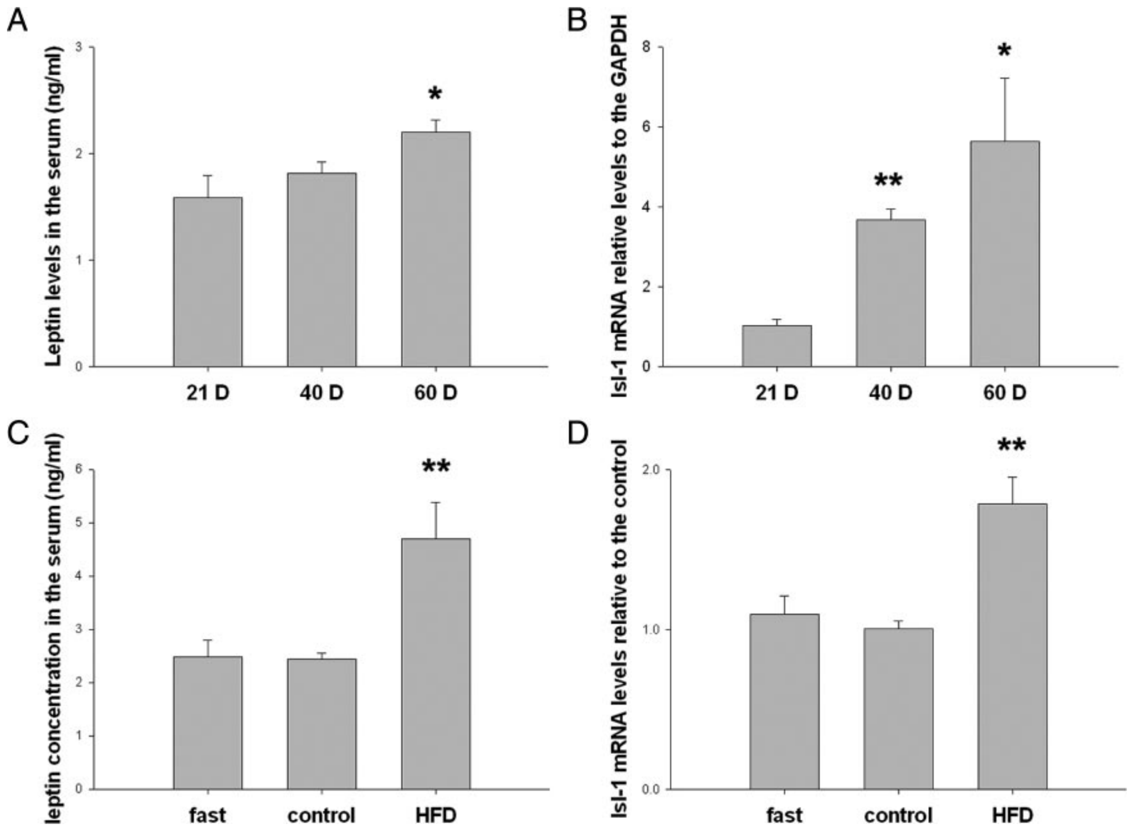

FIG. 8. Changes of pituitary Isl-1 mRNA levels and serum leptin by age and diet. A and B, Pituitary Isl-1 mRNA levels and serum leptin concentrations in mice 21, 40, and 60 d old. Isl-1 mRNA levels were determined by real-time PCR. GAPDH is a reference gene. Each sample was assayed three times in duplicate. Present data of each assay was normalized to that of the 21 D group. The sample numbers for leptin determination were n = 6 (21 D), n = 6 (40 D), and n = 10 (60 D), respectively. C and D, Changes of pituitary Isl-1 mRNA levels and serum leptin concentrations after 2 wk of dietary manipulation. The control group was fed a regular grain diet; the high-fat diet (HFD) group was fed a diet containing 20% fat. Mice in the fast group were starved for the last 48 h with sufficient drinking water. The sample number for each group is 10. Isl-1 mRNA levels were determined by real-time PCR. Each sample was assayed three times in duplicate and normalized to that of control group. All of the data are shown as means ± SEM. *, $P < 0.05$; **, means $P < 0.01$.

tionship with the function of GnRH and activin because their regulatory effects on gonadotropin are not changed by Isl-1 interference. This is reasonable because GnRH has been shown to directly regulate *LHβ* through Egr-1, which synergizes with SF-1 and Pitx1 (65), and activin regulates FSH synthesis by small mothers against decapentaplegic homolog (smad) 2/3, which acts directly on the *FSHβ* promoter (66). However, GnRH and activin increase Isl-1 expression, and the effects of GnRH and activin on FSH and LH do not rely on Isl-1. It is possible that Isl-1 may be involved in other GnRH- and activin-induced effects, *e.g.* cell proliferation, as shown in other cells or tissues (67, 68). This requires further studies.

The results of the present study show that leptin stimulates both FSH and LH synthesis in LβT2 cells, in agreement with some previous reports (27, 28, 69). However, there are conflicting reports about the effect of leptin on gonadotropins and the HPG axis. Administration of leptin *in vitro* reduced LH and FSH secretion in the rat hemipituitary cultures (70) and failed to stimulate FSH in energy-deprived rhesus monkeys (71). Furthermore, in the presence of sufficient energy stores, leptin did not alter reproductive function (19). It is unclear why these discrepancies in the effect of leptin occur. It may be due to the differential effects of leptin in modulating the expression of its own receptors. It may also be due to the dose dependence of leptin. Indeed, the concentration of endogenous leptin is at a comparatively high level in the animals with sufficient energy stores, and this has been confirmed in our results. Thus, leptin may already be acting at maximum. An overdose of leptin was demonstrated to be significantly decreased the expressional levels of hypothalamic OB-Rb (72). Furthermore, there is evidence that leptin's lack of effectiveness on the HPG axis may be due to influences of other neuroendocrine peptides, like neuropeptide Y, GnRH, and estrogen (73–75).

In the reproductive axis, leptin is known to activate signaling via its long- and short-form receptors. And we have now shown that both forms of the receptor are expressed in the hypothalamus, pituitary, and ovary in the mouse, confirming a previous report (22). Studies in cattle (23) and sheep (24) pituitaries have suggested that most of

OB-R-positive cells are gonadotropes. This agrees well with present result that OB-Rs are expressed in LβT2 gonadotropes.

The present work is the first to show, using specific inhibitors, that Isl-1 mRNA levels are regulated by leptin through JAK-STAT's cascade in pituitary gonadotropes, similar to the hypothalamus (76, 77). This, however, differs with a previous report that shows that leptin's action in the reproductive neuroendocrine axis is independent of pSTAT3 signaling as shown by disrupting Tyr^{1138} phosphorylation in the OB-Rb (78). It has been reported that JAK-stimulated Isl-1 could induce Tyr phosphorylation, DNA binding activity, and target gene expression of STAT3 independent of other activators (*e.g.* epithelial growth factor) (79). This suggests that Isl-1 may function as an adaptor protein that brings JAK and STAT3 into proximity and thereby facilitates STAT3 phosphorylation by JAK. This fits well with our results, which indicate that leptin elevates Isl-1 expression, resulting in the induction of STAT3 phosphorylation. However, the inhibitor parthenolide used in our study is a broad inhibitor of phosphorylation of STATs, including STAT1, -3, -5, and -6. We did not check the phosphorylation of other STATs, which may have roles in the present study. This study also shows that leptin activates the ERK pathway in LβT2 gonadotrope cells. It is in agreement with the report that leptin promotes proliferation via the MAPK/ERK signal pathway in endometrial cancer cells (80). This suggests that leptin may function to promote LβT2 cell proliferation by activating ERK. Nevertheless, the molecular mechanism of leptin's effect through STATs and MAPK need to be elucidated further.

In the present study, serum leptin rose from 40 D to 60 D, which agrees with a previous study that showed leptin increases from 20 D to postpuberty in male mouse plasma (81). The expression levels of pituitary Isl-1 also increased over this time. This parallel change suggests that leptin is an important determinative regulator for Isl-1 around puberty. However, the changes in leptin and Isl-1 were not completely consistent with serum LH and FSH levels (82). This is probably related to the complex effects of other regulators like activin, inhibin, and principally GnRH during the prepubertal-pubertal transition. More importantly, our results also show that serum leptin and pituitary Isl-1 display parallel changes under dietary manipulations. This, together with the developmental results, strongly supports the *in vitro* results demonstrating that leptin regulates pituitary Isl-1 expression and reveals their close physiological relationship *in vivo*.

In conclusion, this novel study demonstrates that Isl-1 not only increases the expression of *LH* and *FSH* β-subunit genes but also mediates the regulating functions of leptin on LH and FSH synthesis.

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