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G Proteins and Autocrine Signaling Differentially Regulate Gonadotropin Subunit Expression in Pituitary Gonadotrope*[S]

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Background: The mechanism for differential control of gonadotropin gene induction by GnRH is not established.
Results: GnRH activates $G\alpha_s$ and $G\alpha_{q/11}$, which modulate LH and FSH synthesis, respectively, by a mechanism including secreted factors.
Conclusion: Different G proteins and autocrine signaling regulate the pattern of FSH and LH expression by GnRH.
Significance: A novel G protein and autocrine signaling mechanism has been identified.

Gonadotropin-releasing hormone (GnRH) acts at gonadotropes to direct the synthesis of the gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). The frequency of GnRH pulses determines the pattern of gonadotropin synthesis. Several hypotheses for how the gonadotrope decodes GnRH frequency to regulate gonadotropin subunit genes differentially have been proposed. However, key regulators and underlying mechanisms remain uncertain. We investigated the role of individual G proteins by perturbations using siRNA or bacterial toxins. In LβT2 gonadotrope cells, FSHβ gene induction depended predominantly on $G\alpha_{q/11}$, whereas LHβ expression depended on $G\alpha_s$. Specifically reducing $G\alpha_s$ signaling also disinhibited FSHβ expression, suggesting the presence of a $G\alpha_s$-dependent signal that suppressed FSH biosynthesis. The presence of secreted factors influencing FSHβ expression levels was tested by studying the effects of conditioned media from $G\alpha_s$ knockdown and cholera toxin-treated cells on FSHβ expression. These studies and related Transwell culture experiments implicate $G\alpha_s$-dependent secreted factors in regulating both FSHβ and LHβ gene expression. siRNA studies identify inhibinα as a $G\alpha_s$-dependent GnRH-induced autocrine regulatory factor that contributes to feedback suppression of FSHβ expression. These results uncover differential regulation of the gonadotropin genes by $G\alpha_{q/11}$ and by $G\alpha_s$ and implicate autocrine and gonadotrope-gonadotrope paracrine regulatory loops in the differential induction of gonadotropin genes.

Mammalian reproductive processes are controlled by a complex interplay between functionally and spatially discrete tissues. Pituitary gonadotropes play a central role in the hypothalamic-pituitary-gonadal system and in controlling reproduction. Gonadotropin-releasing hormone (GnRH)[2] is released in discrete pulses by the hypothalamus and acts at the gonadotrope to regulate the synthesis and release of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The gonadotropins regulate various gonadal functions including gametogenesis, sex hormone production, and the phases of the female reproductive cycle.

GnRH is released in discrete pulses. The pattern of gonadotropin synthesis and release in the pituitary gonadotrope is influenced by the frequency of hypothalamic GnRH release. In the female reproductive cycle, for example, lower frequency hypothalamic GnRH promotes preferential FSH synthesis and release that mediate ovarian follicular recruitment and growth in the early follicular phase. Higher frequency hypothalamic GnRH stimulates preferential LH synthesis and release, triggering ovulation in the late follicular phase (1, 2). This frequency encoding is exploited therapeutically to assist reproduction and to cause chemical castration using long acting GnRH analogues for the treatment of gonadal steroid-dependent cancers. Abnormalities in gonadotropin synthesis and release are involved in several reproductive disorders, such as Kallmann syndrome and polycystic ovarian syndrome (1, 3–6). The mechanisms and associated regulatory structures underlying the decoding of GnRH signaling patterns by the gonadotrope are incompletely understood.

An important goal of gonadotropin research over the previous decade has been to define the topology of gonadotropin signaling, *i.e.* the positive and negative signaling and gene regulatory connections and cis-acting gene control processes linking receptor activation to gonadotropin gene regulation (7). There have been several efforts to develop mathematical models of gonadotrope frequency-decoding mechanisms (8–11). Modeling is valuable for understanding information processing in a system of this complexity, and refining the understanding of the regulatory loops and network topology is important for guiding this modeling effort.

The GnRH receptor (GnRHR) is a member of the rhodopsin-like G protein-coupled receptor superfamily that activates heterotrimeric GTP-binding proteins (G proteins) to transmit extracellular information to the intracellular signaling network (12). GnRHR activates G proteins by facilitating GDP to GTP exchange of Gα, which in turn activates downstream effectors

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[2] The abbreviations used are: GnRH, gonadotropin-releasing hormone; CT, cycle threshold; CTX, cholera toxin; GnRHR, GnRH receptor; LH, luteinizing hormone; PTX, pertussis toxin.

(13, 14). There are four Gα subfamilies in the mammalian genome: $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{12/13}$, and $G\alpha_{i/o}$ (15). Each Gα subfamily has been associated with principal downstream effectors. For example, $G\alpha_s$ activates adenylyl cyclase catalyzing cAMP production. $G\alpha_{q/11}$ activates phopholipase Cβ, producing diacylglycerol and inositol triphophate. $G\alpha_{12/13}$ activates RhoGEFs (16). $G\alpha_{i/o}$ inhibits adenylyl cyclase and regulates potassium channels. The GnRHR is believed to regulate several Gα proteins as downstream effectors. However, it has not been fully determined which G proteins are involved in the GnRHR signaling of the gonadotrope. How each G protein contributes to signaling and gonadotropin transcription has not been studied.

Although early studies implicated predominantly $G\alpha_{q/11}$ in gonadotrope GnRHR signaling, recent work from Webster's laboratory based on GTP loading assays and cell-permeable inhibitory peptides suggests that both $G\alpha_q$ and $G\alpha_s$ contribute to regulation of ERK and LHβ expression (17–19). Notably, $G\alpha_s$ and $G\alpha_q$ show differential desensitization in response to pulsatile GnRH stimulation (18). No evidence for involvement of $G\alpha_{i/o}$ in the GnRHR signaling of gonadotropes has been reported (17). On the other hand, in reproductive tumor cells, $G\alpha_{i/o}$ played an important role in GnRHR signaling by regulating MAPK activation and in inhibiting cell proliferation in response to GnRH stimulation (20–23). In the GnRH-producing neurons of the hypothalamus, $G\alpha_i$ and $G\alpha_q$ were involved in the pulsatile GnRH release in response to autocrine/paracrine GnRH stimulation (24). G protein activation by GnRHR may vary, as the GnRHR-activated G protein shifted from $G\alpha_s$ to $G\alpha_i$ under increasing GnRH concentration in hypothalamic neurons (24). The involvement of $G\alpha_{12/13}$ in GnRHR signaling has not been explored. Therefore, GnRHR is capable of activating multiple Gα subfamilies depending on cell type or cell context.

An important technique for studying signaling topology relies on the use of interfering RNA (RNAi). This approach has not been widely utilized in LβT2 cells because of difficulty in inducing a high level of suppression. We have found that nucleofection protocols can provide efficient suppression of specific transcripts and proteins in LβT2 cells (25, 26). In this study we used specific RNAi-mediated suppression of individual G proteins in conjunction with bacterial toxin experiments to probe the topology of GnRHR signaling. These experiments identified differential induction of LHβ and FSHβ by $G\alpha_s$ and $G\alpha_q$ G proteins, respectively. The unexpected discovery of a $G\alpha_s$-dependent suppression of FSH expression led to the identification of autocrine/paracrine signaling mechanisms involved in differential gonadotropin expression.

EXPERIMENTAL PROCEDURES

Materials—G protein antibodies ($G\alpha_s$, $G\alpha_q$, $G\alpha_{11}$, and $G\alpha_{12}$) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-PKCα was from BD Biosciences. Phosphor-JNK, total JNK, phosphor-PKCμ, total PKCμ, and phosphor-PKA substrates antibodies were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-coupled secondary antibodies were from Santa Cruz Biotechnology. Cholera toxin (CTX) and pertussis toxin (PTX) were purchased from Calbiochem. GnRH was purchased from Bachem. Proteinase K was purchased from Roche Applied Science. Molecular weight cutoff filters (Amicon centrifugal filter) and Transwells were purchased from Millipore.

Cell Culture—LβT2 cells obtained from Professor Pamella Mellon (University of California, San Diego) were maintained at 37 °C in 5% CO_2 in humidified air in DMEM (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Gemini, Calabasas, CA).

siRNA Interference—1 million LβT2 cells were transfected with 0.5 μg of siRNA using Amaxa shuttle with SG buffer and DS-137 nucleofection program, following the manufacturer's instructions (Lonza Walkersville Inc., Walkersville, MD). $G\alpha_s$, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{12}$, and $G\alpha_{13}$ siRNAs were ordered from a commercial supplier (On-Target plus siRNA SMARTpool; Dharmacon, Thermo Fisher Scientific). After siRNA transfection, cells were seeded on a cell culture plate supplemented with 10% FBS + DMEM and incubated at 37 °C in 5% CO_2 incubator. 48 h after siRNA transfection, medium was exchanged from 10% FBS + DMEM to serum-free DMEM for an overnight serum starvation. 72 h after siRNA transfection, cells were treated with 1 nM GnRH or 5 μg/ml CTX. Later, cells were harvested for Western blotting or real-time PCR analysis.

Western Blot Analysis—siRNA-transfected LβT2 cells (2 million) were grown in 6-well plates. After Nonidet P-40 lysis (20 mM Tris-HCl, 1% Nonidet P-40, NaCl), cell lysates were centrifuged at 14,000 rpm for 10 min to remove cell debris. Cell lysates were mixed with an equal volume of 2× Laemmli sample buffer and boiled for 5 min at 95 °C. 50 μg of protein/well was loaded onto 10–20% Tris-HCl ready gradient gel (Bio-Rad Laboratories) and underwent electrophoresis for 1.5 h at 100 V. Proteins were transferred to H-Bond membrane (Hybond ECL; Amersham Biosciences), and the membrane was blocked for 40 min with 5% nonfat dry milk (Bio-Rad) in Tris-buffered saline. The membrane was incubated with primary antibody (1:1000) at 4 °C for overnight. Incubation with the secondary antibody (1:5000) coupled to horseradish peroxidase (Santa Cruz Biotechnology) was performed at room temperature for 45 min, which was followed by three 5-min washes with Tris-buffered saline in 1% Tween 20. Target protein bands were visualized by enhanced chemiluminescence (Amersham Biosciences) according to the manufacturer's instructions.

Quantitative Real-time PCR—For quantitative real-time PCR experiments, cells were seeded in 24-well plates at 1 million cells/well. For cell harvest, 300 μl of lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% *N*-lauroyl-sarcosine, and 0.1 M 2-mercaptoethanol) were added to each well of a 24-well plate. Total RNA was isolated using Absolutely RNA 96 micropreparation kit (Stratagene) following the manufacturer's instructions. RNA concentration was measured using NanoDrop (Thermo Scientific), and 1 μg of total RNA was used for reverse transcription using affinity script reverse transcriptase (Agilent, Santa Clara, CA). After reverse transcription, cDNA samples were diluted 1:20 in distilled H_2O. 5 μl of diluted cDNA and 5 μl of master mix (DNA polymerase, primers, dNTP, PCR buffer, and $MgCl_2$) were mixed and used for PCR. Quantitative real-time PCR assays were performed in an ABI Prism 7900HT with SYBR Green

according to the manufacturer's protocol. The results were exported as cycle threshold (CT) values, and CT values of target genes were normalized to that of rps11 in subsequent analysis. Data were expressed as arbitrary units by using the formula, $E = 2500 \times 1.93^{(\text{rps11 CT value} - \text{gene of interest CT value})}$, where E is the expression level in arbitrary units.

Luciferase Assay—0.5 μg of FSHβ or LHβ firefly luciferase reporter, 0.005 μg of thymidine kinase *Renilla* luciferase reporter (internal control), and 0.5 μg of siRNAs (control or Gα_s) were co-transfected into 1 million LβT2 cells using Amaxa shuttle with SG buffer and DS-137 nucleofection program (Lonza Walkersville). After the transfection, 0.5 million LβT2 cells were seeded to each well of 24-well cell culture plates containing 1 ml of DMEM supplemented with 10% FBS. 48 h after transfection, the medium was exchanged to 1 ml of serum-free DMEM for overnight serum starvation. 72 h after transfection, cells were stimulated with 1 nM GnRH for 6 h to induce FSHβ and LHβ firefly luciferase reporter expression. A Sirius single tube luminometer (Berthold Detection Systems, Huntsville, AL) with dual luciferase reporter assay system reagents (Promega) was used for measuring FSHβ and LHβ promoter activity.

Conditioned Media Experiment—1 million LβT2 cells were transfected with either 1 μg of control or Gα_s siRNA using Amaxa shuttle. Then cells were seeded on each well of 24-well plates supplemented with 1 ml of 10% FBS+DMEM. 48 h after the transfection, medium was exchanged to 1 ml of serum-free DMEM for overnight serum starvation. 72 h after the transfection, conditioned medium was harvested and added to naïve cells for 6 h.

Purification of Primary Gonadotropes—H2Kk mice were generously provided by Professor William Miller (University of North Carolina). Gonadotrope purification procedures were described previously (27). Briefly, pituitaries from 10–20 mice were dispersed by digestion with collagenase and pancreatin. Undigested tissue and cell aggregates were removed using a 27-μm pore size nylon mesh. Cells were then incubated with 20 μl of biotin anti-mouse H2Kk antibody (BD Pharmingen) in 180 μl of degassed PBS (BSA/EDTA) at 4 °C for 10 min. Then excess H2Kk antibodies were washed off, and 20 μl of anti-biotin paramagnetic microbeads (Miltenyi Biotec) were added to cells and rotated for 15 min at 4 °C. The cells were added to magnetic separation column (Miltenyi Biotec) and washed three times with 0.5 ml of PBS (BSA/EDTA). Cells attracted to the column were enriched gonadotropes, and other pituitary cells were removed by PBS washing. The enriched gonadotropes were subsequently eluted by removing the magnetic field. The eluted gonadotropes were reapplied to a fresh magnetic separation column, rewashed with 0.5 ml of PBS (BSA/EDTA) three times, and eluted again from the column. This second column purification ensured gonadotropes reaching >90% purity. Then cells were seeded on 96-well plates at 20,000 cells/well in M-199 Complete (Invitrogen) and incubated at 37 °C in humidified air in 5% CO_2.

Inhibinα ELISA—An inhibinα ELISA kit was purchased from a commercial supplier (MyBioSource, San Diego, CA). 1 million LβT2 cells or 80,000 purified primary gonadotropes were stimulated with 1 nM GnRH or 5 μg/ml CTX for 10 h to induce inhibin synthesis and secretion into the medium. Conditioned medium was then harvested and processed for ELISA following the manufacturer's instructions.

Perfusion Experiment—The laminar flow perfusion system was custom designed and built by the laboratory, and its performance has been extensively tested. Internal temperature is maintained at 37 °C by heating blocks. The system holds 16 coverslips in four cassettes. 1 million gonadotrope cells cultured for 2 days on the coverslide were placed in each chamber. Each chamber of the cassette is independently perfused with two electronically controlled microfluidic valves. One valve provided medium (DMEM), and another valve provided 5 nM GnRH in DMEM. Gonadotrope cells were exposed to vehicle (no GnRH control), slow pulse frequency GnRH (5-min GnRH pulse exposure every 2 h), or high pulse frequency GnRH (5-min GnRH pulse exposure every 30 min) for a 10-h period. After 10 h, cells were harvested for RNA extraction.

Statistical Analysis—Statistical calculations were performed using the Prism statistical software package version 5 (GraphPad, San Diego, CA). Data were analyzed for normality followed by calculation of ANOVA.

RESULTS

G Protein Expression in Gonadotropes—In various cell types, the GnRHR has been shown to regulate Gα_s, G$\alpha_{q/11}$, and G$\alpha_{i/o}$ family G proteins (17–24). To guide the study of G protein coding of gonadotropin expression, we determined which G protein mRNAs were expressed in gonadotrope cells. Two independent primer sets were designed for each Gα mRNA. The data from independent primer sets were concordant. In the Gα_s subfamily, Gα_s mRNA was abundantly expressed, but the expression of Gα_l mRNA was not detected (Fig. 1*A*). In the G$\alpha_{q/11}$ subfamily, Gα_q and Gα_{11} mRNAs were moderately expressed, but the expression of Gα_{14} and Gα_{15} mRNAs was not detected (Fig. 1*B*). In the G$\alpha_{12/13}$ subfamily, both Gα_{12} and Gα_{13} mRNAs were detected (Fig. 1*C*). In the G$\alpha_{i/o}$ subfamily, the expression of Gα_{i2}, Gα_{i3}, Gα_o, Gα_{t1}, and Gα_z mRNAs were detected, but not Gα_{i1}, Gα_{t2}, and Gα_{t3} mRNAs (Fig. 1*D*). Overall, among 18 specific Gα genes in the mammalian genome, 10 Gα mRNAs were expressed in LβT2 cells: Gα_s, Gα_q, Gα_{11}, Gα_{12}, Gα_{13}, Gα_{i2}, Gα_{i3}, Gα_{o1}, Gα_{t1}, and Gα_z.

G$\alpha_{i/o}$ Subfamily Is Not Involved in Expression of Either Early Genes or Gonadotropin Genes in Response to GnRH Stimulation—To test the role of G$\alpha_{i/o}$ subfamily G proteins in GnRHR signaling, gonadotropes were treated with PTX, an inhibitor of G$\alpha_{i/o}$ subfamily activation, and the effects on early gene and gonadotropin subunit induction in response to GnRH stimulation were assayed. PTX had no significant effect on early gene induction by GnRH (supplemental Fig. 1). Similarly, the induction of gonadotropin subunits in response to GnRH stimulation was unaltered (Fig. 2). These data indicate that G$\alpha_{i/o}$ subfamily proteins are not significantly involved in GnRHR signaling leading to downstream gene expression in the pituitary gonadotrope. These results are consistent with a previous report (17).

Role of Specific G Proteins in Early Gene Induction—The involvement of Gα_s, G$\alpha_{q/11}$, and G$\alpha_{12/13}$ subfamilies in GnRH-dependent gene expression was investigated using specific siRNAs against individual Gα proteins. We first examined the

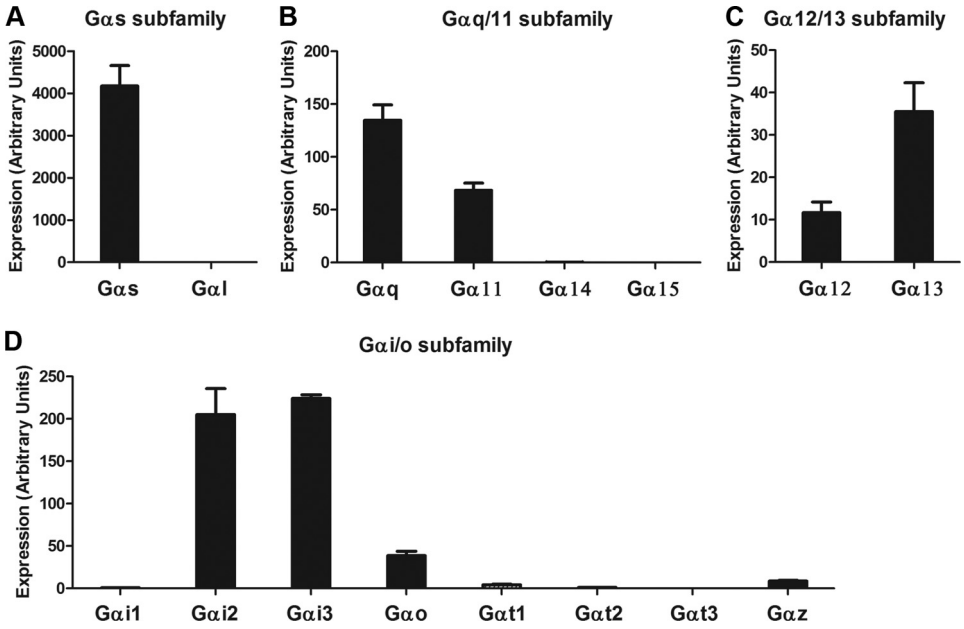

FIGURE 1. **Expression profile of Gα proteins in LβT2 gonadotropes.** mRNA expression levels of each Gα protein were determined by real-time PCR analysis. The mRNA expression of $G\alpha_s$ subfamily (*A*), $G\alpha_{q/11}$ subfamily (*B*), $G\alpha_{12/13}$ subfamily (*C*), and $G\alpha_{i/o}$ subfamily (*D*) is indicated. Two independent primer sets used for each gene gave similar results. One representative dataset is presented. *Error bars*, S.E.

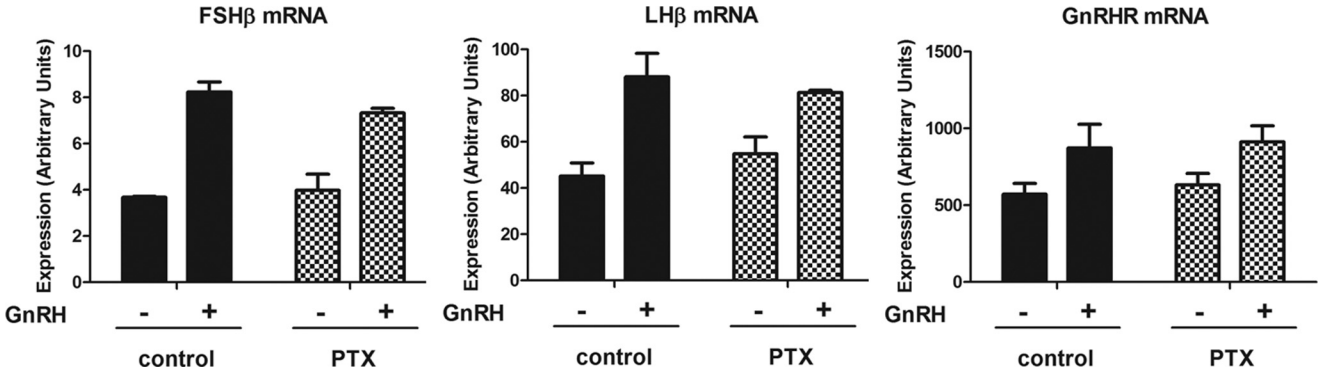

FIGURE 2. **Effect of $G\alpha_{i/o}$ subfamily inhibition on GnRH-induced gonadotropin transcription.** LβT2 gonadotrope cells were pretreated with either vehicle (*control*) or 100 ng/ml PTX for 2 h. Cells were then exposed to 1 nM GnRH for 2 h and harvested 4 h later. FSHβ, LHβ, and GnRHR mRNA expression levels were determined by real-time PCR analysis. For statistical analysis, two-way ANOVA with Bonferroni post test was used, and the effect of PTX was not significant. *Error bars*, S.E.

specificity and the efficiency of siRNA-mediated Gα mRNA knockdown in LβT2 gonadotropes by real-time PCR and Western blot analysis. Chemically modified siRNA pools were used to reduce off-target effects, and nucleofection was employed to increase efficiency. Each Gα siRNA efficiently and specifically down-regulated its target Gα mRNA, and protein. mRNA expression was reduced by 80–90% (Fig. 3*A*), and protein expression was reduced by 60–80% (Fig. 3*B* and supplemental Fig. 2). Importantly, no nonspecific Gα mRNA or protein effects were observed, suggesting that the siRNA-mediated knockdown was both efficient and specific.

GnRHR activation induces various early genes (26, 28). We examined the effects of G protein knockdown on the pattern of early gene induction by GnRH. Either control or Gα siRNAs were transfected into LβT2 gonadotropes, and 3 days after siRNA transfection, cells were exposed to GnRH. $G\alpha_s$ knockdown significantly attenuated the induction by GnRH of several early genes, including *egr3*, *fra1*, *fosB*, *cjun*, *n10*, and *rgs2*. $G\alpha_{q/11}$ knockdown significantly attenuated the induction by GnRH of all early genes assayed. $G\alpha_{12/13}$ knockdown slightly increased the induction of *cfos*, and *pip92* by GnRH suggesting that activation of $G\alpha_{12/13}$ might contribute to suppression of those genes (supplemental Fig. 3).

$G\alpha_s$ and $G\alpha_{q/11}$ Differentially Regulate FSHβ and LHβ Transcription in Response to GnRH—We studied the effects of G protein knockdown on gonadotropin subunit gene induction. $G\alpha_s$ knockdown reduced LHβ mRNA induction by GnRH by 52%. $G\alpha_{q/11}$ and $G\alpha_{12/13}$ knockdown had no significant effect on LHβ induction (Fig. 4). $G\alpha_{q/11}$ knockdown reduced FSHβ mRNA induction by GnRH by 60%, but $G\alpha_{12/13}$ knockdown had no effect on FSHβ mRNA induction by GnRH (Fig. 4). $G\alpha_s$ knockdown strongly increased both basal and GnRH-stimulated FSHβ mRNA expression. G protein knockdown studies using promoter activity reporter constructs for FSHβ and LHβ were not entirely consistent with the effects seen on mRNA expression (supplemental Fig. 4*A*; see "Discussion"). The $G\alpha_s$ knockdown results raised the possibility that $G\alpha_s$ activation leads to the suppression of FSHβ mRNA expression.

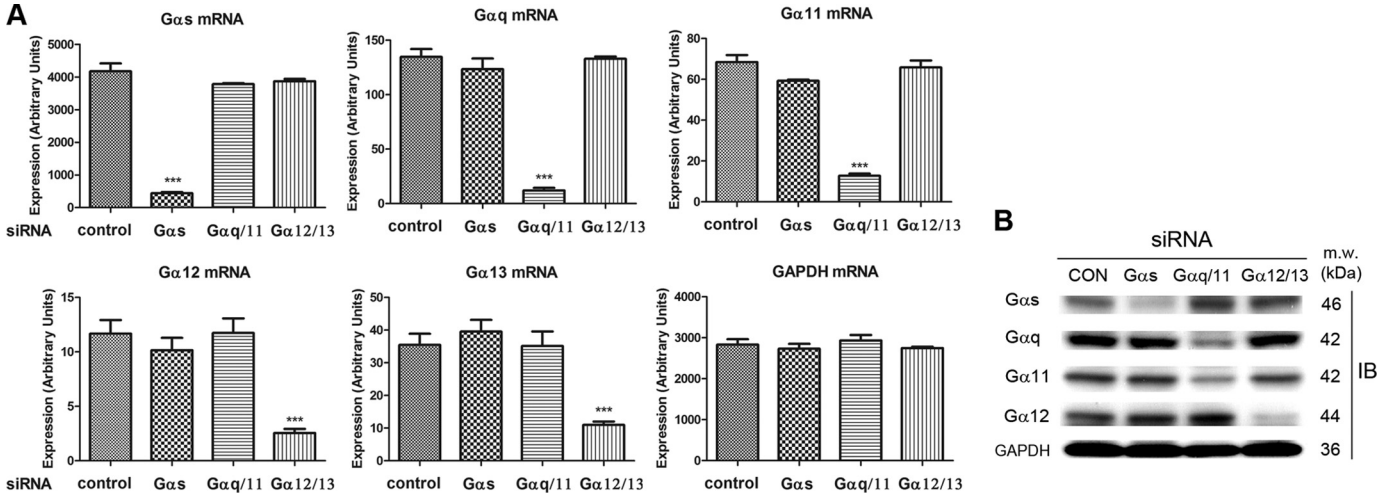

FIGURE 3. **Specificity and efficiency of Gα siRNA-mediated knockdown in LβT2 gonadotrope cells.** LβT2 gonadotrope cells were transfected with control or Gα siRNAs via nucleofection. Cells were harvested, 3 days after siRNA transfection, and the expression levels of individual Gα were monitored by real-time PCR (mRNA) and Western blot (protein) analysis. *A*, real-time PCR quantified the expression level of individual Gα mRNAs under specific Gα siRNA transfections. The mRNA expression of Gα siRNA-transfected samples was compared with that of control siRNA-treated samples. For statistical analysis, one-way ANOVA with Bonferroni post test was used. ***, $p < 0.001$. *Error bars*, S.E. *B*, Western blot analysis determined the expression level of individual Gα proteins with specific Gα siRNA transfections. GAPDH was used as a loading control. One representative dataset from three independent experiments is presented.

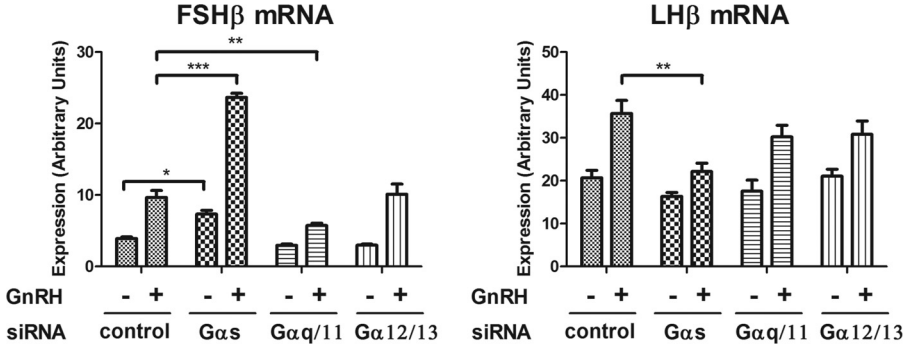

FIGURE 4. **Effect of Gαs, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ subfamily knockdown on GnRH-induced gonadotropin subunit transcription.** LβT2 cells were transfected with control or Gα siRNA by nucleofection. Three days after siRNA transfection, cells were stimulated with either vehicle (no GnRH) or 1 nM GnRH for 2 h, followed by a 4-h incubation without GnRH, to induce gonadotropin subunit expression. The effect of $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ knockdown on GnRH-induced gonadotropin subunit expression was determined by real-time PCR analysis. For statistical analysis, two-way ANOVA with Bonferroni post test was used. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. *Error bars*, S.E.

To test further the surprising observation that $G\alpha_s$ knockdown increased FSHβ mRNA levels, the assays were repeated using four sequence-independent FSHβ primer sets, which generated essentially identical results (supplemental Fig. 5). To exclude the possibility that this effect resulted from off-target knockdown of some other gene, each of the sequence-independent siRNAs comprising the pool used for the previous experiments were tested separately. If the FSHβ mRNA induction resulted from off-target effects, then a similar response would be unlikely using different independent siRNAs. However, each of the four $G\alpha_s$ siRNAs was found to up-regulate FSHβ mRNA expression (Fig. 5*A*). Further supporting the involvement of $G\alpha_s$ in this effect, the efficiency of $G\alpha_s$ knockdown and the increase in basal FSHβ were correlated ($r^2 = 0.88$, $p < 0.01$; Fig. 5*B*). These data indicate that the increased FSHβ mRNA expression was due to $G\alpha_s$ knockdown.

We also studied the effects of $G\alpha_s$ stimulation using CTX, which specifically activates $G\alpha_s$ subfamily proteins via ADP-ribosylation (29). $G\alpha_s$ stimulation by CTX strongly suppressed FSHβ mRNA expression and enhanced LHβ mRNA expression (Fig. 5*C*). These data indicate that $G\alpha_s$ activity suppresses FSHβ expression and induces LHβ expression.

We then tested whether the observations in LβT2 gonadotrope cells could be extended to the primary gonadotrope cells. We purified primary gonadotropes from the pituitaries of H2Kk transgenic mice via affinity purification as described previously (27). Among various cell types in the pituitary, only gonadotropes express GnRHR, thus GnRHR was used to determine the quality of the gonadotrope cell purification. The purification procedures resulted in >90% enrichment in primary gonadotropes (Fig. 5*D*). The purified primary gonadotropes were then tested for $G\alpha_s$ activity dependence on the regulation of FSHβ and LHβ mRNA expression. Consistently, FSHβ mRNA expression was significantly down-regulated by $G\alpha_s$ activation using CTX, whereas LHβ mRNA expression was up-regulated (Fig. 5*E*). These data indicate that $G\alpha_s$ activity suppressed FSHβ mRNA expression and enhanced LHβ mRNA expression in both LβT2 gonadotropes and purified primary gonadotropes.

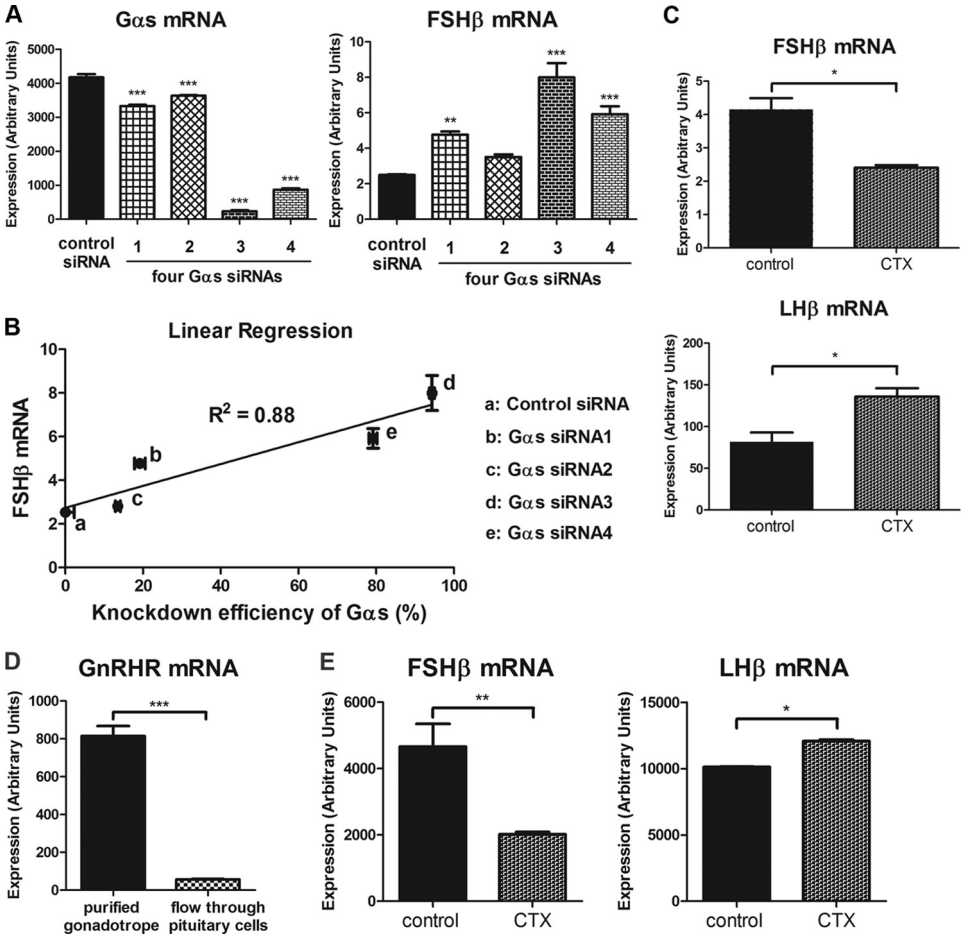

FIGURE 5. **Verification of Gα_s-dependent FSHβ and LHβ mRNA regulation.** LβT2 cells were transfected with control or Gα_s siRNAs via nucleofection. Cells were harvested for real-time PCR analysis, 3 days after siRNA transfection. *A*, four independent Gα_s siRNAs were used to test the specificity of Gα_s siRNA-mediated knockdown and its effect on FSHβ mRNA expression. *B*, linear regression analysis was conducted to examine the correlation between Gα_s knockdown efficiency and increased FSHβ mRNA expression. *C*, LβT2 cells were exposed to 5 μg/ml CTX for 7 h, and the effect of specific Gα_s stimulation on gonadotropin subunit mRNA expression was monitored by real-time PCR. *D* and *E*, primary gonadotrope cells were purified from the pituitaries of H2Kk transgenic mice. *D*, the quality of primary gonadotrope purification was determined by measuring GnRHR mRNA expression of purified gonadotropes and nonpurified flow-through pituitary cells. *E*, purified primary gonadotropes were stimulated with either vehicle (no CTX) or 5 μg/ml CTX for 7 h. FSHβ and LHβ mRNA expression levels were determined by real-time PCR analysis. For statistical analysis, one-way ANOVA (*A*) and two-tailed *t* test with Bonferroni post test (*C–E*) were used. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. *Error bars*, S.E.

Gα_s-dependent FSHβ mRNA Suppression Is Mediated by Secreted Factors—The studies described above show that activation of Gα_s protein by GnRH or CTX suppressed FSHβ gene expression and induced LHβ gene expression. Several secreted proteins are known to regulate gonadotropin expression (30–34). We speculated that the Gα_s-mediated suppression of FSH might result from secreted autocrine and paracrine factors. To test this idea, we first conducted a Transwell experiment (Fig. 6*A*). Gonadotrope cells transfected with control or Gα_s siRNAs were seeded on each side of apical or basolateral chambers. We observed that regardless of cell seeding position (either apical or basolateral) control siRNA-transfected gonadotropes had increased FSHβ mRNA expression, when Gα_s siRNA-transfected cells were seeded on the chamber across the membrane (Fig. 6*B* and supplemental Fig. 6). Gα_s mRNA expression in control-transfected cells was not changed.

We next tested whether the conditioned medium from Gα_s knockdown cells regulated FSHβ mRNA levels in naïve cells. Fresh medium was incubated with control or Gα_s siRNA-transfected cells overnight, and this conditioned medium was transferred to naïve gonadotrope cells. We found that the conditioned medium from Gα_s knockdown cells significantly increased FSHβ mRNA expression in untransfected cells. Also consistent with the Transwell results, Gα_s mRNA expression was not altered by conditioned medium (Fig. 7*A*).

We next characterized the size of the secreted factors using a 3-kDa molecular mass cutoff filter to process conditioned medium from control and Gα_s siRNA-treated cells and exposing naïve cells to each heavy and light fraction. The filtrate fractions did not contain FSHβ regulatory activity, suggesting that the factor or factors were likely to be proteins larger than 3 kDa (Fig. 7*B*). We also found that proteinase K, a broad spectrum endopeptidase, eliminated regulatory activity in conditioned medium (Fig. 7*C*). Proteinase K-treated conditioned medium did not impair the capacity of cells to respond to GnRH (data not shown). Conditioned medium from CTX-treated cells suppressed FSHβ mRNA and stimulated LHβ mRNA (Fig. 7*D*). These results support the presence of secreted, Gα_s-regulated proteins that suppress FSHβ mRNA in an autocrine and paracrine fashion.

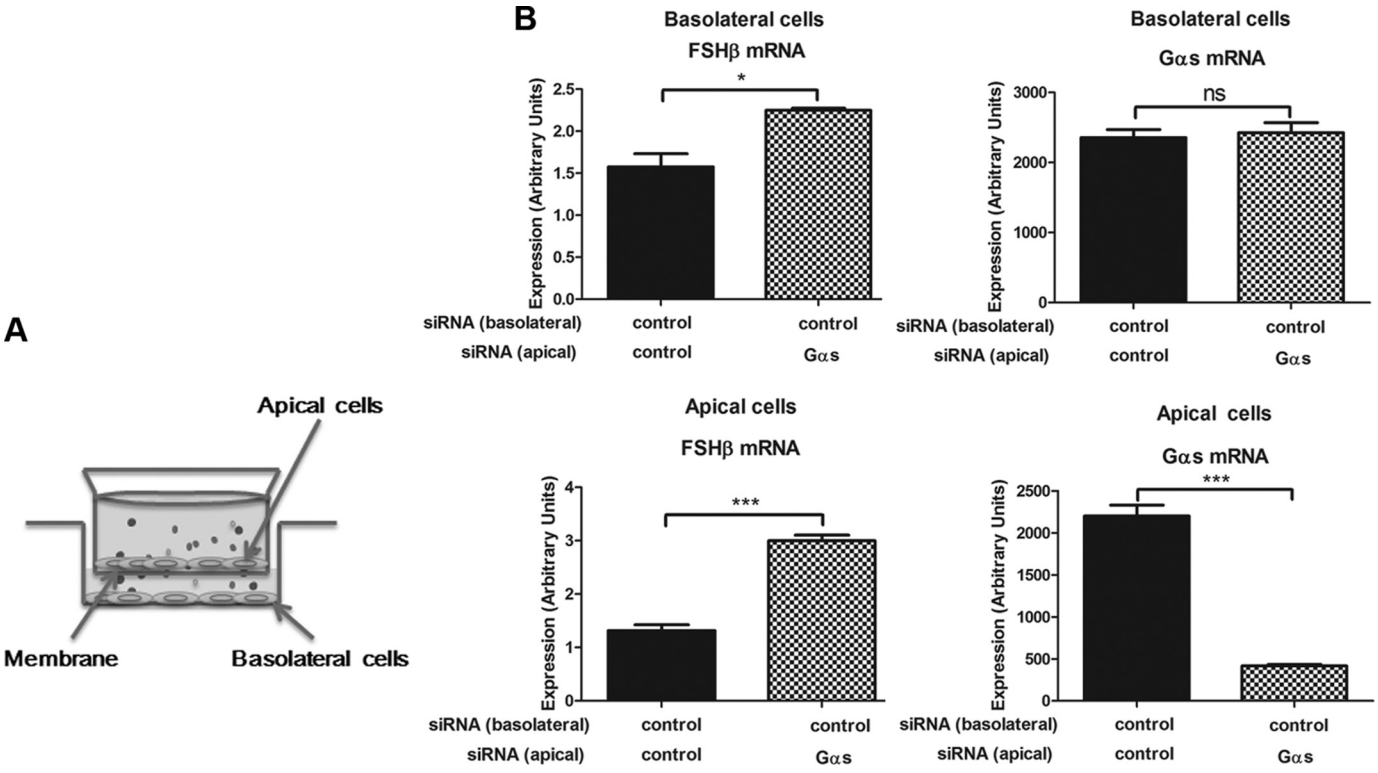

FIGURE 6. **Transwell experiment testing the involvement of Gα_s-dependent secreted factors regulating FSHβ transcription.** LβT2 gonadotrope cells were transfected with control or Gα_s siRNAs via nucleofection. siRNA-transfected gonadotrope cells were then seeded on apical or basolateral chambers of the Transwell. Medium was replaced with fresh medium, 2 days after siRNA transfection. At day 3, cells were harvested, and the expression levels of FSHβ and Gα_s mRNAs were determined by real-time PCR analysis. *A*, schematic shows Transwell experiment. *B*, expression levels of FSHβ and Gα_s mRNAs from apical and basolateral cells in the Transwell were determined by real-time PCR analysis. For statistical analysis, two-tailed *t* test with Bonferroni post test was used. *ns*, nonsignificant. ***, $p < 0.001$; *, $p < 0.05$. *Error bars*, S.E.

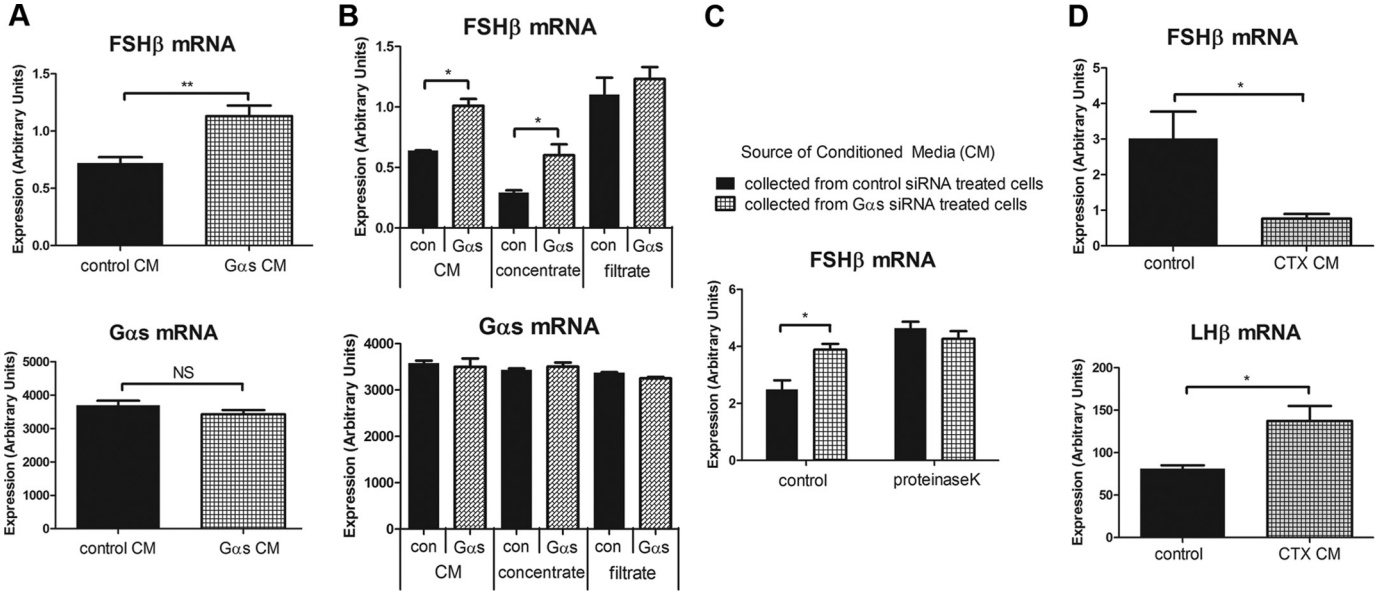

FIGURE 7. **Gα_s-dependent gonadotropin mRNA regulation is mediated by released autocrine/paracrine peptide(s).** *A–C*, LβT2 gonadotrope cells were transfected with control or Gα_s siRNAs via nucleofection. Two days after siRNA transfection, medium was replaced with fresh medium. At day 3, overnight conditioned medium (*CM*) was collected from siRNA-transfected cells, and it was added to non-siRNA-treated recipient cells. *A*, conditioned medium from control and Gα_s knockdown cells was added to the non-siRNA-treated recipient cells for 6 h, and the effect on FSHβ and Gα_s transcription was monitored by real-time PCR analysis. *B*, conditioned medium from control and Gα_s knockdown cells passed through 3-kDa molecular mass cutoff filter. Concentrate fraction (enriched with more than 3-kDa proteins) and filtrate fraction (depleted from more than 3-kDa proteins) were added to non-siRNA-treated recipient cells for 6 h. *C*, conditioned medium from control and Gα_s knockdown cells was treated with proteinase K and then heat-denatured. Both control and proteinase K-treated conditioned media were added to the recipient cells, and the effect on FSHβ transcription was monitored by real-time PCR. *D*, conditioned medium from control and CTX-pretreated cells was collected and was added to non-CTX-treated recipient cells for 6 h. FSHβ and LHβ mRNA expression was monitored by real-time PCR. For statistical analysis, two-tailed *t* test was used. **, $p < 0.01$; *, $p < 0.05$. *Error bars*, S.E.

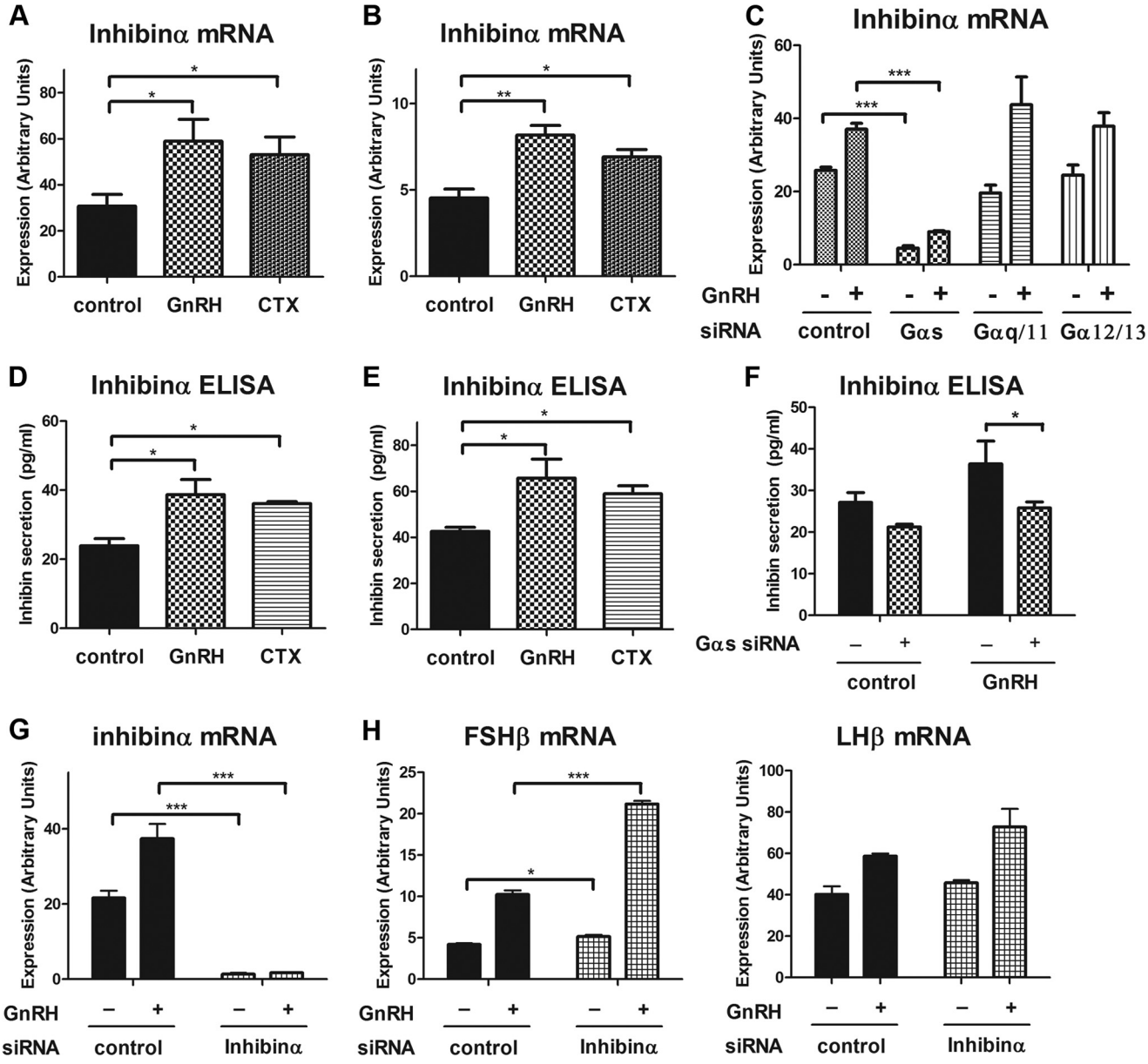

FIGURE 8. **Inhibin is a $G\alpha_s$-dependent autocrine/paracrine factor involved in the feedback suppression of FSHβ expression in response to GnRH stimulation.** *A* and *B*, LβT2 gonadotrope cells (*A*) or purified primary gonadotrope cells (*B*) were stimulated with vehicle, 1 nM GnRH, or 5 μg/ml CTX for 8 h. The level of inhibinα mRNA expression was determined by real-time PCR. *C*, LβT2 gonadotrope cells were transfected with either control siRNA or Gα siRNAs via nucleofection. Three days after siRNA transfection, cells were stimulated with either vehicle or 1 nM GnRH for 6 h. Real-time PCR was used to determine inhibinα mRNA expression. *D* and *E*, LβT2 gonadotropes (*D*) or purified primary gonadotrope cells (*E*) were stimulated with vehicle, 1 nM GnRH, or 5 μg/ml CTX for 10 h. Conditioned medium was harvested, and secreted inhibinα protein was measured by ELISA. *F–H*, LβT2 gonadotrope cells were transfected with either control siRNA or $G\alpha_s$ siRNA (*F*) or inhibinα siRNA (*G* and *H*) via nucleofection. Three days after siRNA transfection, cells were stimulated with either vehicle or 1 nM GnRH for 6 h and tested for inhibinα ELISA (*F*) or the mRNA expression of inhibinα, LHβ, and FSHβ (*G* and *H*) by real-time PCR. For statistical analysis, one-way ANOVA (*A*, *B*, *D*, and *E*) and two-way ANOVA with Bonferroni post test (*C* and *F–H*) were used. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. *Error bars*, S.E.

Role of Inhibin as a $G\alpha_s$-dependent Autocrine/Paracrine Factor Suppressing FSHβ mRNA Expression—We used a candidate approach to search for regulated, secreted factors involved in FSHβ regulation. We first studied whether the TGFβ superfamily member follistatin played a role in $G\alpha_s$-dependent FSHβ suppression. Titrated follistatin experiments looking for competitive inhibition of FSHβ expression and PCR assays for follistatin regulation excluded its role. Follistatin expression level was low, and it was not G_s-dependent (data not shown). We next studied inhibin which is a heterodimeric peptide hormone produced in the ovary that inhibits FSH synthesis in the pituitary. Inhibinα subunit interacts with the activin type I receptor, antagonizes the activin signaling in the pituitary gonadotrope (35), and has been reported to be regulated by GnRH in the gonadotrope (36, 37). Therefore, we tested whether inhibinα expression could be regulated by GnRH stimulation in a $G\alpha_s$-dependent manner. We observed that inhibinα mRNA expression was significantly up-regulated by GnRH stimulation and also by $G\alpha_s$ activation using CTX both in the gonadotrope cell line and in primary gonadotrope cells (Fig. 8, *A* and *B*). Inhibinα mRNA expression was markedly down-regulated in $G\alpha_s$ knockdown gonadotrope cells under basal and GnRH-stimu-

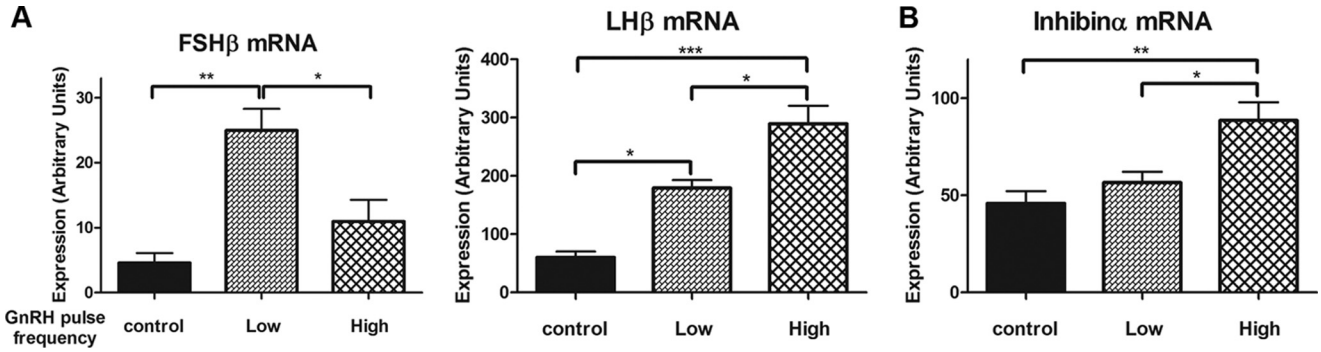

FIGURE 9. **Inhibin expression is regulated in a GnRH pulse frequency-specific manner.** LβT2 gonadotrope cells were stimulated with vehicle or high or low frequency GnRH pulses using a custom designed cell perfusion system. For high pulse frequency GnRH stimulation, gonadotrope cells were exposed to 5 min of 5 nM GnRH pulses every 30 min for a 10-h period; and for low pulse frequency GnRH stimulation, cells were exposed to 5 min of 5 nM GnRH pulses every 2 h for a 10-h period. The levels of FSHβ and LHβ (*A*) and inhibinα (*B*) mRNA expression were monitored by real-time PCR analysis. For statistical analysis, one-way ANOVA with Bonferroni post test was used. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. *Error bars*, S.E.

lated conditions, but it was not affected by the knockdown of other Gα proteins (Fig. 8*C*). In addition, we observed that inhibinα protein secretion was stimulated by GnRH and by $G\alpha_s$ activation both in LβT2 cells and in purified primary gonadotropes (Fig. 8, *D* and *E*). Both basal and GnRH-stimulated inhibinα protein secretion were significantly down-regulated by $G\alpha_s$ knockdown (Fig. 8*F*). Together, these data indicate that inhibinα mRNA expression and protein secretion were induced by GnRH stimulation in a $G\alpha_s$-dependent manner.

To determine whether inhibin acted as an endogenous autocrine/paracrine factor suppressing FSHβ mRNA expression in the gonadotrope, we down-regulated inhibinα expression by inhibinα siRNA and monitored the effect on gonadotropin gene expression. Inhibinα mRNA expression was efficiently down-regulated (>90%) by inhibinα siRNA (Fig. 8*G*). With inhibinα knockdown, both basal and GnRH-induced FSHβ mRNA expression were significantly up-regulated (Fig. 8*H*), supporting the role of inhibin as a GnRH-stimulated, $G\alpha_s$-dependent endogenous autocrine/paracrine regulatory factor.

Inhibin Expression Is Regulated by GnRH in Pulse Frequency-sensitive Manner—We used a custom perfusion system to monitor the mRNA expression patterns of FSHβ, LHβ, and inhibinα in response to pulsatile GnRH stimulation. Consistent with previous studies, the expression of LHβ and FSHβ mRNAs was differentially regulated under low and high pulse frequencies of GnRH stimulation. FSHβ mRNA expression strongly favored low frequency GnRH pulses, whereas LHβ mRNA expression favored high frequency GnRH pulses (Fig. 9*A*). Notably, inhibinα mRNA expression is specifically up-regulated under high pulse frequency GnRH stimulation, but not under slow pulse frequency GnRH stimulation (Fig. 9*B*). Overall, our data suggest that inhibin contributes to the autocrine/paracrine-mediated negative feedback control of FSHβ expression in response to GnRH stimulation and to the suppression of FSHβ expression under high pulse frequency GnRH stimulation.

DISCUSSION

Specific perturbation of $G\alpha_s$ and $G\alpha_{q/11}$ differentially affected the GnRH-stimulated early gene and gonadotropin gene expression patterns. $G\alpha_{i/o}$ and $G\alpha_{12/13}$ played relatively minor roles. Our results are consistent with previous reports suggesting that GnRH signaling in gonadotrope cells involves mainly $G\alpha_{q/11}$ and $G\alpha_s$ (17, 18, 38, 39), whereas $G\alpha_{i/o}$ proteins are less important (17). Notably, we found distinct roles for $G\alpha_s$ and $G\alpha_{q/11}$ in controlling gonadotropin subunit expression. $G\alpha_{q/11}$ predominantly mediated FSHβ transcription, whereas $G\alpha_s$ mediated LHβ transcription and suppressed FSHβ transcription.

The finding that $G\alpha_s$ contributed to suppression of FSHβ mRNA was unexpected, as the FSHβ promoter has been reported to have activating CREB sites that could be stimulated by $G\alpha_s$ signaling (40). Experiments using separate individual $G\alpha_s$ siRNAs and stimulatory experiments in the gonadotrope cell line and in primary mouse gonadotrope cells using CTX confirmed that the major effect of $G\alpha_s$ signaling on FSHβ expression was inhibitory. Notably, a previous study indicated that $G\alpha_s$ activation using CTX in dispersed rat pituitary cells significantly down-regulated FSH secretion and up-regulated LH secretion (41).

The regulation of gonadotropin gene promoter activity, as determined using reporter assays, often diverged from the regulation of the endogenous mRNA levels. For example, $G\alpha_s$ knockdown enhanced GnRH-stimulated FSHβ mRNA expression, whereas it reduced FSHβ reporter activity (see supplemental Fig. 4*A*). LHβ mRNA induction was eliminated by $G\alpha_s$ knockdown, whereas the induction of LHβ reporter activity persisted (supplemental Fig. 4*A*). Furthermore, the concentration response curve of LHβ and FSHβ mRNA induction by GnRH was U-shaped whereas the response of the each promoter construct was sigmoidal (supplemental Fig. 4*B*). These results suggest that the mechanisms underlying control of mRNA levels are more complex than can be accounted for by regulation of the proximal promoter. Expression and stability of mRNAs may be controlled by diverse mechanisms such as upstream promoter region, distant enhancer elements, chromatin structure (42), mRNA processing (43), and mRNA stability and degradation (44, 45). mRNA processing has been implicated in regulating gonadotropin mRNA levels (46).

Several studies have suggested paracrine factors that influence gonadotropin expression (30–34),45–50). We studied whether the $G\alpha_s$-mediated suppression of FSH might result from secreted autocrine factors. Transwell and conditioned

media experiments indicated that secreted proteinase K-sensitive factors larger than 3 kDa suppressed FSHβ expression. Several known autocrine/paracrine factors may be involved. Using a candidate approach, we have identified inhibin as a GnRH-induced, Gα_s-dependent autocrine/paracrine factor that mediates FSHβ suppression in gonadotropes.

We identified expression of all Gα G protein genes in gonadotrope cells except Gα_l, Gα_{14}, G$\alpha_{15/16}$, Gα_{i1}, Gα_{t2}, and Gα_{t3}. Many nonexpressed G proteins are tissue-specific genes, thus the absence of their expression in gonadotropes is not surprising. For example, Gα_l is involved in odorant transduction and is exclusively expressed in the olfactory receptor neurons (47), and Gα_{t2} and Gα_{t3} are involved in light detection and found predominantly in photoreceptor cells of the eye (48). G$\alpha_{15/16}$ expression is restricted to tissues rich in hematopoietic cell types such as spleen, thymus, and bone marrow (49, 50).

Experimentally, high frequency stimulation favors LH gene induction, whereas low frequency favors FSH gene induction. The differential induction of inhibin by Gα_s and FSHβ by Gα_q provides a simple, yet novel mechanism to explain the preferential induction of FSHβ by low frequency GnRH stimulation of the gonadotrope. High frequency stimulation favors inhibin expression which consequently suppresses FSHβ gene induction. Low frequency stimulation reduces the inhibin level and allows higher levels of FSHβ expression.

Our results suggest that the signaling network between the cell surface GnRHR and the gonadotropin genes includes previously unrecognized extracellular regulatory loops. We propose that differential targeting of LH and FSH gene regulation by distinct G protein pathways and autocrine factors such as inhibin underlies the frequency-dependent regulation of the gonadotropin genes by GnRH. Further experimental studies and mathematical simulations will be required to test this hypothesis.

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