ERK Signaling in the Pituitary Is Required for Female But Not Male Fertility

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Males and females require different patterns of pituitary gonadotropin secretion for fertility. The mechanisms underlying these gender-specific profiles of pituitary hormone production are unknown; however, they are fundamental to understanding the sexually dimorphic control of reproductive function at the molecular level. Several studies suggest that ERK1 and -2 are essential modulators of hypothalamic GnRH-mediated regulation of pituitary gonadotropin production and fertility. To test this hypothesis, we generated mice with a pituitary-specific depletion of ERK1 and 2 and examined a range of physiological parameters including fertility. We find that ERK signaling is required in females for ovulation and fertility, whereas male reproductive function is unaffected by this signaling deficiency. The effects of ERK pathway ablation on LH biosynthesis underlie this gender-specific phenotype, and the molecular mechanism involves a requirement for ERK-dependent up-regulation of the transcription factor Egr1, which is necessary for LHβ expression. Together, these findings represent a significant advance in elucidating the molecular basis of gender-specific regulation of the hypothalamic-pituitary-gonadal axis and sexually dimorphic control of fertility. **(*Molecular Endocrinology* 23: 1092–1101, 2009)**

The MAPK signaling pathways comprise a conserved set of signal transduction modules that are activated in response to a variety of extracellular stimuli (1). The ERK pathway is the most thoroughly characterized of the MAPK systems and consists of a three-level phosphorylation cascade, which, in its canonical form, includes the MAPK-kinase-kinase Raf-1, the MAPK kinases MEK1 and MEK2, and the MAPK's ERK1 (MAPK3) and ERK2 (MAPK1). Activated ERKs phosphorylate a multitude of targets throughout the cell, exerting broad regulatory influence over a wide range of processes including transcription, translation, cell cycle regulation, cytoskeletal remodeling, and apoptosis (1).

ERK1 and 2 are generally thought to serve overlapping functions; however, it has also been demonstrated that ERK1 and 2 may play quite distinct roles in some differentiated cells (2). In addition, the ERK1 null mouse is viable and fertile whereas the ERK2 null is embryonic lethal (3, 4). Thus, ERK1 and 2 clearly serve highly divergent functions during development (5). A genetic model that would allow for systematic analysis of the functions of ERK1 and 2 within specific cellular or developmental contexts is highly desirable; however, the embryonic lethality of the ERK2 null mouse necessitates a conditional approach to the ablation of ERK2 and has presumably hampered development of such a model.

The pituitary gland is a complex endocrine organ that regulates many aspects of mammalian homeostasis. The anterior pituitary is composed of several major hormone-producing cell types including thyrotropes that produce TSH and gonadotropes that produce FSH and LH. TSH, LH, and FSH are heterodimeric hormones that have distinct β-subunits but share a common α-subunit (glycoprotein hormone α-subunit, αGSU). The αGSU is the first differentiated cell marker to appear during pituitary development (6). Reporter assays suggest that the *αGSU* promoter may become activated as early as embryonic d 9.5 (e9.5) throughout the pituitary primordium; however, subsequent expression of αGSU becomes restricted to thyrotropes and gonadotropes (7–9).

Thyrotropes and gonadotropes play important roles in the regulation of metabolic and reproductive function, respectively.

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Abbreviations: DKO, double knockout; EGR1, early growth response factor-1; GnRHR, GnRH receptor; αGSU, glycoprotein hormone α-subunit; MEK, MAPK kinase; qPCR, quantitative PCR.

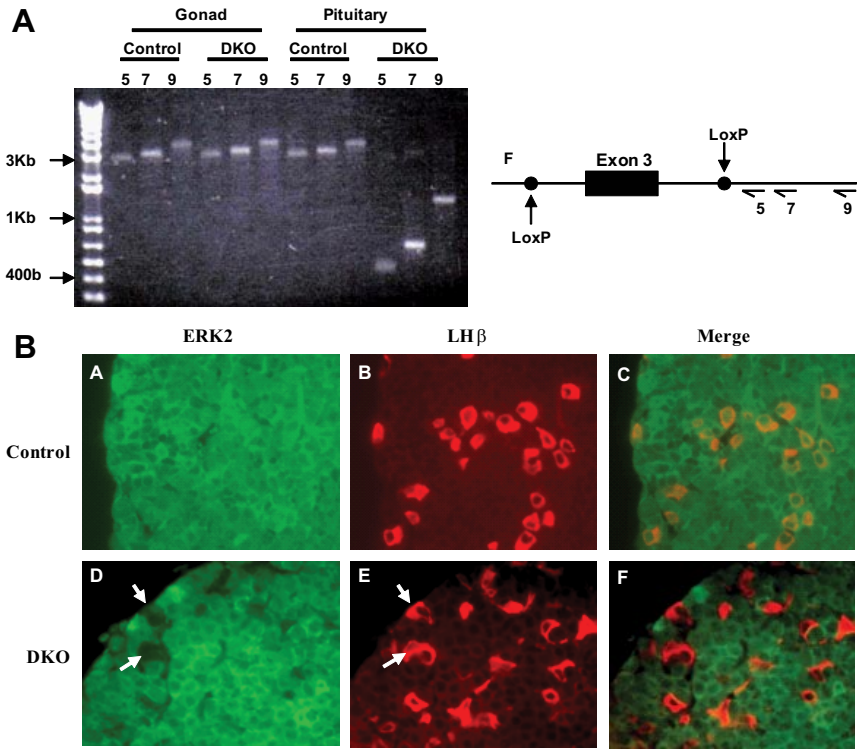

FIG. 1. Validation of the ERK1/2 DKO mouse. A, Cre-mediated recombination at the *ERK2* locus within the pituitary was detected by PCR using genomic DNA from the specified tissues. For each DNA sample, the forward primer designated "F" was paired individually with reverse primers labeled "5," "7," and "9" spanning the floxed genomic region as indicated in the *schematic*. Lanes are labeled with the specified reverse primer used for the reaction. Molecular weight marker is shown in the *left lane*. Equivalent results were obtained from males and females. B, Cell type-specific loss of ERK protein in the ERK1/2 DKO was determined by coimmunofluorescent labeling of pituitary sections from control (panels A–C) and DKO (panels D–F) male animals using fluorescein isothiocyanate-conjugated antibodies against ERK1/2 and Texas Red-conjugated antibodies against LH-β. ERK (A and D), LHβ (B and E), and simultaneous visualization of both wavelengths (C and F) are shown. *Arrows* indicate representative LHβ-positive cells with distinct lack of ERK labeling. *Bars*, 100 μm.

Stimulation of these cells by the hypothalamic releasing hormones TRH or GnRH leads to synthesis and secretion of TSH by thyrotropes, and FSH and LH by gonadotropes. The ERK pathway is activated in both thyrotropes and gonadotropes after stimulation by TRH or GnRH. Little is known regarding the importance of the ERK pathway in thyrotropes. In contrast, many studies suggest that ERK signaling is critical for the expression of several genes essential for the function of gonadotropes, including the *αGSU*, *LHβ*, and a regulatory MAPK phosphatase (*MKP2/DUSP4*) (10–12). Data supporting the importance of ERK signaling for gonadotrope function have been generated primarily through study of the αT3–1 and LβT2 gonadotrope-derived cell lines. These cell lines have been useful tools in the molecular dissection of gonadotrope function; however, they are isolated from the complex endocrine environment of the living animal. In light of the need for an *in vivo* model for study of the role of ERK signaling in the pituitary, we used Cre-LoxP technology to generate mice in which ablation of ERK1 and 2 were targeted to the αGSU-expressing cells of the anterior pituitary. Our results demonstrate that ERK signaling is required for fertility only in the female and provide new insight into the mechanisms underlying sexually dimorphic regulation of reproductive function.

Results

Generation and validation of the pituitary-targeted ERK1/2 double-knockout mouse

To define the physiological importance of ERK signaling in pituitary gonadotropes and thyrotropes, we began by generating mice with a conditional, pituitary-targeted ablation of ERK2. Mice homozygous for a floxed mutation at the *ERK2* locus (*ERK2$^{fl/fl}$*), were crossed with αGSU:Cre mice, in which Cre recombinase is expressed under the regulatory control of a 4.6-kb fragment of the murine αGSU promoter. *ERK2$^{fl/fl}$*, *αGSU:Cre* mice were then mated to ERK1 null mice to generate *ERK2$^{fl/fl}$,ERK1$^{-/-}$*, *αGSU:CRE*, hereafter designated DKO (double knockout). Pituitary-specific ERK2 null males and females (with normal ERK1) were viable and fertile. They were born at expected Mendelian frequencies and were grossly and histologically unremarkable at 3 months of age (data not shown). In contrast, at 3 wk of age, DKO animals were represented at less than the expected frequency (expected: 0.5, observed: 0.24; n = 327). Within litters of neonates, some DKO animals were frequently identified either dead, or as unthrifty pups that failed to nurse and that died shortly after birth.

To validate the fidelity of αGSU:Cre-mediated recombination, we crossed *αGSU:Cre* males with Rosa26 reporter (R26R) females and assayed β-galactosidase activity in tissue lysates from both Cre-expressing, and nonexpressing offspring. β-Galactosidase activity was significantly increased in pituitary lysates from Cre-expressing animals as compared with Cre-negative controls (data not shown). Differences in β-galactosidase activity were not observed in lysates from other tissues (lung, kidney, heart, and brain). These data support the pituitary-enriched activity of the αGSU:Cre. Cre-dependent recombination specifically at the *ERK2* locus was verified by PCR from *ERK2$^{fl/fl}$,CRE^{+}* and *ERK2$^{fl/fl}$,CRE^{-}* animals using primers flanking the floxed region of the *ERK2* locus (Fig. 1A, *schematic*). Products indicating excision of the floxed region of the *ERK2* gene were only produced from pituitary genomic DNA of Cre^{+} individuals, indicating tissue-specific susceptibility of the *ERK2* locus to Cre-mediated recombination (Fig. 1A, *left panel*).

Immunohistochemical colabeling of pituitary sections from adult DKO and control animals using antibodies against ERK2 and LHβ confirmed a high level of colocalization between LHβ and negative ERK labeling, indicating that ERK deficiency was relatively penetrant within the gonadotropes of DKO animals (Fig. 1B). Immunohistochemical identification of TSHβ within ERK-deficient, LHβ-negative cells in these sections confirmed the prediction that αGSU-expressing thyrotropes were also rendered ERK

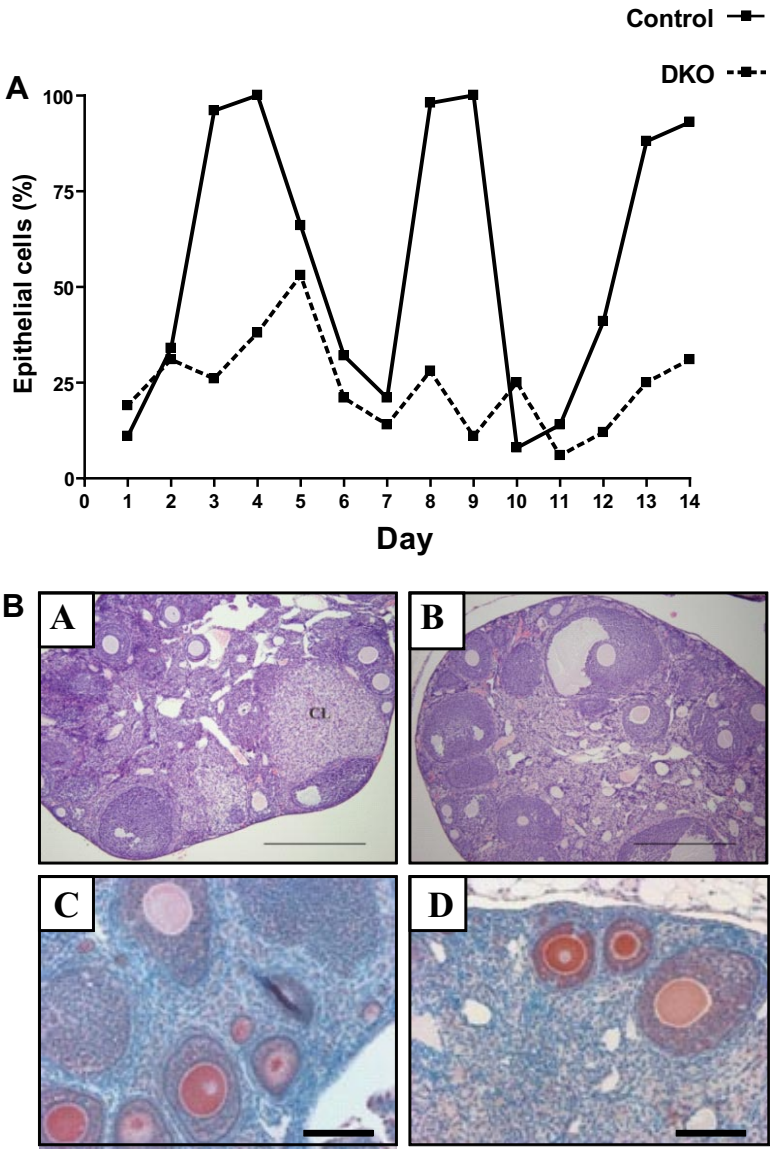

FIG. 2. Assessment of estrous cycle activity in the ERK1/2 DKO mouse. A, Vaginal cytology was performed at 24-h intervals by microscopic examination of Wright's stained vaginal lavage effluents. One hundred cells were counted, and the percentage of epithelial cells at each time point was determined. Data are shown for a single animal; however, similar results were obtained from three animals of each genotype. B, Hematoxylin and eosin-stained histological sections of ovaries from control (panel A) and DKO (panel B) animals are shown. A representative corpus luteum is indicated (CL). Microscopic sections from control (panel C), and DKO (panel D) ovaries were immunohistochemically stained using an antibody against ERK1/2. Both control and DKO animals were ERK1 null; thus all specific labeling represents ERK2 protein. A and B, *bars*, 200 μm; C and D, *bars*, 100 μm.

deficient within this line (data not shown). Overall, these results support the fidelity of the compound genetic deletion within the adult DKO animals.

Phenotypic characterization of the ERK1/2 DKO mouse

DKO animals of both genders grew at the same rate as Cre$^-$ littermate controls and were grossly indistinguishable from controls from birth through 8 months of age (data not shown). Test matings indicated that DKO males were fertile, whereas females showed no evidence of estrous cycle activity and did not mate. To further assess estrous cycle activity, vaginal cytology was performed in control and DKO mice. Cyclic cytological changes indicative of estrous cycle activity were observed in control mice (Fig. 2A). In contrast, vaginal cytology in the DKO females showed a preponderance of polymorphonuclear leukocytes with occasional basal epithelial cells over the course of the study, consistent with anestrous behavior.

Histological examination of a variety of tissues including the reproductive tract from DKO and control males revealed no abnormalities. In contrast, ovaries from DKO females contained follicles at various stages of maturation (primary through large antral follicles) but were specifically devoid of luteal tissue, whereas ovaries from control females were histologically unremarkable (Fig. 2B, panels A and B). The combined data from cytological analysis of estrous cycle behavior and histological evidence of lack of luteal tissue within the ovary in DKO female mice supported the preliminary conclusion that the DKO mice were anovulatory. To address whether the ovarian lesion in DKO animals may reflect promiscuous Cre expression and lack of ERK2 protein in the ovary, we examined ERK2 expression in ovaries from DKO and control females immunohistochemically. Expression of ERK2 was observed in both DKO and control ovaries, indicating that the anovulatory ovarian lesion was secondary to the primary pituitary defect (Fig. 2B, panels C and D).

ERK1/2 DKO mice are euthyroid

Because αGSU is expressed in thyrotropes as well as in gonadotropes, our model would predict fully penetrant Cre-dependent disruption of ERK signaling in the thyrotropes of DKO animals. To evaluate thyroid function in these animals, we used quantitative PCR (qPCR) to measure whole pituitary content of TSHβ mRNA in DKO and control animals. Pituitary content of TSHβ protein was examined by immunoblot analysis, and T4 levels in serum were examined using an ELISA. Pituitary TSHβ mRNA, TSHβ protein in whole pituitary lysates or serum T4 concentrations did not differ between DKO and control mice (Fig. 3). These results confirm that DKO animals that survived to adulthood were euthyroid and effectively rule out thyroid dysfunction as a contributing factor to the anovulatory phenotype.

Expression of LHβ is reduced in pituitaries of ERK1/2 DKO animals

The lack of luteal tissue in the ovaries of DKO females suggests that DKO females were anovulatory and implicates a gonadotropin deficiency in these animals. To test this, we used qPCR to measure transcript levels of the gonadotropin subunits (LHβ, FSHβ, and αGSU), as well as the GnRH receptor (GnRHR) in the pituitaries of DKO and control animals. In

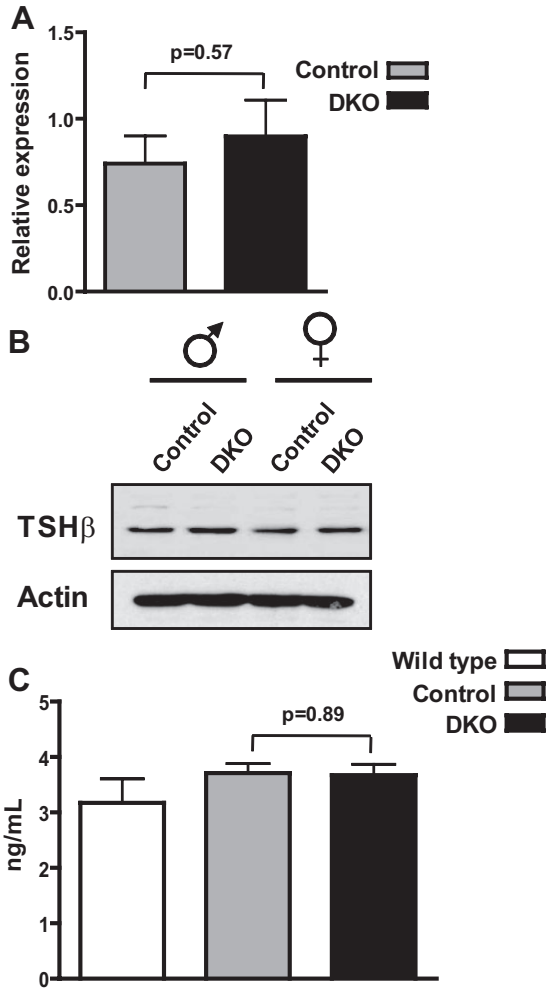

FIG. 3. Evaluation of thyroid function in the ERK1/2 DKO mouse. A, Relative transcript levels of *TSHβ* from whole pituitaries of female control and DKO animals were determined by quantitative PCR. Results were calibrated to levels of *TSHβ* mRNA from pituitaries of randomly cycling female wild-type mice. *Bars* represent mean ± SEM for five animals of each genotype. Means were compared by two-tailed *t* test. B, Levels of TSHβ in whole pituitary lysates from control and DKO animals were compared by immunoblot. Actin is shown as a lane-loading control. C, Serum levels of total T4 from randomly cycling wild-type, control, and DKO females were determined by ELISA. *Bars* represent mean ± SEM for three (wild type) or 11 (control and DKO) animals in the respective groups. Means for control and DKO groups were compared by two-tailed *t* test.

females, LHβ mRNA levels were significantly lower in DKOs as compared with randomly cycling control animals (Fig. 4A). In males, LHβ levels were not statistically significantly different between DKO and control animals (Fig. 4A). Transcript levels of the other gonadotropin subunits, as well as the GnRHR, did not differ between DKO and control animals for either gender (Fig. 4A).

To assess the effects of ERK deletion on pituitary stores of gonadotropin subunits, we examined pituitary lysates by immunoblotting using antibodies against LHβ and FSHβ. LHβ was less abundant in pituitaries of DKO animals of both genders, as compared with controls, and was essentially undetectable in the pituitaries of DKO females (Fig. 4B). Levels of FSHβ were similar between DKO and control animals of both genders (Fig. 4B).

The anovulatory phenotype of ERK1/2 DKO females is rescued by pharmacological superovulation

Our histological findings and gene profiling data suggest that the anovulatory infertility in the DKO mice was a direct consequence of LH deficiency. If correct, we reasoned that the ovaries of DKO females should remain capable of mounting an ovulatory response to an appropriate supraovarian gonadotropic stimulus. To test this, we subjected DKO and control females to conventional pharmacological superovulation. Corpora lutea were present in the ovaries of gonadotropin-treated DKO animals albeit at a frequency (number of corpora lutea per ovary) less than that observed in superovulated control females (Fig. 5). These results confirm the intrinsic ability of DKO ovaries to respond to an ovulatory signal.

ERK signaling is required for the response of the gonadotropin subunits after gonadectomy

Gonadal steroids regulate the expression of the gonadotropin subunit genes within the pituitary through negative feedback inhibition of GnRH release from the hypothalamus. Accordingly, gonadectomy leads to increased expression of the β-subunits of LH and FSH through disinhibition of the hypothalamic GnRH system (13, 14). To determine the role of ERK signaling in mediating the gonadotrope response to endogenous GnRH hyperstimulation, we subjected control and DKO mice of both genders to gonadectomy. After 7 d, pituitary expression of the gonadotropin subunits and the GnRHR were assayed by qPCR. After gonadectomy, significant increases in LHβ transcript levels were observed in control mice of both genders; however, the inductive effect of gonadectomy on LHβ transcript levels was blocked in DKOs (Fig. 6, A and B, *upper left panels*). In males, castration had no significant effect on FSHβ transcript levels in either DKOs or controls (Fig. 6A, *upper right panel*). In contrast, ovariectomy led to an approximately 20-fold increase in FSHβ transcript levels in the control females (Fig. 6B, *upper right panel*). In DKO females, ovariectomy led to a more modest elevation in FSHβ transcript levels, which did not reach statistical significance (Fig. 6B, *upper right panel*). Gonadectomy led to significant increases in αGSU transcript levels in both control males and females; however, this response was blocked in DKO animals of both genders (Fig. 6, A and B, *lower left panels*). Transcript levels of the GnRHR in males did not differ between either genotype or treatment, whereas in females, statistically significant but quantitatively negligible differences were observed in GnRHR transcript levels between sham-operated control animals and all other groups (Fig. 6, A and B, *lower right panels*).

In parallel to the gene profiling results, serum levels of both LHβ and FSHβ rose significantly after gonadectomy in control animals of both genders (Fig. 7, A and B, *left panels*). Baseline serum FSHβ levels were not different between sham-operated DKO and control animals of either gender. In contrast, baseline serum levels of LHβ were significantly lower in sham-operated DKOs as compared with controls, and significant increases in serum LHβ did not occur after gonadectomy in DKO animals of

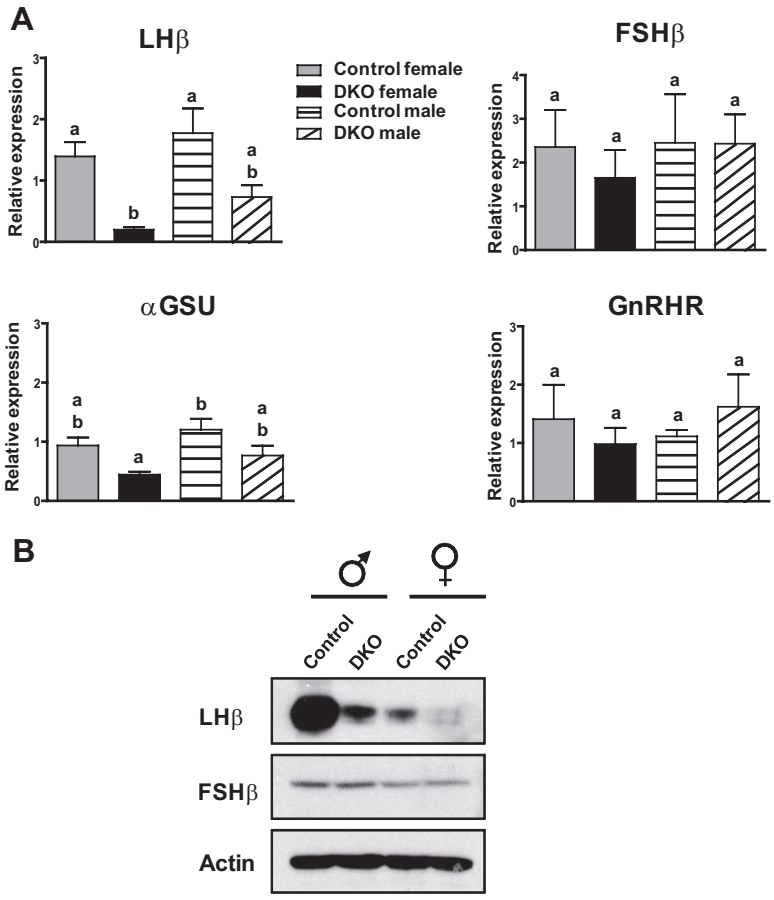

FIG. 4. Basal expression of gonadotropin subunit and GnRHR genes in the ERK1/2 DKO mouse. A, Whole pituitary relative transcript levels of the specified genes were determined by quantitative PCR. Results were calibrated to corresponding transcript levels in pituitaries of randomly cycling female wild-type mice. *Bars* represent mean ± SEM for five animals of each gender and genotype. *Bars not sharing common letter designations* represent mean values that are statistically significantly different ($P < 0.05$). B, Levels of LHβ and FSHβ protein in whole pituitary lysates from control and DKO animals were compared by immunoblotting. Actin is shown as a lane-loading control.

either gender. These results support the conclusion that ERK signaling plays a critical role in mediating up-regulation of both subunits of LH in response to GnRH stimulation *in vivo*.

ERK-deficient gonadotropes fail to up-regulate the immediate early response gene *Egr1* after GnRH stimulation

Early growth response factor-1 (EGR1) is a transcription factor encoded by an ERK-dependent immediate early gene that has been shown to play a key role in the transcriptional regulation of LHβ (15). To determine the effect of ERK ablation on the ability of gonadotropes to up-regulate EGR1 in response to GnRH stimulation, we subjected control and DKO mice of both genders to gonadectomy and after 7 d, measured pituitary expression of *Egr1* by qPCR. In the control animals, mean *Egr1* transcript levels rose approximately 3-fold and 5-fold after gonadectomy in males and females, respectively; however, due to the high variability in transcript levels observed in the gonadectomized animals, these differences were not statistically different (Fig. 8A). No differences were observed in Egr1 transcript levels after gonadectomy in DKO animals of either gender (Fig. 8A).

Given the dynamic nature of the *Egr1* transcriptional response to GnRH stimulation, we considered that the lack of statistically significant differences between the sham operated and gonadectomized control animals may reflect type II error. However, power analysis indicated that to achieve a power of 0.8 for detection of a 4-fold difference in *Egr1* transcript levels within the context of this experimental design would require sample sizes of eight and 71 animals per group, for males and females, respectively. To circumvent this limitation, we undertook an alternative strategy and measured *Egr1* and *LHβ* transcript levels by qPCR in primary pituitary cell cultures from DKO and control animals after stimulation with vehicle or the GnRH agonist buserelin (GnRHa). We reasoned that this experimental approach would allow synchronization of experimental units with respect to the onset and duration of the GnRH signal in a manner that cannot be accomplished within our *in vivo* paradigm. Baseline *Egr1* transcript levels were significantly reduced in cells from DKO females as compared with controls (Fig. 8B, *lower left panel*). *Egr1* transcripts were significantly up-regulated after GnRHa exposure in cells from control animals of both genders; however, up-regulation of *Egr1* mRNA was not observed in cells from DKO animals of either gender (Fig. 8B, *left panels*). *LHβ* transcripts were significantly reduced in cultures of vehicle-treated cells from DKO animals of both genders, as compared with controls. After GnRH stimulation, a statistically significant, but quantitatively negligible, increase in *LHβ* transcripts was observed in cells from control females. Overall, the reduction in GnRH-induced *Egr1* transcription in ERK-deficient gonadotropes underscores a key mechanism linking ERK signaling to LH biosynthesis and highlights the unique importance of the ERK pathway for female fertility.

Discussion

Using a genetic approach, we show here that ablation of ERK1 and 2 in the anterior pituitary leads to infertility in females, but not males. The anovulatory infertility in the ERK-deficient females in this study was associated with marked reductions in pituitary levels of LHβ. Our studies on the pituitary response to gonadectomy indicate further that ERK-deficient gonadotropes are incapable of transcriptional up-regulation of either LHβ or αGSU after pulsatile hyperstimulation by hypothalamic GnRH within the intact endocrine milieu of the living animal. Thyroid function remained normal in animals with pituitary ERK deficiency, suggesting that the function of the thyroid axis does not depend upon ERK signaling at the level of the thyrotrope.

Although our model reveals that ERK signaling is absolutely required for appropriate LH biosynthesis, our data also suggest that the ERK pathway may play a partial role in mediating

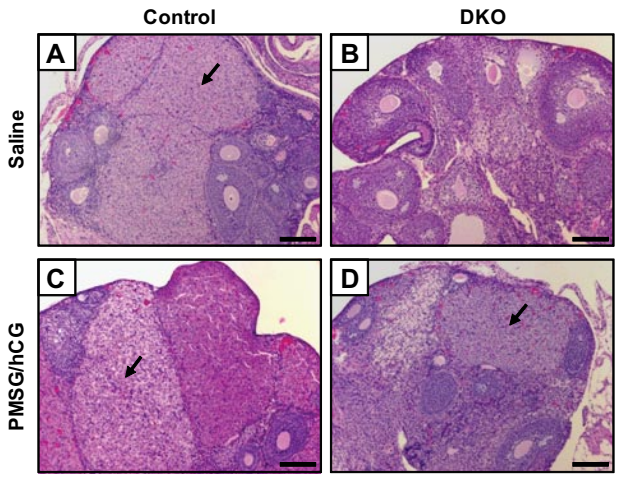

FIG. 5. Pharmacological superovulation rescues the anovulatory phenotype of the ERK1/2 DKO mouse. Control and DKO females were injected ip with vehicle or 5 U pregnant mare serum gonadotropin, followed in 48 h by vehicle or 5 U of human chorionic gonadotropin. After 9 d, ovaries were collected and examined histologically. Ovarian sections from vehicle-treated (A and B) and superovulated (C and D) control (A and C) and DKO (B and D) animals are shown. *Arrows* indicate corpora lutea. *Bars*, 200 μm.

transcriptional up-regulation of FSHβ in the female. Nevertheless, our observations of whole pituitary and serum FSH levels would suggest that overall expression of FSHβ is largely ERK independent. Moreover, the presence of antral follicles in the ovaries of the DKO females, along with the absence of corpora lutea, strongly implicates LH deficiency as the primary cause of infertility in these animals and further supports the relative ERK independence of FSH biosynthesis. This is further corroborated by the observation that the anovulatory phenotype could be rescued in the DKO females by administration of exogenous gonadotropin receptor ligands. Our model thus confirms the ERK pathway as a dominant link between the activation of the GnRHR and LHβ biosynthesis within the gonadotrope *in vivo*. Gender differences in the transcriptional response of the gonadotropin subunits to GnRH stimulation have been reported previously (16–19); however, the sexually dimorphic nature of the requirement for the ERK signaling pathway that is disclosed by our model is a novel observation that has not previously been appreciated.

In contrast to the reproductive phenotype displayed within the adult DKO females, the majority of DKOs predicted to occur within this pedigree remained unaccounted for at 3 wk of age. The regular finding of dead DKO neonates suggests that pre- or perinatal mortality may represent a distinct phenotype resulting from pituitary-targeted disruption of the ERK pathway. Perinatal mortality has been described after deletion of several genes that interfere with appropriate development of anterior pituitary cell lineages (20–22). The pathophysiological basis of perinatal mortality has perhaps been most clearly defined in the Prop1-deficient mouse, in which severe congenital hypothyroidism secondary to hypoplasia of the Pit1-dependent thyrotrope lineage leads to surfactant deficiency and respiratory failure in neonatal pups (23). Whether ERK signaling plays a role in thyrotrope development through modification of either the expression or function of Prop1 or Pit1 is unknown. However, as previously reported, some variability has been observed in the timing of onset and penetrance of αGSU-Cre-mediated target gene recombination within the developing pituitary (24). Studies are currently underway to define the underlying mechanism of perinatal mortality within this model and to clarify the role of the ERK pathway in development of the Pit1-dependent anterior pituitary cell lineages.

The transcriptional regulation of *LHβ* has been studied in a variety of experimental systems (15, 25, 26). The *LHβ* promoter contains binding sites for several transcription factors, including the zinc-finger transcription factor EGR1 (15, 27). EGR1 has been shown to play a critical role in *LHβ* transcription and appears to function primarily as an amplifier of *LHβ* promoter activity, contributing to the responsiveness of the promoter to changes in the amplitude or pulse frequency of GnRH stimulation (26, 28). Interestingly, despite the broad pattern of expression of EGR1, the predominant phenotype of the EGR1 null mouse consists of female anovulatory infertility associated with LH deficiency (29). The phenotype of the female ERK DKO mouse reported here bears a striking similarity to that of the EGR1 null, and the inability of ERK-deficient gonadotropes to up-regulate EGR1 in response to GnRH stimulation indicates that ERK-dependent induction of EGR1 is a dominant mechanism by which GnRHR occupancy is linked to transcription of the *LHβ* gene *in vivo*. However, whether the LH deficiency in the ERK DKO is due solely to a failure of Egr1 induction, or whether the phenotype may also reflect a lack of ERK-dependent EGR1 phosphorylation is not clear. For example, in one study, the GnRH-induced transcriptional activity of constitutively expressed *Egr1* was attenuated in transiently transfected gonadotrope cells by treatment with the MEK inhibitor PD98059 (30). This observation raises the possibility that the ERK pathway may modulate LHβ gene transcription at multiple levels involving not only gene induction but also direct phosphorylation of LHβ transactivators such as EGR1. Ultimately, full clarification of the role of ERK activation in LH biosynthesis will require more comprehensive genomic and proteomic approaches aimed at identification of the entire complement of ERK-dependent genes that are expressed in the gonadotrope, as well as relevant targets of ERK-dependent phosphorylation.

In contrast to the females, ERK DKO males retained normal reproductive function and gonadal histology. This observation provides further interesting comparison with the EGR1 null mouse because EGR1 null males are also reproductively competent (29). However, despite their fertility, EGR1 null males did display Leydig cell atrophy whereas testicular histology in the ERK-deficient males in this study was unremarkable (29). The testicular lesion in the EGR1 null male undoubtedly reflects a more severe impairment of LHβ production than in the ERK DKO. Thus, despite the measurable decrease in pituitary LHβ protein content in the DKO males in this study, production of LH in these animals was clearly sufficient to maintain normal testicular function and structure as well as fertility. The inability of gonadotropes from ERK-deficient males to up-regulate EGR1 in response to GnRH stimulation suggests further that basal EGR1 expression is sufficient to support this level of LHβ production. Overall, our data support a model in which the ERK pathway functions in both genders as a dominant link

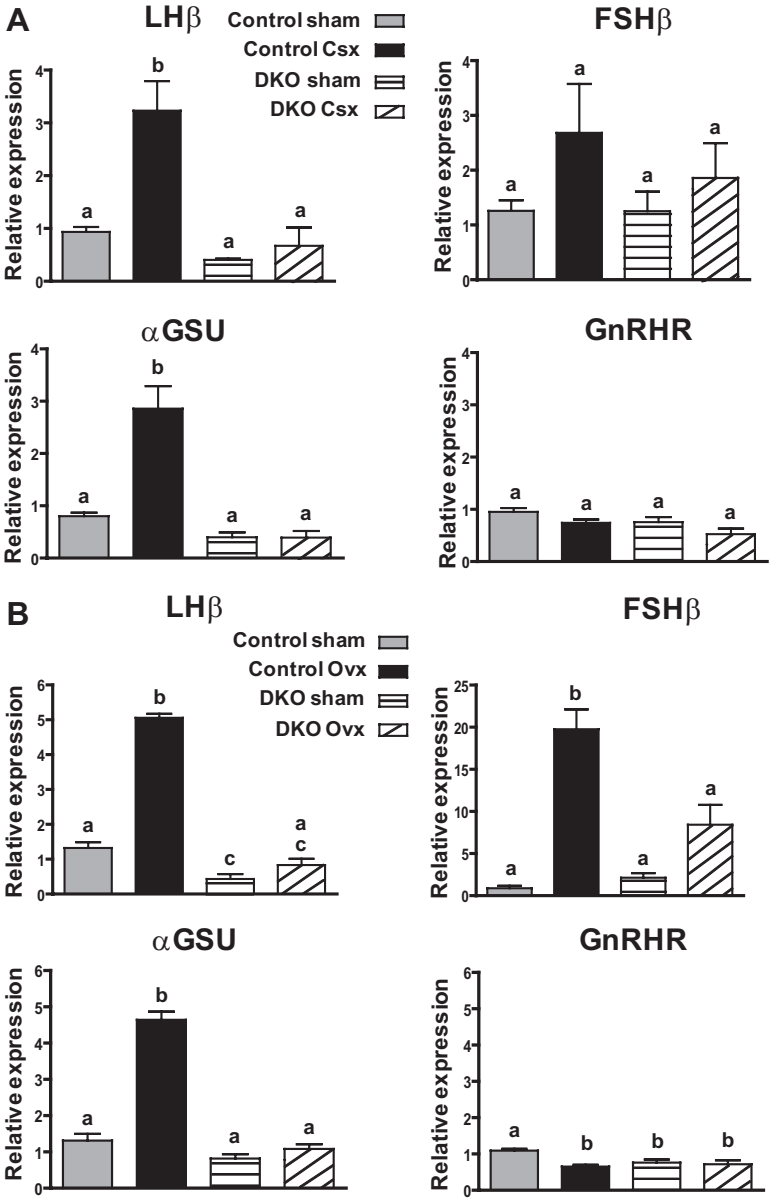

FIG. 6. ERK1/2 DKO animals of both genders fail to up-regulate LHβ after gonadectomy. Control and DKO animals were either castrated (Csx), ovariectomized (Ovx), or sham operated (sham). After 7 d, pituitaries were collected and analyzed by qPCR for relative transcript levels of the indicated gonadotropin subunit genes and the *GnRHR*. Data are shown separately for males (A) and females (B). Results were calibrated to corresponding transcript levels in pituitaries of randomly cycling female wild-type mice. *Bars* represent mean ± SEM for six males or seven females per group and represent pooled results from two separate experiments. *Bars* not sharing *common letter* designations represent mean values that are statistically significantly different ($P < 0.05$). Note the differences in scaling of the y-axis for the FSHβ results in the female.

between the GnRHR and the *Egr1* locus. In turn, EGR1 serves as a primary mediator of GnRH-induced up-regulation of LHβ. The gender difference in the requirement for ERK signaling in the gonadotrope would thus seem to reflect the relatively modest levels of LH that are required to support Leydig cell function in the male, as compared with the high levels of LH that are required for ovulation in the female. Consistent with that notion, transcriptional regulation of the gonadotropin subunit genes is highly dependent upon the frequency of the pulsatile hypothalamic GnRH signal; rapid pulsatility favors LHβ production whereas slower GnRH pulsing appears to favor production of FSHβ (31). Moreover, studies using the LβT2 cell model indicate that the ability of the gonadotrope to interpret variations in the GnRH interpulse interval may depend upon a functional ERK signaling module (32). During the estrous cycle, regular variations occur in the frequency of GnRH pulsatility; pulsatility increases markedly during proestrus, eventually leading to a preovulatory GnRH/LH surge and ovulation. We propose that the ERK module plays a central role in the ability of the gonadotrope to respond to variations in the pulse pattern of the GnRH signal, thereby linking changes in GnRH pulse frequency with the coordinated physiological outcomes associated with the female reproductive cycle. The fact that males have no requirement for cyclic variations in GnRH pulsatility is consistent with our observations that steroidogenesis and spermatogenesis are unaffected by disruption of the ERK signaling pathway at the level of the gonadotrope.

In summary, we have shown that ablation of the ERK signaling pathway in pituitary gonadotropes leads to infertility in female but not male mice. Our data establish an essential role for the ERK pathway in mediating transcriptional up-regulation of LH in the gonadotrope after GnRH stimulation *in vivo* and lend insight into the molecular mechanisms underlying sexually dimorphic control of reproductive function.

Materials and Methods

Animals

ERK1 null (*ERK1*$^{-/-}$) and ERK2 floxed (*ERK2*$^{fl/fl}$) mice have been described previously (33, 34). *αGSU:CRE* mice were purchased from Jackson Laboratories (Bar Harbor, ME). Swiss Webster mice used as wild-type controls were obtained from Taconic Farms (Germantown, NY). Rosa 26 reporter (R26R) mice were generously provided by Dr. Michael Kotlikoff (Cornell University, Ithaca, NY). Animals were handled in compliance with the Cornell University Institutional Animal Care and Use Committee. For assessment of fertility, females were individually housed with males of proven fertility and examined daily for vaginal plugs. Upon identification of a plug, females were isolated and observed for pregnancy. Females in which vaginal plugs were not identified, or that failed to develop pregnancy after identification of a plug, were reintroduced to new males, for a total of 21 d of breeding opportunity. After the breeding challenge, females were isolated for 14 d and then killed and examined for pregnancy post mortem. For superovulation, mice were injected ip with either 5 IU pregnant mare serum gonadotropin or vehicle, followed in 48 h by injection of 5 IU of either human chorionic gonadotropin or vehicle. After 72 h, mice were killed and ovaries were collected for histological examination. Ovariectomy and castration were performed via ventral midline celiotomy under isoflurane anesthesia using standard aseptic conditions.

Genotyping

Genomic DNA was isolated from tail snips (3 mm), or an equivalent quantity of other tissues as indicated, using a commercial kit (Dneasy;

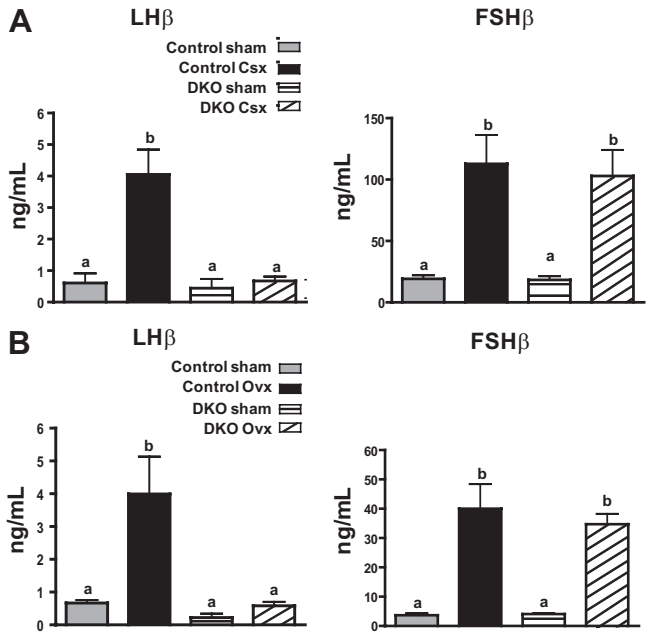

FIG. 7. Serum gonadotropin levels do not increase in DKO animals of either gender after gonadectomy**.** Control and DKO animals were either castrated (Csx), ovariectomized (Ovx), or sham operated (sham). After 7 d, animals were euthanized, and serum levels of LHβ and FSHβ were determined by ELISA. Data are shown separately for males (A) and females (B). *Bars* represent mean ± SEM for six males or seven females per group and represent pooled results from two separate experiments. *Bars* not sharing *common letter* designations represent mean values that are statistically significantly different ($P < 0.05$).

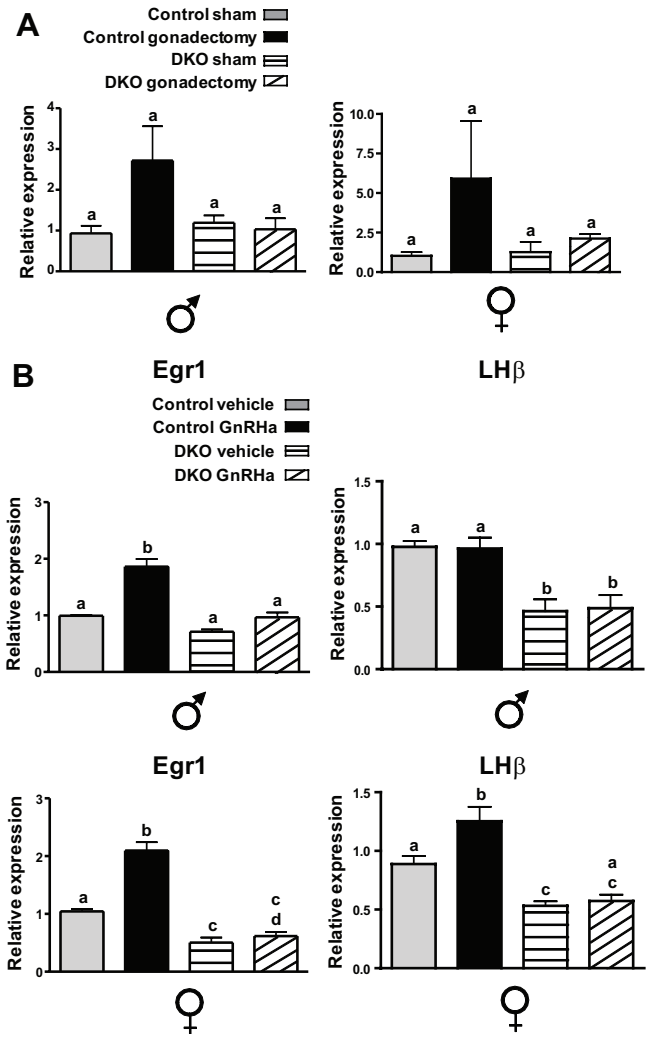

FIG. 8. ERK-deficient gonadotropes fail to up-regulate the immediate early gene Egr1 in response to GnRH stimulation. A, Control and DKO animals of both genders were either gonadectomized (gonadectomy) or sham operated (sham). After 7 d, pituitaries were collected and analyzed by qPCR for relative transcript levels of *Egr1*. Data are shown separately for males (*left panel*) and females (*right panel*). Results were calibrated to corresponding transcript levels in pituitaries of randomly cycling female wild-type mice. *Bars* represent mean ± SEM for six animals per group and represent pooled results from two separate experiments for each gender. *Bars* not sharing *common letter* designations represent mean values that are statistically significantly different ($P < 0.05$). Pituitaries from control and DKO animals were dispersed into primary culture (two pituitaries per well) and treated for 20 min with vehicle or with 100 nM of the GnRH agonist buserelin (GnRHa). Relative transcript levels of Egr1 were measured in each sample by qPCR. *Bars* represent mean ± SEM for six wells representing pooled results from two separate experiments. Data are shown separately for males (*left*) and females (*right*). For these experiments, the expression level of the vehicle-treated control animals was arbitrary assigned as the calibrator.

QIAGEN, Valencia, CA) as per the manufacturer's instructions. Routine genotyping of animals was performed by PCR as previously described (5). PCR for confirmation of recombination at the *ERK2* locus was performed using primer F: 5′-AGCCAACAATCCCAAACCTG-3′; primer 5: 5′-GCTGCCTAGAAACATGGAGCTGC-3′; primer 7: 5-GCTCTTTAACCTCCACTGCCTAAGC-3′; and primer 9: 5′-GCCAGCTGCTCACACTTAGCAAAGC-3′. The identities of PCR products were confirmed by direct nucleotide sequencing for all founder animals.

Histology, immunohistochemistry, and immunofluorescent labeling

For histological examination, tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin using standard histological technique, and examined by light microscopy. In some studies, ovaries were serially sectioned, and every fourth section was examined microscopically for identification of luteal tissue.

For ERK2 immunohistochemistry, sections were deparaffinized in xylene, rehydrated through EtOH dilution series to distilled H_20. Antigen retrieval was performed by boiling the slides in 0.01 M Citrate buffer (pH 6.0). Sections were washed in PBS and blocked with 10% normal rabbit serum/10% nonfat dry milk in PBS for 20 min at room temperature. Sections were then incubated in goat anti-ERK2 primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:60 in PBS/1× casein (Vector Laboratories, Inc., Burlingame, CA), for 2 h at 37 C. Sections were further washed in PBS, and incubated at room temperature for 20 min with biotinylated rabbit-antigoat IgG (Invitrogen, Carlsbad, CA). Sections were then treated with ABC reagent and diaminobenzidine as per the manufacturer's recommendations (Vector).

For immunofluorescent colabeling of ERK2 and LHβ, 5-μm pituitary sections were deparaffinized and rehydrated as described above. Antigen retrieval and incubation in anti-ERK2 primary antibody were also performed as above, substituting normal goat IgG at equivalent concentration (micrograms/ml) as negative control. After incubation with biotinylated rabbit-antigoat IgG and further washing, sections were incubated with Streptavidin Alexa Fluor 488 (Invitrogen) for 20 min at room temperature in the dark. Stained slides were washed further with PBS and stored in distilled water overnight at 4 C. On d 2, sections were blocked with Fab Fragment goat antirabbit IgG H + L (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:50 in PBS for 30 min at 37 C, and reblocked for 20 min with 10% goat serum/2× casein (Vector) in PBS. Rabbit anti-LHβ primary antibody (National Hormone and Peptide Program; NIDDK) was reconstituted in PBS at a concentration of 1 μg/μl, and applied at a 1:50 dilution for 2 h at 37 C, substituting normal rabbit IgG (Vector) at an equivalent concentration (micrograms/ml) as a negative control. LHβ was detected with Texas Red goat-antirabbit IgG H&L (Vector) at a 1:80 dilution in PBS for 20

min at room temperature in the dark. Slides were washed and mounted in Vectashield 4′,6-diamidino-2-phenylindole (Vector). Images were obtained on a Nikon E400 epifluorescence microscope using the appropriate filters.

Vaginal cytology

Vaginal lavage was performed with 30 μl PBS, and the effluent was used to make routine cytological smears. Slides were fixed in methanol and stained with Wright's stain. Slides were examined by light microscopy, and epithelial cells and leukocytes were differentiated on the basis of cell morphology.

Serum hormone measurements

Immediately after euthanasia, mice were exsanguinated by cardiac puncture. Blood samples were allowed to clot at room temperature for 2 h and were then centrifuged at 8000 rpm for 10 min. Serum supernatants were collected and stored at −80 C until assayed. Total serum T4 concentrations were determined by ELISA using a commercial kit according to the manufacturer's instructions (Alpha Diagnostics, San Antonio, TX) with samples run in duplicate. Measurements of LHβ and FSHβ were performed by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core facility with samples run in singlet.

Immunoblotting

Tissues were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 130 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 2 mM EDTA, 5 mM sodium vanadate, 0.2 mM phenylmethysulfonylfluoride, 5 mM benzamidine. Lysates were cleared by centrifugation, and protein concentrations were determined by Bradford assay. Protein samples were boiled for 5 min in sodium dodecyl sulfate load buffer, resolved by SDS-PAGE, and transferred to polyvinylidine difluoride membranes by electroblotting. Membranes were blocked with 5% nonfat dry milk in TBST (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.05% Tween 20) and then incubated with specified antisera (anti-LHβ, FSHβ, and TSHβ from the National Hormone and Peptide Program; NIDDK; and antiactin and horseradish peroxidase-conjugated secondary antibodies from Santa Cruz). Protein bands were visualized using enhanced chemiluminescence according to the manufacturer's instructions (PerkinElmer, Boston, MA).

Pituitary cell primary culture

Mice were euthanized and pituitaries were placed in DMEM containing 10% fetal bovine serum on ice. Tissues were digested with agitation at 37 C for 10 min in DMEM containing 0.5 mg/ml each of collagenase and hyaluronidase (Sigma Chemical Co.; St Louis, MO). After tritiuration, tissue remnants were allowed to settle by gravity. The supernatant was removed and the tissue pellet was resuspended in DMEM containing 0.25 mg/ml each of the same enzymes and digested for an additional 10 min. After a second round of tritiuration and sedimentation, the supernatants were combined. Cells were washed once in DMEM containing 10% fetal bovine serum, and plated in like medium on 15-mm diameter poly-L-lysine coated dishes at a density of two pituitary equivalents per well. Cells were maintained at 37 C in 5% CO_2 for 12 h before treatment.

RNA isolation and quantitative PCR

After the indicated treatments, total RNA was isolated from cultured cells or whole pituitaries using the RNeasy kit (QIAGEN) according to the manufacturer's instructions. Reverse transcription was carried out in 40 μl reaction volumes using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Taqman primer-probe sets for mouse LHβ, αGSU, FSHβ, GnRHR, TSHβ, and Egr1, were purchased commercially (Applied Biosystems). Amplification was performed under standard conditions using the ABI Prism 7500 Sequence Detection System. Transcript levels were normalized to corresponding levels of β-actin and were calibrated to corresponding transcript levels represented in a pooled cDNA stock derived from wild-type female mice, or to levels of the control group, as indicated.

Data analysis

Quantitative PCR and ELISA results were analyzed by *t* test or one-way ANOVA. *Post hoc* tests were performed with Bonferroni all-pairwise comparisons. All differences were considered significant at $P \leq 0.05$.

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