Luteinizing Hormone Deficiency and Female Infertility in Mice Lacking the Transcription Factor NGFI-A (Egr-1)

Stephen L. Lee, Yoel Sadovsky, Alexander H. Swirnoff, Jeffrey A. Polish, Pam Goda, Galina Gavrilina, Jeffrey Milbrandt*

The immediate-early transcription factor NGFI-A (also called Egr-1, zif/268, or Krox-24) is thought to couple extracellular signals to changes in gene expression. Although activins and inhibins regulate follicle-stimulating hormone (FSH) synthesis, no factor has been identified that exclusively regulates luteinizing hormone (LH) synthesis. An analysis of NGFI-A–deficient mice derived from embryonic stem cells demonstrated female infertility that was secondary to LH-β deficiency. Ovariectomy led to increased amounts of FSH-β but not LH-β messenger RNA, which suggested a pituitary defect. A conserved, canonical NGFI-A site in the LH-β promoter was required for synergistic activation by NGFI-A and steroidogenic factor–1 (SF-1). NGFI-A apparently influences female reproductive capacity through its regulation of LH-β transcription.

Reproduction in vertebrates requires hormonal regulation by the hypothalamic-pituitary-gonadal axis to coordinate germ cell development, reproductive organ function, and sexual behavior (*1*). The pituitary glycoproteins LH and FSH function to promote growth, differentiation, steroidogenesis, and germ cell development. They are composed of a common α subunit and a hormone-specific β subunit. The LH-β subunit is regulated at the levels of mRNA transcription (*2*), mRNA polyadenylation (*3*), and protein glycosylation (*4*). The LH-β promoter contains binding sites for the estrogen receptor, the adenosine 3′,5′-monophosphate (cAMP)-response-element–binding factor, and the orphan nuclear receptor SF-1 (Ad4BP) (*5*). The expression of LH-β requires SF-1 (*6*).

NGFI-A encodes a zinc-finger transcription factor that recognizes the sequence GCG(T/G)GGGCG and is rapidly induced in many cell types by growth, differentiation, and apoptotic stimuli (*7*). To investigate its function, we created mice bearing a targeted disruption of NGFI-A (*8*). These NGFI-A$^{-/-}$ mice exhibited no obvious developmental or behavioral defects. However, test matings revealed that NGFI-A$^{-/-}$ females were infertile, whereas wild-type and heterozygous females were productive with similar frequency and average litter sizes. Homozygous males were fertile. Cytological analysis indicated that NGFI-A$^{-/-}$ females were anestrous (Fig. 1A). Ovaries were of similar weight to those of wild-type mice, but the uterus was only 30% of the weight of uteri from wild-type or heterozygous littermates (Fig. 1B). Histological examination revealed an intact inner circular and outer longitudinal myometrium and an intact endometrial layer with uterine glands (Fig. 2, A and B). The ovaries contained a similar number of follicles at all stages of maturation but exhibited a marked absence of corpora lutea (Fig. 2, C and D). Consistent with this observation, basal concentrations of serum progesterone were lower in NGFI-A–deficient mice (11.3 ± 3.6 ng/ml, n = 5 wild-type; 6.3 ± 2.4 ng/ml, n = 5 NGFI-A$^{-/-}$; $P < 0.05$, Student's t test). In contrast, no difference was detected in the concentration of serum estradiol (52.0 ± 34.3 pg/ml, n = 10 wild-type or heterozygous; 50.7 ± 36.9 pg/ml, n = 8 NGFI-A$^{-/-}$).

To determine whether these observations reflected a primary or secondary defect, we examined the ovarian and uterine response to exogenous gonadotropins. Administration of pregnant mare serum (PMS) gonadotropin induced an increase in uterine weight and cellularity similar to that observed in heterozygous and wild-type mice (Fig. 1B). Ovarian response was intact; administration of PMS gonadotropin and human chorionic gonadotropin (hCG) resulted in ovulation of similar numbers of oocytes and subsequent formation of corpora lutea, which were positive for mRNA expression for cholesterol side-chain cleavage enzyme (P450scc), a key regulatory enzyme in progesterone synthesis (Fig. 2, E and F) (*9*).

To assess pituitary function, we used a semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) to measure the amount of gonadotropin mRNA. Decreased amounts of mRNA encoding LH-β, but not FSH-β, were observed in NGFI-A$^{-/-}$ males and females (Fig. 3A). No differences were observed in the amount of mRNA for another pituitary hormone, prolactin, or for proteins important for gonadotropin expression, such as the gonadotropin-releasing hormone (GnRH) receptor and the type II activin receptor (*9*, *10*). Analysis of pituitary proteins by immunoblotting with antisera to LH confirmed that LH-β expression was decreased in NGFI-A$^{-/-}$ males and was undetectable in NGFI-A$^{-/-}$ females (Fig. 3B). The lack of LH-β protein was not attributable to a loss of gonadotropes, however, because the number of gonadotropes detected with FSH antisera was similar between wild-type and NGFI-A$^{-/-}$ mice (Fig. 2, I to L).

The decreased amount of LH-β in the male pituitary prompted further examina-

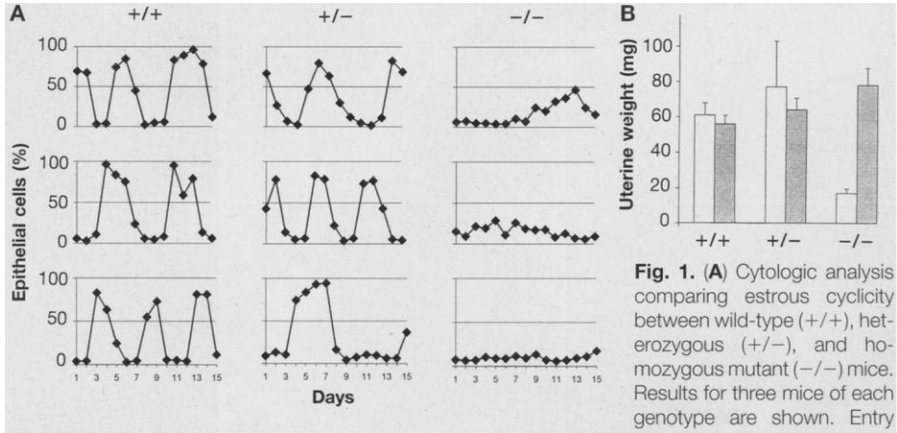

Fig. 1. (**A**) Cytologic analysis comparing estrous cyclicity between wild-type (+/+), heterozygous (+/−), and homozygous mutant (−/−) mice. Results for three mice of each genotype are shown. Entry into the estrous cycle is indicated by an increase in the percentage of epithelial cells in vaginal samples obtained in 0.5 ml of phosphate-buffered saline (*18*). Epithelial cells were distinguished from leukocytes on a flow cytometer (FACScan, Becton Dickinson) on the basis of forward scatter and green fluorescence. (**B**) Uterine weights of basally cycling mice (open bars) and mice treated with PMS gonadotropin 2 days before killing (shaded bars) (*19*). Means ± SD (n = 3 per genotype) are shown.

S. L. Lee, A. H. Swirnoff, P. Goda, G. Gavrilina, J. Milbrandt, Departments of Pathology and Internal Medicine, Washington University School of Medicine, St. Louis, MO 63110, USA.
Y. Sadovsky and J. A. Polish, Department of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, MO 63110, USA.
*To whom correspondence should be addressed.

tion of the male reproductive axis. Histological analysis revealed atrophic steroidogenic interstitial (Leydig) cells, which are the primary targets of LH (Fig. 2, G and H); however, seminal vesicles were normal compared with those of controls (9). Testicular weights of mice 10 to 20 weeks of age were comparable (236 ± 20 mg, $n = 7$ wild-type; 226 ± 36 mg, $n = 10$ NGFI-A$^{-/-}$). Serum testosterone concentrations, although highly variable (*11*), were also similar (0.3 to 4.3 ng/ml). Protein immunoblot analysis for LH confirmed that males had greater amounts of LH than did females (*12*), and this difference was also observed in homozygous mutant mice (Fig. 3B). Thus, the amount of LH, although decreased in NGFI-A$^{-/-}$ males, was adequate for spermatogenesis and male fertility.

The removal of gonadal feedback inhibition by ovariectomy has been shown to increase the amounts of LH-β and FSH-β mRNA in the pituitary, in part by increasing the GnRH stimulus from the hypothalamus (*13*). Seven days after ovariectomy, the amounts of FSH-β mRNA in wild-type and NGFI-A$^{-/-}$ mice exhibited a comparable fivefold increase (Fig. 3C). In contrast, a fivefold increase in the amount of LH-β mRNA seen in wild-type mice was not observed in NGFI-A$^{-/-}$ mice. Thus, the decreased LH-β expression in NGFI-A$^{-/-}$ mice is not the result of excessive negative feedback from the ovary. Moreover, because FSH-β was increased and because GnRH neurons were present and appeared intact in the mutant mice (9), these results indicate that the primary defect in NGFI-A$^{-/-}$ mice is at the level of the pituitary gonadotrope.

One explanation of our results is that NGFI-A directly regulates the LH-β gene. A search of the LH-β promoter revealed canonical binding sites for NGFI-A and SF-1 (*14*) that were conserved across all vertebrate species examined (Fig. 4A). To test the possibility that NGFI-A regulates the LH-β promoter, we transfected the αT3 pituitary gonadotrope cell line (*15*), which constitutively expresses SF-1 (6), with an NGFI-A expression construct and a reporter plasmid that contains nucleotides –156 to +7 of the rat LH-β promoter. The observed luciferase activity was four times that induced by reporter activity alone (Fig. 4B).

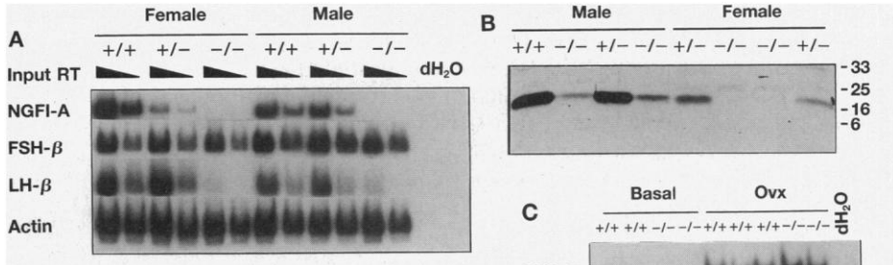

Fig. 3. (**A**) RT-PCR analysis of NGFI-A, FSH-β, and LH-β expression in pituitaries of wild-type, heterozygous, and NGFI-A$^{-/-}$ mice (*21*). (**B**) Immunoblot analysis of pituitary proteins (30 μg) probed with rabbit polyclonal antibody to rat LH (*21*). (**C**) Expression of FSH-β and LH-β in pituitaries of basal and ovariectomized (Ovx) wild-type and NGFI-A$^{-/-}$ mice. Seven days after ovariectomy, mice were analyzed for gonadotropin expression as in (A). Each lane represents an RT-PCR analysis of one mouse with the genotype and treatment indicated. Two independent experiments produced similar results.

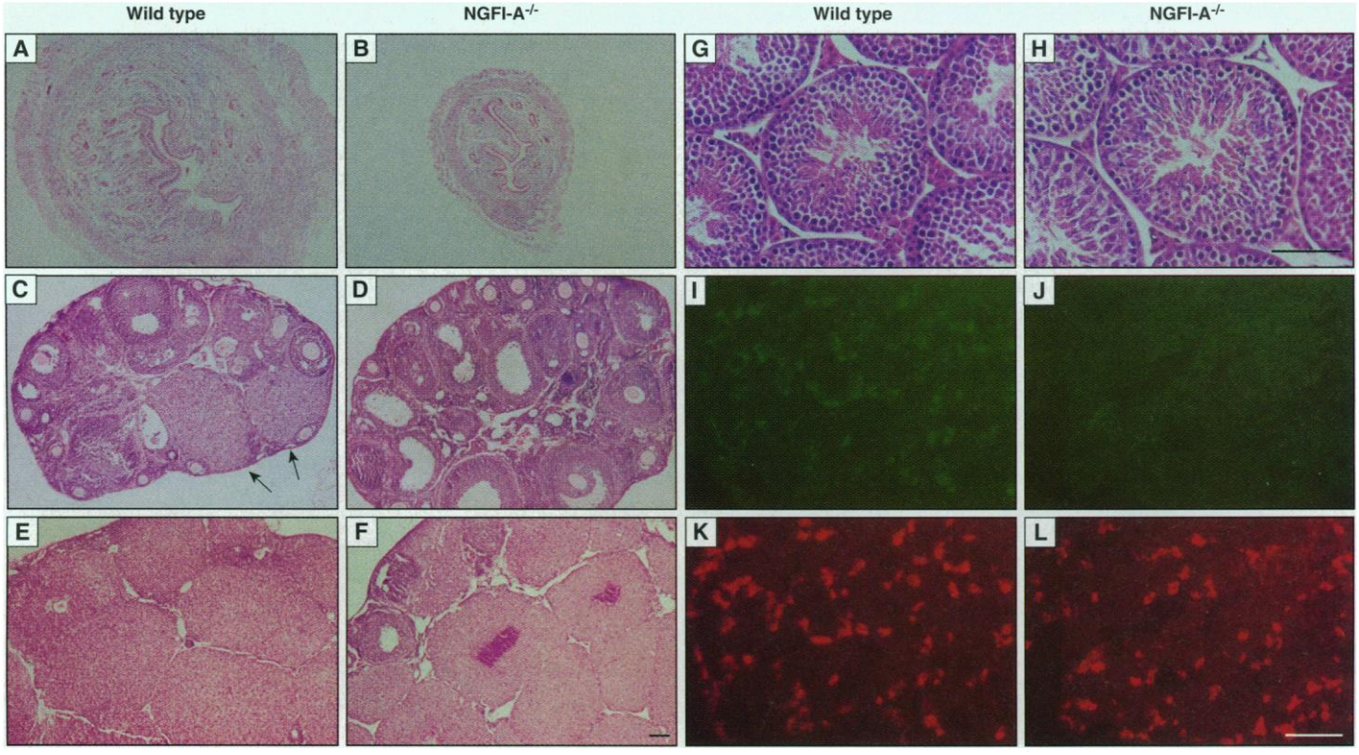

Fig. 2. Analysis of 8-week-old wild-type and NGFI-A$^{-/-}$ mice (*20*). (**A** to **H**) Hematoxylin and eosin–stained sections of uteri (A and B) and ovaries (C and D) from basally cycling mice (corpora lutea denoted by arrows), ovaries from gonadotropin-treated mice (E and F), and testes (G and H). (**I** to **L**) Double-label indirect immunofluorescent staining of pituitary with antibodies to LH-β (I and J) or to FSH-β (K and L). Scale bars, 100 μm.

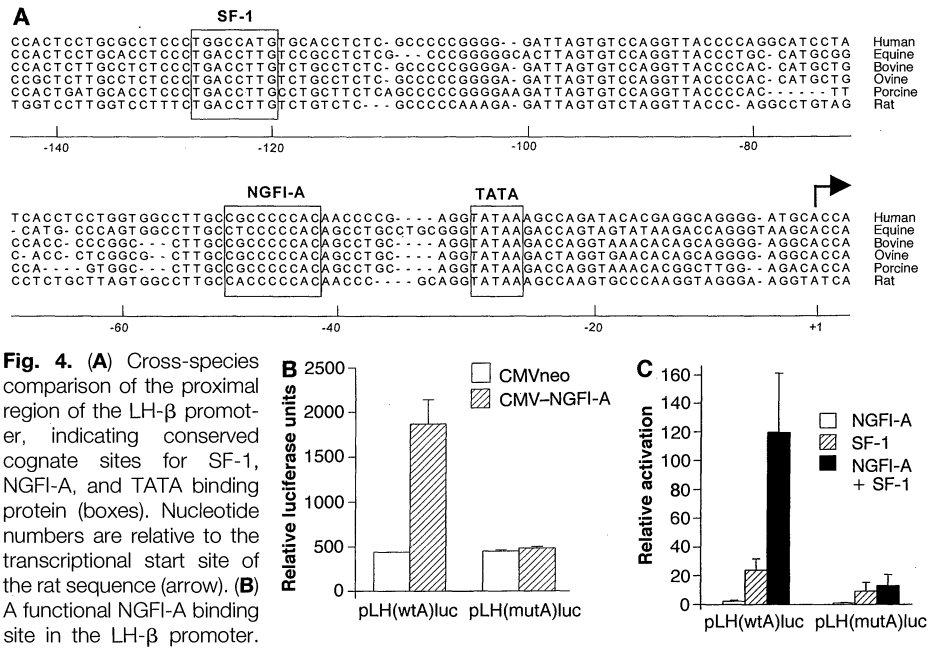

Fig. 4. (**A**) Cross-species comparison of the proximal region of the LH-β promoter, indicating conserved cognate sites for SF-1, NGFI-A, and TATA binding protein (boxes). Nucleotide numbers are relative to the transcriptional start site of the rat sequence (arrow). (**B**) A functional NGFI-A binding site in the LH-β promoter. Transient transfection assays (*22*) in the αT3 pituitary cell line were done with the rat LH-β promoter–luciferase reporter construct (500 ng) containing a wild-type [pLH(wtA)luc] or mutated [pLH(mutA)luc] NGFI-A site and 50 ng of either an NGFI-A expression vector (CMV–NGFI-A) or the expression vector alone (CMVneo). Relative luciferase units were determined by normalizing luciferase activity to that of an internal β-Gal expression vector (*22*). Data shown are means ± SD of duplicate samples from one experiment representative of four that yielded similar results. (**C**) NGFI-A and SF-1 synergistically activate the LH-β promoter in CV-1 cells. Data shown are means ± SD of at least five independent experiments.

Mutation of the binding site from GTGGGGGTG to CTAAGAATA, which replaces the guanosine residues required for recognition by the NGFI-A zinc fingers (*7*), abolished the ability of NGFI-A to activate transcription from this promoter (Fig. 4B). We also tested the individual and cooperative contributions of NGFI-A and SF-1 to LH-β promoter activity in CV-1 monkey kidney cells, which lack SF-1. Although NGFI-A weakly activated the LH-β promoter and SF-1 strongly activated the promoter when tested separately, the combination of NGFI-A and SF-1 resulted in synergistic activation of the LH-β gene (Fig. 4C).

The regulation of the LH-β promoter by NGFI-A and SF-1 through two evolutionarily conserved regions provides insight into the requirements of LH-β gene expression. Cell type specificity in gonadotrope cells is fulfilled by the restricted pattern of SF-1 expression (6), whereas NGFI-A, an inducible, widely expressed transcription factor, can be viewed as a regulatable amplifier of gene expression. The synergism between NGFI-A and SF-1 may provide a means to achieve the degree of biosynthetic activity required for gonadotrope function, especially during menstrual cycles (which depend on a LH surge). The loss of either of these factors results in LH-β deficiency and could underlie pituitary causes of human reproductive dysfunction.

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19. Mice were housed and bred in a specific pathogen-free facility with a 12-hour light-dark cycle. Procedures were conducted in compliance with the guidelines of the Animal Studies Committee of Washington University and the National Institutes of Health. Adult mice (older than 6 weeks) were given an intraperitoneal injection of PMS gonadotropin (5 IU) (Sigma). Where indicated, hCG (5 IU) was given 48 hours later. Progesterone, estrogen, and testosterone concentrations were determined by radioimmunoassay.
20. Preparation of mouse tissue for histology was done as described (*8*). For immunofluorescence, frozen pituitary sections were incubated in buffer [0.5 M tris (pH 7.5) and 0.3% Triton X-100] containing rabbit antibody to rat FSH (lot AFP-C0972881; 1:300) and guinea pig antibody to rat LH-β (lot AFP-22238790; 1:300) for 2 hours at 25°C, and then in buffer containing indocarbocyanine-conjugated donkey antibody to rabbit immunoglobulin G (IgG) (1:100) and fluorescein isothiocyanate–conjugated donkey antibody to guinea pig IgG (1:50) (Jackson ImmunoResearch, West Grove, PA).
21. Total RNA and protein were prepared with TriReagent according to the manufacturer's protocol. RT-PCR was done as described (*16*) in the presence of 0.1 μCi of [α-^{32}P]deoxycytidine 5′-triphosphate, with primers for the following genes (GenBank accession numbers are in parentheses): rat LH-β (D00576), 5′-GCCGGCCTGTCAACGCAACC-3′, 5′-GAGGGCCACAGGGAAGGAGA-3′; rat FSH-β (D00577), 5′-TGAACTGACCAACATCACC-3′, 5′-ACTATCACACTTGCCACAGT-3′; rat β-actin (J00691), 5′-TATGGAGAAGATTTGGCACC-3′, 5′-GTCCAGACGCAGGATGGCAT-3′; and mouse NGFI-A (M20157), 5′-ACCCGGCCCAGCAAGACACC-3′, 5′-GGGCACAGGGGATGGGAATG-3′. Portions of the reaction were subjected to electrophoresis in a 5% nondenaturing polyacrylamide gel and were quantified by Phosphorimager (Molecular Dynamics) or x-ray film (Kodak). Control experiments verified a linear relation between the input RNA and the radioactive signal. For protein blots, pituitary lysates were resolved on an SDS-polyacrylamide gel (12%) and analyzed essentially as described (*16*). Rabbit antiserum to rat LH (NIDDK-rLH-I-9) was used at a dilution of 1:1000. Horseradish peroxidase–conjugated goat antibody to rabbit IgG (Cappel, Durham, NC) was used at a dilution of 1:10,000 and detected by enhanced chemiluminescence (Amersham).
22. Reporter constructs of the LH-β promoter containing a wild-type or mutated NGFI-A site were constructed in pL(KS)b-LUC (*17*) with standard methods. CMV–NGFI-A (*17*), CMV-SF1 (*14*), and an LH-β reporter construct were transfected along with a constitutive β-galactosidase reporter (RSV-βGal) by means of the calcium phosphate method (*17*) in six-well plates containing αT3 or CV-1 cells. Luciferase activity was determined 48 hours later, as described (*17*), and normalized to β-Gal activity (Galacto-Light; Tropix, Bedford, MA) according to the manufacturer's protocol.

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