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Reproductive Deficiencies in Transgenic Mice Expressing the Rat Inhibin α-Subunit Gene

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Inhibin is an important modulator of reproductive function at both the endocrine level, through its regulation of pituitary FSH biosynthesis, and at the paracrine and autocrine levels, as an intragonadal regulatory factor. To investigate the *in vivo* actions of inhibin in FSH regulation and gonadal function, transgenic mice that overexpress the rat inhibin α-subunit gene were generated. A transgene that includes the mouse metallothionein-I gene promoter (MT-α) fused to the rat inhibin α-subunit precursor coding sequences was used to produce three lines of transgenic mice. Transgene mRNA is expressed in numerous tissues, including the pituitary, liver, testis, ovary, and kidney. Inhibin α-subunit protein was also increased in transgenic pituitary and ovary. Serum inhibin α-subunit levels are highly increased compared with control mice. Inhibin β_A- and β_B-subunit protein amounts are lower in transgenic ovaries compared with wild type, although serum levels of activin A are not significantly reduced in transgenic female mice. FSH levels are reduced in both male and female transgenic mice, whereas LH levels are increased in MT-α female mice. MT-α transgenic females are subfertile and exhibit a 52% reduction in litter size compared with wild-type females. The smaller litter size of MT-α female mice was correlated with a reduction in the number of oocytes ovulated during a normal cycle. Treatment of the transgenic females with exogenous gonadotropins resulted in an ovulation rate similar to that of stimulated wild-type animals, suggesting that altered gonadotropin levels may be responsible for the decreased ovulation rates. MT-α transgenic male mice are fertile and sire litters of equivalent size to those sired by wild-type males, despite an approximately 50% reduction in sperm numbers. These results indicate that overexpression of the rat inhibin α-subunit gene in mice leads to a disruption of the normal inhibin-to-activin ratio and to reproductive deficiencies, and they support the hypothesis that inhibin and activin act to regulate FSH secretion *in vivo* and are essential for normal gonadal function. (*Endocrinology* 142: 4994–5004, 2001)

INHIBIN AND ACTIVIN are multifunctional hormones belonging to the TGF-β superfamily of proteins. Inhibin is a heterodimer composed of an inhibin-specific α-subunit and one of two related β-subunits (β_A and β_B) (1–4), whereas activin is a dimer of two of the β-subunits (5, 6). The subunit RNAs are individually expressed at varying levels in tissues, including the ovary, testis, adrenal, pituitary, brain, placenta, and spleen (7). Inhibin α-subunit mRNA is primarily expressed in the granulosa cells of the ovary (8), the Sertoli cells of the testis (9), and the adrenal cortex (10). Inhibin and activin were identified for their ability to inhibit and stimulate, respectively, FSH secretion and synthesis from cultured anterior pituitary cells (1–6, 11). The inhibins and/or activins have also been shown to regulate diverse physiological functions, including early embryonic development (12–14), erythroid differentiation (15), pituitary GH biosynthesis (16), hypothalamic oxytocin secretion (17), and neuronal survival (18).

Inhibin production is tightly regulated throughout the female reproductive cycle. On the afternoon of proestrus of the rodent estrus cycle, just before the preovulatory FSH and LH surges, the granulosa cells of developing follicles produce high levels of inhibin A (8, 19). After the gonadotropin surges, ovarian inhibin production declines (20). Low inhibin levels persist through the morning of estrus, providing an environment permissive to increased FSH and the generation of the secondary FSH surge. Treatment of proestrus rats with charcoal-extracted follicular fluid as a source of inhibin prevents the secondary FSH surge (21). The secondary FSH surge is responsible for the recruitment of new follicles into the antral pool (22, 23), and FSH stimulates inhibin expression in newly recruited follicles (19, 24). Thus, appropriate inhibin levels are important to maintain the estrus cycle and for normal follicle development.

Mice deficient in inhibin after the disruption of the inhibin α-subunit gene illustrate the *in vivo* importance of inhibin for normal gonadal function. Inhibin α-subunit-deficient mice develop ovarian and testicular stromal tumors (25), and if gonadectomized before gonadal tumorigenesis, they develop adrenal tumors (26). Maturing oocytes and spermatozoa are initially present in the gonads of inhibin α-subunit-deficient mice, although the development of gonadal stromal tumors leads to the arrest of gametogenesis (25). These animals die prematurely at 3–4 months of age and thus do not permit an extensive analysis of inhibin in reproductive function in the adult. An alternative approach for establishing the importance of inhibin and its appropriate regulation in the reproductive axis is to investigate phenotypes that arise as a consequence of overexpression of the inhibin α-subunit in transgenic mice. To determine the effects of inhibin misregulation on activin expression, on FSH production, and on gonadal function, we generated transgenic mice that overexpress the rat inhibin α-subunit cDNA. This paper reports

Abbreviations: hCG, Human CG; MT-α, metallothionein-I promoter inhibin α-subunit; SSC, standard sodium citrate; TBS, Tris-buffered saline.

on the reproductive phenotypes of these mice, and the accompanying paper describes gonadal pathologies related to inhibin α-subunit overexpression in transgenic mice.

Materials and Methods

Generation and maintenance of transgenic mice

A 1.4-kb rat inhibin α cDNA was cloned into the vector pEV-142 (provided by Dr. Richard Palmiter, University of Washington, Seattle), which includes a mouse metallothionein-I promoter (MT-α) and a human GH RNA processing and polyadenylation site (27–29). The vector was digested with *Eco*RI to linearize the transgene, and the linear DNA was subsequently isolated from an agarose gel. Transgenic animals were produced at the Northwestern University-Markey Developmental Biology Center core facility under the direction of Dr. Phillip Iannoccone. Transgene DNA at a concentration of 1–10 ng/μl was injected into the pronucleus of CD-1 (Swiss Webster albino outbred mice, Charles River Laboratories, Inc., Wilmington, MA) zygotes. Microinjected eggs were transferred to the oviducts of pseudopregnant foster mothers. Genomic DNA was isolated from tail biopsies of the 11 potential founder mice born. DNA (20 μg) was digested with *Kpn*I, fractionated on a 0.7% agarose gel, and hybridized with the 1.4-kb inhibin α cDNA probe. Three founder male mice were identified and used to establish three separate transgenic lines (lines MT-α A–C). Transgene copy number was determined by Southern blot analysis with 10 μg of DNA prepared as described (29). Founder males were crossed with CD-1 females, and all subsequent generations were raised in a room with a controlled photoperiod (14 h of light, 10 h of dark) and temperature (22–25 C). All lines stably transmit the transgene at the expected 50% Mendelian frequency. Animals were tested for the presence of the transgene by dot blot analysis of 10 μg of genomic (tail) DNA using the 1.4-kb rat inhibin α cDNA as a hybridization probe.

Transgene RNA analysis

RNA used for RT-PCR was isolated by homogenization of tissues in guanidinium thiocyanate and ultracentrifugation through a CsCl cushion (30). Total RNA yield was estimated by A_{260}. RNA (5 μg) was reverse transcribed at 42 C using random hexamer primers and avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI) in a total volume of 20 μl. A separate negative control RT reaction was performed with 5 μg of RNA from each sample (excluding avian mosaic virus reverse transcriptase) to ensure the absence of genomic DNA contamination. The PCR reaction mix included 5 μl of the RT reaction, [α-^{32}P]dCTP (2 μCi at 3000 Ci/mmol), *Taq* DNA polymerase (Perkin-Elmer Corp., Norwalk, CT), and 70 ng of each oligonucleotide primer. The oligonucleotide primers used to detect the MT-α transgene included MT-I (5′-CCA CGA CTT CAA CGT CCT GAG-3′) and MI-3 (5′-CCT GTA CCA AGG ACA CAG GC-3′), which amplified a 580-bp transgene mRNA product. In a separate PCR reaction, the oligonucleotide primers L19-A (5′-CTG AAG GTC AAA GGG AAT GTG-3′) and L19-B (5′-GGA CAG AGT CTT GAT GAT CTC-3′) were used to amplify a 194-bp region of ribosomal protein L19 as an internal control (31). Amplification was carried out for 24 cycles using an annealing temperature of 58 C in a Perkin-Elmer Corp. thermal cycler. The PCR products were separated by size using electrophoresis on 6% polyacrylamide gels and exposed to Kodak X-Omat film (Eastman Kodak Co., Rochester, NY).

In situ hybridization

Ovaries were removed from storage at −80 C and brought to −20 C in a Reichert 820 cryostat (Buffalo, NY). Sections were cut at 20 μm and mounted onto VWR Scientific Superfrost Plus slides (West Chester, PA) for *in situ* hybridization. Ovarian sections were fixed in 5% paraformaldehyde (pH 7.8) for 5 min, rinsed in 2× standard sodium citrate (SSC) for 5 min, washed in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min, and then dipped in 2× SSC. Sections were dehydrated through an ethanol series and vacuum dried until hybridization. Antisense and sense [^{35}S]UTP-labeled RNA probes were synthesized using T7 or SP6 polymerase. The probe (1.1×10^7 cpm/ml with a specific activity of $3.0–6.0 \times 10^7$ cpm/μg) in hybridization buffer [50% formamide, 0.3 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 1× Denhardt's reagent, 10% dextran sulfate, 0.1% SDS, 10 mM dithiothreitol, 600 μg/ml yeast tRNA, and 600 μg/ml poly(A)] was applied to the tissue sections, and the sections were overlaid with a coverslip. Slides were hybridized in a humidified incubator at 47 C for 16–20 h. After hybridization, the coverslips were removed in 4× SSC, and sections were treated with 20 μg/ml RNase-A at 37 C for 1 h, washed in decreasing concentrations of SSC down to 0.1× SSC at 65 C, and dehydrated in an ethanol series. The slides were exposed to Kodak Biomax MR film for 3–5 d and then processed for liquid emulsion autoradiography using Kodak NTB-2 emulsion. Slides were developed after 2 wk and stained with hematoxylin.

Protein blot analysis

Tissues were removed and immediately frozen on dry ice. Tissues were ground by mortar and pestle and collected in 40 μl of protein extraction solution [10 mM Tris (pH 7.5), 0.5 M NaCl, 1 mM $MgCl_2$, 0.1% Triton X-100, and one complete mini protease inhibitor cocktail tablet per 10 ml (Roche, Indianapolis, IN)]. Homogenates were exposed to two freeze-thaw cycles (dry ice, +37 C) and briefly centrifuged, and the supernatant was transferred. Protein concentration was determined by a bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL) using 1-μl dilutions of protein on microtiter plates. Protein (60 μg) was equally loaded into each lane for each tissue. Protein was mixed with 4× NuPAGE LDS (Novex, Carlsbad, CA) at a final concentration of 0.1 M dithiothreitol and heated at 70 C for 10 min before loading. Proteins were electrophoresed in NuPAGE 4–12% Bis-Tris gels (Novex), immersed in 1× MES buffer [20× = 1 M 2-(*N*-morpholino)-ethanesulfonic acid, 1 M Tris Base, 70 mM SDS, 20.5 mM EDTA; 200 μl of NuPAGE antioxidant between gels in each apparatus; Novex, Carlsbad, CA], and subsequently transferred in 1× transfer buffer (10× = 0.25 M Tris Base, 0.5 M glycine) mixed with 20% methanol. Blots were incubated overnight with shaking in 10% milk-Tris-buffered saline (TBS)-Tween [20 mM Tris (pH 7.5), 500 mM NaCl, 0.1% Tween] blocking solution at 4 C. Blots were incubated with α, β_A, or β_B primary antibodies at the following concentrations in 5% milk-TBS-Tween: α (1:2000) for 1 h [anti-α 1–26 GY affinity purified], β_A (1:1000) for 4 h [anti-β_A 81–113 affinity purified], and β_B (1:1000) for 4 h [anti-β_B 80–112 affinity purified]. Antibodies were provided by Dr. Wylie Vale (Salk Institute, San Diego, CA). Blots were washed in milk-TBS-Tween and incubated with secondary antirabbit Ig horseradish peroxidase-linked whole antibody (from donkey) (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) (1:10,000) in 5% milk-TBS-Tween for 30 min. Secondary antibody-specific signal was detected with an ECL kit (Amersham Pharmacia Biotech) using an overnight exposure.

Hormone measurements

All intact female mice were cycled before collection of serum for hormone analysis. Estrous cycle stages were determined by daily examination of vaginal cytology. Those animals demonstrating a minimum of two consecutive 4- to 5-d cycles were killed on the morning of metestrus or diestrus. Gonadectomized animals were maintained for 1 wk before serum collection. To examine potential alterations in hormone levels based on age, measurements were performed on mice ranging from 3 to 12 months in age. Serum hormone measurements were determined by RIA at the Northwestern University P30 Center RIA Core Facility under the direction of Drs. John Levine and Neena Schwartz. National Institute of Diabetes and Digestive and Kidney Diseases antiserum and standards (rLH-RP-3 standard/rLH-S-11 antibody and rFSH-RP-2 standard/rFSH-S-11 antibody) were used for LH and FSH measurements. FSH and LH results are expressed as nanograms per milliliter. FSH assay sensitivity was 0.05 ng/sample or 1.0 ng/ml, and LH assay sensitivity was 0.01 ng/sample or 0.2 ng/ml. The interassay coefficients of variation were 7.9% and 17.5% for female serum FSH and LH measurements, respectively. The interassay coefficients of variation were 15.8% and 9.9% for male serum FSH and LH measurements, respectively. Reagents provided by Dr. Wylie Vale (Tyr 27 rat inhibin α 1–27 standard/sheep anti-Tyr 27 rat inhibin α 1–27 antibody 795) were used for the inhibin α-chain assay as described (32, 33). The RIA measures total inhibin (free α and dimeric inhibin) (32, 33), and inhibin α results are expressed as picomoles per milliliter. The interassay coefficient of variation for all inhibin measurements was 9.3%. We attempted to mea-

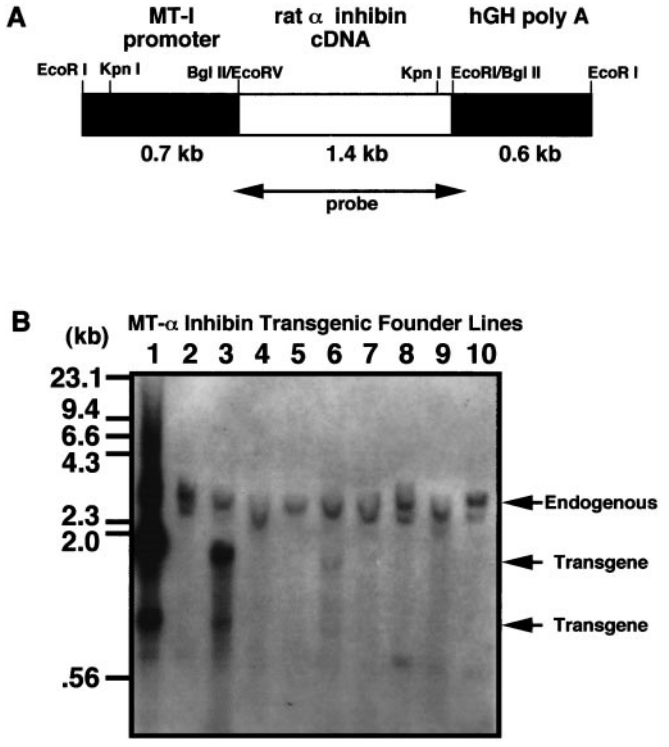

FIG. 1. Map of the MT-α inhibin transgene construct and Southern blot analysis of founder lines. A, The transgene construct includes a 0.7-kb promoter region of the mouse metallothionein-I gene fused to the rat inhibin α cDNA and a human GH polyadenylation signal. The probe template used for identification of transgenic founders and offspring is the 1.4-kb rat inhibin α cDNA. B, DNA (20 μg) was digested with *Kpn*I, fractionated on a 0.7% agarose gel, and blotted onto a nylon membrane. The filter was hybridized with an inhibin α cDNA probe and exposed to x-ray film. Lanes 1–10 include DNA from 10 potential transgenic founder mice. Lane 1 represents the line C founder, lane 3 represents line A, and lane 6 represents line B. The positions of the endogenous gene and the transgene bands are indicated by the *arrows*. Line C (20 copies), line A (12 copies), and line B (2 copies) represent MT-α inhibin transgenic mouse lines. Transgene copy number was determined by dot blot analysis.

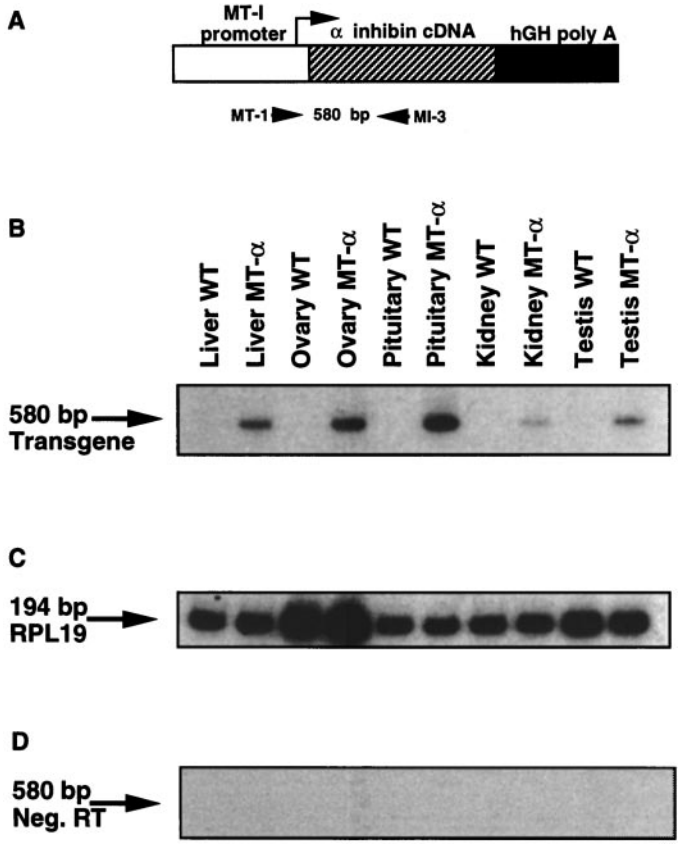

FIG. 2. Expression of MT-α inhibin transgene mRNA in mouse tissues. A, Primers for RT-PCR were designed such that the expected transgene-specific product would be 580 bp, as shown in the scheme. B, Tissue RNAs from transgenic (MT-α) and wild-type (WT) mice were subjected to RT-PCR with the above primers. MT-α line C mRNA expression results are shown. A similar mRNA expression pattern was observed for MT-α line A. RNAs (5 μg) from the indicated tissues were treated with RNase-free DNase and used for RT-PCR as described in *Materials and Methods*. Amplification was carried out for 24 cycles using an annealing temperature of 58 C. The samples were electrophoresed on a 6% polyacrylamide gel, and the dried gel was exposed to x-ray film. C, A 194-bp RT-PCR control product with primers specific for ribosomal protein L19 is shown. D, A negative control for the reverse transcriptase reaction of all sample RNAs is shown.

sure serum dimeric inhibin levels using the human inhibin A dimer ELISA (Serotec, Oxford, UK); however, sera from MT-α or wild-type littermate mice were nonparallel in the assay. Sera from other mouse strains such as C57BL16 were parallel in the assay, suggesting that serum from the CD-1 mouse strain specifically interferes with this human inhibin A dimeric ELISA. Serum dimeric activin A levels were measured with the human activin A dimer ELISA (Serotec) according to the manufacturer's protocol with undiluted serum.

Fertility measurements

MT-α (transgenic) mice and control (nontransgenic littermate) mice were paired with wild-type (CD-1; Charles River Laboratories, Inc.) mice of the opposite sex. Each mating pair was housed together until three to eight litters of offspring were produced. The litter sizes of each mating pair were averaged (lines A and C), and the time interval between births was recorded (line A). Female age varied from 3–6 months at the beginning of the study to 7–11 months by the end. Wild-type or transgenic females in this age range (3–11 months) did not display significant variance in litter size as a result of age. Males used in this study ranged from 3 to 10 months of age.

Ovulation analysis

Female MT-α (transgenic) mice and female control (nontransgenic littermate) mice were housed with wild-type (CD-1; Charles River Laboratories, Inc.) vasectomized males. Females were examined every morning for the presence of a copulatory plug. Those females with copulatory plugs were killed at midday, and the oviducts were removed and transferred into room temperature M2 medium (Sigma, St. Louis, MO). Oviducts were flushed and opened to release the ova/cumulus cell complexes. After removal of the cumulus cells with hyaluronidase, the ova were counted. Gonadotropin-stimulated females were injected with 5 IU of PMSG followed by 3 IU of hCG 45 h later. After the hCG injection, females were housed and ova were recovered under the same conditions described above. Those females with copulatory plugs were killed 20–22 h after hCG treatment.

Sperm parameters

The epididymis was dissected from the scrotum of 2- to 3-month-old adult male mice. The sperm were squeezed from the epididymis with watchmaker's forceps. The sperm were incubated in 2 ml of M2 medium (Sigma) at 37 C for 15–30 min, diluted 1:10, counted, and examined for motility (based on flagellar movement) on a hemocytometer as described (29, 34).

Results

Generation of transgenic mice overexpressing the inhibin α-subunit

A transgene composed of the mouse MT-α (27) fused to the rat inhibin α-subunit cDNA was used to generate transgenic mice that overexpress the rat inhibin α-subunit gene product (Fig. 1A). Southern blot analysis with 10 μg of genomic DNA isolated from the F_0 offspring identified three transgenic founder males (Fig. 1B) that were bred to establish three separate MT-α transgenic lines. The *Kpn*I-digested genomic DNA resulted in an approximately 2.0-kb band and a 0.7-kb band when probed with the 1.4-kb rat inhibin α-subunit cDNA (Fig. 1A). Lanes 1, 3, and 6 represent MT-α transgenic lines C, A, and B, respectively (Fig. 1B). The pattern of hybridization indicates that multiple copies of the transgene are inserted in tandem. Copy number was determined by comparing 10 μg of genomic DNA with a series of copy number controls using dot blot analysis (data not shown) (29). Line A has approximately 12 copies of the transgene (per diploid genome), line B has approximately 2 copies, and line C has approximately 20 copies. Southern blot analysis of genomic DNA isolated from offspring indicates that the transgene is passed to 50% of the offspring, as expected for all three lines. MT-α transgenic mouse lines A and C were used for all of the studies reported here.

Expression and protein analysis of the transgene

To determine the pattern of transgene expression, RT-PCR was performed on RNA samples extracted from various tissues of both control and transgenic animals. The primers used for the RT-PCR included one primer specific for the metallothionein-I 5′ nontranslated sequence and a second primer specific for the rat inhibin α coding sequence (Fig. 2A). Transgene mRNA expression was found in all transgenic tissues examined, including the ovary, testis, pituitary, kidney, and liver of line A and line C MT-α transgenic mice (Fig. 2B). Although the RT-PCR is not quantitative, based on Northern analysis, transgene mRNA levels are particularly high in the pituitary (data not shown). As expected, no transgene mRNA expression was observed in wild-type littermates. *In situ* hybridization of MT-α and wild-type mouse ovaries revealed that inhibin expression is detected in the granulosa cells of maturing follicles, as expected (Fig. 3, *left*). Additional inhibin α-subunit gene expression is observed in the interstitial and stromal cells of MT-α transgenic ovaries, indicating increased levels of inhibin α-subunit gene mRNA expression in these cell types of the transgenic ovary compared with controls. Inhibin β_A-subunit expression is localized to the granulosa cells of mature antral follicles in both MT-α transgenic and wild-type control ovaries, and there do not appear to be substantial changes in β_A mRNA levels in the MT-α ovaries (Fig. 3, *right*).

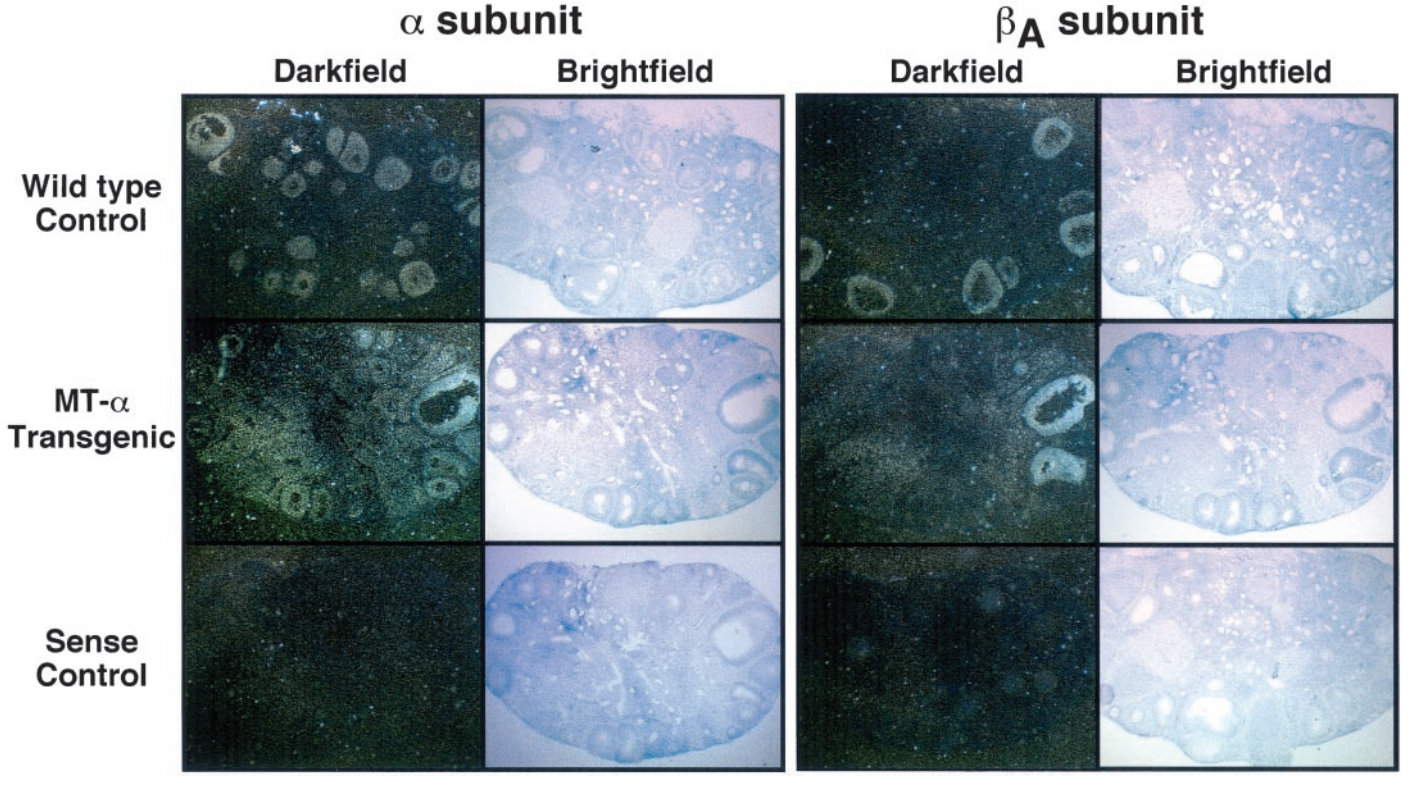

FIG. 3. *In situ* hybridization showing the distribution of inhibin α- and β_A-subunit mRNAs in wild-type and MT-α transgenic ovaries. Ovaries collected from a 4-month-old wild-type littermate mouse (*top panels*) and a MT-α transgenic mouse (*middle panels*) were hybridized with a ^{35}S-labeled rat inhibin α antisense riboprobe (*left*) or with a ^{35}S-labeled rat inhibin β_A antisense riboprobe (*right*). Corresponding sense riboprobe controls are shown (*bottom panels*). After high stringency washing, the sections were processed for liquid emulsion autoradiography. Sections were photographed at ×28 magnification under dark-field and light-field microscopy.

To examine the relative levels of the inhibin subunit proteins (α, β_A, and β_B) in transgenic tissues, Western analysis was performed with antibodies specific for the individual subunit proteins. The Western blots shown in Fig. 4 are representative of results from three different experiments. An abundance of mature α-subunit protein (18 kDa) was present in MT-α transgenic ovary and pituitary (Fig. 4A). The protein levels in the transgenic pituitary are consistent with the Northern and RT-PCR RNA expression studies. No α-subunit protein was observed in the wild-type ovary in the blot shown; however, with increased incubation time of primary α antibody, α-subunit protein was observed in wild-type ovary (data not shown). Mature β_A-subunit protein (14 kDa) was detected in the ovary of wild-type mice, and levels were slightly reduced in the MT-α ovary (Fig. 4B). Very small amounts of β_B-subunit protein (14 kDa) were also detected in the ovary of wild-type mice, and this level was reduced in the ovary of MT-α transgenic mice (Fig. 4C). No mature inhibin/activin subunit proteins were detected in the negative control tissue (liver), although a larger molecular mass band that might represent the precursor protein was observed.

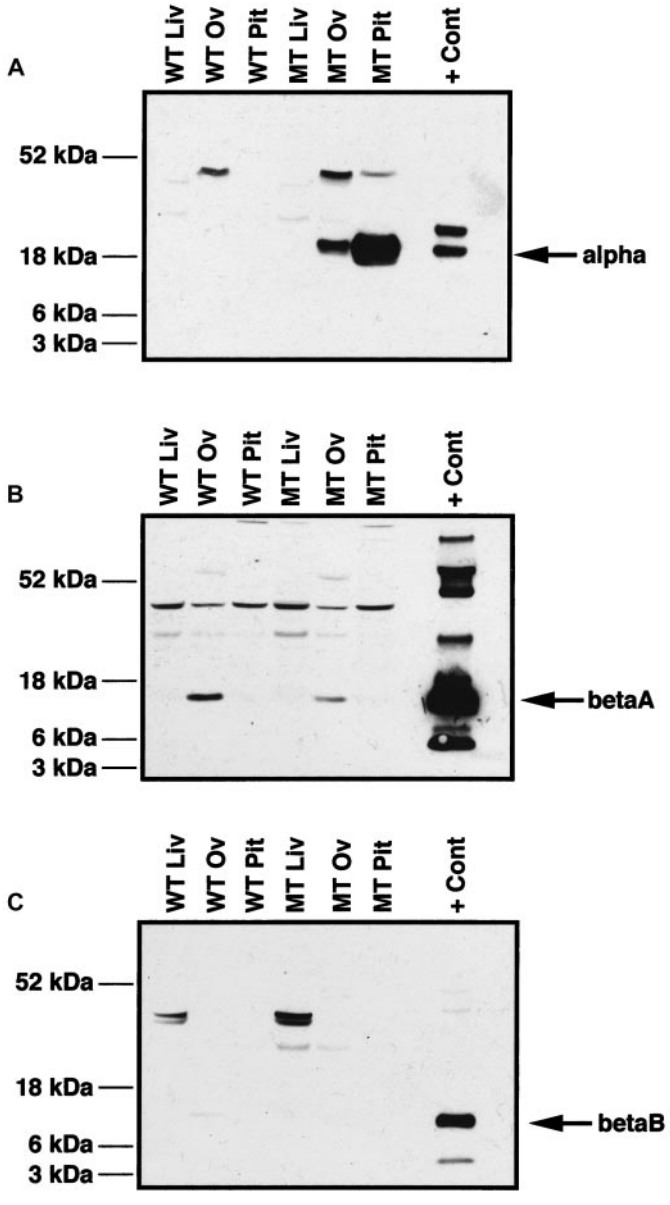

FIG. 4. A, Protein blot of wild-type and transgenic tissues incubated with an inhibin α-subunit-specific antibody. The positive control exhibited the mature glycosylated 20-kDa and nonglycosylated 18-kDa (*arrow*) forms of the inhibin α-subunit. The nonglycosylated form of inhibin α-subunit is present in excess in MT-α transgenic ovary and pituitary. B, Protein blot of wild-type and transgenic tissues incubated with an inhibin β_A-subunit-specific antibody. The positive control showed the 14-kDa mature inhibin β_A-subunit (*arrow*). Wild-type and MT-α transgenic ovaries showed small amounts of mature inhibin β_A-subunit protein. C, Protein blot of wild-type and transgenic tissues incubated with an inhibin β_B-subunit-specific antibody. The positive control showed the 14-kDa mature inhibin β_B-subunit (*arrow*). Wild-type and MT-α transgenic ovaries showed mature inhibin β_B-subunit protein. Tissues analyzed in these studies were from MT-α line C female mice. Western blots shown are representative of results obtained from three experiments. Positive controls were recombinant human inhibin subunit proteins.

Serum hormone measurements

To assess transgene protein expression, a RIA specific for the rat inhibin α-chain (measures free α-subunit and dimeric inhibin) was performed on sera samples collected from MT-α transgenic and control mice (32, 33). Serum total inhibin levels were significantly increased in MT-α transgenic male and female mice compared with wild-type mice (Fig. 5, A and B). After ovariectomy, there was a significant reduction in serum total inhibin in wild-type female mice. In contrast, total inhibin levels remained increased in the MT-α female mice, suggesting nongonadal expression of the transgene. This is consistent with the RT-PCR results, which show transgene expression in numerous nongonadal tissues. Basal FSH levels (Fig. 6, A and B) were significantly reduced in the MT-α mice compared with wild-type controls, consistent with the idea that biologically active inhibin is produced that is able to suppress pituitary FSH synthesis or secretion. In addition, FSH levels after gonadectomy were significantly repressed in MT-α female mice compared with controls (wild type, 24 ± 1.2 ng/ml; line A, 15.9 ± 1.2 ng/ml; line C, 17.4 ± 1.0 ng/ml; $P < 0.01$). Serum FSH levels were measured for female mice ranging from 3 to 12 months in age, and reduced FSH levels remained constant in transgenic females despite age, whereas FSH levels increased slightly in older wild-type females (data not shown). In contrast to serum FSH levels, MT-α female mice showed a significant increase in serum LH levels (Fig. 6C), and serum LH levels increased with advancing age. In contrast to MT-α female mice, MT-α male mice displayed a decrease in serum LH, although this was not significant in either line (Fig. 6D). Dimeric activin A levels were similar among young transgenic and wild-type female mice (Table 1), but activin A levels decreased slightly in older transgenic female mice.

Fertility studies

To establish the effects of transgene expression on male and female fertility, MT-α transgenic mice from lines A and C were mated with wild-type animals. Successive matings of MT-α female mice with wild-type male mice revealed a 52% decrease in litter size for MT-α female mice compared with

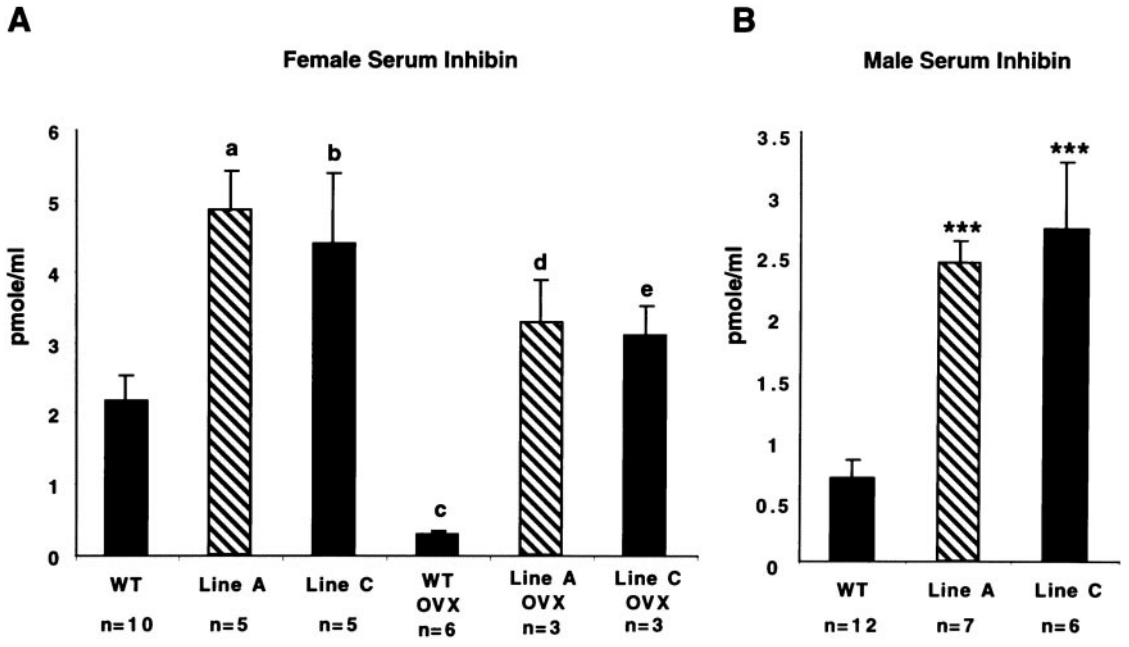

FIG. 5. Serum levels of inhibin α-subunit in wild-type and MT-α transgenic mice. A, Serum inhibin levels for female MT-α transgenic mice *vs.* wild-type littermates. Intact MT-α female serum inhibin levels are significantly greater than inhibin levels of intact wild-type littermates (^{a,b}, $P < 0.01$). Inhibin levels of ovariectomized wild-type mice compared with intact wild-type mice are significantly different (^{c}, $P < 0.01$). Inhibin levels of ovariectomized MT-α female mice are significantly greater than ovariectomized wild-type littermates (^{d,e}, $P < 0.001$). B, Serum inhibin levels for male MT-α transgenic mice *vs.* wild-type littermates. Line A and line C, MT-α transgenic mice; WT, wild-type control mice (CD-1; Charles River Laboratories, Inc.); OVX, ovariectomized. All statistical analyses were performed using one-way and two-way ANOVA with variance indicated by SEM. ***, $P < 0.001$.

litters produced by control (nontransgenic littermate) female mice (Fig. 7). Line A MT-α transgenic females gave birth to an average of 6.5 $\pm$ 0.6 pups, line C MT-α transgenic females had an average litter size of 5.0 $\pm$ 0.3 pups, and nontransgenic female littermates had an average litter size of 11.5 $\pm$ 0.6 pups ($P < 0.001$). The time interval between births was also slightly greater in MT-α transgenic female mice, with an average of 29.5 d between births, compared with 25.7 d between births for nontransgenic littermates.

No significant impairment of fertility was observed when MT-α transgenic males were mated to control females. MT-α males sired litters (line A, 13.3 $\pm$ 0.8 pups; line C, 12.0 $\pm$ 0.2 pups) of comparable size to litters sired by nontransgenic males (11.6 $\pm$ 0.6 pups) (Fig. 7). Although MT-α male fertility does not appear to be impaired, MT-α males have reduced sperm production. Analysis of sperm parameters showed epididymal sperm numbers to be decreased by approximately 55–60% in MT-α transgenic males compared with nontransgenic males (Table 2). However, there was no significant difference in sperm motility, as measured by the percentage of sperm showing flagellar movement (Table 2).

Reduced female fertility could be attributable to one or more reproductive dysfunctions, such as a follicular defect, decreased ovulation, fertilization, or implantation, and embryo survival. The gross morphology of MT-α transgenic ovaries revealed fewer antral follicles and corpora lutea than wild-type ovaries, as described in the accompanying paper, suggesting a follicular defect and/or a potential decrease in ovulation rates. To test this, the number of oocytes released from a natural ovulatory cycle was determined for MT-α transgenic female mice and control female mice. The number of oocytes recovered from MT-α transgenic female mice was 54% the number of oocytes recovered from control female mice. MT-α transgenic females have an average ovulation rate of 8.2 $\pm$ 0.9 oocytes for line A and 8.0 $\pm$ 1.8 oocytes for line C, whereas control female mice have an average of 15.1 $\pm$ 0.2 oocytes per ovulation ($P < 0.001$) (Fig. 8A). Thus, the reduction in ovulation rate for transgenic female mice is similar to the approximately 50% reduction in litter size (Fig. 7).

The decreased ovulations for MT-α transgenic females could be secondary to abnormal gonadotropin levels. To determine if transgenic females were capable of responding to exogenous gonadotropins to increase ovulation rate, MT-α transgenic females and control females were treated with PMSG and hCG. After superovulation, transgenic females ovulated an approximately equal number of oocytes (line A, 50.5 $\pm$ 5.8; line C, 34.5 $\pm$ 4.4) as control females (46.3 $\pm$ 5.7) (Fig. 8B).

Discussion

To further understand the physiological function of inhibin in mammalian reproduction, we generated a "gain-of-function" mouse model, MT-α inhibin transgenic mice. The mouse metallothionein promoter was used to produce broad expression of the rat inhibin α-subunit cDNA in transgenic mice. Of the 11 mice produced after pronuclear injection, 3 MT-α transgenic founder male mice were identified (Fig. 1B), thus permitting the establishment of MT-α transgenic lines A, B, and C.

In the adult rodent, inhibin α-subunit gene expression is

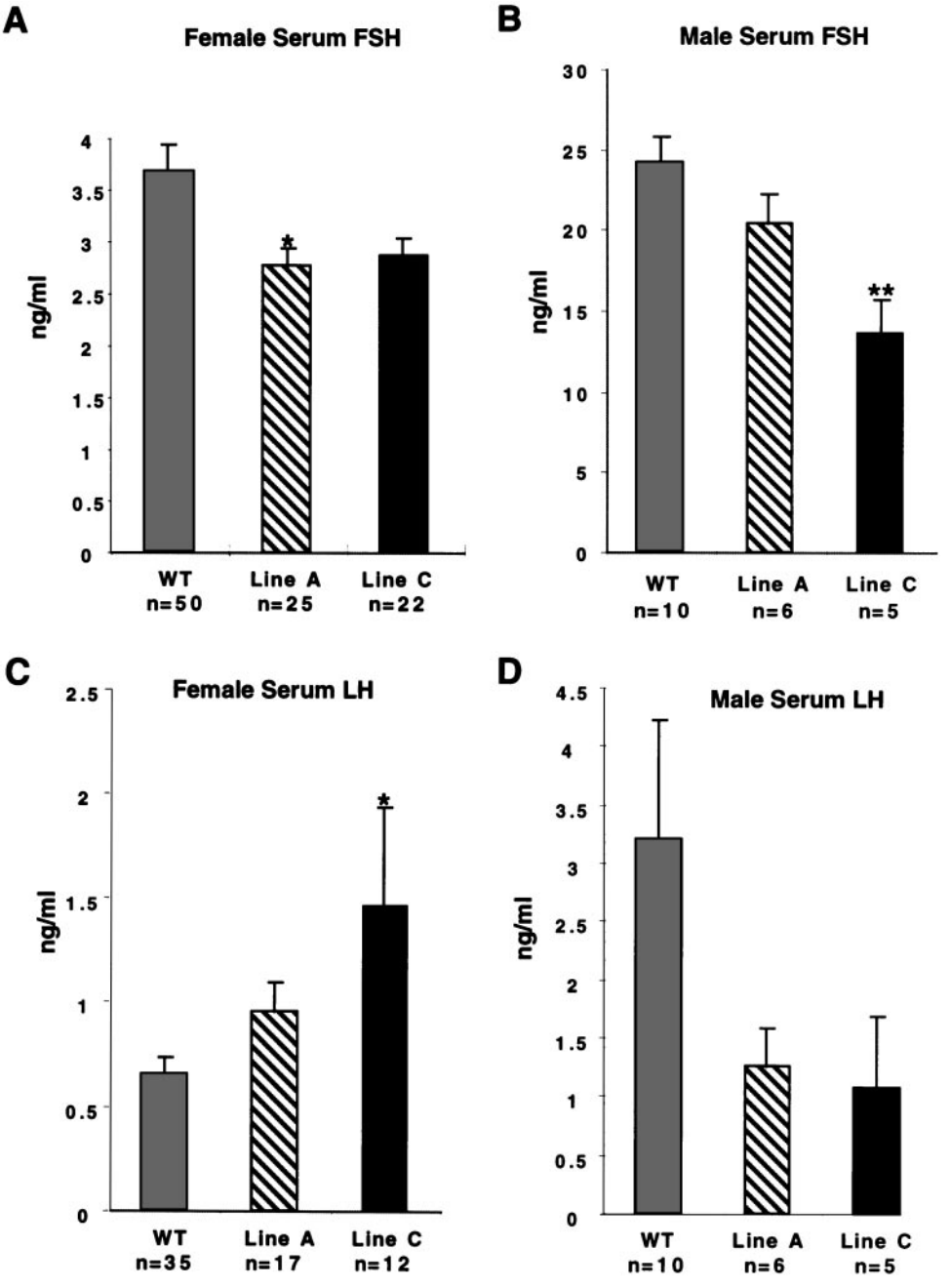

FIG. 6. Serum levels of FSH and LH in MT-α transgenic and wild-type mice. A, Serum FSH levels for female MT-α transgenic mice *vs.* wild-type littermates; B, serum FSH levels for male MT-α transgenic mice *vs.* wild-type littermates; C, serum LH levels for female MT-α transgenic mice *vs.* wild-type littermates; D, serum LH levels for male MT-α transgenic mice *vs.* wild-type littermates. Line A and line C, MT-α transgenic mice; WT, wild-type control mice (CD-1; Charles River Laboratories, Inc.). All statistical analyses were performed using one-way ANOVA comparing MT-α transgenic mice to their wild-type littermates with variance indicated by SEM. *, $P < 0.05$; **, $P < 0.01$.

TABLE 1. Serum activin A levels in MT-α transgenic mice (pg/ml)

Wild type (n = 19)	MT-α line A (n = 10)	MT-α line C (n = 9)
172.0 ± 15.0	127.0 ± 17.0	138.0 ± 18.0

Results are means ± SEM. Statistical analysis was performed using one-way ANOVA, comparing MT-α transgenic mice to wild-type littermates. There are no significant differences between groups ($P > 0.05$).

both spatially and temporally regulated (7, 8). The inhibin α-subunit is primarily expressed in the ovary, testis, adrenal, and pituitary, whereas the tissue-specific expression patterns of the inhibin β-subunits, β_A and β_B, are less restricted (7). The metallothionein-I gene is expressed in almost all tissues except thymus (35). This promoter region of mouse metallothionein-I was originally used to generate transgenic MT-human-GH mice (28) and transgene expression in these mice was observed in many different tissues (35). Similarly, in our MT-α transgenic mice, the expression of the MT-α transgene was achieved in all tissues examined (Fig. 2B). The expression of the MT-α transgenic rat inhibin α-subunit in a broad range of tissues, in addition to endogenous mouse inhibin α-subunit expression, should permit the increased production and assembly of dimeric inhibin in those tissues that also express the β-subunits. The suppression of serum FSH in MT-α intact and MT-α gonadectomized mice suggests that increased bioactive dimeric inhibin production was achieved *in vivo* (Fig. 6, A and B). Ovariectomized wild-type mice

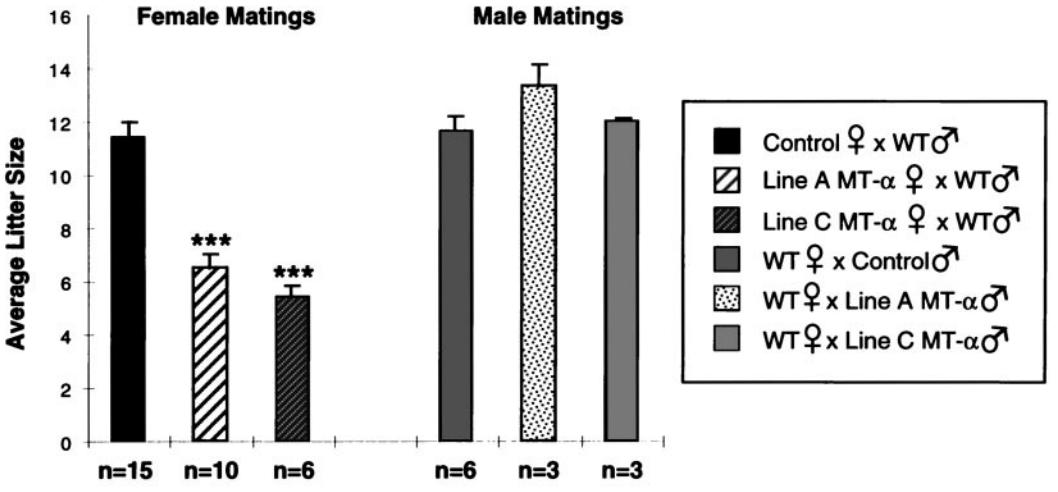

FIG. 7. Mean litter size of MT-α transgenic mice. Individual MT-α transgenic mice or control (nontransgenic littermate) mice were continually housed with a wild-type animal of the opposite sex. One male and one female defined a mating pair, and each mating pair was caged together until three to five litters were born. The litter sizes of each mating pair were averaged to yield the mean litter size per mating pair. n, Number of mating pairs analyzed in each group; control, nontransgenic littermate mice; MT-α, transgenic mice; WT, wild-type control mice (CD-1; Charles River Laboratories, Inc.). The average litter size from MT-α female mice was significantly different from the average litter size from control female mice. All statistical analyses were performed using one-way ANOVA with variance indicated by SEM. ***, $P < 0.001$.

TABLE 2. Sperm count and motility in MT-α transgenic mice

	Wild type (n = 12)	MT-α line A (n = 8)	MT-α line C (n = 5)
Sperm count	76.6×10^6/ml	31.6×10^6/ml	34.7×10^6/ml
Sperm motility	44.0 ± 1.7%	48.0 ± 2.9%	37.0 ± 7.6%

Sperm cells were collected from the epididymis of 4-month-old MT-α transgenic mice or age-matched littermates. Sperm numbers were significantly reduced in transgenic males ($P < 0.0001$) based on one-way ANOVA. Sperm motility refers to the percentage of sperm that exhibited flagellar movement.

exhibited very low inhibin α levels, whereas serum inhibin levels for ovariectomized MT-α female mice remained increased, indicating nongonadal expression of the MT-α transgene (Fig. 5A). These data support the RT-PCR findings that demonstrate expression of the transgene mRNA in nongonadal tissues.

An alternative explanation for the reduced FSH levels in these mice is that dimerization of the excess free α-subunit with endogenous β-subunits, or effects of α-subunit expression on β-subunit synthesis, might reduce the levels of activin dimers and thus adversely affect activin actions. Consistent with this possibility, we observed small reductions in the levels of β-subunit proteins in transgenic mouse ovaries, and serum levels of activin A were slightly reduced compared with control mice, although this change does not reach statistical significance. It may be that both increased inhibin production and decreased activin production contribute to an altered inhibin-to-activin ratio and to the observed hormonal and reproductive phenotypes in MT-α transgenic mice.

MT-α transgenic mice provide a useful *in vivo* model for examining the effects of inhibin on the hypothalamic-pituitary-gonadal axis. The gonadotropin ratio is significantly altered in MT-α transgenic female mice. FSH levels are repressed in both male and female MT-α transgenic mice, which supports the roles of inhibin and activin as important negative regulators of FSH secretion *in vivo*. The treatment of anterior pituitary cell cultures with inhibin results in a significant reduction in FSH production (1–4). *In vivo* studies that support the role of inhibin as a negative regulator of FSH include an early experiment performed with proestrus rats treated with porcine follicular fluid as a source of inhibin. After treatment, these animals exhibited suppression of the secondary FSH surge (21). Additionally, the infusion of female rats with an inhibin polyclonal antiserum caused an increase in plasma FSH, thus supporting the importance of inhibin negative feedback in FSH regulation (36).

It is perhaps surprising that MT-α transgenic mice are fertile and that FSH secretion is not further reduced in these animals. *In vivo*, the ability of inhibin to suppress FSH may be dependent on additional factors. An *in vivo* study was performed with cycling female rats treated with recombinant human inhibin A at a dosage in excess of available circulating inhibin (37). Rats were treated with inhibin during proestrus, estrus, metestrus, or diestrus, and serum FSH was measured each hour after treatment. Animals in proestrus demonstrated a reduction in FSH, although generally no more than 2-fold. Rats in estrus, metestrus, and diestrus treated with recombinant inhibin showed no reductions in serum FSH, suggesting that *in vivo* regulation of FSH production by inhibin is limited and cycle dependent (37). These *in vivo* data suggest that inhibin may not act alone but instead likely acts with other factors, such as E, in mediating the down-regulation of FSH production in a cycle-dependent manner. *In vivo* studies performed with E and inhibin further support the coordinated actions of these hormones in mediating FSH suppression (38). Ovariectomized rats treated with charcoal-extracted porcine follicular fluid as a source of inhibin, as well as rats treated with E, exhibited a decrease in serum FSH (38). Rats treated with both follicular fluid (inhibin) and E exhibited an added suppression in serum FSH compared with either treatment alone (38), suggesting that inhibin and

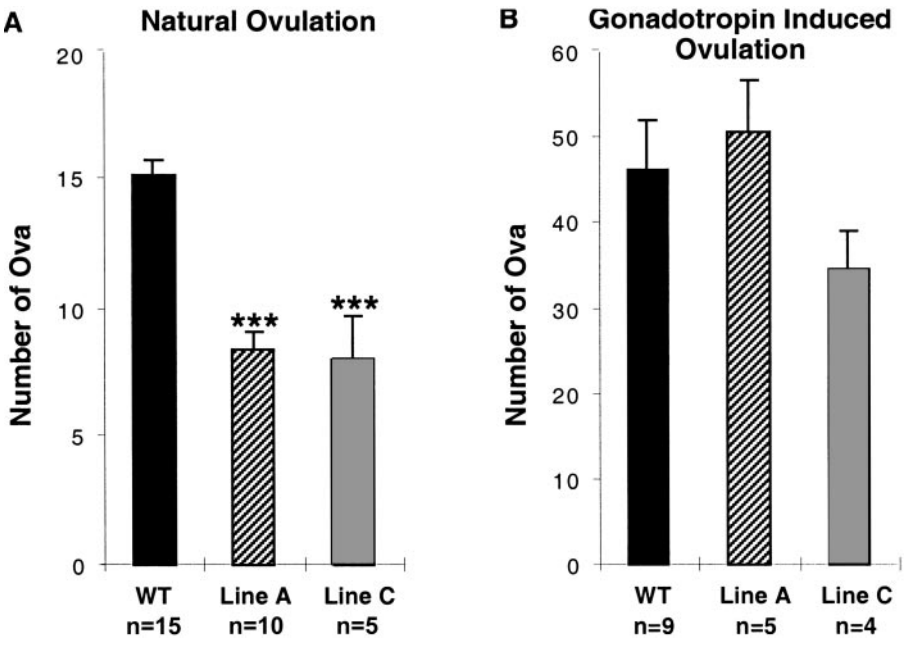

FIG. 8. Natural ovulation rates and gonadotropin-induced ovulation rates of MT-α transgenic female mice and wild-type control female mice. Female mice were paired with vasectomized control males. Females with copulatory plugs were killed and ova were collected. A, Natural ovulation rates for MT-α transgenic mice *vs.* wild-type littermates. The average number of ova released per ovulation from naturally ovulating MT-α females was significantly different from the average number of ova released per ovulation from naturally ovulating wild-type females. B, Gonadotropin-induced ovulation rates for MT-α transgenic mice *vs.* gonadotropin-induced ovulation rates for wild-type littermates. Gonadotropin-induced MT-α mice did not display a statistically significant difference in ovulation rates *vs.* gonadotropin-induced wild-type females. Females ranged in age from 3 to 6 months. All statistical analyses were performed using one-way ANOVA with variance indicated by SEM. ***, $P < 0.001$.

E act in a synergistic manner to reduce FSH secretion *in vivo*. E levels are reduced in MT-α female mice, as described in the accompanying paper. This lack of adequate E negative feedback could limit the suppression of FSH by inhibin in MT-α transgenic female mice.

The basis for increased LH levels in MT-α females (Fig. 6C) is not clear because there is no known direct mechanism of LH regulation by inhibin. However, the inappropriate expression of inhibin α may contribute indirectly to the increase of LH. Exposing cultured thecal cells to increased levels of inhibin has been shown to stimulate thecal cell androgen production *in vitro* (39). The local overexpression of inhibin α in MT-α transgenic mice may be responsible for the increase in androgen production, as reflected by increased T levels in transgenic females reported in the accompanying paper. High levels of T may desensitize the pituitary and hypothalamus to the negative feedback of the steroid hormones, causing increases in LH. In women who suffer from virilizing congenital adrenal hyperplasia, exposure to high levels of androgens at birth is associated with high LH levels by the time they reach sexual maturity (40). If MT-α female mice are exposed to increased androgens during development, this exposure could result in hypothalamic/pituitary insensitivity and continued increases in serum LH.

In MT-α transgenic male mice, the observed decrease in sperm numbers may be secondary to increased inhibin or suppressed activin, and it is likely to be a direct consequence of the altered gonadotropin environment. The treatment of adult mice and hamsters with purified inhibin has been shown to reduce spermatogonial numbers (41). However, it is difficult to determine if inhibin directly suppressed sperm production, because inhibin treatment also slightly reduced serum FSH levels (41). Reduced or absent FSH has been shown to decrease sperm numbers in rodents. For example, FSH-deficient male mice exhibit a 75% reduction in sperm numbers but are still fertile (42), and FSH receptor-deficient mice similarly display a significant reduction in sperm numbers and remain fertile (43). Thus, the likely basis for decreased sperm production in MT-α transgenic male mice is suppressed FSH levels.

Similarly, the observed reduction in MT-α female ovulation rates is probably attributable to the altered gonadotropin ratios in these animals and not to a follicular defect (Fig. 8A). During the normal estrus cycle, the GnRH-independent secondary FSH surge is important for the recruitment of maturing follicles into the preovulatory pool (23, 44). In MT-α transgenic mice, total inhibin α-subunit levels are high and FSH levels are suppressed. The possible reduction in FSH levels during the morning of estrus, the time of the secondary FSH surge, could limit the number of immature follicles recruited for subsequent ovulation. To determine if the ovulation rate of MT-α females could be rescued, we treated transgenic female mice with exogenous gonadotropins. The exogenous gonadotropin stimulation of MT-α females resulted in an ovulation rate almost equal to that of wild-type gonadotropin-stimulated females (Fig. 8B), suggesting that reduced FSH, or the abnormal gonadotropin ratio, inhibits follicle recruitment in MT-α ovaries.

It is unlikely that reduced oocyte competence is responsible for reduced MT-α fertility. The combined decrease in birth rate of MT-α female mice compared with their wild-type littermates was 52%. The combined reduction in the ovulation rate was 54%. Thus, the reduction in offspring number mirrors the observed reduction in ovulation rates, indicating that ovulatory defect is the primary factor in reduced MT-α female fertility.

The loss of the inhibin α-subunit in inhibin-deficient mice results in significant increases in serum activin levels (26), indicating that the inhibin β-subunits are up-regulated in the absence of the α-subunit. With the overexpression of the inhibin α-subunit in MT-α transgenic mice, a decrease in inhibin β-subunit expression and/or activin dimer assembly might be expected. We observed small reductions in ovarian β-subunit protein expression and in serum activin levels in

older female mice. In contrast, *in situ* analysis performed on ovaries from MT-α transgenic mice with an inhibin β_A riboprobe showed no substantial change in β-subunit RNA per follicle in the presence of excess α-subunit (Fig. 3). Activin-deficient mice (45, 46) exhibit a markedly different phenotype than MT-α transgenic mice. Although metallothionein is expressed early in development (47), MT-α transgenic mice mature normally, whereas activin β_A-deficient mice or β_A/β_B-deficient mice die postnatally from cleft palate defects (46). Activin β_B-deficient mice are viable, subfertile because of a failure to nurse their young, and show a 20% increase in serum FSH levels (45). This suggests that the phenotypes of the MT-α transgenic mice are more likely to be associated with the increased inhibin levels than with decreased activin levels, although both are likely to be contributory.

In conclusion, the overexpression of inhibin α in transgenic mice results in reduced serum FSH and an overall alteration in gonadotropin ratios. MT-α transgenic female mice exhibit reduced fertility as a result of reduced ovulations, whereas MT-α transgenic male mice are fertile despite having significantly lower epididymal sperm numbers. These findings support a role for inhibin and activin as key *in vivo* modulators of FSH production, and they suggest that inhibin and activin are essential for normal fertility and gametogenesis. The accompanying paper focuses on potential autocrine/paracrine actions of the inhibin transgene product within the ovary and describes the effects of increased inhibin α-subunit on gonadal morphology and ovarian steroidogenesis in these transgenic mice.

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