Mol. Cells, Vol. 18, No. 1, pp. 79-86

Molecules and Cells

Changes in the Reproductive Functions of Mice due to Injection of a Plasmid Expressing an Inhibin α-Subunit into Muscle: a Transient Transgenic Model

Jung Mi Ahn†, Hoi Kyung Jung†, Chunghee Cho[1], Donchan Choi[2], Kelly E Mayo[3], and Byung-Nam Cho*

Department of Life Science, The Catholic University of Korea, Buchon 421-743, Korea;

[1] Department of Life Science, Gwangju Institute of Science and Technology, Gwangju 500-712, Korea;

[2] Division of Environmental and Biological Science, College of Natural Science, Yong In University, Yongin 449-714, Korea;

[3] Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL 60208, USA.

(Received March 19, 2004; Accepted April 28, 2004)

Inhibin is a gonadal hormone composed of an α-subunit and one of two β-subunits (β_A, β_B), and its primary role is to inhibit FSH secretion by the pituitary. To investigate the roles of inhibin α in the reproductive system, an expression plasmid, pCMV-rINA, with the rat inhibin α cDNA fused to the cytomegalovirus promoter, was introduced into muscle by direct injection. Inhibin α mRNA was detected in the muscle by RT-PCR and Southern blot analysis. Inhibin protein was also detected, and Western blot analysis revealed a relatively high level of serum inhibin, but not of activin β_A. The estrous cycle of the pCMV-rINA-injected mice was extended, but there was no change in levels of pituitary FSH mRNA or serum FSH and no ovarian cysts were observed. When injected female mice were mated with males of proven fertility, litter size increased. Surprisingly, the embryos of pregnant females injected with pCMV-rINA, were retarded in growth and had defects in internal organs. When male mice were injected, testicle weight increased slightly without any noticeable change in the histology of the seminiferous tubules. Taken together, our data indicate that the inhibin α subunit influences a number of the reproductive functions of female mice.

Keywords: Abnormal Reproduction; Inhibin α; Small Embryo.

Introduction

Inhibin and activin are multifunctional hormones belonging to the TGF-β superfamily of proteins. Inhibin and activin were originally identified by their ability to inhibit and stimulate, respectively, FSH secretion and synthesis in cultured anterior pituitary cells (Ling *et al*., 1985; 1986a; 1986b; Miyamoto *et al*., 1985; Rivier *et al*., 1985; Robertson *et al*., 1985; Vale *et al*., 1986). Inhibin is a heterodimer composed of an inhibin-specific α subunit and either one of two related β subunits (β_A, β_B) (Ling *et al*., 1985; Miyamoto *et al*., 1985; Rivier *et al*., 1985; Robertson *et al*., 1985), whereas activin is a dimer of β subunits (Ling *et al*., 1986b; Vale *et al*., 1986). Inhibin α mRNA is primarily expressed in the granulosa cells of the ovary (Meunier *et al*., 1988b), the Sertoli cells of the testis (Roberts *et al*., 1989), and the adrenal cortex (Crawford *et al*., 1987).

Inhibin production is tightly regulated throughout the female reproductive cycle. On the afternoon of proestrus of the rodent estrus cycle, just prior to the preovulatory FSH and LH surge, the granulosa cells of developing follicles produce high levels of inhibin A (Meunier *et al*., 1988a; 1988b). Following the gonadotropin surges, ovarian inhibin production declines (Woodruff *et al*., 1989), and low inhibin levels persist through the morning of estrus, providing an environment permissive for elevated FSH and for generating the secondary FSH surge. Treatment of proestrus rats with charcoal-extracted follicular fluid as a source of inhibin prevents the secondary FSH surge (Schwartz and Channing, 1977). The latter is responsible for recruiting new follicles into the antral pool (Lintern-Moore, 1977; Neal and Baker, 1973), and FSH stimulates inhibin expression in newly recruited follicles (Bicsak *et al*., 1986; Meunier *et al*., 1988a). Thus, appro-

† These first two authors contributed equally to this work.

* To whom correspondence should be addressed.
Tel: 82-2-2164-4358; Fax: 82-2-2164-4765
E-mail: ocean@catholic.ac.kr

priate inhibin levels are important for maintaining the estrous cycle and for normal follicle development.

The importance of inhibin for normal gonadal function was revealed by inhibin α-deficient (Matzuk *et al*., 1992), inhibin A-overexpressing (Pierson *et al*., 2000), and inhibin α-overexpressing transgenic mice (Cho *et al*., 2001; McMullen *et al*., 2001). Inhibin α-deficient mice develop ovarian and testicular stromal tumors (Matzuk *et al*., 1992), and, if they are gonadectomized prior to gonadal tumorogenesis, they develop adrenal tumors (Matzuk *et al*., 1994). Maturing oocytes and spermatozoa are initially present in the gonads of inhibin α subunit-deficient mice; however, the development of gonadal stromal tumors leads to the arrest of gametogenesis (Matzuk *et al*., 1992). These animals die prematurely at 3–4 months of age and thus do not permit an extensive analysis of the role of inhibin in adult reproductive function. Transgenic mice overexpressing inhibin A have small testes and blocked folliculogenesis (Pierson *et al*., 2000), whereas transgenic mice overexpressing the inhibin α subunit have reproductive deficiencies, including reduced ovulation, reduced litter size, explicit ovarian cysts, and abnormal follicles containing two oocytes (Cho *et al*., 2001; McMullen *et al*., 2001).

Inhibin α mRNA was found to be down-regulated in prostate cancer (Mellor *et al*., 1998) and up-regulated in the aging rodent (Jih *et al*., 1993; Pal *et al*., 1991). Inhibin α is present in follicular fluid (Sugino *et al*., 1989) and its precursor can alter FSH binding to the FSH receptor (Schneyer *et al*., 1991). Thus, there is clearly a need to investigate the diverse activities of inhibin α. In the present study, we have investigated its function in mice by expressing it in muscle, using a method of direct gene transfer that permits rapid expression of foreign genes (Danko *et al*., 1997); the inhibin produced by the muscle gains access to the circulation and affects reproductive function.

Materials and Methods

Animals and experimental design ICR mice at 2 months of age were purchased from the Daehan Animal Center and maintained under 14 h light, 10 h dark illumination at 23°C, with food and water *ad libitum*. Plasmid DNA was purified and injected as described (Ko *et al*., 2003). To measure inhibin α mRNA and protein (Figs. 1B and 2A), muscle tissue was harvested 4 days after a single injection of DNA. For the reproductive studies (Figs. 2B–2C, Figs. 3–5, and Fig. 8), two injections of 300 μg pCMV-rINA in 50 μl of 10% sucrose in saline were made into the quadriceps of mice at 10:00 A.M., 7 days apart. For the developmental studies (Figs. 6–7), the first injection was performed at 10:00 A.M. on diestrus II and the second 7 days later. Control mice were injected with the same amount of pcDNA3 vector. All experiments were performed at least four

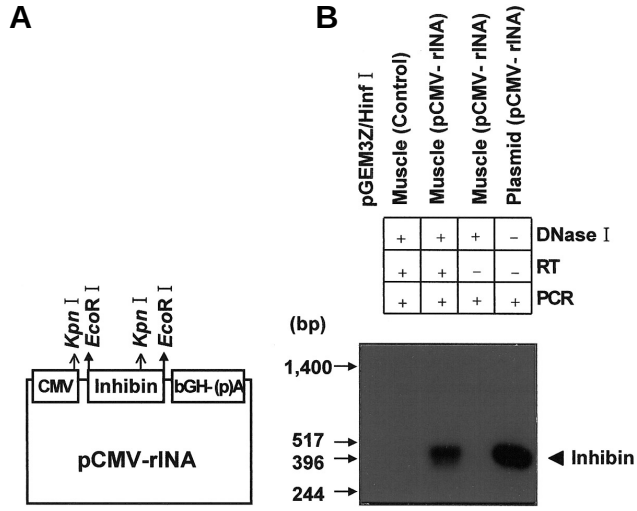

Fig. 1. pCMV-rINA structure and inhibin gene expression. **A.** Diagram of the pCMV-rINA construct. Functional elements include the cytomegalovirus (CMV) promoter, the rat inhibin α cDNA, and the human growth hormone (hGH) poly(A). **B.** RT-PCR was performed as described in **Materials and Methods**. RNAs from control, and from pCMV-rINA-injected mice without reverse transcription, were used as normal and internal controls, respectively. The pCMV-rINA plasmid was used as a positive control.

times if not otherwise noted, and representative results are shown.

Construction of the pCMV-rINA expression vector To generate pCMV-rINA (7.02 kb), a 1.572-kb rat inhibin α cDNA was digested with *Eco*RI and cloned into the *Eco*RI site of vector pcDNA3 (Invitrogen, USA) which has a CMV early promoter and a bovine growth hormone polyadenylation site (Fig. 1A). The correct insertion of inhibin α cDNA was confirmed by digestion with *Kpn*I.

Reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot hybridization RT-PCR and Southern blot hybridization were performed as described (Cho *et al*., 2001). Briefly, muscles were homogenized with denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% N-lauryl sarcosine, and 0.1 M 2-mercaptoethanol). The homogenate was phenol/chloroform extracted, and the RNA precipitated and quantified with a U.V. 2000 spectrophotometer (Pharmacia, USA). A_{260}/A_{280} ranged from 1.8 to 2.0. Ten micrograms of total RNA were used in duplicate. The RNA was then treated with DNase I (5U, Promega, USA) at 37°C for 10 min in order to remove genomic and plasmid DNA, and reverse transcribed at 42°C with random hexamer primers and AMV reverse transcriptase (Promega, USA) in a 20 μl reaction. A mixture of oligonucleotide primers (500 ng each), dNTP, and *Taq* DNA polymerase (2.5 U) was added to each reaction, the total volume was brought to 100 μl with 1× PCR buffer [10 mM

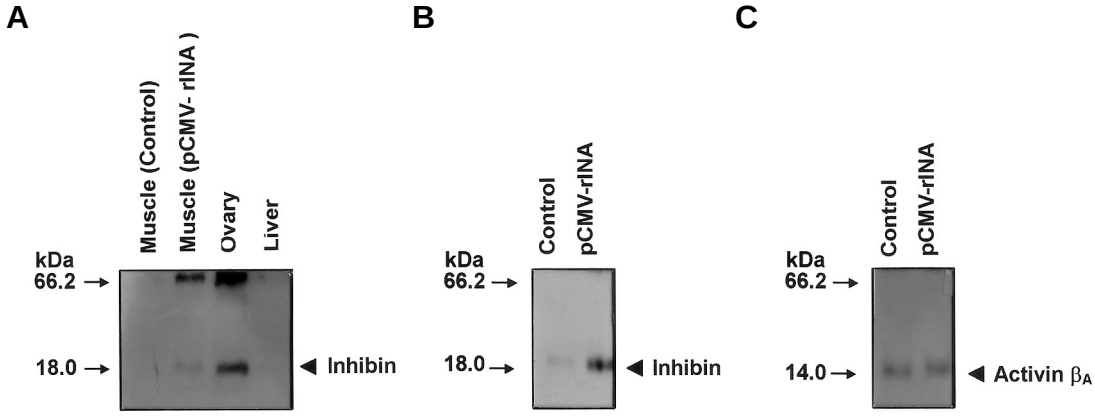

Fig. 2. Inhibin detection. (A) Western blot analysis was performed as described in **Materials and Methods**. Proteins from the muscle, ovary, and liver of control mice were used as normal sample, positive control, and negative control, respectively. The Western blot shown is representative of four independent experiments. pCMV-rINA: pCMV-rINA-injected mice. Inhibin (B) and activin β_A (C) were detected by Western blot analysis in 1 μl serum 4 days after the second injection of pCMV-rINA plasmid.

Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin] and the sample was overlaid with light mineral oil. Amplification was performed for 30 cycles using an annealing temperature of 65°C on an Omn-E thermal cycler (Hybaid Limited, UK). The primers for the inhibin α gene were designed to generate a 341 bp fragment. The 5′ primer was 5′-GTCCTGCCTCGAAGACATGCC-3′ and the 3′ primer 5′-CTGTACCAAG GACACAGGCA-3′. After amplification, the samples were chloroform extracted, dried, resuspended in 10 μl TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), and electrophoresed on a 1.2% agarose gel. The gel was photographed after ethidium bromide staining. The PCR products were then denatured with sodium hydroxide and transferred to Nytran filters (0.45 μm, Schleicher & Schuell, Germany) under vacuum. They were hybridized with ^{32}P-labeled rat inhibin α cDNA, washed, and processed for autoradiography.

Northern blot analysis Northern blot analysis was carried out as previously described (Cho *et al*., 1993). Briefly, total RNA (10 μg) was electrophoresed on a 1.2% agarose gel at 70 V for 1.5 h. After transferring the RNA onto Nytran filters (0.45 μm, Schleicher & Schuell, Germany) by diffusion blotting, the filters were hybridized with ^{32}P-labeled rat FSHβ cDNA, washed, and autoradiographed with X-ray film (Konica AX film) at –70°C for 1 d.

Protein blot analysis Tissues were removed, homogenized in 400 μl of protein extraction buffer [0.1 M NaCl, 0.01M Tris-Cl (pH 7.6), 1 mM EDTA (pH 8.0), 0.1% TritonX-100, 1 μg/ml aprotinin, and 100 ng/ml phenylmethylsulfonyl fluoride], and centrifuged four times. The homogenates were mixed with an equal volume of 2× SDS-loading buffer [100 mM Tris-Cl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% BPB, 20% glycerol], placed in boiling water for 10 min, and centrifuged. The supernatants were transferred to fresh tubes. Samples of each extract containing 10 μg protein were heated at 70°C for 10 min, electrophoresed on a 12% acrylamide gel and transferred onto Nytran filters in 1× transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol). The blots were incubated overnight in blocking solution (5% nonfat dried milk, 0.02% sodium azide, 0.02% Tween 20) with shaking at 4°C, followed by exposure to primary inhibin α antibodies (1:400) (Serotec, UK) or activin β_A antibodies (1:400) (Serotec, UK) overnight. They were washed in milk-TBS-Tween for 30 min and incubated with secondary anti-rabbit Ig horseradish peroxidase-linked whole donkey antibody (1:100) (Amersham Pharmacia Biotech, USA) in azide-free blocking solution [5% nonfat dried milk, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5)] for 2 h. The secondary antibody was detected with an ECL kit (Amersham Pharmacia Biotech, USA).

Hormone measurement Serum hormone levels were measured at the Northwestern University RIA Core Facility under the direction of Dr John Levine. For FSH measurement National Institute of Diabetes and Digestive and Kidney Disease antiserum and standard (rFSH-RP-2 standard/rFSH-S-11) antibody were used. FSH results are expressed as nanograms per milliliter, and the assay sensitivity was 0.05 ng/sample or 1.0 ng/ml. The interassay coefficient of variation was 7.9% for female serum FSH measurements.

Histology and estrous cycle analysis The gross appearance of excised tissues from injected and control mice were examined, and the tissues immediately fixed in fresh 4% paraformaldehyde in PBS, pH 7.4. Following overnight fixation, they were dehydrated in ethanol and embedded in paraffin, and seven-micrometer sections were prepared with a microtome (Nippon Optical Works, Japan). The sections were de-paraffinized with xylene, dehydrated in absolute ethanol, and rehydrated in water. Sections were stained with hematoxylin, counterstained with eosin, and observed under a light microscope (Olympus IX70, Japan) or a steromicroscope (Leica ME Apo, Switzerland).

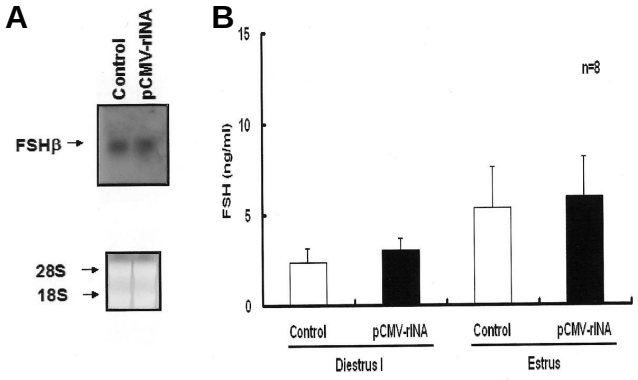

Fig. 3. Change in FSH. **A.** FSH mRNA in the pituitary was detected at the first estrus after injection of the pCMV-rIINA plasmid by Northern blotting with ^{32}P-labled FSHβ cDNA. The amount of RNA was normalized to the content of 28S and 18S ribosomal RNA. **B.** Serum FSH was detected by RIA at the first diestrus I and estrus after pCMV-rINA plasmid injection.

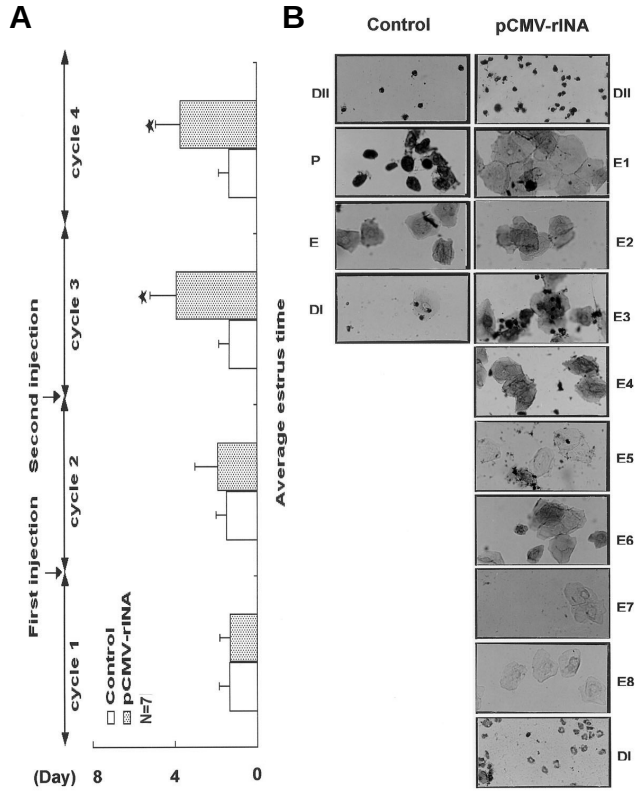

Fig. 4. Change in the estrous cycle in inhibin α-expressing mice. **A.** Each stage of the estrous cycle was identified by daily examination of vaginal cytology at 9:30 A.M. at a 100× magnification (n = 7). The first injection was carried out after confirming at least two normal estrous cycles, and the second injection followed 4 days later. Note that estrus was extended after the second injection. Asterisks denote values that are significantly different from the mean control value (Student's *t*-test, $P < 0.01$). Values shown are means ± standard deviations. **B.** A photograph of one example. Note that few leukocytes were observed at times corresponding to the diestrus I or diestrus II stages. DI, diestrus I; DII, diestrus II; P, proestrus; E(1)–E(8), day 1–day 8 of estrus.

Results

Inhibin α gene expression in muscle In initial studies we tested whether intramuscular injection was effective in expressing the inhibin α subunit in mice. Using RT-PCR with inhibin α-specific primers and Southern blot analysis, we detected a 341 bp inhibin α mRNA in mouse muscle after injecting the pCMV-rINA construct (Fig. 1A). Hybridization with labeled inhibin α cDNA confirmed that the PCR product was inhibin α DNA (Fig. 1B). Western blot analysis revealed mature inhibin α protein (18 kDa) in injected muscle (Fig. 2A), along with a larger species that probably corresponds to the inhibin precursor (Fig. 2A). The level of serum inhibin α protein was substantially elevated in the pCMV-rINA-injected mice (Fig. 2B). Thus, inhibin α mRNA and protein are expressed in muscle and secreted into the serum by this approach. No change of serum activin β_A (14 kDa) was observed in the pCMV-rINA-injected mice (Fig. 2C).

Effects of inhibin α on reproductive functions Inhibin was initially described and named for its ability to inhibit FSH secretion in the female reproductive axis. To investigate whether the ectopically expressed inhibin α influences FSH gene expression, we measured FSH-β mRNA by Northern blot hybridization and found no change in FSH mRNA or in FSH itself in the injected mice (Figs. 3A and 3B). However, when we examined individual stages of the estrous cycle after injecting pCMV-rINA DNA into female mice, we found that their estrus was extended (Fig. 4A). After two injections of pCMV-rINA into mice at 10:00 A.M., the estrus stage in the subsequent two cycles, cycles 3 and 4, was approximately twice the average length (Fig. 4A). In one case it was extended to 8 days (Fig. 4B). It is noteworthy that few leukocytes were observed at times corresponding to diestrus I and II [Fig. 4B, E(3)–E(5)]. Within the ovary, each follicle had a small antrum and a normal-appearing oocyte (Fig. 5). However, no cysts were observed in the pCMV-rINA-injected mice [rate of cyst formation: 0.0% (pCMV-rINA) vs 0.0% (control), n = 15]. Because estrus was extended, presumably due to the increased estrogen and resulting increase in mature follicles, we asked whether this was associated with any change in litter size. There was indeed an increased number of embryos in the inhibin α-expressing female mice (Fig. 6), suggesting that the extended estrus and the preliminary finding of increased numbers of corpora lutea (Fig. 5) were correlated with an increased litter size. This was somewhat unexpected since inhibin is

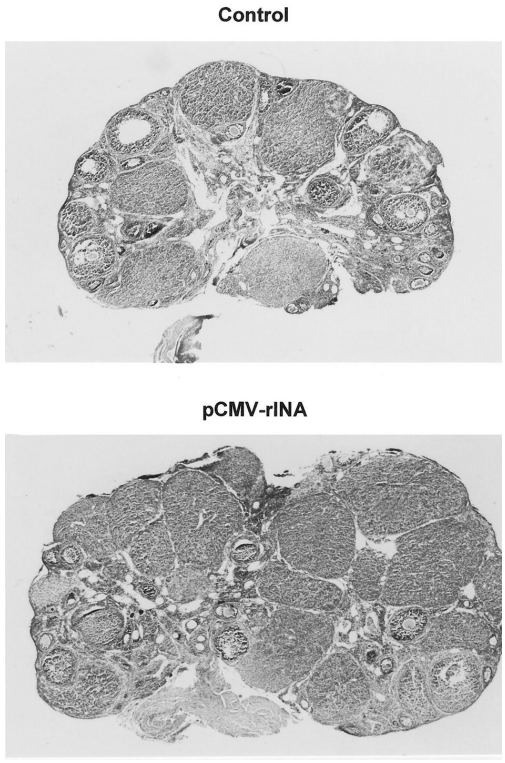

Fig. 5. Histology of the ovary. Tissues were prepared as described in **Materials and Methods** and observed at 40× magnification. No cysts were observed in the ovaries of pCMV-rINA-injected mice.

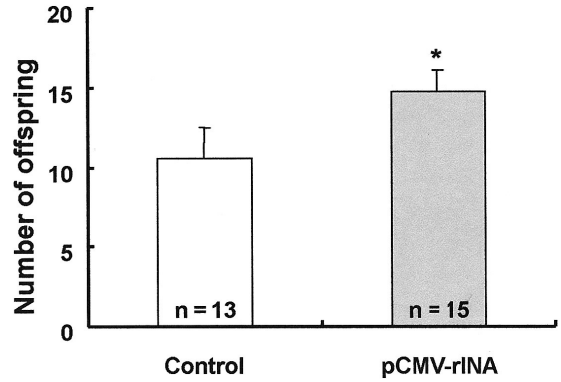

Fig. 6. Litter size in pCMV-rINA-injected pregnant mice. Numbers of offspring in control (n = 13) and pCMV-rINA-injected mice (n = 15). Asterisks denote values that are significantly different from the mean control value (Student's *t*-test, $P < 0.05$). Values shown are means ± the standard deviations.

known to inhibit FSH synthesis and secretion, although levels of the pituitary FSH mRNA and serum FSH were unchanged in our study. Finally, we investigated the possible role of inhibin α in embryonic development since inhibin α has a variety of developmental functions and inhibin α, like activin, belongs to the TGF-β superfamily whose members are mainly involved in embryonic development (Matzuk *et al*., 1995). When we investigated embryos from female mice that were injected with pCMV-rINA while pregnant, they proved to be surprisingly small; day E15.5 embryos were about the same size as day E10.5 embryos in normal mice (Fig. 7A). Histological examination of these small embryos revealed retarded growth of every organ, specially the lung, with no particular abnormalities other than size (Fig. 7B). In addition, testicular weight was slightly increased in male mice (Fig. 8A) without noticeable change in size and histology of the seminiferous tubules (Fig. 8B).

Discussion

To further understand the physiological function of inhibin α in mammalian reproduction and development, we applied a "transient gain of function" mouse model, using naked DNA injection as a gene transfer method. Direct injection of pCMV-rINA DNA into mouse muscle led to the appearance of inhibin α mRNA and protein, and had a variety of consequences, including changes in estrous cycle, litter size, and embryo size.

The transient inhibin α expression approach provides a useful *in vivo* model for examining the effects of inhibin α on the hyphothalamic-pituitary-gonadal axis in addition to established transgenic mouse models. In the adult rodent, inhibin α expression is both spatially and temporally regulated and the inhibin α subunit is primarily expressed in the ovary, testis, and adrenal and pituitary glands (Meunier *et al*., 1988b; Woodruff *et al*., 1988). With our method, inhibin α is conditionally expressed within a restricted tissue, muscle. Thus, this approach provides a relatively simple means of examining the roles of the inhibin α subunit that could be extended to other endocrine genes.

Inhibin was initially identified based on its ability to inhibit FSH secretion; treatment of anterior pituitary cell cultures with inhibin resulted in a reduction of FSH production (Ling *et al*., 1985; Miyamoto *et al*., 1985; Rivier *et al*., 1985; Robertson *et al*., 1985). Whereas treatment of proestrus rats with porcine follicular fluid reduced FSH production (Schwartz and Channing, 1977) infusion with inhibin polyclonal antiserum caused an increase in plasma FSH (Rivier *et al*., 1986). Recent reports that inhibin α-overexpressing transgenic mice have reduced FSH levels (Cho *et al*., 2001) whereas inhibin α-deficient mice have increased levels (Matzuk *et al*., 1992) confirm the importance of inhibin negative feedback in FSH regulation. However, FSH secretion is little affected in inhibin α-overexpressing transgenic mice and, similarly, levels of FSH mRNA in the pituitary and of FSH in serum were not significantly affected in our pCMV-rINA injected mice. Moreover, follicular fluid (inhibin) and estrogen act syn-

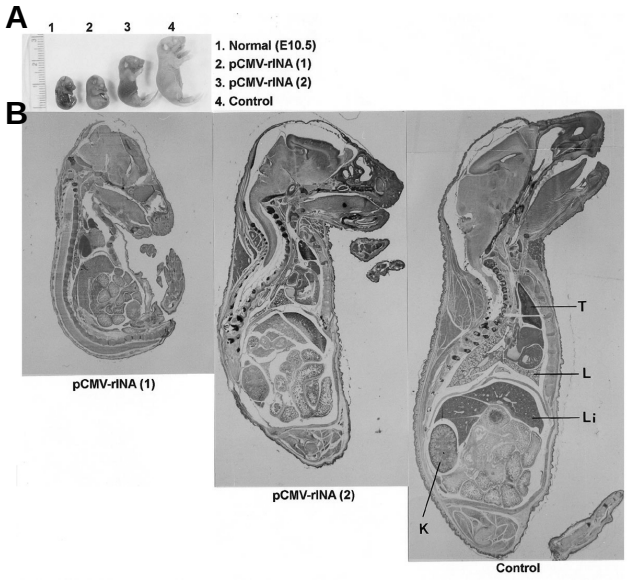

Fig. 7. Gross morphology and histology of the embryos. **A.** Embryos were obtained at E15.5 after two injections of pCMV-rINA. **B.** Histology of a mid sagital section of the embryos. Note that the lungs of embryos (pCMV-rINA(1), pCMV-rINA(2)) from pCMV-rINA injected mothers are poorly developed. T, thymus; L, lung; Li, liver; K, kidney.

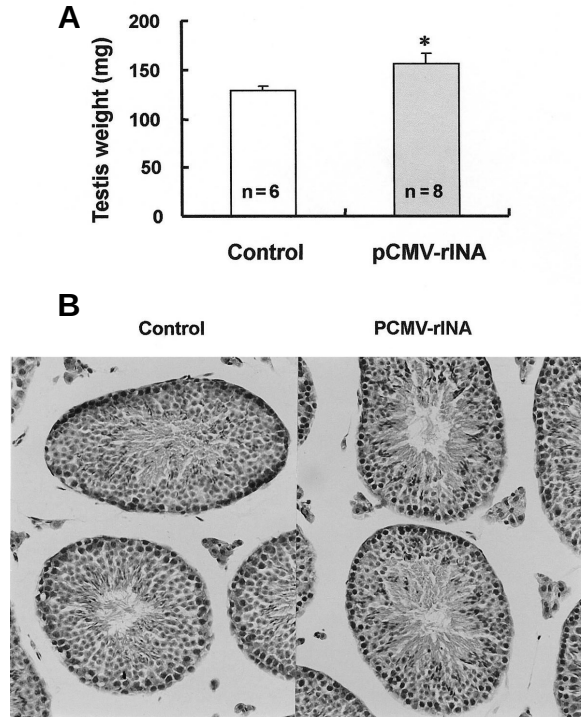

Fig. 8. Histology of the testis. **A.** Testis weight was measured after isolation of testes from control and pCMV-rINA-injected mice. **B.** Tissues were prepared as described in **Materials and Methods** and observed at 200× magnification.

ergistically in suppressing serum FSH in rats (Grady *et al*., 1982). Together these findings indicate that FSH synthesis and secretion are under complex regulation *in vivo*.

In contrast to its lack of effect on FSH in this model, inhibin α overexpression markedly affected the estrous cycle, particularly the uterine cycle. The estrous cycle represents the integration of the ovarian, uterine and cervical cycles. The state of the uterus is primarily influenced by estrogen produced in developing and mature follicles within the ovary, and follicle maturation within the ovary is stimulated by intraovarian injection of recombinant inhibin (Woodruff *et al*., 1990). Thus the state of the ovary, which is primarily influenced by FSH, may be changed independently by inhibin without a change in FSH. A previous report of a relatively large interval between pregnancies in inhibin α-overexpressing transgenic mice indirectly supports the ability of inhibin to influence the estrous cycle (Cho *et al*., 2001). In addition, the reduced, but not disappeared, leukocytes in number observed at times corresponding to diestrus I and II [Figs. 4B, E(3)–E(5)] suggest that the populations of leukocyte and epithelial cells, two important cell types found in uterine fluid, are regulated in different ways.

When we examined ovarian histology in the pCMV-rINA injected mice, we observed no cysts or abnormal follicular structures such as those seen in inhibin α-overexpressing transgenic mice (McMullen *et al*., 2001). The transient expression of inhibin α in the present model, as opposed to chronic expression in the transgenic model, probably accounts for this difference. Inhibin α overexpressing transgenic male and female mice are fertile (Cho *et al*., 2001). However, the females have reduced fertility and this worsens with age. In the pCMV-rINA injected mice, the females showed no such fertility change and the males were also fully fertile. The decreased fertility in the inhibin α-overexpressing transgenic female is thought to be partially caused by cyst formation and the ensuing reduction in the pool of follicles. In the pCMV-rINA injected female mice, fertility increased rather than decreased, and cysts were not observed. Once again, this probably reflects differences in the timing and duration of inhibin α overexpression in the two models.

The embryos obtained from pCMV-rINA injected pregnant mice were growth-deficient. Although an involvement of inhibin in pancreatic function has not been reported, our preliminary studies reveal that the glucose level in blood is severely lowered in pCMV-rINA injected female mice (data not shown). This may in turn affect fetal growth and development. Further studies are required to ascertain if this is the cause of the growth retardation.

Inhibin α-overexpressing transgenic males exhibited a reduction in testis volume and sperm numbers that is quite similar to the phenotype of both FSH-deficient male mice (Kumar *et al*., 1997) and FSH receptor-deficient males

(Dierich *et al*., 1998). On the other hand, we observed a slight increase in testis weight and almost no change in histology in our pCMV-rINA injected mice.

The loss of the inhibin α-subunit in inhibin-deficient mice results in a significant increase in serum activin levels (Matzuk *et al*., 1994), indicating that inhibin β-subunits are up-regulated in the absence of α-subunits. In inhibin α-overexpressing transgenic mice, small reductions in ovarian β-subunit protein expression and in serum activin levels were observed in older female mice, but there was no substantial change in β-subunit RNA per follicle (Cho *et al*., 2001). It is known that parietal cells in the stomach are lost in response to elevated activin β_A in inhibin α-deficient mice (Matuzk *et al*., 1994). When we examined the stomach in the present study, we found no loss of parietal cells in the pCMV-rINA-injected mice (data not shown) nor was there any change in serum activin β_A (Fig. 2C). We therefore believe that activin levels are not down-regulated in this model.

In addition to the implications of this work for understanding inhibin α subunit function, our studies point to significant technical advantages and potential clinical applications of intramuscular injection. It is an approach that can be applied to many different species, as it does not require the use of specific genetic strains. It is also easy to apply, in that expression of the targeted gene can be achieved at essentially any stage of development, as well as in the adult. Finally, this approach could be used for *in vivo* screening of genetically engineered proteins for therapeutic efficacy and side effects in the case of secreted proteins without the need for the time-consuming production of transgenic mice.

Acknowledgments The authors thank Young il Kim for technical assistance and Oye-sun Seok for help in preparing the manuscript. We thank Brigitte Mann for performing the RIA and the director of the RIA Core Facility, Dr. John Levine. We also thank the National Hormone and Pituitary Program for FSH RIA reagents. We thank professor Teresa Woodruff (Northwestern University) for helpful discussion. This work was supported by a grant (R01-2000-00158) from the Basic Research Program of the Korea Science & Engineering Foundation (B-N. C.).

References

Bicsak, T. A., Tucker, E. M., Cappel, S., Vaughan, J., Rivier, J., Vale, W., and Hsueh, A. J. (1986) Hormonal regulation of granulosa cell inhibin biosynthesis. *Endocrinology* **119**, 2711–2719.

Cho, B.-N., Suh, Y. H., Yoon, Y. D., Lee, C. C., and Kim, K. J. (1993) Progesterone inhibits the estrogen-induced prolactin gene expression in the rat pituitary. *Mol. Cell. Endocrinol.* **93**, 47–52.

Cho, B.-N., McMullen, M. L., Pei, L., Yates, J., and Mayo, K. (2001) Reproductive deficiencies in transgenic mice expressing the rat inhibin α-subunit gene. *Endocrinology* **142**, 4994–5004.

Crawford, R. J., Hammond, V. E., Evans, B. A., Coghan, J. P., Haralambidis, J., Hudson, B., Penschow, J., Richards, R., and Treagear, G. (1987) α-inhibin gene expression occurs in the ovine adrenal cortex, and is regulated by adrenocorticotropin. *Mol. Endocrinol.* **1**, 699–706.

Danko, I., Williams, P., Herweijer, H., Zhang, G., Latendresse, J. S., Bock, I., and Wolff, J. A. (1997) High expression of naked plasmid DNA in muscles of young rodents. *Hum. Mol. Genet.* **6**, 1435–1443.

Dierich, A., Sairam, M. R., Monaco, L., Fimia, G. M., Gansmuller, A., Lemeur, M., and Sassone-Corsi, P. (1998) Impairing follicle-stimulating hormone (FSH) signaling *in vivo*: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc. Natl. Acad. Sci. USA* **95**, 13612–13617.

Grady, R., Charlesworth, M. C., and Schwartz, N. B. (1982) Characterization of the FSH-suppressing activity in follicular fluid. *Recent Prog. Horm. Res.* **38**, 409–456.

Jih, M. H., Lu, J. K., Wan, Y. J., and Wu, T. C. (1993) Enhanced ovarian inhibin subunit gene expression in aging rats is due to chronic anovulation. *Biol. Reprod.* **49**, 1208–1214.

Ko, J.-Y., Ahn, Y.-L., and Cho, B.-N. (2003) Angiogenesis and white blood cell proliferation induced in mice by injection of a prolactin-expressing plasmid into muscle. *Mol. Cells* **15**, 262–270.

Kumar, T. R., Wang, Y., Lu, N., and Matzuk, M. M. (1997) Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat. Genet.* **15**, 201–204.

Ling, N., Ying, S. Y., Ueno, N., Esch, F., Denoroy, L., and Guillemin, R. (1985) Isolation and partial characterization of a Mr 32,000 protein with inhibin activity from porcine follicular fluid. *Proc. Natl. Acad. Sci. USA* **82**, 7217–7221.

Ling, N., Ying, S. Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., and Guillemin, R. (1986a) Pituitary FSH is released by a heterodimer of the β subunits from the two forms of inhibin. *Nature* **321**, 779–782.

Ling, N., Ying, S. Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., and Guillemin, R. (1986b) A homodimer of the β-subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. *Biochem. Biophys. Res. Commun.* **138**, 1129–1137.

Lintern-Moore, S. (1977) Initiation of follicular growth in the infant mouse ovary by exogenous gonadotrophin. *Biol. Reprod.* **17**, 635–639.

Matzuk, M. M., Finegold, M. J., Su, J. G., Hsueh, A. J., and Bradley, A. (1992) α-inhibin is a tumour-suppressor gene with gonadal specificity in mice. *Nature* **360**, 313–319.

Matzuk, M. M., Finegold, M. J., Mather, J. P., Kremmen, K., Lu, H., and Bradley, A. (1994) Development of cancer cachexia-like syndrome and adrenal tumors in inhibin-deficient mice. *Proc. Natl. Acad. Sci. USA* **91**, 8817–8821.

Matzuk, M. M., Kumar, T. R., Vassali, A., Bickenbach, J. R., Roop, D. R., Jaenish, R., and Bradley, A. (1995) Functional analysis of activins during mammalian development. *Nature* **374**, 354–356.

McMullen, M. L., Cho, B.-N., Yates, C. J., and Mayo, K. E. (2001) Gonadal pathologies in transgenic mice expressing

the rat inhibin α-subunit. *Endocrinology* **142**, 5005–5014.

Mellor, S. L., Richards, M. G., Pedersen, J. S., Robertson, D. M., and Risbridger, G. P. (1998) Loss of the expression and localization of inhibin α-subunit in high grade prostate cancer. *J. Clin. Endocrinol. Metab.* **83**, 969–975.

Meunier, H., Cajander, S., Roberts, V., Riveir, C., Sawchenko, P., Hsueh, A., and Vale, W. (1988a) Rapid changes in the expression of inhibin α, β_A and β_B subunits in ovarian cell types during the rat estrous cycle. *Mol. Endocrinol.* **2**, 1352–1363.

Meunier, H., Rivier, C., Evans, R. M., and Vale, W. (1988b) Gonadal and extragonadal expression of inhibin α, β_A, and β_B subunits in various tissues predicts diverse functions. *Proc. Natl. Acad. Sci. USA* **85**, 247–251.

Miyamoto, K., Hasegawa, Y., Fukuda, M., Nomura, M., Igarashi, M., Kangawa, K., and Matsuo, H. (1985) Isolation of porcine follicular fluid inhibin of 32K daltons. *Biochem. Biophys. Res. Commun.* **129**, 396–403.

Neal, P. and Baker, T. G. (1973) Response of mouse ovaries *in vivo* an in organ culture to pregnant mares serum gonadotrophin and human chorionic gonadotrophin. *J. Reprod. Fert.* **33**, 399–410.

Pal, D., Collins, T. J., and Parkening, T. A. (1991) Levels of α-inhibin in aging female mice. *Biol. Reprod.* **45**, 869-875.

Pierson, T. M., Wang, Y., DeMayo, F. J., Matzuk, M. M., Tsai, S. Y., and O'Malley, B. W. (2000) Regulable expression of inhibin A in wild-type and inhibin α null mice. *Mol. Endocrinol.* **14**, 1075–1085.

Rivier, J., Spiess, J., McClintock, R., Vaughan, J., and Vale, W. (1985) Purification and partial characterization of inhibin from porcine follicular fluid. *Biochem. Biophys. Res. Commun.* **133**, 120–127.

Rivier, C., Rivier, J., and Vale, W. (1986) Inhibin mediated feedback control of follicle-stimulating hormone secretion in the female rat. *Science* **234**, 205–208.

Roberts, V., Meunier, H., Sawchenko, P. E., and Vale, W. (1989) Differential production and regulation of inhibin subunits in rat testicular cell types. *Endocrinology* **125**, 3122–3129.

Robertson, D. M., Foulds, L. M., Leversha, L., Morgan, F. J., Hearn, M. T., Burger, H. G., Wettenhall, R. E., and de Krester, D. M. (1985) Isolation of inhibin from bovine follicular fluid. *Biochem. Biophys. Res. Commun.* **126**, 220–226.

Schneyer, A., Sluss, P. M., Whitcomb, R. W., Martin, K. A., Sprengel, R., and Crowley, W. F. (1991) Precursor of α-inhibin modulate follicle-stimulating hormone receptor binding and biological activity. *Endocrinology* **129**, 1987–1991.

Schwartz, N. B. and Channing, C. P. (1977) Evidence for ovarian inhibin: suppression of the secondary rise in serum follicle stimulating hormone levels in proestrous rats by injection of porcine follicular fluid. *Proc. Natl. Acad. Sci. USA* **74**, 5721–5724.

Sugino, K., Nakamura, T., Takio, K., Titani, K., Miyamoto, K., Hasegawa, Y., Igarashi, M., and Sugino, H. (1989) Inhibin alpha-subunit monomer is present in bovine follicular fluid. *Biochem. Biophys. Res. Commun.* **159**, 1323–1329.

Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D., and Spiess, J. (1986) Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature* **321**, 776–779.

Woodruff, T. K., D'Agostino, J., Schwatrz, N. B., and Mayo, K. E. (1988) Dynamic changes in inhibin messenger RNAs in rat ovarian follicles during the reproductive cycle. *Science* **239**, 1296–1299.

Woodruff, T. K., D'Agostino, J., Schwartz, N. B., and Mayo, K. E. (1989) Decreased inhibin gene expression in preovulatory follicles requires primary gonadotropin surges. *Endocrinology* **124**, 2193–2199.

Woodruff, T. K., Lyon, R. J., Hansen, S. E., Rice, G. C., and Mather, J. P. (1990) Inhibin and activin locally regulate rat ovarian folliculogenesis. *Endocrinology* **127**, 3196–3205.