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Secretion of Inhibin and Testicular Expression of Inhibin Subunits in Male Duck Embryos and Newly Hatched Ducks

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Concentrations of immunoreactive (ir-) inhibin in circulation, amniotic fluid, and testes of embryos and newly hatched ducks were determined from d 21 of incubation to d 1 of age. Plasma concentrations of FSH and LH were also determined by chicken radioimmunoassay (RIA) systems. In addition, gene expression and cellular source of inhibin were investigated by *in situ* hybridization and immunohistochemistry. The results showed that plasma ir-inhibin gradually declined from d 21 to d 24, followed by an increase on d 25 and remained high until d 1 after hatching. FSH in plasma was high on d 21 followed by a sharp decline toward d 25 after which FSH levels stabilized. A reverse relationship was observed between inhibin and FSH during the late stage of incubation. Embryonic testes contained high ir-inhibin levels. Testicular ir-inhibin levels were relatively high at early time points with a peak on d 23, and significantly decreased from d 23 to d 24 and stabilized thereafter. Amniotic fluid concentrations of ir-inhibin were relatively low and remained constant between d 21 and d 25. *In situ* hybridization demonstrated that the expression of inhibin α- and βA-subunit mRNA was coexisted in the cells in the seminiferous tubules of testes on d 25. The immunoreactivity of inhibin βA- and βB-subunits was colocalized in the cells in the seminiferous tubules of testes on d 25. The results of dimeric inhibins determined by the ELISA method showed that inhibin B can be measured in embryonic testicular homogenate and pooled embryonic plasma. Although inhibin A was detected in testicular homogenate, it was under the detection limit in pooled embryonic plasma. In conclusion, these results indicate that cells in the seminiferous tubules of embryonic testes in ducks may secrete dimeric (bioactive) inhibins to circulation and that the FSH–inhibin feedback loop may become operational during the late stage of the incubation.

Key Words: Gonadotropin level; inhibin expression; male duck embryo.

Introduction

In mammals, inhibin, a dimeric glycoprotein hormone, is produced primarily by the testicular Sertoli cells in the male *(1)* and by the follicular granulosa cells in the female *(2)*. Inhibin is composed of an α-subunit and one of two β-subunits, βA or βB, which give rise to inhibin A (α+βA) or inhibin B (α+βA) *(3)*. Activins are dimers of two β subunits and the three possible activin dimers have been designated, activin A (βA βA), activin B (βB βB), and activin AB (βA βB) *(4)*. Inhibin selectively suppresses FSH secretion *(4)*, whereas activin stimulates FSH release *(5,6)*. Evidence of the presence of inhibin bioactive and immunoreactive material has been reported in several species during fetal development *(7–18)*. In chicken, the presence of bioactive inhibin has been determined in follicular preparation *(19)*, plasma of domestic fowl *(20)* and cockerel *(21)*. The mRNA expression pattern of the three inhibin/activin subunits has been demonstrated in immature and mature testes *(22,23)*.

Avian embryos are good models for studies on the secretion, source, and regulation of inhibin as described in chicken by Rombauts et al. *(24)*. Chicken embryonic testes contain the highest concentration of immunoreactive (ir)-inhibin among a variety of tissues examined *(24)*. However, subsequence studies showed that the main source of circulating ir-inhibin was the adrenal gland and not the gonads in chicken embryo *(25)*. In chicken embryos, testicular inhibin mainly played intergonadal roles as influence on steroidogesis other than endocrine roles as inhibition on FSH secretion *(26)*. Cumulative evidence has been given to support the existence of functional FSH–inhibin feedback in male ovine fetus *(7,27)*. Different species have shown different patterns of

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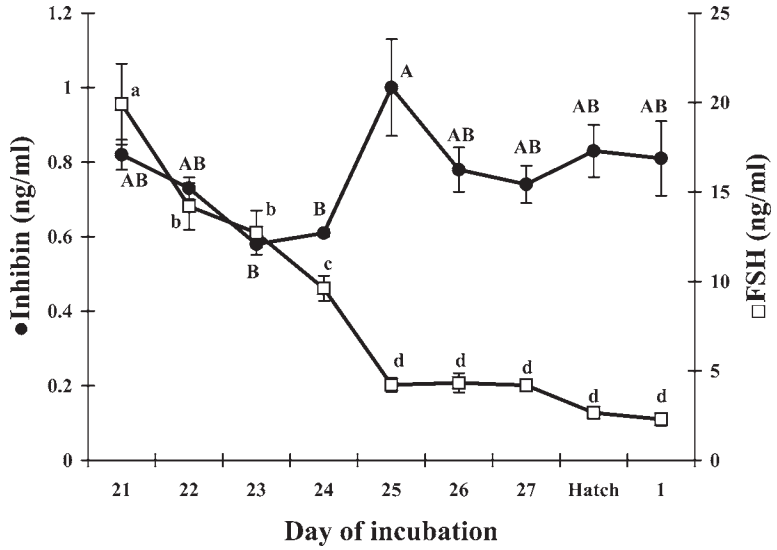

Fig. 1. Changes of plasma immunoreactive inhibin (-●-) and FSH (-□-) concentrations in male duck embryos and newly hatched ducks from d 21 of incubation to d 1 of age. Values are mean ± SEM of five animals. Significant differences ($p < 0.05$) at different time points are indicated by different letters.

inhibin secretion. Duck embryos have a longer developmental period, 28-d incubation, compared to that of chicken. The correlation between secretion of inhibin and FSH is not clear in duck embryos. Also, gene expression and cellular source of inhibin subunits in embryos of ducks have not yet been determined.

In the present study, changes in plasma concentrations of inhibin were investigated and compared to changes in testicular and amniotic inhibin and to pituitary gonadotropins in duck embryos during the late stage of development. Furthermore, cellular expression of the inhibin α- and βA-subunit mRNA in d 25 testis was investigated by *in situ* hybridization using chicken α and βA riboprobes, and to determine the cellular source of testicular inhibin, immunolocalization of the two inhibin/activin β subunits in d 25 testis was conducted.

Results

Plasma Concentrations of Ir-Inhibin and FSH

Concentrations of ir-inhibin and FSH measured in plasma from d 21 of incubation to d 1 of age are shown in Fig. 1. Ir-inhibin gradually declined from d 21 of incubation to d 24, followed by an increase on d 25 and remained high until one day after hatching. Plasma FSH sharply decreased with the development of embryo toward d 25 and remained low until d 1 of age. An inverse relationship between ir-inhibin and FSH was present during the late stage of the incubation and d 1of age ($r = –0.77$; $p < 0.05$).

Concentrations of Ir-Inhibin in Testicular Homogenate and Amniotic Fluid

Testicular inhibin content from d 21 of incubation to d 1 of age and amniotic fluid inhibin concentrations are shown in Figs. 2A,B. Testicular inhibin content expressed per testis pair decreased about 2.5-fold during the period of study ($p < 0.05$). Concentrations on d 21 and d 23 were significantly higher than other days ($p < 0.05$). There was no significant decrease observed from d 24 to d 26. Testicular inhibin content expressed per milligram of the wet weights of testes demonstrated a similar time course as those expressed per testis pair. There were significantly higher levels ($p < 0.05$) on d 21 and d 23 compared with other time points. There was no significant change from d 24 to hatch. Amniotic fluid concentrations of ir-inhibin were relatively constant from d 21 to d 25.

Plasma Concentrations of LH

Plasma concentration of LH from d 21 of incubation to 1 d of age was shown in Fig. 3. The maximum plasma LH level was observed on d 21, and then progressively declined toward d 23, after which LH levels stabilized.

Cellular Expression of Inhibin α, βA-Subunit Gene and Immunolocalization of Inhibin βA βB-Subunits on D 25 Testes

Localization of inhibin α, βA-subunit transcripts in the testes from d 25 embryos was achieved by nonradioactive *in situ* hybridization, using digoxigenin-labeled inhibin-antisense cRNAs as positive and the corresponding sense cRNAs as negative hybridization probes. With the antisense inhibin α-subunit probe, strong staining was observed in the cells in the seminiferous tubules (Fig. 4A). Expression of inhibin βA-subunit was also detected in cells in the seminiferous tubules of d 25 testes (Fig. 4B). The specificity of these reactions was shown by a comparison with adjacent sections through the same embryonic testis that had been hybridized with the respective labeled sense riboprobes (Figs. 4C,D).

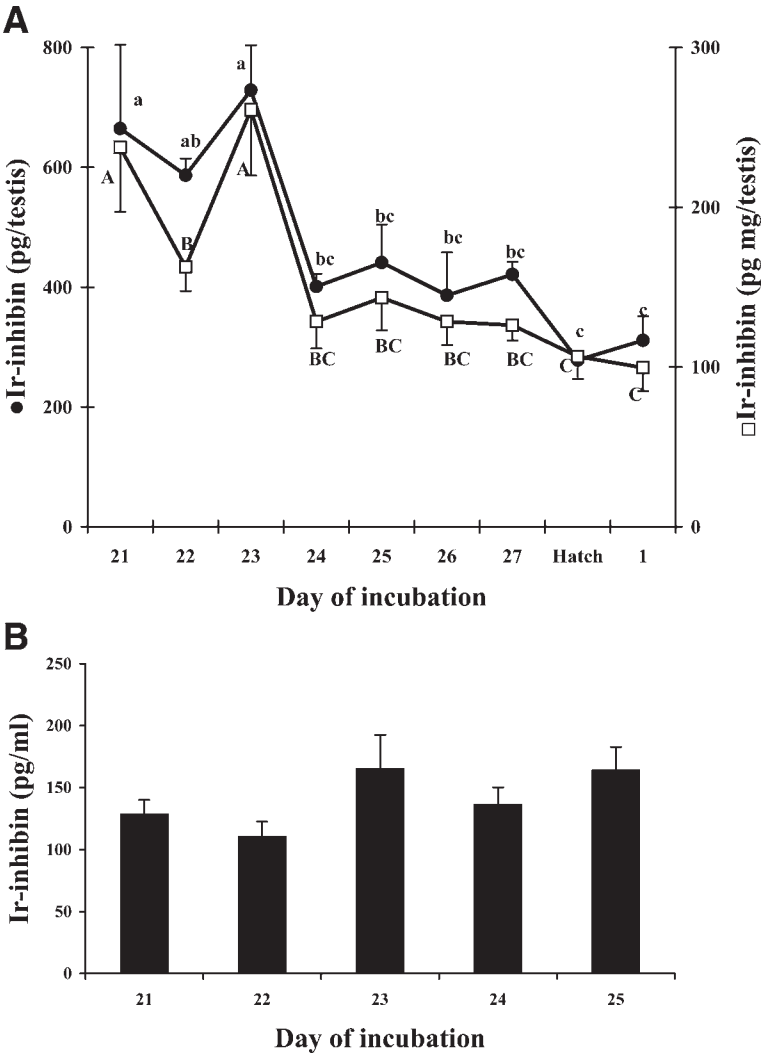

Fig. 2. Immunoreactive inhibin contents in testes from d 21 of incubation to d 1 of age (**A**) and inhibin concentrations in amniotic fluid from d 21 to d 25 of incubation (**B**). Values are expressed per organ (**A**, -●-) or per milligram of wet weight (**A**, -□-). Results are mean ± SEM of five animals. Significant differences ($p < 0.05$) at different time points are indicated by different letters.

Immunohistochemistry with the βA- and βB-subunit antisera showed a positive immunostaining in cytoplasm of cells in the seminiferous tubules of d 25 testes (Figs. 4E,F).

Characterization of the Dimeric Inhibin ELISA System

Serial dilutions of pooled testicular homogenates and plasma from embryos gave response curves in both inhibin A and inhibin B ELISAs that were parallel to the respective standard curve (Figs. 5A,B). It was undetectable for inhibin A concentrations in embryonic pooled plasma. These curves were parallel with the human inhibin A or inhibin B standard curves, indicating that bioactive dimeric inhibin B can be detected in both plasma and testicular homogenate, whereas inhibin A was detected only in testicular homogenate in duck embryos.

Discussion

In the absence of maternal and placental influence, avian embryos provide a good model system to study the role of the inhibin during fetal development. In the present study, we measured ir-inhibin in plasma, amniotic fluid, and testicular homogenates during the last week of incubation in male duck embryos. In an attempt to determine the exact source of inhibin secretion in the duck fetal testis, we had undertaken *in situ* hybridization and immunohistochemical studies. To our knowledge, this is the first report about gene expression and immunolocalization of inhibin subunits in the male duck embryonic gonad.

In the present study, high concentrations of ir-inhibin were detected in circulation and testicular homogenates. In agreement with the present results, high concentrations of ir-inhibin were found in plasma and gonads of male chick embryos *(24)*. The main component of the high testicular ir-inhibin at early time points may be the inhibin α-subunit. In common with most other inhibin RIAs, the radioimmunoassay system used in the present study cross-reacts extensively with free α-subunit forms *(28)* that lack inhibin-like biological activity. Plasma ir-inhibin reached the highest

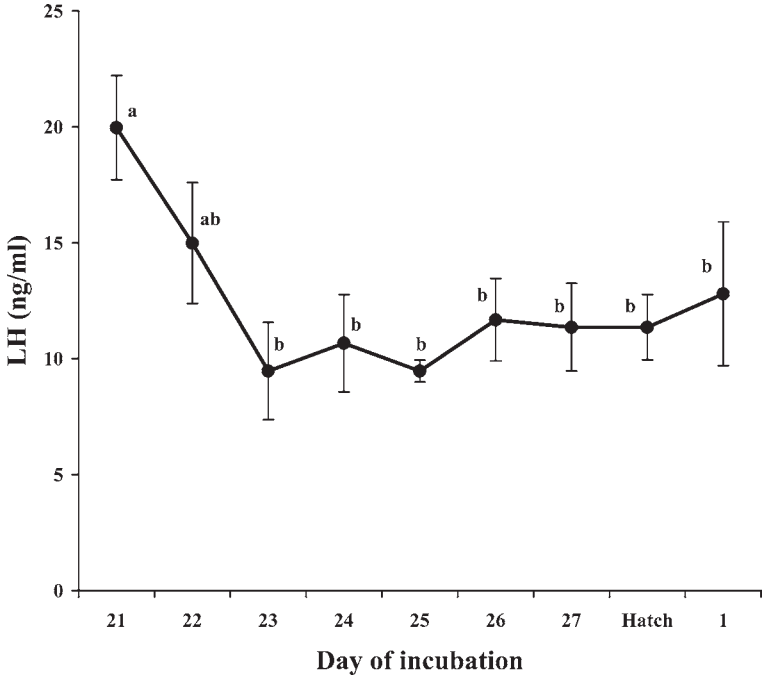

Fig. 3. Changes of plasma LH concentrations in male duck embryos and newly hatched ducks from d 21 of incubation to d 1 of age. Values are the mean ± SEM of five animals. Significant differences ($p < 0.05$) at different time points are indicated by different letters.

value on d 25 of incubation, while testicular ir-inhibin contents were highest on d 23, demonstrating that circulating ir-inhibin and testicular ir-inhibin followed different time course patterns during duck male embryo development. This suggests that another tissue source of inhibin apart from the gonad was contributing to the plasma ir-inhibin, especially at the early time points examined in the present study. In agreement with the present results, a reverse relationship was clearly observed between the inhibin content of the testis and the plasma concentration of inhibin during chick fetal growth *(24)*. In addition, a significant portion of plasma ir-inhibin appeared to escape control by FSH in chick embryos *(29)*. Subsequent studies showed that chicken fetal adrenal was the main source of circulating inhibin in the chicken embryo *(25)*. Nonetheless, high testicular contents of ir-inhibin was found in the present study as reported in the chicken fetal testis *(29)*, indicating that testicular inhibin may play an inhibiting effect on FSH secretion and/or some important intragonadal roles in the duck embryo.

The highest concentration of plasma FSH was observed on d 21 of incubation and then decreased during incubation. High plasma FSH at these earlier time points may have stimulatory effects on testicular production of inhibin. This hypothesis is in accordance with the observation of Rombauts et al. *(30)* that showed a dose-related stimulation of inhibin secretion in chicken fetal testicular cell culture by FSH. From d 24 of incubation onward, FSH decreased while plasma ir-inhibin reached its highest level, suggesting that an inverse relationship between FSH and ir-inhibin is present during this period. In accordance with the present results, the increase of FSH concentrations by gonadectomy in male fetuses but not in female fetuses *(31,32)* and the suppression of plasma FSH levels in the fetus by the administration of charcoal-treated porcine follicular fluid *(7)* have demonstrated that the inhibin–FSH feedback mechanism is functional in the ovine male fetuses. In contrast to the present observation, no obvious inverse relationship was observed between FSH and ir-inhibin in the chicken embryo, although estradiol may be an important inhibitor of pituitary FSH secretion during the embryonic development *(29)*.

Immunoreactive inhibin was also detected in amniotic fluid in the present study. These levels remained relatively constant and did not reflect the changes in plasma inhibin concentration as reported in chick embryo *(24)*, suggesting that the secretion of inhibin into the amniotic fluid is controlled independently. Moreover, no sex differences were observed in amniotic fluid inhibin (data not shown).

Gene expression of inhibin α- and βA-subunit in embryonic testes of duck embryo is reported here for the first time. The present results show that the expression of inhibin α- and βA-subunit genes is in cells in the seminiferous tubules of testes from d 25 embryos. Thus it is reasonable to suggest that embryonic testes possess the ability to produce inhibin dimers from this time point onward. Direct evidence about the presence of inhibin dimers was demonstrated, and inhibin A was detected in homogenates of pooled embryonic testes by using two-site immunoabsorbent enzyme assay. The sharp decline of plasma FSH during the late stage of incubation may be primarily due to the initiation of the

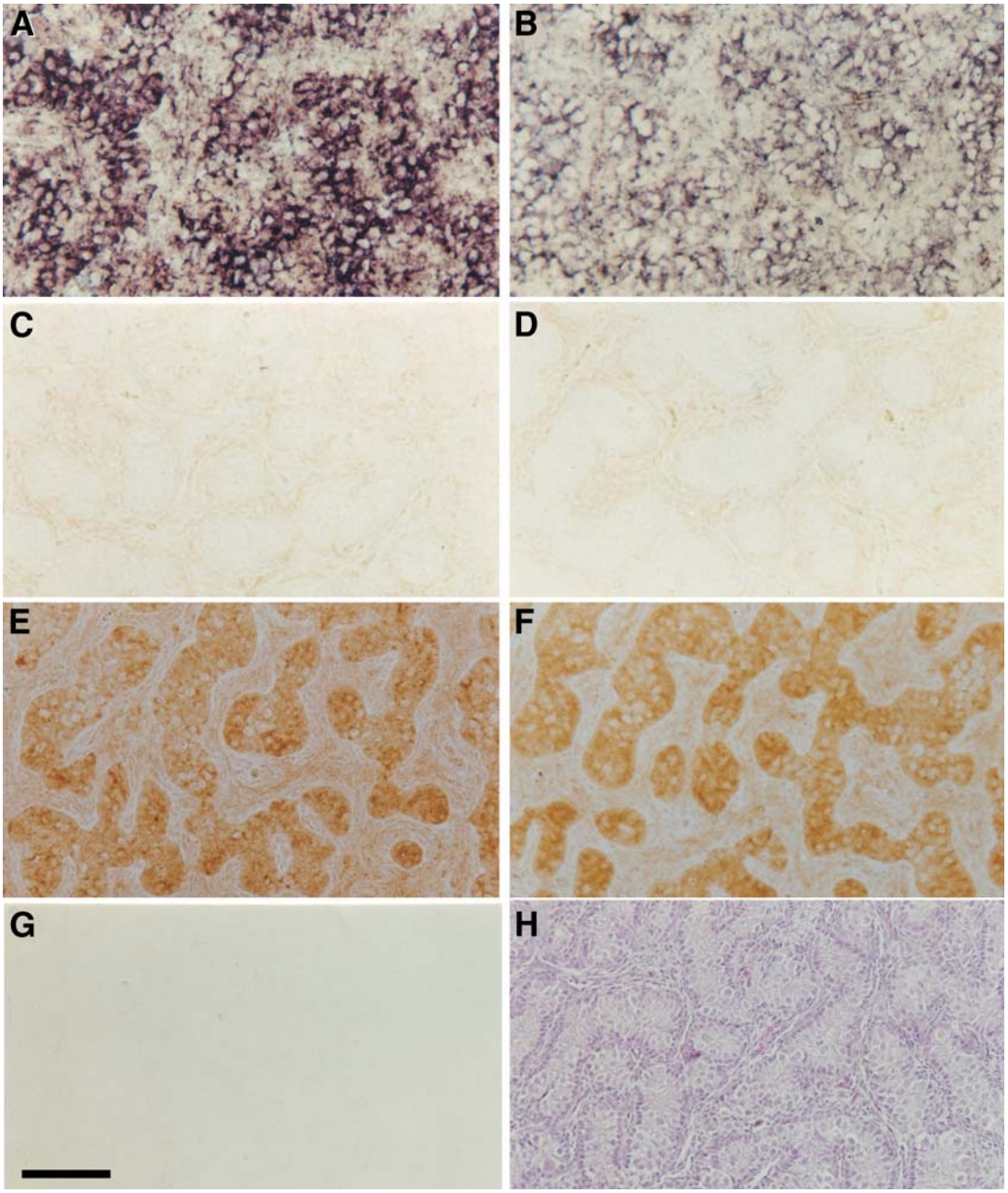

Fig. 4. Localization of inhibin in cross-sections through the d 25 embryonic testes using nonradioactive *in situ* hybridization. Sections hybridized with digoxigenin-labeled antisense-inhibin α cRNA (**A**) and β_A cRNA (**B**). Adjacent sections hybridized with the corresponding sense-cRNA, α (**C**), β_A (**D**). Hybrids detected by an immunostaining procedure using anti-digoxigenin antibodies were visualized by standard brightfield microscopy. Immunohistochemical localization of inhibin β_A- and β_B-subunits in the d 25 embryonic testes. (**E, F**) Stained with anti-inhibin β_A-, β_B-subunit sera, respectively. (**G**) Stained with normal rabbit serum (NRS). (**H**) Stained with hematoxylin and eosin. Scale bar = 100 μm.

secretion of testicular inhibin dimers. Testicular inhibin dimers contributed to circulation and played a role in inhibiting the secretion of pituitary FSH.

The presence of inhibin α- and βA-subunits in the embryonic duck testis is in accordance with previous reports in rats *(33)*, fetal sheep *(34)*, and horses *(35)*. In contrast, the reports from human and primates *(36)*, cows *(9)*, and rats *(37,38)* demonstrated that the expression of α-subunit, but not βA-subunit occurred in Sertoli cells within the seminiferous cords and also in a small proportion of Leydig cells during gestation. A previous study has reported the immunoreactivity of inhibin subunits in the chick embryo during morphogenesis by using antibodies against the human inhibin α- and βA-subunits *(15)*. All of the three inhibin/activin subunit

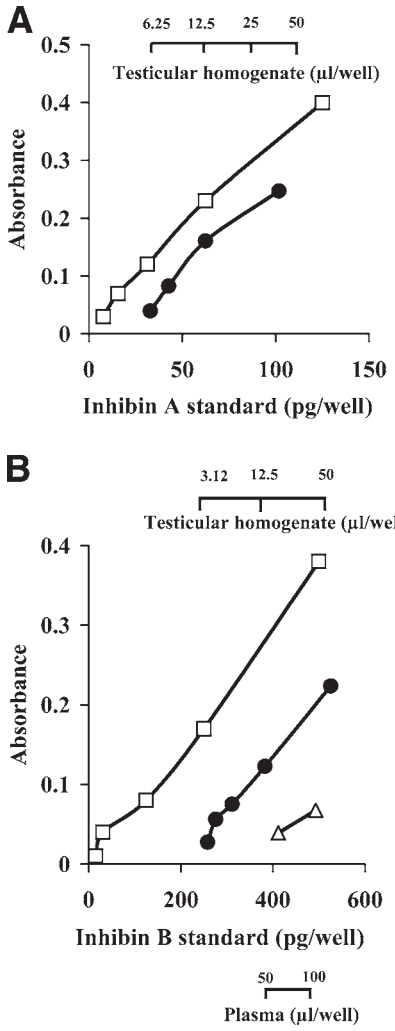

Fig. 5. Dose–response curves in the two-site ELISA for inhibin A (**A**) and inhibin B (**B**), showing parallelism between the respective standard (-□-), serial dilutions of embryonic testicular homogenate (-●-) and plasma from duck embryos (-△-). Values are the means of duplicate determinations and experiments were repeated twice.

mRNAs were detected in the testes from immature roosters by using probes made from the same DNA clones *(22)*. The present data suggest that the embryonic duck Sertoli cells have the capacity to produce all forms of inhibins and activins, i.e., inhibin A and B, and activins A, AB, and B.

High concentrations of plasma LH at early time points may have the stimulating effect on testicular inhibin secretion. In chick embryonic testicular cell culture, LH has the same stimulative effect on inhibin secretion as FSH *(30)*. The change pattern of plasma LH during the development of duck embryo is consistent with the previous reports from chick embryo *(39)*. In chick embryo, evidence *(40,41)* shows that androgen secretion becomes dependent on LH after 2 wk of incubation and seems to decrease in parallel with plasma LH after d 13.5.

In conclusion, we have shown that developmental changes in circulating ir-inhibin show a negative correlation with plasma FSH during the late stage of incubation, and that embryonic testes contain high ir-inhibin, indicating that the main source of circulating ir-inhibin may be the testis. We have also demonstrated that the inhibin α and inhibin/activin βA subunit gene coexist, and the two inhibin/activin β subunits are colocalized within the cells in the seminiferous tubules in duck embryonic testes of d 25 incubation, suggesting that cells in the seminiferous tubules from duck embryonic testes in the late stage incubation may secrete dimeric inhibin to circulation and that the FSH–inhibin feedback loop may be functional during this period.

Materials and Methods

Animals and Tissues

Fertilized eggs of British Khaki Campbell duck (obtained from Tokyo Metropolitan Livestock Experimental Station, Tokyo 198-0024, Japan) were incubated at 37.5 ± 0.2°C and 70% relative humidity. Amniotic fluid, blood, and testes were collected from embryos starting from d 21 of incubation to one day after hatching. Amniotic fluid was aspirated through the intact amniotic membrane. After 25 d of incubation too little fluid remained to allow adequate collection. Embryos were put on ice, dissected, and sexed. After hatching, neonatal ducks were anesthetized by ether before being sacrificed. In the immunohistochemical experiment, embryos were sacrificed on d 25 of incubation. Blood samples were collected from the heart into heparinized tubes. After centrifugation at 1700*g* for 10 min at 4°C, plasma and amniotic fluid were stored at –20°C. Testes were dissected from embryos, weighed, and stored in 100 µL saline at –20°C until homogenization. Testicular samples were homogenized in 500 µL saline. All the homogenates were centrifuged at 20,000*g* for 30 min at 4°C. The supernatants were collected and stored at –20°C until assay.

RIAs for Inhibin, FSH and LH

Concentrations of immunoreactive (ir)-inhibin in plasma were measured in triplicate (50 µL per sample) using rabbit antiserum against bovine inhibin (TNDH-1) and ^{125}I-labeled 32-kDa bovine inhibin, by a previously validated RIA *(42)*. Partially purified bovine follicular fluid inhibin was used for immunization in an adult castrated Japanese white rabbit. The inhibin antiserum (TNDH-1) showed no significant cross reaction with LH, FSH, or prolactin of rats, cattle, and sheep, or with gonadotrophin releasing hormone (GnRH), transforming growth factor, or activin, whereas the antiserum cross-reacts with inhibin Pro-α and free inhibin α-subunit *(28)*. Results were expressed in terms of 32-kDa bovine inhibin. The intra- and interassay coefficients of variation were 6.4 and 7.2, respectively.

Highly purified chicken FSH (AGCQSQ 111232D) and anti-chicken rabbit FSH serum were kindly supplied by Dr. S. Ishii (Waseda University, Tokyo, Japan). The FSH radioimmunoassay has been modified from that originally

described by Sakai and Ishii *(43)*. Iodination was accomplished by the chloramine-T procedure. The standard used was chicken gonadotropin standard fraction (AGC112B), the relative potencies of this standard to the highly purified chicken FSH and LH are 0.0075 and 0.13, respectively *(44)*. Results were expressed in term of chicken FSH (AGCQSQ 111232D). The intra- and interassay coefficients of variation were 6.0 and 7.4%, respectively.

Plasma concentrations of LH were determined in duplicate by a double-antibody RIA system using ^{125}I-labeled radioligands, chicken LH preparation (AGMS1122F, provided by Dr. S. Ishii, Waseda University), as described previously *(45)*. Antiserum against chicken LH (HAC-CH27-01RBP75) was kindly provided by Dr. K. Wakabayashi, Guma University, Maebashi, Japan. Results were expressed in term of nanograms of AGMS1122F LH per milliliter of plasma. The intra- and interassay coefficients of variation were 5.4 and 8.2%, respectively.

Tissue Fixation and Preparation

Animals were operated on ice. Testes were immediately removed and fixed in 4% paraformaldehyde (PFA; Sigma Chemical Co., St. Louis, MO, USA) in 0.01 *M* phosphate-buffered saline (PBS, pH 7.6) for 24 h. After fixation, the testes were dehydrated through a graded series of ethanol solutions ending with xylene. After dehydration, testes were embedded in paraffin. The paraffin-embedded testes were serially sectioned at 6 µm thickness and mounted on microscope slides coated with poly-L-lysine solution (Sigma Chemical Co.) and dried 24 h at 32°C. Some sections were stained with hematoxylin and eosin for general histological observation and some sections were used for *in situ* hybridization and immunohistochemistry.

Probe Preparation

The 1600 basepair (bp) chicken inhibin and 1540-bp cDNA inserted in plasmid pBlueScript KS+ were donated by P.A. Johnson (Cornell University) and used as templates for the in vitro synthesis of RNA. Antisense and sense digoxigenin-labeled single-strand RNA was transcribed (SP6/T7 transcription kit; Boehringer Mannheim, Mannheim) and the DNA template removed by incubation with DNase I (Sigma). The RNA was purified by phenol/chloroform extraction and precipitated with 3 *M* sodium acetate and ethanol, resuspended in 10 m*M* dithiothreitol (DTT) and stored at –70°C.

In Situ *Hybridization*

Prior to hybridization, sections were deparaffinized in xylene, rehydrated in a graded series of alcohols, and rinsed in diethyl pyrocarbonate-treated water. All the sections were sequentially incubated, at room temperature unless otherwise specified, with each of the following regents: (a) 0.3% Triton X-100 for 5 min, (b) 2X SSC (1X SSC is 150 m*M* sodium chloride, 15 m*M* sodium citrate) for 30 min, (c) proteinase K (Sigma) at a final concentration of 20 µg/mL in 50 mM Tris-HCl (pH 7.5), 2 m*M* $CaCl_2$ for 10 min at 37°C, (d) 2X SSC twice for 5 min, (e) 4% PFA in PBS for 5 min, (f) 0.2% glycine in PBS for 10 min. Sections were completely dried and prehybridized in hybridization buffer contained 50% formamide, 0.3 *M* NaCl, 10 m*M* Tris-HCl (pH 6.8), 10 m*M* sodium phosphate (pH 6.8), 5 m*M* EDTA (pH8.0), 1X Denhardt's solution (0.02%) each of BSA, Ficoll and polyvinylpyrrolidone), 10% dextran sulfate, 50 m*M* DTT, 1 mg tRNA/mL. The hybridization mixture containing the probe was denatured at 65°C for 10 min and 15–20 µL were applied to the pretreated and dried tissue sections. Hybridization was performed for 18 h at 48°C in a sealed container humidified with 50% formamide.

After hybridization, the sections were washed in 5X SSC, 1% β-mercaptoethanol (β-ME) for 30 min at 50°C, 2X SSC, 50% formamide, 1% β-ME for 30 min at 65°C, 2X SSC three times for 5 min at 37°C. Tissue sections were treated with 2 µg RNase A/mL in 0.1 *M* Tris-HCl (pH 7.5), 0.4 *M* NaCl, 5 m*M* EDTA for 30 min at 37°C. The RNase was removed by washing the slides in 50% formamide, 2X SSC for 30 min at 65°C, followed by 2X SSC at 37°C for 15 min and 0.2X SSC at 37°C for 15 min. The sections were then dehydrated by passing through a graded series of alcohols containing 0.3 *M* ammonium acetate and air-dried. Two to four serial sections for each probe from testes of 25 d of incubation were run as a batch and hybridization was repeated at least three times for each probe.

Antiserum and Peptides

Antisera raised in rabbits, against porcine inhibin β_A-subunit-(81-113)-NH_2 and human inhibin β_B-subunit-(80-112)-NH_2, were kindly donated by Dr. Wylie Vale (The Salk Institute for Biological Studies, La Jolla CA, USA).

Immunohistochemistry

After deparaffinization, sections were autoclaved for 15 min at 121°C in sodium citrate buffer (0.01 *M*, pH 6.0) to retrieve antigen. Sections were incubated in 3% H_2O_2 in methanol for 30 min to quench endogenous peroxide activity and then washed three times for 5 min in Tris-buffered saline (TBS) (0.05 *M* Tris-HCl pH 7.6 with 0.15 *M* NaCl) and then incubated with Block Ace (Dsinippon Pharmaceutical Co. Ltd., Osaka, Japan) for 30 min at 37°C in humidified chamber to prevent nonspecific reactions. Subsequently, sections were incubated with inhibin β_A and β_B subunit antiserum (diluted 1:2000) at 4°C for 24 h. After removing excess antibody with three 5-min washes with TBS, sections were further incubated with 0.5% biotinylated goat anti-rabbit secondary antibody (Vector, Burlingame, CA, USA) diluted in TBS containing 10% Block Ace. Afterward, the sections were washed with TBS three times for 5 min each time. The sections were then treated with 2% avidin–biotin complex (Vector) for 30 min at 37°C. After washing with TBS, the sections were reacted with 0.5% 3,3'-

diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) and 0.01% H_2O_2 in TBS for visualization of the bound antibody. Some sections were stained with hematoxylin–eosin for observation of morphology structure.

Two-Site Enzyme-Linked Immunosorbent Assay (ELISA) for Inhibin A and Inhibin B

Inhibin A and inhibin B were measured using the ELISA kits (Serotec, Oxford, England) *(46,47)* for the measurement of human inhibin A and inhibin B without modifications. Serial dilutions of plasma and testicular homogenate of embryos were assayed to test for parallelism.

Statistical Analyses

Values are presented as means ± SEM. The statistical significance between means was assessed by one-way ANOVA followed by Duncan's multiple range test. Canonical correlation analysis was performed between inhibin and FSH and statistical significance was obtained by using Wilks' Lambda test. A probability value (p) of less than 0.05 was considered to be significant. All statistical analyses were performed using the SAS computer package *(48)*.

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