Expression of Inhibin α -Subunit in Horse Testis

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(Received 22 December 1997/Accepted 24 April 1998)

ABSTRACT. Inhibin is believed to play roles in the pituitary secretion of FSH and in the paracrine regulation of testicular function. Although it has been generally accepted that inhibin is produced in Sertoli cells, there was a recent evidence for the localization of inhibin in Leydig cells of primates, rat and sheep. However, there is no report on the expression of inhibin in the adult horse testis. Therefore, using immunohistochemistry, western blotting and *in situ* hybridization techniques, the present study examined inhibin α -subunit (Ih- α) expression in the adult horse testis. For the detection of Ih- α protein, we used anti-porcine Ih- α antibody in immunohistochemistry and western blotting. Furthermore, digoxigenin-labeled complementary RNA probes were prepared to detect intracellular messenger RNA (mRNA) of Ih- α . Immunostainings for Ih- α were found not only in Leydig cells but also in Sertoli cells. The intensity in Leydig cells was stronger than in Sertoli cells. Immunoreactivities for Ih- α were found at approximately 46 kDa, 56 kDa and 90 kDa in the homogenates from testicular interstitial tissues. The bands at 56 kDa and 90 kDa agree with previous report, but not at 46 kDa. Signals for mRNA of Ih- α by *in situ* hybridization were detected in Leydig cells and in the basal region of seminiferous epithelium including Sertoli cells. These results suggest that Ih- α is expressed in Leydig cells and Sertoli cells of horse testis, and the expression level should be higher in Leydig cells than Sertoli cells. — **KEY WORDS:** horse testis, inhibin α -subunit, *in situ* hybridization, Leydig cell, Sertoli cell.

J. Vet. Med. Sci. 60(8): 937–942, 1998

Inhibin is a glycoprotein hormone which is composed of two disulfide-linked polypeptide chains designated as the α and β subunits [25]. Two different β -subunit forms of inhibin were described as β_A and β_B , which gave rise to two forms of inhibin, i.e., inhibin A and inhibin B [16, 29]. The two β -subunits combine to form three dimeric forms, activin A (β_A, β_A), activin B (β_B, β_B), and activin AB (β_A, β_B) [16, 18, 29]. It was generally accepted that inhibin suppresses [7, 35, 38] and activin stimulates [16, 29] the pituitary secretion of FSH. Recently, however, it was reported that inhibin was an endocrine hormone functioning primarily on reproductive tissues in paracrine and autocrine fashions [5, 15, 19]. In the testis, inhibin appeared to decrease spermatogonial mitotic activity [9, 30] and to regulate Leydig cell function [11, 15]. It also enhanced LH-mediated androgen production in Leydig cells [11, 15]. Although it has been generally accepted that inhibin is produced in Sertoli cells [2, 6, 20, 24, 26] and activin in Leydig cells [14], there was a recent evidence for the localization of inhibin and inhibin-like peptides in Leydig cells of primates [1, 4, 31, 34], rats [17, 24, 26] and sheep [32]. Furthermore, inhibin α -subunit (Ih- α) messenger RNA (mRNA) was localized in Leydig cells of rats [8, 23, 24]. Also in the

horse, Leydig cells are thought to be the major inhibin secreting cells in the testis, as demonstrated by immunolocalization of the protein [22].

However, there is no report on the expression of Ih- α in adult horse testis. The goal of this report was to confirm the expression of Ih- α in the adult horse testis at both the protein and mRNA levels.

MATERIALS AND METHODS

Animals and tissue preparations: Samples used in this study were shown in Table 1. Under sodium thiamylate anesthesia, horses were exsanguinated. For western blotting, excised testes were immediately followed by the separation of interstitial tissues. For histological analysis, testes were immersed in one of the following fixatives; Bouin's solution, phosphate buffered 10% formalin (pH 7.4), phosphate buffered 10% formalin (pH 7.4) containing 0.5% picric acid and methanol Carnoy's solution (Table 1). Specimens were then dehydrated in a graded series of ethanol, cleared in xylene, embedded in paraffin, cut at 5 μ m and mounted on slides. Sections were stained by hematoxylin-eosin for general histological observation.

Immunohistochemistry: Prior to the incubation with primary antibody, sections were deparaffinized in xylene, rehydrated in a graded series of ethanol, washed in distilled water (DW), and treated with 6 M urea/50 mM Tris-HCl (pH 7.4) for 30 min at room temperature (RT).

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Subsequently, sections were treated with 10% H_2O_2 in methanol for 10 min at RT, washed in 0.01 M phosphate buffered saline (pH 7.4) (PBS), then blocked with normal goat serum for 30 min at RT. Specimens were incubated with rabbit anti-porcine $Ih-\alpha$ 1–32 (Peninsula Laboratories, Inc., Belmont, CA, U. S. A.) diluted 1:1,000 in PBS overnight at 4°C. For the identification of Leydig cells, we performed immunohistochemistry involving anti- 3β -hydroxysteroid dehydrogenase (3β -HSD) antibody against oligopeptides corresponding to the middle region of the amino acid sequence predicted from rainbow trout (dilution 1:1,600) [12]. Immunohistochemistry was performed following the same procedures mentioned above. Sections were then washed in PBS, incubated with biotinylated goat anti-rabbit IgG diluted 1:400 in PBS for 30 min at RT, and then washed in PBS. Horseradish peroxidase (HRP) conjugated avidin-biotin complex (Vector Laboratories, Burlingame, CA, U. S. A.) was combined with secondary antibody at RT for 30 min. Immunoreactivities were visualized with 0.02% 3, 3'-diaminobenzidine tetrahydrochloride (DAB) in 50 mM Tris-HCl (pH 7.4) containing 0.1% H_2O_2 . Sections were washed in DW, dehydrated in a graded series of ethanol, cleared in xylene, and coverslipped. Two controls were set up in which (1) the primary antibody was replaced by PBS or normal rabbit serum, and (2) the primary antibody was pre-incubated overnight at 4°C with an excess amount of respective antigens.

Western blotting: To detect $Ih-\alpha$ proteins in the interstitium, excised testes were immersed in 2.5 mg/ml collagenase (Table 1), and the interstitial tissues were separated under the dissecting microscope. The interstitial and remaining tissues (which consisted mainly of seminiferous tubules) were then homogenized. Extracts prepared from each homogenized tissue in a buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate (SDS), and 0.125% bromophenol blue were boiled for 5 min. Each protein was fractionated by electrophoresis in 10% polyacrylamide gel containing 0.1% SDS. Separated proteins were transferred onto nitrocellulose filters and blocked in 10% skimmed milk

(Snow Brand Milk Products, Co., Ltd., Tokyo, Japan) and 0.5% Tween 20/PBS overnight at 4°C. Subsequently, filters were incubated with rabbit anti- $Ih-\alpha$ polyclonal antibody diluted 1:2,000 in PBS containing 0.5% Tween 20 for 2 hr at RT, and conjugated HRP goat anti-rabbit IgG diluted 1:1,000 for 1 hr at RT. Color reaction product was developed in DAB containing 0.1% H_2O_2 . For the identification of Leydig cells in the separated interstitial tissues, the same procedures were performed using anti- 3β -HSD antibody.

In situ hybridization: Specific complementary RNA (cRNA) probes for horse $Ih-\alpha$ mRNA were prepared. The template was approximately 1.2 kbps and was obtained in the cDNA library from rat ovaries [37]. Sense and anti-sense cRNA probes were prepared using digoxigenin (DIG) RNA Labeling Mixture (Boehringer Mannheim, Mannheim, Germany) and an RNA Transcription Kit (Stratagene, La Jolla, CA, U. S. A.). Prior to hybridization, sections were deparaffinized in xylene, rehydrated in a graded series of ethanol, and subjected to the following treatment; 0.3% Triton X-100/PBS for 5 min at RT, 20 μ g/ml proteinase K/ 2 mM $CaCl_2$ /50 mM Tris-HCl (pH 7.5) for 10 min at RT, 4% paraformaldehyde/PBS for 5 min at RT, 0.2% glycine/PBS for 10 min at RT. Specimens were then prehybridized with 50% formamide/2 $\times$ saline-sodium citrate (SSC) for 60 min at RT. Sections were hybridized for 48 hr with a solution consisting of 50% formamide, 20% dextran sulfate, 5 $\times$ SSC, 1 $\times$ Denhardt's solution, 10 mM dithiothreitol and sense or anti-sense cRNA probes at 45°C. Sections were then washed in 2 $\times$ SSC, 0.1 $\times$ SSC, 0.1 $\times$ SSC each for 30 min at 50°C and dried. Subsequently, signals were visualized by the use of a DIG Nucleic Acid Detection Kit (Boehringer Mannheim).

RESULTS

Immunohistochemistry: Active spermatogenesis was recognized throughout our samples except for the 1 year old horse (Table 1). All sections revealed immunopositive reactions for $Ih-\alpha$ in the interstitium and the seminiferous epithelium (Fig. 1a, 1c). The intensity of immunoreaction

Table 1. Used samples and tissue treatment

Ages (years)	Month	Spermatogenesis	Treatment		
			Immunohistochemistry	Western blotting	<i>In situ</i> hybridization
1	Sep.	I	BS	*	—
3	Mar.	A	PF	*	—
4	Mar.	A	PF-P	—	—
4	Nov.	A	BS	—	—
6	Jul.	A	BS	*	—
7	Mar.	A	PF-P	—	—
7	Apr.	A	MCS	—	MCS
9	Dec.	A	MCS	—	MCS

I: inactive, A: active, BS: Bouin's solution, PF: phosphate buffered 10% formalin, PF-P: phosphate buffered 10% formalin containing 0.5% picric acid, MCS: methanol Carnoy's solution, *: 2.5 mg/ml collagenase, —: not investigated

Fig. 1. (a) Immunohistochemistry for $Ih-\alpha$ in the 7-year old testis. Positive immunostainings were clear in the interstitium. $\times 400$. (b) Immunohistochemistry for 3β -HSD in the 7-year old testis. The immunostainings were clear in the interstitium. $\times 370$. (c) Immunohistochemistry for $Ih-\alpha$ in the 7-year old testis. The immunostainings were found clearly in interstitial cells (asterisk) and Sertoli cells (arrows). S; seminiferous tubule. $\times 2,000$.

was stronger in the interstitium than in the seminiferous epithelium. The immunopositive cells in the interstitium were round or polygonal as well as large and rich in cytoplasm (Fig. 1c). These cells were also immunopositive for 3β -HSD (Fig. 1b). This double-immunopositive pattern was confirmed in the serial sections stained in turn for $Ih-\alpha$ and 3β -HSD. In the general histological observation of the sections stained by hematoxylin-eosin, these cells showed

the histological characteristics of Leydig cells, namely, large round or polygonal cells rich in eosinophilic cytoplasm and with a clear and large nucleus. The immunopositive cells for $Ih-\alpha$ in the seminiferous epithelium were Sertoli cells (Fig. 1c). In the present study, there were no differences in immunoreactivities among the ages and seasons examined.

Western blotting: As for western blotting, the immunoreactivities for $Ih-\alpha$ were found at approximately

Fig. 2. (a) Western blotting for Ih- α in the 1-year old testis. Positive reactions for Ih- α were detectable at approximately 90, 56 and 46 kDa (arrow heads) in both preparations from the interstitial (I) and remaining (R) tissues. (b) Western blotting for 3 β -HSD in the 1-year old testis. Positive reaction for 3 β -HSD was detectable at approximately 45 kDa in the preparation from the interstitial tissue.

Fig. 3. (a) *In situ* hybridization with anti-sense RNA probes for Ih- α in the 9-year old testis. Positive mRNA signals for Ih- α were present in the interstitium (arrow heads) and the basal region of seminiferous epithelium (arrows). $\times 800$. (b) *In situ* hybridization with sense RNA probes for Ih- α in the 9-year old testis. Few positive mRNA signals for Ih- α were present. $\times 700$.

46 kDa, 56 kDa and 90 kDa in both preparations from the interstitial and remaining tissues (Fig. 2a). In the detection of 3 β -HSD, immunoreactivity was found as a single band at approximately 45 kDa in the preparation from the interstitial tissues (Fig. 2b).

***In situ* hybridization:** *In situ* hybridization with anti-sense RNA probes showed positive signals for the Ih- α in the interstitial cells and the basal region of the seminiferous

epithelium (Fig. 3a). The positive cells in the interstitium were present constitutively as cell groups among the seminiferous tubules and were round with a large amount of cytoplasm. All positive cells in the seminiferous epithelium were located in the basal region. Some of them were apparently Sertoli cells, although some spermatogonia appeared to show positive signals. *In situ* hybridization with sense RNA probes showed few positive signals (Fig. 3b).

DISCUSSION

The present study revealed positive immunostainings for Ih- α in the interstitium and in the seminiferous epithelium of adult horse testis. The intensity of immunoreactivity was stronger in the interstitium than in the seminiferous epithelium. All interstitial cell types which were immunoreactive for Ih- α were also immunoreactive for 3β -HSD. This indicated that the cells which were immunoreactive for Ih- α in the interstitium were steroidogenically active Leydig cells. Ih- α immunoreactive cells in the seminiferous epithelium were regarded as Sertoli cells from their form and location. There were no detectable changes in the immunohistochemical characteristics of Leydig and Sertoli cells in the present study.

Immunoreactivities for Ih- α were found at approximately 46 kDa, 56 kDa and 90 kDa. Immunoreactivity for 3β -HSD was found at approximately 45 kDa. It is reported that there are five sizes of horse Ih- α : 90, 80, 56, 40 and 32 kDa [21]. The bands at 90 kDa and 56 kDa detected in the present study agree with the report, but not those at 80 kDa, 40 kDa and 32 kDa. The reason for this inconsistency should be determined in any future investigation. The immunoreactivity for 3β -HSD in the interstitial tissues should result from steroidogenic Leydig cells because the seminiferous tubules were never found in the interstitial fraction.

In situ hybridization with anti-sense RNA probes revealed positive signals for mRNA of Ih- α in the interstitium and the basal region of the seminiferous epithelium. The positive interstitial cells were present constitutively as cell groups among seminiferous tubules and were round with large amounts of cytoplasm. These same cells in the serial sections also revealed positive immunoreactivity for 3β -HSD. This indicated that the cells expressing mRNA of Ih- α in the interstitium are Leydig cells. The present study could not identify positive cells in the seminiferous epithelium as Sertoli cells, spermatogonia or both. However, Sertoli cells should be one of the positive cells. These assumptions are strongly supported by the results of immunohistochemistry and immunoblotting.

It is reported that Leydig cells are immunostained for Ih- α in the testes of adult, immature and fetal rats [17, 24, 26], adult and fetal primates [1, 4, 31, 34], adult sheep [32] and adult horses [22]. On the other hand, Leydig cell also showed a positive signal for Ih- α mRNA in adult and immature rats [8, 23, 24] and fetal sheep [28] testes. Recently, Yamanouchi *et al.* [36] reported the presence of mRNA for Ih- α in fetal horse gonads. In fetal testis, mRNA for Ih- α is almost exclusively localized in the interstitial cells surrounding the developing seminiferous cords and in Sertoli cells within the cords. These reports and the present results suggest that Leydig cells produce inhibin.

Several studies have suggested that inhibin not only suppresses pituitary FSH secretion, but also plays an important paracrine regulatory role in several tissues/organs during fetal, neonatal and adult lives [5, 10]. In testis, it is

reported that inhibin produced in Sertoli cells affects spermatogonial proliferation [9, 30] and is related to the modulation of LH-stimulated androgen biosynthesis [3, 27, 33]. In fact, inhibin was taken up by spermatocytes in human testis [31], and inhibin-binding sites are localized in rat Leydig cells [13]. These reports suggest that inhibin produced in Leydig cells might play a paracrine/autocrine role in the regulation of androgen production. Therefore, it is possible that Leydig cells in horse testis produce inhibin which may play such a role in the regulation of androgen production. However, we did not refer to the inhibin- β -subunit in the present study. Further studies should be undertaken to clarify the function of inhibin in horse testis.

In conclusion, Ih- α was precisely expressed not only in Sertoli cells but also in Leydig cells in horse testis, and the expression level should be higher in Leydig cells than in Sertoli cells.

REFERENCES

1. Balboni, G. C., Barni, T., Serio, M. and Vannelli, G. C. 1991. Plasminogen activator and inhibin in the human testis. An immunohistochemical investigation. *Bull. Assoc. Anat. (Nancy)* 75: 123–126.
2. Bardin, C. W., Morris, P. L., Chen, C.-L. C., Shaha, C., Voglmayr, J., Rivier, J., Spiess, J. and Vale, W. W. 1987. Testicular inhibin: structure and regulation by FSH, androgens and EGF. p. 179. *In: Inhibin: Nonsteroidal Regulation of Follicle Stimulating Hormone Secretion*. Serono Symposia. 42 (Burger, H. G., de Kretser, D. M., Findlay, J. K. and Igarashi, M. eds.), Raven Press, New York.
3. Benahmed, M., Grenot, C., Tabone, E., Sanchez, P. and Morera, A. M. 1985. FSH regulates cultured Leydig cell function via Sertoli cell proteins: an *in vitro* study. *Biochem. Biophys. Res. Commun.* 132: 729–734.
4. Bergh, A. and Cajander, S. 1990. Immunohistochemical localization of inhibin- α in the testes of normal men and in men with testicular disorders. *Int. J. Androl.* 13: 463–469.
5. Chen, C. L. 1993. Inhibin and activin as paracrine/autocrine factors. *Endocrinology* 132: 4–5.
6. Cuevas, P., Ying, S. Y., Ling, N., Ueno, N., Esch, F., Guillemin, R., Healy, D. and Ta, S. 1987. Immunohistochemical detection of inhibin in the gonad. *Biochem. Biophys. Res. Commun.* 142: 23–30.
7. de Kretser, D. M. and Robertson, D. M. 1989. The isolation and physiology of inhibin and related proteins. *Biol. Reprod.* 40: 33–47.
8. de Winter, J. P., Timmerman, M. A., Vanderstichele, H. M., Klaij, I. A., Grootenhuys, A. J., Rommerts, F. F. and de Jong, F. H. 1992. Testicular Leydig cells *in vitro* secrete only inhibin α -subunits, whereas Leydig cell tumors can secrete bioactive inhibin. *Mol. Cell. Endocrinol.* 83: 105–115.
9. Franchimont, P., Croze, F., Demoulin, A., Bologne, R. and Hustin, J. 1981. Effect of inhibin on rat testicular desoxyribonucleic acid (DNA) synthesis *in vivo* and *in vitro*. *Acta Endocrinol. (Copenh.)* 98: 312–320.
10. Halvorson, L. M. and DeCherney, A. H. 1996. Inhibin, activin and follistatin in reproductive medicine. *Fertil. Steril.* 65: 459–469.
11. Hsueh, A. J., Dahl, K. D., Vaughan, J. Tucker, E., Rivier, J., Bardin, C. W. and Vale, W. 1987. Heterodimers and

- homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* 84: 5082–5086.
12. Kobayashi, T., Chang, X.-T., Nakamura, M., Kajiura, H. and Nagahama, Y. 1996. Fish 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase: antibody production and their use for the immunohistochemical detection of fish steroidogenic tissues. *Zoolog. Sci.* 13: 909–914.
 13. Krummen, L. A., Moore, A., Woodruff, T. K., Covello, R., Taylor, R., Working, P. and Mather, J.P. 1994. Localization of inhibin and activin binding sites in the testis during development by *in situ* ligand binding. *Biol. Reprod.* 50: 734–744.
 14. Lee, W., Mason, A. J., Schwall, R., Szonyi, E. and Mather, J. P. 1989. Secretion of activin by interstitial cells in the testis. *Science* 243: 396–398.
 15. Lin, T., Calkins, J. K., Morris, P. L., Vale, W. and Bardin C. W. 1989. Regulation of Leydig cell function in primary culture by inhibin and activin. *Endocrinology* 125: 2134–2140.
 16. Ling, N., Ying, S.-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M. and Guillemin, R. 1986. Pituitary FSH is released by a heterodimer of the β -subunits from the two forms of inhibin. *Nature (Lond.)* 321: 779–782.
 17. Majdic, G., McNeilly, A. S., Sharpe, R. M., Evans, L. R., Groome, N. P. and Saunders, P. T. 1997. Testicular expression of inhibin and activin subunits and follistatin in the rat and human fetus and neonate and during postnatal development in the rat. *Endocrinology* 138: 2136–2147.
 18. Mason, A. J., Berkemeier, L. M., Schmelzer, C. H. and Schwall, R. H. 1989. Activin B: precursor sequences, genomic structure and *in vitro* activities. *Mol. Endocrinol.* 3: 1352–1358.
 19. Mather, J. P. and Krummen, L. A. 1992. Inhibin, activin and growth factors: paracrine regulators of testicular function. pp. 169–200. In: *Spermatogenesis-Fertilization-Contraception: Molecular, Cellular and Endocrine Events in Male Reproduction* (Nieschlag, E. and Habenicht, U.-F. eds.), Springer-Verlag, New York.
 20. Merchenthaler, I., Culler, M. D., Petrusz, P. and Negro-Vilar, A. 1987. Immunocytochemical localization of inhibin in rat and human reproductive tissues. *Mol. Cell. Endocrinol.* 54: 239–243.
 21. Moore, K. H., Dunbar, B. S., Bousfield, G. R. and Ward, D. N. 1994. Initial characterization of equine inhibin. *Biol. Reprod.* 51: 63–71.
 22. Nagata, S., Miyake, Y.-I., Nambo, Y., Nagamine, N., Watanabe, G., Tsunoda, N., Taniyama, H., Hondo, E., Yamada, J. and Taya, K. 1997. Inhibin secretion in the stallion. *Equine Vet. J.* (in press).
 23. Risbridger, G. P., Clements, J., Robertson, D. M., Drummond, A. E., Muir, J., Burger, H. G. and de Kretser, D. M. 1989. Immuno- and bioactive inhibin and inhibin α -subunit expression in rat Leydig cell cultures. *Mol. Cell. Endocrinol.* 66: 119–122.
 24. 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: 2350–2359.
 25. Robertson, D. M., Foulds, L. M., Leversha, L., Morgan, F. J., Hearn, M. T., Burger, H. G., Wettenhall, R. E. and de Kretser, D. M. 1985. Isolation of inhibin from bovine follicular fluid. *Biochem. Biophys. Res. Commun.* 126: 220–226.
 26. Shaha, C., Morris, P. L., Chen, C. L., Vale, W. and Bardin, C. W. 1989. Immunostainable inhibin subunits are in multiple types of testicular cells. *Endocrinology* 125: 1941–1950.
 27. Sharpe, R. M. and Cooper, I. 1984. Intratesticular secretion of a factor (s) with major stimulatory effects on Leydig cell testosterone secretion *in vitro*. *Mol. Cell. Endocrinol.* 37: 159–168.
 28. Thomas, G. B., Davidson, E. J., Engelhardt, H., Baird, D. T., McNeilly, A. S. and Brooks, A. N. 1995. Expression of mRNA and immunocytochemical localization of inhibin α - and inhibin β A-subunits in the fetal sheep testis. *J. Endocrinol.* 145: 35–42.
 29. 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 (Lond.)* 321: 776–779.
 30. van Dissel-Emiliani, F. M., Grootenhuys, A. J., de Jong, F. H. and Rooij, D. G. 1989. Inhibin reduces spermatogonial numbers in testes of adult mice and Chinese hamsters. *Endocrinology* 125: 1899–1903.
 31. Vannelli, G. B., Barni, T., Forti, G., Negro-Vilar, A., Vale, W., Serio, M. and Balboni, G. C. 1992. Immunolocalization of inhibin α -subunit in the human testis. A light- and electron-microscopy study. *Cell Tissue Res.* 269: 221–227.
 32. Veeramachaneni, D. N., Schanbacher, B. D. and Amann, R. P. 1989. Immunolocalization and concentrations of inhibin α in the ovine testis and excurrent duct system. *Biol. Reprod.* 41: 499–503.
 33. Verhoeven, G. and Cailleau, J. 1985. A factor in spent media from Sertoli-cell-enriched cultures that stimulates steroidogenesis in Leydig cells. *Mol. Cell. Endocrinol.* 40: 57–68.
 34. Vliegen, M. K., Schlatt, S., Weinbauer, G. F., Bergmann, M., Groome, N. P. and Nieschlag, E. 1993. Localization of inhibin/activin subunits in the testis of adult nonhuman primates and men. *Cell Tissue Res.* 273: 261–268.
 35. Weinbauer, G. F., Bartlett, J. M., Fingscheidt, U., Tsonis, C. G., de Kretser, D. M. and Nieschlag, E. 1989. Evidence for a major role of inhibin in the feedback control of FSH in the male rat. *J. Reprod. Fertil.* 85: 355–362.
 36. Yamanouchi, K., Hirose, K., Hondo, E., Hasegawa, T., Ikeda, A., Sugawara, Y., Matsuyama, S., Miyazawa, K., Sawasaki, T., Tojo, H., Tachi, C. and Takahashi, M. 1997. Expression and cellular localization of inhibin α -subunit mRNA in equine fetal gonads. *J. Vet. Med. Sci.* 59: 569–573.
 37. Yamanouchi, K., Yoshida, S., Hasegawa, T., Ikeda, A., Chang, K.-T., Matsuyama, S., Nishihara, M., Miyazawa, K. and Takahashi, M. 1995. Molecular cloning of cDNA for inhibin α -subunit from equine ovary. *J. Vet. Med. Sci.* 57: 905–909.
 38. Ying, S. Y. 1988. Inhibins, activins and follistatins. Gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocrinol. Rev.* 9: 267–293.