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Authors: Koike, Satoshi, and Noumura, Tetsuo

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Effects of Inhibin on Rat Gonadal Differentiation and Development *In Vitro*

Satoshi Koike[1,2] and Tetsuo Noumura[1]

[1]*Department of Regulation Biology, Faculty of Science, Saitama University, Urawa, Saitama 338 and* [2]*Drug Safety Research, Tsukuba Research Laboratories, Pharmacia & Upjohn, Inc., Tsukuba, Ibaraki 300-42, Japan*

ABSTRACT—Previously we examined that inhibin-α subunit, transforming growth factor-β_1 (TGF-β_1) and epidermal growth factor (EGF) were expressed in sex-, cell- and stage-specific manners in perinatal rat gonads. To clarify effects of these growth factors on the rat gonadal differentiation and development, indifferent gonadal primordia with mesonephric tubules on gestational day 13 were cultured *in vitro* for 4 days in serum-free CMRL-1066 medium with inhibin, TGF-β_1, EGF, anti-sera against these growth factors, testosterone or estradiol-17β, and then morphologically examined with reference to seminiferous tubule formation, germ cell division, Wolffian and Müllerian duct development. In male gonads, anti-inhibin-α serum suppressed the seminiferous tubule formation but inhibin, TGF-β_1, EGF or steroid hormones did not affect on the tubule formation, germ cell proliferation nor gonoduct development. Seminiferous tubules in male gonads cultured in the medium containing anti-inhibin-α serum were incomplete and irregular in shape. On the other hand, in female gonads, inhibin suppressed the germ cell division and anti-inhibin-α serum led to the necrosis of germ cells, but other factors affected to neither sex cord formation nor germ cell division. Testosterone and estradiol-17β stimulated female Wolffian and Müllerian duct development, respectively. These results indicate that inhibin induces the seminiferous tubule formation and suppresses the female germ cell division in developing rat gonads *in vitro*.

INTRODUCTION

Sex differentiation in mammals is a sequential process beginning with the genetic sex differentiation, progressing to the gonadal sex differentiation, and then proceeding to the phenotypic sex differentiation. The initial genetic masculinizing trigger, which overrides the constitutive program towards femaleness, resides in a sex-determining region of the Y chromosome (Sry in mice) (Gubby *et al.*, 1990; Koopman *et al.*, 1990; Sinclair *et al.*, 1990). Sry is believed to act directly by regulating proximal upstream regions of Y chromosome (Nasrin *et al.*, 1991; Harley *et al.*, 1992; Haqq *et al.*, 1993). Expression of Sry gene triggers indifferent gonads to develop into testes, while indifferent gonads not carrying Sry develop into ovaries. However, in contrast to a differentiation process of the genetic sex, it is little known about factors participating in differentiation processes of the gonadal sex.

Growth factors have a wide range of roles for embryonic and neonatal development (for reviews, Bellvé and Zheng, 1989; Lee and Han, 1991; Ackland *et al.*, 1992; Verhoeven, 1992). Expression of growth factor genes and proteins in the pre- and postnatal rat gonads has been reported by many investigators (Merchenthaler *et al.*, 1987; Lehnert and Akhurst, 1988; Ogawa *et al.*, 1991; Pelton *et al.*, 1991; Burton *et al.*, 1993; Teerds and Dorrington, 1993; Gautier *et al.*, 1994). Our previous findings also showed that growth factors were immunohistochemically detected in the developing perinatal rat gonads: inhibin-α subunit was expressed in the male Sertoli cells on gestational days (GDs) 14 and 15 (Koike and Noumura, 1993a), transforming growth factor beta (TGF-β) in the germ cells in both sexes from GD 13 and in the Leydig/interstitial cells from GD 15 (Koike and Noumura, 1993b), and epidermal growth factor (EGF) in almost all cell types of male gonads from GD 15 and female interstitial/stromal cells from GD 16 (Kanno *et al.*, 1994). These results, together with other experimental findings, indicate that these growth factors are concerned with the rat gonadal development *in vivo*.

Gonadal primordia taken from the fetuses and explanted in a defined medium,are able to complete their morphological and endocrine differentiation within three days after the culture (Taketo and Koide, 1981; Agelopoulou *et al.*, 1984; Magre and Jost, 1984; Jost and Magre, 1988). Though growth of these gonads is limited *in vitro*, some seminiferous tubules are formed and the Leydig cells subsequently differentiate

[2] Address for correspondence: Drug Safety Research, Tsukuba Research Laboratories, Pharmacia & Upjohn, Inc. 23 Wadai, Tsukuba, Ibaraki 300-42, Japan.

(Charpentier and Magre, 1990). Production of Müllerian inhibiting substance (MIS) was confirmed by the *in vitro* test (Magre and Jost, 1984), and testosterone and androstenedione were detected in the medium with a radioimmunoassay (Patsavoudi *et al.*, 1985). Therefore, *in vitro* organ culture is an useful tool to study the development of fetal gonads and to examine effects of the supplements added in the medium, on the gonadal development.

In order to investigate effects of growth factors on the gonadal differentiation and development, indifferent gonads on GD 13, just before the seminiferous tubule formation, were cultured for four days in a defined medium with or without growth factors or steroid hormones, and then the formation of seminiferous tubules and the division of germ cells in the gonads and the development of Wolffian and Müllerian ducts were examined morphologically.

MATERIALS AND METHODS

Experimental animals

Crj:CD (Sprague-Dawley) rats in 13 to 20 weeks of age were housed in constant temperature (22±2°C), relative humidity (55±10%) and light-dark cycle (lights on 7:00-19:00) conditions. The animals were fed purina chow and took tap water *ad libitum*. Cohabitation was done during the vaginal proestrous evening in the 1:1 basis of male:female. In the next morning, copulation was checked by the presence of sperm in the vaginal smear. The day when sperm-positive smear was found was designated as GD 0. Dams were sacrificed on GD 13 by carbon dioxide.

Sexing the fetus

The fetal masses with intact fetal membrane and placenta were aseptically removed from the uterus of dams. The sex of the GD 13 fetuses was determined by the Y-chromatin: a piece of amniotic membrane was carefully spread over a slide and treated with Clarke's solution consisting of methanol and glacial acetic acid (Sigma Chemical Co.), 3:1 in volume. The piece of membrane was incubated with 0.005% quinacrine mustard (Sigma Chemical Co.) solution for 20 min at room temperature in a light-shade box and then washed twice in McIlvaine's buffer (1.231 g citric acid and 1.75 g Na_2HPO_4 in 100 ml DDW, pH 4.1). The Y-chromatin was observed as a fluorescent body under the fluorescent microscope.

Explants

The fetuses were aseptically dissected under a dissecting microscope. After the intestine, mesentery, aorta and cardinal veins were discarded from the fetus, the mesonephroi with gonadal primordia were removed as explants, together with the adhering dorsal wall tissues. In such preparations, gonadal primordia remained untouched and were permitted further differentiation. In order to minimize variation of fetal development because the developmental stage of fetuses is critical, fetuses with no or slight digit formation in the right fore paw were used as GD 13 (Agelopoulou *et al.*, 1984).

In vitro organ culture

The explants were placed on 1% agar-coated stainless steel grids (60 meshes, Ikeda Rika Co.) in organ culture dishes (#3037, Falcon) containing approximately 0.75 ml medium, and cultured for 4 days in a humidified atmosphere of 5% CO_2 in air at 36±1°C. Some explants were cultured for 1 to 6 days. The gonads were maintained in good, at least, for 4 days, but most of explants became necrotic in 5 and 6 days of culture (unpublished data).

The basic medium was CMRL-1066 supplemented with 250 mM glutamine (Gibco Lab. Co.), 100 IU/ml penicillin (Sigma Chemical Co.) and 100 μg/ml streptomycin (Sigma Chemical Co.). The growth factors examined were inhibin/inhibin-α subunit purified from bovine follicular fluid by using affinity chromatography (a generous gift from Professor S. Sasamoto, Tokyo University of Agriculture and Technology), porcine inhibin (Sigma Chemical Co.), porcine platelet TGF-β_1 (R&D Systems, Inc.) and mouse submandibular glands EGF(Chemicon International, Inc.). Anti-sera examined were the polyclonal antibody against (Tyr^{30}) porcine inhibin α-chain (1-30)NH_2 raised in the goat (a generous gift from Professor S. Sasamoto), the antibody against bovine inhibin-α subunit raised in the rabbit (East Acres Farm Inc.) and the antibody against native porcine platelet TGF-β_1 raised in the rabbit (R&D Systems). Steroid hormones examined were testosterone (Serdary Research Lab.) and estradiol-17β (Sigma Chemical Co.). Normal goat and rabbit sera were purchased from Vector Lab., Inc. The concentrations and dilutions of the growth factors and their antibodies were shown in the corresponding tables (Table 1 to 3). The culture media were renewed every two days.

Histology

At the end of culture period, explants were fixed overnight with G.P.A. fixative mixture consisting of 25% glutaraldehyde, saturated aqueous picric acid and glacial acetic acid, 100:300:1 in volume (Solcia *et al.*, 1968). And then the explants were dehydrated through a series of graded concentration of ethanol and xylene, embedded in paraffin and sectioned at 5 μm thickness. The section was mounted on a slide and stained with Mayer's hematoxylin and eosin. Cells were designated as germ or somatic cells by their morphological features: germ cells were relatively large and had clear cytoplasm and large spherical nuclei (Orth, 1993).

RESULTS

Gonadal development in CMRL-1066 medium

In male gonadal explants, cultured for 4 days in CMRL-1066 medium, many seminiferous tubules were formed and the Wolffian ducts developed, but most of germ cells did not divide and the Müllerian ducts were degenerating (Fig. 1A, Table 1). On the other hand, in female gonadal explants, both the Wolffian and Müllerian ducts developed and some germ cells divided, but no tubule was formed (Fig. 1B, Table 1).

Effects of growth factors

Effects of growth factors on the gonadal development *in vitro* are summarized in Table 1. Inhibin and purified inhibin/inhibin-α affected neither male gonadal nor duct development in males (Fig. 1C, E), but inhibited the female germ cell division (Fig. 1D, F). Meiotic division of female germ cells was observed in both conditions, *in utero* (data not shown) and organ culture *in vitro* in the CMRL-1066 medium, but not in the media containing porcine inhibin and bovine inhibin/inhibin-α (Fig. 1D, F). Seminiferous tubule formation, germ cell division or gonoduct development was not influenced by TGF-β_1 or EGF (Table 1).

Effects of anti-growth factor antibodies

Effects of anti-growth factor antibodies are summarized in Tables 2 and 3. In male gonads, both anti-inhibin-α sera raised in the goat and the rabbit suppressed the seminiferous tubule formation in males (Fig. 2C): the seminiferous tubules were formed in incomplete and irregular shape at all

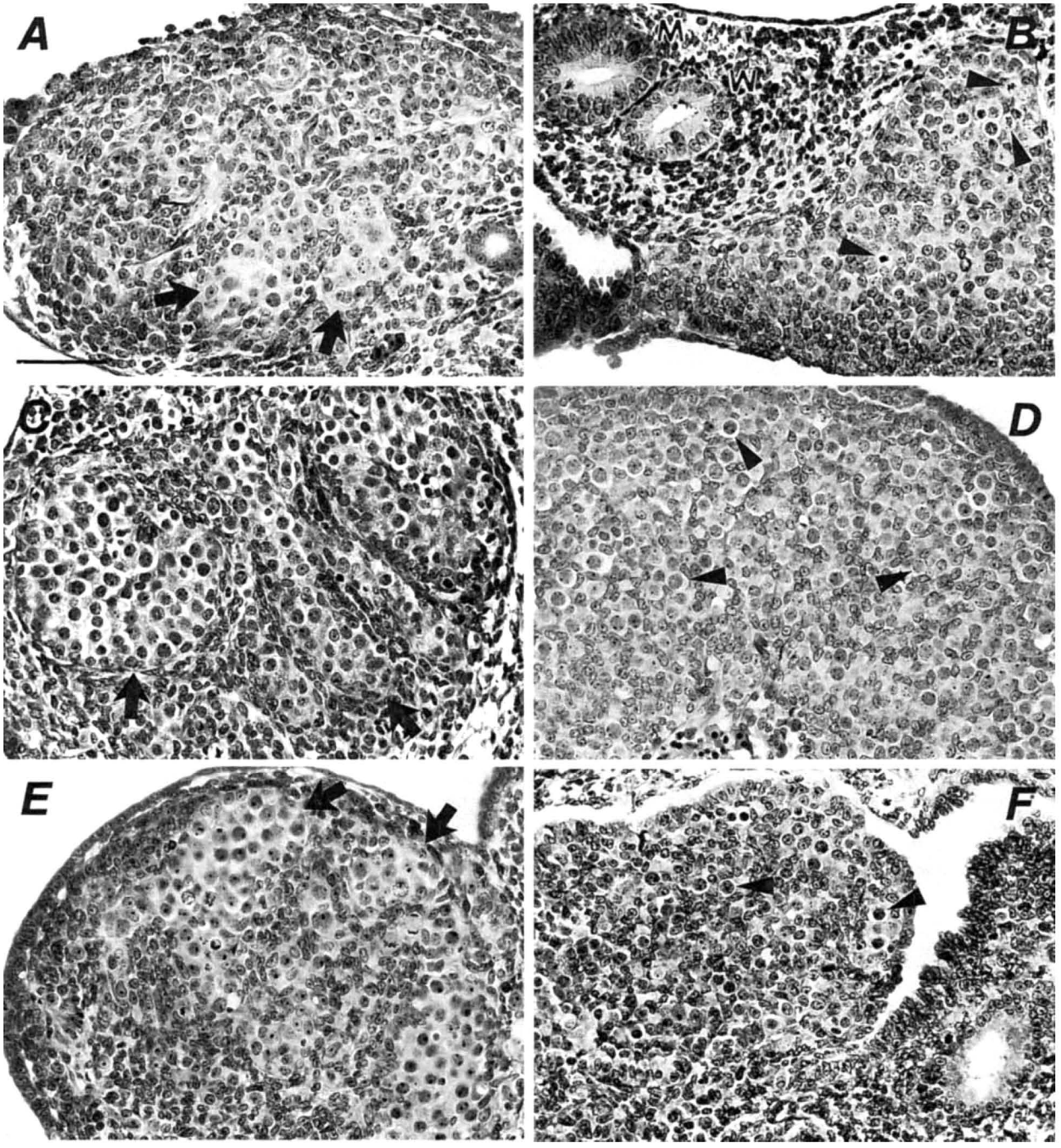

Fig. 1. Histological sections of gonads from gestational day 13 cultured in CMRL-1066 medium with or without porcine inhibin or purified bovine inhibin/inhibin-α subunit for 4 days. A, C and E show male gonads. B, D and F show female gonads. In a male gonad cultured in CMRL-1066 medium (A), many seminiferous tubules (arrow) have differentiated. In a female gonad (B), germ cells (arrow head), the Wolffian (W) and Müllerian (M) ducts well develop. Inhibin supplement (C and D, 10 μg/ml porcine inhibin: E and F, 0.4 μg/ml purified bovine inhibin/inhibin-α) to the medium affects neither male gonadal nor gonoduct development (C and E), but inhibits the germ cell division in female gonads (D and F). Bar: 50 μm.

concentrations examined (Table 2). However, germ cell division or gonoduct development was not affected by anti-inhibin-α serum. In females, some germ cells showed necrotic figures by the addition of anti-inhibin-α-serum (Fig. 2D). Number of necrotic germ cells increased in the dose-dependent manner (Table 2). But either sex cord formation or the gonoduct development was not influenced (Fig. 2D). While anti-TGF-β_1 serum did not affect on the gonadal or gonoduct development in both sexes, at all concentrations used in this experiment (Table 3). The fetal gonads and gonoducts from both sexes normally developed in normal goat and rabbit sera (Fig. 2A, B).

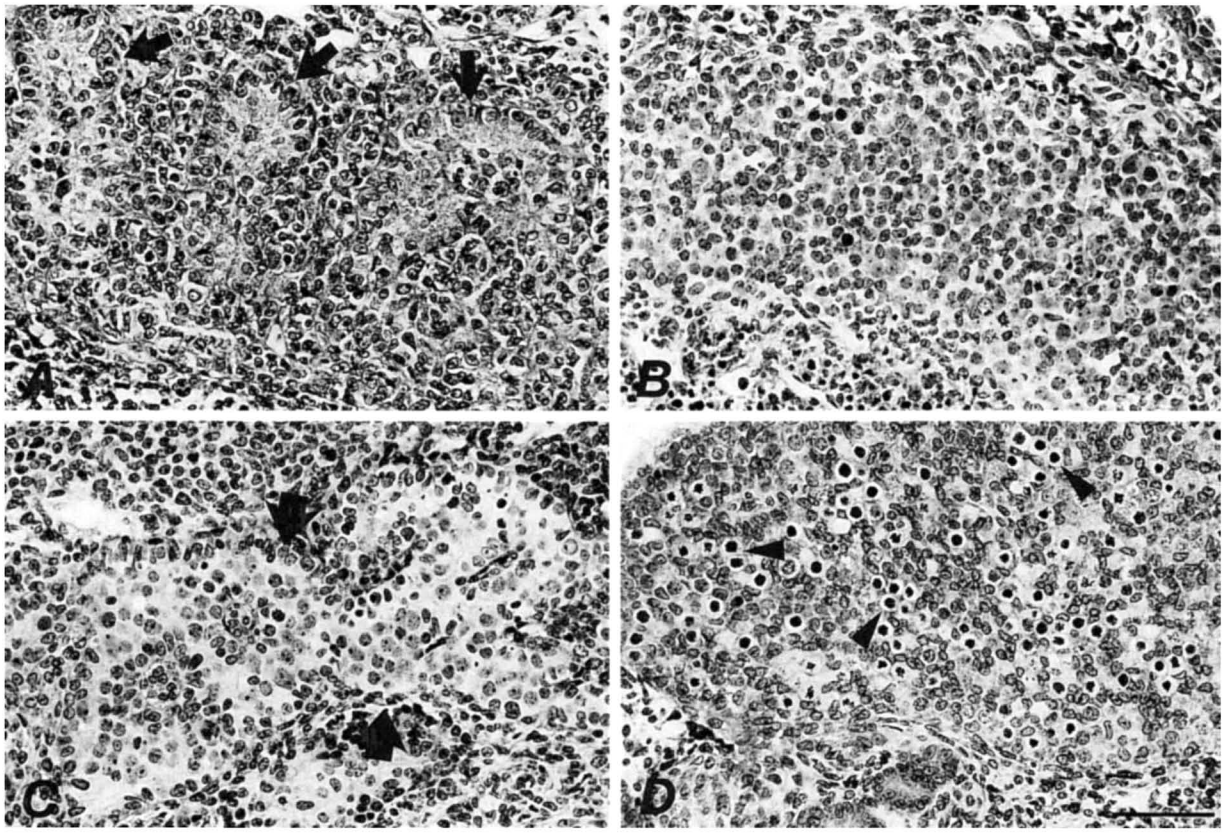

Fig. 2. Histological sections of gonads from gestational day 13 cultured in CMRL-1066 medium supplemented with normal and anti-inhibin-α goat serum for 4 days. A and C show male gonads. B and D show female gonads. Gonadal development in the culture medium supplemented with normal serum (10 μg/ml, A and B) is comparable to those in the CMRL-1066 medium only (Fig. 1 A and B). In a male gonad, anti-inhibin-α serum (10 μg/ml, C and D) suppresses the seminiferous tubule formation (arrow); tubules show incomplete formation and irregular shape (C). In a female gonad, anti-inhibin-α serum induces the necrosis to germ cells (arrow head in D). Bar: 50 μm.

Table 1. Results of *in vitro* 4-day culture of indifferent rat gonads with growth factors

Medium type (Concentration)	Gonadal Sex	No.	Tubule formation	Germ cell division	Wolffian duct development	Müllerian duct development
CMRL-1066	♂	4	S.T. (many)	No[b]	+++	+/-
	♀	9	No	Dividing	++/+	++/+
Inhibin (100 ng/ml)	♂	7	S.T. (many)	No[b]	++	+/-
	♀	5	No	No	++/+	+++/++
Inhibin/Inhibin-α[a] (0.4 ng/ml)	♂	6	S.T. (many)	No[b]	+++/++	-
	♀	4	No	No	++/+	+++/++
TGF-β_1 (1, 10, 20, 50 ng/ml)	♂	3 to 5	S.T. (many, some: large)	No[b]	+++/++	+/-
	♀	3 or 4	No	Dividing	++/+	+++/++
EGF (0.1, 1, 10 ng/ml)	♂	2 to 4	S.T. (many)	No[b]	++/+	+/-
	♀	2 to 9	No	Dividing	++/+	++/+

[a] Inhibin/inhibin-α was purified from bovine follicular fluid by using affinity chromatography.
[b] Dividing a few germ cells not within the seminiferous tubules. S.T.: Seminiferous tubule
Grade of gonoduct development: -, Degenerating; +, Maintaining; ++, Developing; +++, Well developing/differentiating

Table 2. Results of *in vitro* 4-day culture of indifferent rat gonads with anti-inhibin-α sera

Medium type (concentration)	Gonadal Sex	No.	Tubule formation	Germ cell division	Wolffian duct development	Müllerian duct development
Anti-Inhibin-α Goat Serum (1/10,000, 1/1,000, 1/100)	♂	3 to 7	No and some S.T. (incomplete, irregular shape)	No[a)]	+++/++	-
	♀	3 to 5	No	Dividing (some: necrosis)	++	+++/++
Normal Goat Serum (1/10,000, 1/1,000, 1/100)	♂	2 to 5	many S.T. (some: large)	No[a)]	+++/++	+/-
	♀	3 to 5	No	Dividing	++/+	+++
Anti-Inhibin-α Rabbit Serum (1, 10, 100 ng/ml)	♂	4 to 8	S.T. (incomplete, irregular shape)	No[a)]	+++/++	++/+/-
	♀	6 to 16	No	Dividing (some: necrosis)	+++/++/+	+++/++
Normal Rabbit Serum (100 ng/ml)	♂	5	many S.T.	No[a)]	+++/++	++/-
	♀	2	No	Dividing	++	+++/++

[a)] Dividing a few germ cells not within the seminiferous tubules. S.T.: Seminiferous tubule
Grade of gonoduct development: -, Degenerating; +, Maintaining; ++, Developing; +++, Well developing/differentiating

Table 3. Results of *in vitro* 4-day culture of indifferent rat gonads with anti-TGF-β and steroids

Medium type (concentration)	Gonadal Sex	No.	Tubule formation	Germ cell division	Wolffian duct development	Müllerian duct development
Anti-TGF-β (5 μg/ml)	♂	3	many S.T. (some: large)	No	+++/++	-
	♀	10	No	Dividing	++/+	+++
Normal Rabbit IgG (5 μg/ml)	♂	5	many S.T. (some: large)	No[b)]	++	-
	♀	3	No	Dividing	++/+	+++
Testosterone (0.1, 1, 10, 100 ng/ml)	♂	2 to 6	many S.T. (some: large)	No[b)]	+++/++	-
	♀	2 to 4	No	Dividing	+++/++	+++/++
Estradiol-17β (0.1, 1, 10, 100[a)] ng/ml)	♂	2 to 4	many S.T.	No[b)]	+++/++	+/-
	♀	3 to 6	No	Dividing	+++/++	+++

[a)] Supplement of 100 ng/ml estradiol-17β induced the necrotic figures to the *in vitro* gonads.
[b)] Dividing a few germ cells not within the seminiferous tubules. S.T.: Seminiferous tubule
Grade of gonoduct development: -, Degenerating; +, Maintaining; ++, Developing; +++, Well developing/differentiating

Effects of steroids

Both sex steroid hormones, testosterone and estradiol-17β (E_2), did not influence the gonadal differentiation or development in both sexes at all concentrations applied (Table 3). In females, the development of Wolffian ducts was stimulated by testosterone at a concentration of 10 ng/ml or more, and that of Müllerian ducts by E_2 in levels of 0.1 to 10 ng/ml. The Müllerian ducts in males degenerated regardless of the supplement of testosterone or E_2. E_2 at 100 ng/ml induced necrosis of the cells in most of the explants.

DISCUSSION

Gonadal development *in vitro* was influenced by the application of inhibin or anti-inhibin-α serum. Inhibin suppressed the germ cell division in female gonads, while anti-inhibin-α suppressed the seminiferous tubule formation in males and led to germ cell necrosis in females. The immunohistochemical findings in developing rat gonads showed that inhibin-α expressed in male Sertoli/supporting cells on GD 14 and GD 15, but not in females (Koike and Noumura, 1993a). These results suggest that inhibin and/or inhibin-α involve in the seminiferous tubule formation in male gonads. However, in contrast to effectiveness of anti-inhibin-α serum on the suppression of the seminiferous tubule formation in male gonads, inhibin/inhibin-α did not induce any tubular formation in females. This inconsistency may be due to that the concentration used of inhibin/inhibin-α is insufficient to induce the morphogenetic activity, or that inhibin receptor(s) is not existed in the female gonadal cells during this experimental period. When the fetal ovaries exposed to MIS, they develop seminiferous tubules (Vigier *et al.*, 1987; Behringer *et al.*, 1990). MIS is also a member of TGF-β superfamily, a group of protein that includes the TGF-βs, inhibin and activin. The C-terminal domains of all family members share homology, ranging from 23 to 80% (Massagué, 1990; Roberts and Sporn, 1990). If anti-inhibin-α antibody binds to the C-terminal domain of MIS and blocks the biological activity of MIS, the tubular formation may be suppressed indirectly.

On the other hand, female germ cells *in vitro* became necrotic by the addition of anti-inhibin-α serum to the culture medium, but not by normal goat or rabbit serum. During fetal period, oogonia enter into the prophase of meiosis to become oocytes (Haffen, 1977; Merchant-Larios and Taketo, 1991), whereas male germ cells do not divide meiotically (Hilscher *et al.*, 1974). Therefore, the speculation that inhibin and/or inhibin-α may contribute to suppress the meiotic division of the germ cells is supported by the results that purified inhibin/inhibin-α ceased the female germ cell division and that anti-inhibin-α induced necrotic changes in female germ cells. Male germ cell division is thought to be regulated by factor(s) secreted from the neighboring cells, i.e. MIS from Sertoli cells (Jost, 1972; Ozdzenski *et al.*, 1976; Takahashi *et al.*, 1986; Vigier *et al.*, 1987; Merchant-Larios and Taketo, 1991). Inhibin/inhibin-α also exists in Sertoli cells on the stage of seminiferous tubule formation (Koike and Noumura, 1993a). Therefore, inhibin may be a candidate to prevent male germ cells from the entrance into meiotic phase during the fetal life.

TGF-β_1 or EGF had no effect on gonadal development in both sexes. The concentrations used were enough to exert biological actions of these growth factors, because the concentrations for producing biological actions, such as cell proliferation and functional differentiation, in cell- and organ-culture are less than 10 ng/ml for both TGF-β (Avallet *et al.*, 1987; Knecht *et al.*, 1987; Benahmed *et al.*, 1989) and EGF (Ascoli *et al.*, 1987; Bendell and Dorrington, 1990; Sordoillet *et al.*, 1991). Therefore, the results that neither TGF-β_1 nor EGF produced any morphological changes in developing gonads *in vitro* indicate that these growth factors have not ability to act on gonadal development in fetal rats or that corresponding receptors may not be expressed in the gonads during this experimental period. This is also supported by the results that anti-TGF-β_1 serum did not influence the gonadal development *in vitro*.

Testosterone and E_2 also stimulate the development of the Wolffian and Müllerian ducts in females. It is well known that testosterone induces the Wolffian duct development (Jost, 1972, 1985; Goldstein and Wilson, 1975) and that E_2 induces the Müllerian duct development and differentiation (Newbold *et al.*, 1984). On the other hand, Müllerian ducts in males degenerate during the fetal life by MIS derived from the Sertoli cells in the testis, regardless of the presence or absence of testosterone (Josso *et al.*, 1977; Tran *et al.*, 1977). Therefore, these *in vitro* findings also suggest the general concept for hormonal regulation of the gonoduct development.

Activin-A, which is a homodimer of inhibin-β_A subunit, has a sexually dimorphic roles in fetal rat gonadal differentiation (Kaipia *et al.*, 1994). Activin-A stimulated DNA synthesis in the ovary but inhibited in the testis on GD 14. Inhibin-β_A mRNA was expressed in the fetal testes but not in the fetal ovaries on GD 15 and 18. Activin receptors (ActR and ActRII) were found in both fetal gonads from GD 15 (Kaipia *et al.*, 1994). These results suggest that activin-A might suppress meiotic division of the male gonocyte in the gonadal sexual differentiation. MIS is also produced in the immature Sertoli cells on GD 13, prior to the seminiferous tubule formation (Kuroda *et al.*, 1990; Hirobe *et al.*, 1992) and this expression is correlated with the expression of Sry gene (Haqq *et al.*, 1993). Taken together with our results, TGF-β members, inhibin, activin and MIS, have important roles for the fetal gonadal differentiation and development in rats.

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