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·*Original Article*·

17beta-estradiol stimulates proliferation of spermatogonia in experimental cryptorchid mice

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Abstract

Aim: To investigate whether estrogen stimulates the proliferation of spermatogonia or induces spermatogenesis in cryptorchid mice. **Methods:** Mice were surgically rendered cryptorchid, then treated with different doses of 17β-estradiol (E2) s.c. once a day. Mice were killed at sexual maturity (45 days of age), and histological analysis and immunofluorescence were performed. Serum follicle stimulating hormone (FSH), estradiol, testosterone and luteinizing hormone (LH) were measured. **Results:** Low doses of E2 had no notable effect on spermatogonia, but at higher doses, E2 stimulated the proliferation of spermatogonia. **Conclusion:** E2 has a dose-related mitogenic effect on spermatogonia. *(Asian J Androl 2007 Sep; 9: 659-667)*

Keywords: 17β-estradiol; cryptorchid mice; proliferation; spermatogonia

1 Introduction

Millions of spermatozoa are produced daily in the mammalian testis from spermatogonial stem cells (SSC) and the germ line stem cells in the testis. To maintain normal spermatogenesis, the balance of self-renewal and differentiation of SSC must be precisely regulated by intrinsic gene expression in the stem cells and extrinsic signals, including soluble factors or adhesion molecules, from the surrounding microenvironment, the stem cell niche [1, 2]. SSC are a kind of unique cell in postnatal animals that both undergoes self-renewal and contributes genes to subsequent generations. Therefore, SSC are essential for species continuity. SSC are also valuable for genome modification and production of transgenic animals. However, SSC are rare in testis tissue, comprising approximately 1 in 5 000 cells in adult mouse testis [1]. Enrichment and purification of SSC are vital to obtain sufficient numbers of cells for study. Therefore, if we could find a better method of obtaining SSC, we

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could more readily study the cells' development and spermatogenesis. Cryptorchid mice make a likely model for studying spermatogonia *in vivo* as spermatogenesis is arrested and the spermatogonia are enriched in the spermtogenic epithelium [3]. Estrogen decreases the rate of apoptosis and stimulates proliferation of human spermatogonia *in vitro* [4], and that of rat spermatogonia *in vivo* [5, 6], and induces the renewal of SSC in Japanese eels, *Anguilla Japonica*, *in vitro* [7] and in lizards (*Podarcis S. Sicula*) *in vivo* [8]. We hypothesize that we can study the effects of 17β-estradiol (E2) stimulation of spermatogonia in cryptorchid mice. If E2 stimulates renewal of spermatogonia in cryptorchid mice, then these mice could serve as a source of enriched and expanded spermatogonia for studies on the mechanisms controlling spermatogonia proliferation and differentiation.

2 Materials and methods

2.1 Animals

For the present study, fourty 10-day-old male and eight 45-day-old male Kunming mice were obtained from the Experimental Animal Center of the Academy of Military Medical Sciences (Beijing, China). They were maintained under standard mouse laboratory conditions in a temperature-controlled and light-controlled room (temperature 23 ± 2ºC; 12 h:8 h Light:Dark cycle [lighted approximately 7:00 to 19:00]). The 10-day-old mice remained with their mothers and were allowed to nurse *ad libitum*. The 45-day-old mice were also kept in the controlled environment with free access to food and water. The experimental protocol was approved by the Animal Experimental Committee of the Beijing Academy of Medical Sciences (Beijing, China).

2.2 Experimental cryptorchidism

The 10-day-old male KM mice were surgically rendered cryptorchid via a modification of a procedure reported by Nishimune and colleagues [9]. A midline lower abdominal incision was made under ether anesthesia. The gubernacula were bilaterally cut and the adipose tissue of the capita epididymidum on each side was sutured to the inner peritoneal wall, pulling the testes into the abdomen. Eight of the 10-day-old mice and all of the 45-day-old mice remained as intact controls.

2.3 Hormone treatment

Surgical cryptorchid mice were randomly divided into four groups of 8 mice each and were treated with E2 (Sigma-Aldrich, St. Louis, MO, USA) dissolved in olive oil at different dosages s.c. Group I received 0.25 μg/g body weight (bw) of E2 (low-estradiol-treated group) per day; Group II, 0.5 μg/g bw (mid-estradiol-treated group) per day; and Group III, 1 μg/g bw (high-estradiol-treated group) per day. Group IV was the control, and was given 10 μL/mouse of olive oil (vehicle only) per day. Each mouse was injected once a day, between 8:00 and 9:00, commencing just after the operation.

2.4 Hormone measurement

The animals were killed with sodium pentobarbital on post-surgical day 35, and blood was collected by removing an eyeball. The serum was separated and stored at –20ºC until analyses of follicle stimulating hormone (FSH), estradiol, testosterone and luteinizing hormone (LH) level could be done. Levels of serum FSH and LH were measured using an enyzme-linked immunosorbent assay (ELISA) kits (RapidBio, West Hills, CA, USA). Intra-assay and inter-assay coefficients of variation for FSH were not more than 1% and were not more than 2% for LH. Serum estradiol and testosterone were measured using the Automated Chemiluminescence System (Bayer, New York, USA). The assay kits were also purchased from Bayer. The sample number is 6–8 for each examination.

2.5 Inhibin B measurement

At the time of death, the right testes of the mice were removed, weighed and stored in liquid nitrogen. They was then homogenized in 0.5 mL deionized water using a Teflon homogenizer that fits into a microfuge tubule. Each sample was centrifuged at 4ºC (9 000 × *g*, 10 min). The supernatant was frozen for later assay of inhibin B. Plasma and testis concentrations of inhibin B were determined by a two-site ELISA, as previously described [10], with a sensitivity of 30 ng inhibin B/mL and intra-assay and inter-assay coefficients of variation of 4.2% and 9.8%, respectively (n = 6–8). The kit was purchased from Diagnostic Systems Laboratories (Webster, TX, USA).

2.6 Histological analysis

After the right testes were removed, the experimental cryptorchid mice were perfused with Bouin's fluid, and the left testes samples were fixed for 12 h in Bouin's fluid. After washing with ethanol (50%) for three times

(30 min each time), the samples were dehydrated, embedded and cut into 6 μm sections at 60 μm intervals. The 6 μm sections were divided into two parts and every other formed into one part. One part was used for hematoxylin eosin (HE), and the other for immunofluorescence analysis. The slides in part I were deparaffinized and hydrated in a graded ethanol series. Slides were stained with HE. If one of the testes had re-descended into the scrotum, neither testis was analyzed histologically. In addition, the eight intact 10-day-old mice and the eight intact 45-day-old mice were killed, and their testes were subjected to the same analyses as the experimental and control (Group IV) groups' testes.

2.7 Immunofluorescence

Both Thy-1 and integrin-β1 are surface markers of SSC in mouse testes [11, 12, 14]. Therefore, immunofluorescence with the two molecules as antigens was performed to identify spermatogonia. The 6-μm slides in Group II were analyzed via immunofluorescence. Deparaffinized sections were hydrated in a graded ethanol series and 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. Antigen was retrieved by microwave oven in 10 mmol/L citrate buffer (pH 6.0), at 95–98ºC for 15 min, and were then treated with 1% normal goat serum for 30 min at 37ºC. The primary antibody (anti-Thy-1 monoclonal antibody, rat anti-mouse [Lab Vision, Fremont, CA, USA] used at a 1:200 dilution and anti-integrin-β1 polyclonal antibody, rabbit anti-mouse [Boster, Wuhan, Hubei, China] used at a 1:200 dilution) was incubated with the sections at 4ºC overnight (approximately 18 h). After washing in phosphate buffered saline (PBS) (0.01 mol/mL), the sections were incubated with FITC-goat-anti-rat and Cy3-goat-anti-rabbit immunoglobulin (Boster, Wuhan, Hubei, China) for 20 min each at 37ºC. The sections were again washed with PBS (0.01 mol/mL) for three times, and once for 5 min and observed under fluorescence microscopy. The negative control sections were treated using the same procedure as above, except that the primary antibody was replaced by PBS. The numbers of Thy-1^+ and integrin-$\beta1^+$ cells per 100 μm tubule width and per 100 μm tubule perimeter were counted, respectively. The numbers of positive cells were all expressed as number/100 μm tubule width plus number/100 μm tubule perimeter.

2.8 Statistical analysis

The results of number/tubule of Thy-1^+ and integrin-$\beta1^+$ cells were both expressed as mean ± SD. Serum FSH, estradiol, testosterone and LH concentration and inhibin B level were also expressed as mean ± SD. Statistical comparison between means was analyzed by one-way analysis of variance.

3 Results

3.1 Histological analysis

On histological analysis, testes from 45-day-old mice contained germ cells at every stage of development, from SSC to sperm. This indicates that the animals were fully sexually mature at that age [11]. The degree of differentiation of germ cells was much lower in 10-day-old mice than that in 45-day-old mice; in 10-day-old mice only spermatogonia and primary spermatocytes were present. Furthermore, the spermatogonia were relatively greater in number. In the experimental cryptorchid mice (including estradiol-treated groups and the control group), the types of germ cells were similar to those found in 10-day-old mice. The proportions of spermatogonia to all germ cells in the seminiferous tubules differed in the animals in the various cryptorchid groups. The proportions found in Group IV and in Group I were similar to those found in 10-day-old mice. Comparison among the various estradiol-treated cryptorchid groups showed that the number of spermatogonia present in the testis increased in the presence of estrogen in a dose-related manner. Mice treated with a high dose of E2 had the most testis cells that appeared morphologically similar to spermatogonia (Figure 1).

3.2 Immunofluorescence

Immunofluorescence examination with the Thy-1 antigen showed that the number of cells that reacted to Thy-1 antibody (Thy-1^+ cells) increased directly with the dose of E2. Group II and Group III both had more than one layer of Thy-1^+ cells. In contrast, only the cells along the basal membrane of the seminiferous tubules were Thy-1^+ in 45-day-old intact mice. In 10-day-old mice, Thy-1^+ cells were also present only along the seminiferous tubules' basal membrane; however, there were more of those cells in 45-day-old mice (Figure 2). Mice in groups I and IV were similar to 10-day-old mice. In addition, immunofluorescence examination with the integrin-β1 marker yielded similar results (Figure 2). In the negative control, peritubular cells were also

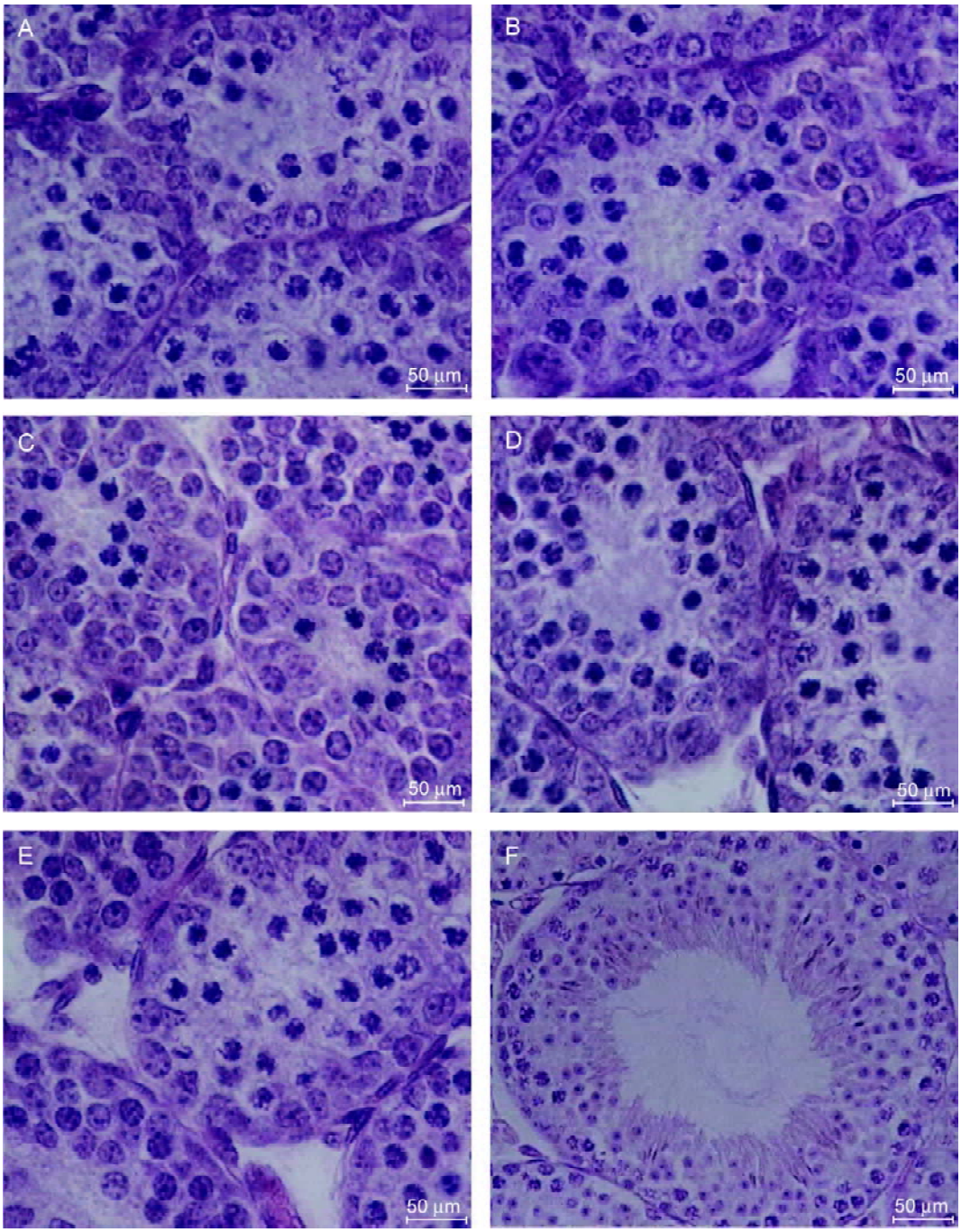

Figure 1. Hematoxylin eosin (HE) staining of mouse testis related to estrogen treatment. (A): Low dose (0.25 μg/g bw) of estradiol; (B): Mid-dose (0.5 μg/g bw) of estradiol; (C): High-dose (1.0 μg/g bw) of estradiol; (D): Control group (olive oil-treated group); (E): 10-day-old mice; (F): 45-day-old mice. (A)–(D): The mice were subjected to experimental cryptorchid, and received different treatment once a day for 35 days.

immunostained, both for Thy-1 and for integrin-β1. These might result from unspecific staining.

Statistical analysis showed significant increases in the number of Thy-1^+ and integrin-$\beta1^+$ cells as the dose of exogenous estradiol increased (Table 1). Table 1 illustrates the relationship between the amount of estra-

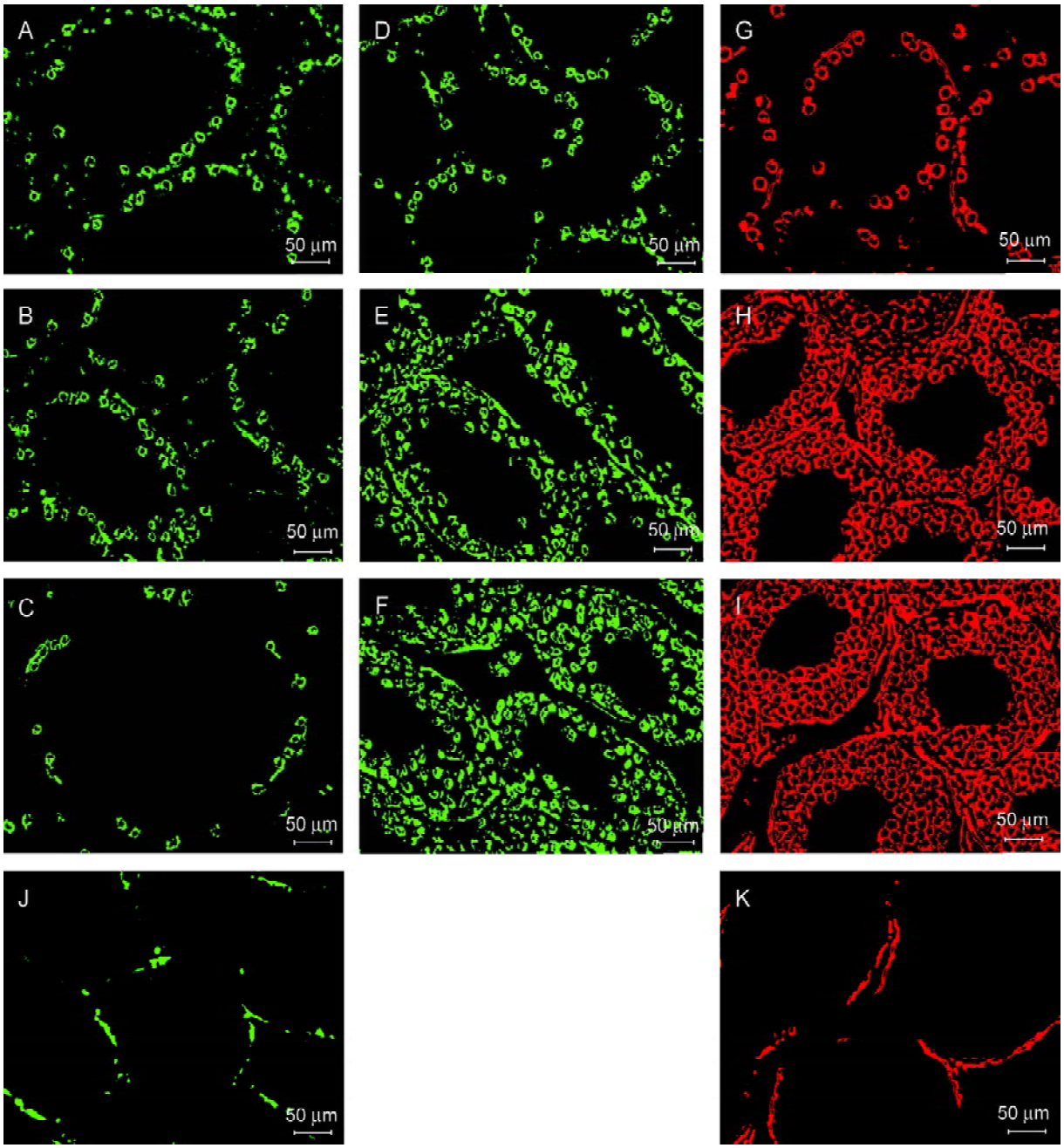

Figure 2. Immunofluorescence of testis of experimental cryptorchid mice. (A): Control (olive oil-treated group); (B): 10-day-old mice; (C): 45-day-old mice; (D) and (G): Low dose (0.25 μg/g bw) of estradiol group; (E) and (H): Mid-dose (0.5 μg/g bw) of estradiol group; (F) and (I) high dose (1.0 μg/g bw) of estradiol group; (A), (D)–(I) the mice were subjected to experimental cryptorchid, and received different treatment once a day for 35 days. (A)–(F), (J), Thy-1 was marker and (G)–(I), (K) integrin-β1 was marker molecule. Only the cells along the basal membrane of the seminiferous tubule were Thy-1^+ in the control group. Thy-1^+ and integrin-$\beta1^+$ cells related to the dose of exogenous estradiol in the estradiol-treated groups. (J) and (K): negative control.

diol given and the numbers of Thy-1^+ and integrin-$\beta1^+$ cells present.

3.3 Hormone level

To further understand the endocrine basis of these

Table 1. Number/tubule of Thy-1$^+$ and integrin-β1$^+$ cells in different groups (number/50). All mice, except those in 10- and 45-day-old groups were subjected to experimental cryptorchid, and received different treatment once a day for 35 days. [a]$P < 0.05$, compared with each other; [b]$P < 0.05$, compared with control group. There was no statistically significant difference between any two of the 10-day-old mice, low-dose-estradiol-treated and control groups. E2, 17β-estradiol.

	E2 (low)	E2 (middle)	E2 (high)	Control	10-day-old mice	45-day-old mice
Thy-1$^+$ cells	6.4 ± 0.4[a]	11.2 ± 0.5[a,b]	14.3 ± 0.3[a,b]	6.3 ± 0.3	5.9 ± 0.5	3.1 ± 0.6
Integrin-β1$^+$ cells	6.5 ± 0.2[a]	12.0 ± 0.3[a,b]	14.6 ± 0.2[a,b]	6.5 ± 0.5	6.1 ± 0.3	3.0 ± 0.5

Table 2. Serum estradiol and testosterone level in different groups. All animals except those in 10- and 45-day-old groups were subjected to experimental cryptorchid, and received different treatment once a day for 35 days. [a]$P < 0.05$, compared with each other; [b]$P < 0.05$, compared with control group; [c]$P < 0.05$ compared with mid-dose-estradiol-treated group (group II) and high-dose-estradiol-treated group (Group III). Serum testosterone was no statistically significant difference between any two of 10-day-old mice group, Group I and Group II.

Groups	Group I	Group II	Group III	Group IV	10-day-old-mice	45-day-old-mice
Estradiol (pg/mL)	170.3 ± 4.6[a,b]	469.0 ± 7.5[a,b]	1046.0 ± 8.2[a,b]	32.5 ± 1.02	30.80 ± 0.32	60.9 ± 1.9
Testosterone (nmol/L)	0.92 ± 0.03[c]	0.21 ± 0.02[b]	0.09 ± 0.01[b]	0.69 ± 0.03	0.87 ± 0.07	2.10 ± 0.04

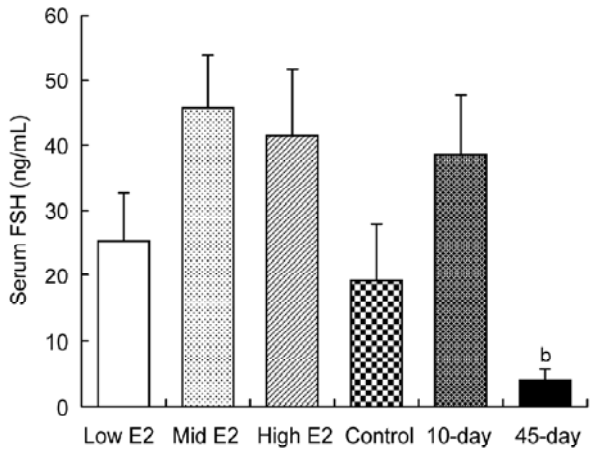

Figure 3. Serum follicle stimulating hormone (FSH) level in different groups. Animals except 10-day-old and 45-day-old mice were subjected to experimental cryptorchid, and received different treatment once a day for 35 days. [a]$P < 0.05$ *vs.* 10-day-old mice, mid-dose and high-dose 17β-estradiol (E2) treated groups; [b]$P < 0.05$ *vs.* any other groups; there is no statistically significant difference between any two of 10-day-old mice, mid-dose and high-dose-E2-treated groups.

changes, serum FSH, estradiol, testosterone and LH were measured. As shown in Figure 3, the mature mice (45-day-old mice) had the lowest FSH levels (3.95 ± 1.65 ng/mL); in comparison, serum FSH of mice in Group IV (control group), which was up to 19.1 ± 8.8 ng/mL, was significantly higher ($P < 0.05$). There were no significant difference in FSH level between any two of the 10-day mice, Group II (38.6 ± 9.2 ng/mL, 45.7 ± 8.1 ng/mL) and Group III (41.5 ± 10.3 ng/mL). However, these levels were all significantly higher than those for Group IV ($P < 0.05$). Animals treated with a low dose of E2 showed FSH concentrations (25.3 ± 7.3 ng/mL) approximately the same as those of the control group ($P = 0.11$). LH was not significantly different between any two of the different treated groups (results not shown). Estradiol was significantly different between any two of the E2-treated groups ($P < 0.05$). Testosterone levels descended with increasing doses of estradiol (Table 2). Serum testosterone levels of mice in Groups II and III were not different from each other, but both higher than those of Group I ($P < 0.05$), and lower than the control (Group IV) ($P < 0.05$).

3.4 Inhibin B measurement

The results of the ELISA for testis inhibin B are presented in Figure 4. The plasma inhibin B of normal mature mice (45-day-old) was the highest (409 ± 36 pg/mL), whereas the levels in 10-day-old mice and mid-dose and high-dose E2-treated mice were the lowest (176 ± 30 pg/mL, 119 ± 16 pg/mL and 134 ± 17 pg/mL, respectively) and did not significantly different from each other. Group I (199 ± 21 pg/mL) had higher levels of plasma inhibin B than Group II and Group III ($P < 0.05$), but no higher level than that the 10-day-old mice had. Inhibin B was higher than any estradiol-treated cryptorchid group ($P < 0.05$) in olive

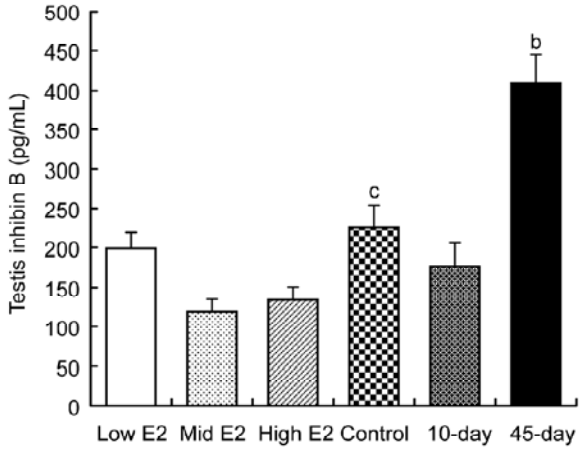

Figure 4. Plasma inhibin B level in testis of different treated groups. All mice except for the 10- and 45-day-old groups were subjected to experimental cryptorchid, and received different treatment once a day for 35 days. [a]$P < 0.05$ *vs.* mid-dose and high-dose-17β-estradiol (E2)-treated groups; [b]$P < 0.05$ *vs.* any other groups; [c]$P < 0.05$ *vs.* 10-day-old mice; there is no statistically significant difference between mid-dose and high-dose-estradiol-treated groups.

oil-treated cryptorchid mice (226 ± 28 pg/mL). On the other hand, serum inhibin B levels were not different between any two of the surgical cryptorchid groups (E2-treated groups and the control group), but were lower than that in 45-day-old mice (results not shown).

4 Discussion

A scrotal temperature approximately 5–7ºC lower than abdominal temperature is required for normal spermatogenesis [12]. Cryptorchidism has detrimental effects on spermatogenesis [12]. In the present study, cryptorchidism showed a striking influence on testis development in KM mice. In these mice, cryptorchidism led to spermatogenesis being arrested at the primary spermatocyte stage (Figure 1D).

In men, estrogen is secreted by Sertoli and Leydig cells, as well as by the adrenal gland. Estrogen receptors are expressed in the male reproductive organ. Estrogen is important for male reproduction [13]. In mammals, it is generally thought that estrogen appears to regulate the synthesis of testosterone and sexual behavior, in addition to spermatogenesis [14]. In recent years, there have been increasingly frequent reports investigating the functions of estrogen in the testis, but the actual effects of estrogen in male reproduction still remain unknown. One of interesting aspects of estrogen function is its role in the regulation of spermatogonia proliferation. Therefore, it struck us as logical to study the role of E2 on SSC development. The numbers and types of germ cells found were similar in Groups I and IV (control). This indicates that low doses of E2 have no remarkable effect on proliferation and differentiation of germ cells in cryptorchid mice. However, the number of germ cells in Groups II and III were both much greater than in Group IV. The increase in cell number was in direct proportion to the dosage of exogenous E2. This suggests that E2 promotes germ cell division in a dose-related fashion.

We used Thy-1 antibody for immunofluorescence examination of the testis to determine if E2 can stimulate spermatogonia proliferation. Thy-1 (CD90) was first identified on the T-cell surface in the thymus. Recent studies have found that Thy-1 is also a unique surface marker of SSC in mouse testes [15–17]. In the present study, more cells in the seminiferous tubules of surgical cryptorchid mice treated with estradiol were Thy-1$^+$ cells, especially in the mid-dose and high-dose-estradiol-treated groups. Furthermore, comparison of HE staining (Figure 1F) and immunofluorescence (Figure 2C) of the same field showed that in the 45-day-old mice, only the cells along the basal membrane of the seminiferous tubules were Thy-1$^+$. Therefore, it appears that high doses of E2 can promote spermatogonia proliferation.

Reports have established that integrin-β1 is the surface marker on mouse SSC [16, 18]. Therefore, in testis seminiferous tubules, the integrin-β1$^+$ cells are SSC. To further confirm that E2 stimulates SSC division toward mitosis, but not meiosis, immunofluorescence was conducted using integrin-β1 as a marker. The results showed that the Thy-1$^+$ cells were also integrin-β1$^+$. In the present study, the Thy-1$^+$ and integrin-β1$^+$ cells were regard as spermatogonia.

According to this study, the most effective doses of E2 for producing spermatogonia are 0.5–1.0 μg/g bw. Our data are consistent with those previously reported by Pentikainen *et al.* [4] and Li *et al.* [19], in which E2 was observed to stimulate proliferation of spermatogonia and gonocytes *in vitro*, and a report by Miura *et al.* [7], in which they found that 10 pg/mL of E2 was sufficient to induce SSC division in cultured testicular tissue.

Proliferation and differentiation of SSC is a complex process that is tightly controlled by both endocrine and paracrine factors. The mechanisms by which estrogen

induces the renewal of SSC are not well understood. Estrogen regulation may occur indirectly at the pituitary level by regulating gonadotropin (FSH and LH) secretion. FSH and LH are the primary tropic hormones that regulate testicular function. FSH is well known to regulate spermatogonia; for instance, FSH has been shown to increase the number of spermatogonia in hypogonadal (hpg) mice [20]. Stimulation of FSH by estradiol treatment has been noted in normal and transgenic rodents, notably hpg mice [14].

Inhibins are a group of glycoprotein hormones secreted by the gonads in men and women. The inhibin α subunit heterodimerizes with inhibin β subunits to form inhibin A (α and β_A) and inhibin B (α and β_B), which are the bioactive forms of inhibin in the general circulation. These bioactive forms have been well-characterized as negatively regulating pituitary FSH secretion through direct action on the pituitary gonadotrophs in mature female and male rats [21].

To elucidate the mechanism by which E2 stimulates SSC in surgical cryptorchid mice, we measured serum FSH and plasma and testis inhibin B. Results showed that FSH in surgical cryptorchid mice (including E2-treated groups and the control group) were higher than in normal, 45-day-old mice. This suggests that cryptorchidism causes an increase in serum FSH. There were more spermatogonia (Thy-1^+ and integrin-$\beta1^+$) in all of these cryptorchid groups than in normal mature mice. This indicates that FSH induces spermatogonial proliferation in cryptorchid mice, consistent with the observation in hypogonadal mice [18]. However, a statistical analysis of FSH level and spermatogonia in the estradiol-treated groups showed that spermatogonia present in the testis increased in a dose-related manner, but FSH level did not. When compared, Group II and III FSH levels (45.7 ± 8.1 ng/mL *vs*. 41.5 ± 10.3 ng/mL) were not significantly different; but their number of Thy-1^+ cells (11.2 ± 0.5 and 14.3 ± 0.3, respectively) and integrin-$\beta1^+$ cells (12.0 ± 0.3 and 14.6 ± 0.2, respectively) differed significantly ($P < 0.05$). Plasma inhibin B levels were not different among the cryptorchid groups, although testis inhibin B levels were different. In addition, we found that estradiol has no significant effect on serum LH (result not shown). These findings suggest that one, but not all, of the pathways by which E2 regulates SSC is via FSH.

In the present study, we found that estrogen inhibits testosterone secretion. This finding suggests that another pathway through which E2 promotes spermatogonia proliferation is by estrogen inhibiting the effect of testosterone on SSC differentiation [22].

There might be two other pathways through which estrogen stimulates SSC proliferation. The first possible pathway concerns the complex of estrogen and its receptors. The estrogen receptor family is composed of ERα and ERβ. The bulk of reports point out that ERα is expressed in Leydig cells and peritubular myoid cells, whereas ERβ is present in both Sertoli cells and germ cells [23, 24]. Subsequently, experimental evidence shows that ERβ regulates germ cell development [25]. ERβ has been shown to mediate estrogen action in much the same way as ERα [26]. Therefore, estrogen might bind to ERβ to activate the genes that promote SSC self-renewal.

The second pathway relates to the ability of estrogen to induce expression of growth factors that stimulate SSC self-renewal. Because estrogen has the ability to bind and activate ERs, it affects testicular gene expression [27]. Possibly, the complex of estrogen and its receptor (ERα) induces Sertoli or Leydig cells to secrete growth factors, such as glial cell derived neurotrophic factor (GDNF), the same way that stem cell factor is produced in Sertoli cells when induced by FSH [28]. Perhaps this is just like the way that E2 stimulates GDNF expression in hypothalamic neurons [29]. In cryptorchid mice, this possible pathway has been implicated in the restoration of spermatogenesis [30]. Furthermore, bovine serum albumin-conjugated E2 decreases testicular androgen production *in vitro* [31].

All results above indicate that E2 can stimulate spermatogonia proliferation in cryptorchid mice. Treatment of cryptorchid mice with 0.5 μg/g bw and 1.0 μg/g bw of E2 can be used to enrich spermatogonia and produce mice that have more spermatogonia. The mechanism by which E2 stimulate spermatogonia are complex, in which many factors are involved.

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References

1 Brinster RL. Germline stem cell transplantation and transgenesis. Science 2002; 296: 2174–6.
2 Huleihel M, Lunenfeld E. Regulation of spermatogenesis by paracrine/autocrine testicular factors. Asian J Androl 2004; 6: 259–68.
3 Shinohara T, Avarbock MR, Brinster RL. Functional analysis of spermatogonial stem cells in Steel and cryptorchid infertile mouse models. Dev Biol 2000; 220: 401–11.
4 Pentikainen V, Erkkila K, Suomalainen L, Parvinen M, Dunkel L. Estradiol acts as a germ cell survival factor in the human testis *in vitro*. J Clin Endocrinol Metab 2000; 85: 2057–67.
5 Kula K. Induction of precocious maturation of spermatogenesis in infant rats by human menopausal gonadotropin and inhibition by simultaneous administration of gonadotropins and testosterone. Endocrinology 1988; 122: 34–9.
6 Wang Y, Thuillier R, Culty M. Prenatal estrogen exposure differentially affects estrogen receptor-associated proteins in rat testis gonocytes. Biol Reprod 2004; 71: 1652–64.
7 Miura T, Miura C, Ohta T, Nader MR, Todo T, Yamauchi K. Estradiol-17beta stimulates the renewal of spermatogonial stem cells in males. Biochem Biophys Res Commun 1999; 264: 230–4.
8 Chieffi P, Colucci D'Amato L, Guarino F, Salvatore G, Angelini F. 17beta-estradiol induces spermatogonial proliferation through mitogen-activated protein kinase (extracellular signal-regulated kinase 1/2) activity in the lizard (*Podarcis s. sicula*). Mol Reprod Dev 2002; 61: 218–25.
9 Nishimune Y, Aizawa S, Komatsu T. Testicular germ cell differentiation *in vivo*. Fertil Steril 1978; 29: 95–102.
10 Groome NP, Illingworth PJ, O'Brien M, Pai R, Rodger FE, Mather JP, *et al*. Measurement of dimeric inhibin-B throughout the human menstrual cycle. J Clin Endocrinol Metab 1996; 81: 1401–5.
11 Wan X, Zhao XJ. Laboratory Animal Science. Beijing: Monograph Literature Press, 1998.
12 Regadera J, Martinez-Garcia F, Gonzalez-Peramato P, Serrano A, Nistal M, Suarez-Quian C. Androgen receptor expression in sertoli cells as a function of seminiferous tubule maturation in the human cryptorchid testis. J Clin Endocrinol Metab 2001; 86: 413–21.
13 Carreau S, Delalande C, Silandre D, Bourguiba S, Lambard S. Aromatase and estrogen receptors in male reproduction. Mol Cell Endocrinol 2006; 246: 65–8.
14 O'Donnell L, Robertson KM, Jones ME, Simpson ER. Estrogen and spermatogenesis. Endocr Rev 2001; 22: 289–318.
15 Kubota H, Avarbock MR, Brinster RL. Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod 2004; 71: 722–31.
16 Kubota H, Avarbock MR, Brinster RL. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci U S A 2003; 100: 6487–92.
17 Kubota H, Avarbock MR, Brinster RL. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 2004; 101: 16489–94.
18 Shinohara T, Avarbock MR, Brinster RL. β1- and α6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 1999; 96: 5504–9.
19 Li H, Papadopoulos V, Vidic B, Dym M, Culty M. Regulation of rat testis gonocyte proliferation by platelet-derived growth factor and estradiol: identification of signaling mechanisms involved. Endocrinology 1997; 138: 1289–98.
20 Haywood M, Spaliviero J, Jimemez M, King NJ, Handelsman DJ, Allan CM. Sertoli and germ cell development in hypogonadal (hpg) mice expressing transgenic follicle-stimulating hormone alone or in combination with testosterone. Endocrinology 2003; 144: 509–17.
21 De Jong FH. Inhibin. Physiol Rev 1988; 68: 555–607.
22 D'Souza R, Gill-Sharma MK, Pathak S, Kedia N, Kumar R, Balasinor N. Effect of high intratesticular estrogen on the seminiferous epithelium in adult male rats. Mol Cell Endocrinol 2005; 241: 41–8.
23 Selva DM, Tirado OM, Toran N, Suarez-Quian CA, Reventos J, Munell F. Estrogen receptor beta expression and apoptosis of spermatocytes of mice overexpressing a rat androgen-binding protein transgene. Biol Reprod 2004; 71: 1461–8.
24 Lambard S, Carreau S. Aromatase and oestrogens in human male germ cells. Int J Androl 2005; 28: 254–9.
25 Delbes G, Levacher C, Pairault C, Racine C, Duquenne C, Krust A, *et al*. Estrogen receptor beta-mediated inhibition of male germ cell line development in mice by endogenous estrogens during perinatal life. Endocrinology 2004; 145: 3395–403.
26 Bigsby RM, Caperell-Grant A, Berry N, Nephew K, Lubahn D. Estrogen induces a systemic growth factor through an estrogen receptor-alpha-dependent mechanism. Biol Reprod 2004; 70: 178–83.
27 Akingbemi BT. Estrogen regulation of testicular function. Reprod Biol Endocrinol 2005; 3: 51.
28 Kotaja N, Sassone-Corsi P. Plzf pushes stem cells. Nat Genet 2004; 36: 551–3.
29 Ivanova T, Karolczak M, Beyer C. Estradiol stimulates GDNF expression in developing hypothalamic neurons. Endocrinology 2002; 143: 3175–8.
30 Shetty G, Weng CC. Cryptorchidism rescues spermatogonial differentiation in juvenile spermatogonial depletion (jsd) mice. Endocrinology 2004; 145: 126–33.
31 Loomis AK, Thomas P. Effects of estrogens and xenoestrogens on androgen production by Atlantic croaker testes *in vitro*: evidence for a non-genomic action mediated by an estrogen membrane receptor. Biol Reprod 2000; 62: 995–1004.

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