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Estradiol-17β Stimulates the Renewal of Spermatogonial Stem Cells in Males

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In this work, we examined the functions of the female hormone "estrogen" on spermatogenesis of the Japanese eel (*Anguilla japonica*). Estradiol-17β (E_2), a natural estrogen in vertebrates, was present in the serum and its receptor was expressed in the testis during the whole process of spermatogenesis. Spermatogonial stem cell renewal was promoted by E_2 implantation but was suppressed by tamoxifen (an antagonist of estrogen). *In vitro,* 10 pg/ml of E_2 was sufficient to induce spermatogonial stem cell division in cultured testicular tissue, therefore confirming the *in vivo* observations. These results clearly show that estrogen is an indispensable "male hormone" in the early spermatogenetic cycle.

It is widely accepted that "estrogen" is a "female" hormone. However, it has been reported that estrogen is present in some male vertebrates (1–3) and that it's receptors are expressed in the male reproductive organs (4, 5). In mammals, estrogen appears to regulate the reabsorption of luminal fluid in the epididymis (6) and to affect sexual behavior (7, 8). Even though there has been a noticeable increase in reports analyzing the actions of estrogen in the testis recently, the clear functions of estrogens in male reproduction have not been established.

Recently, environmental pollution by chemicals—collectively known as endocrine disrupters—have been shown to stimulate or block biological processes (9), and have been recognized to interfere with sensitive hormone pathways that regulate reproductive functions. This diverse group of chemicals includes environmental estrogens or exestrogens, compounds that evoke estrogenic responses by mimicking the actions of endogenous estrogen including estradiol-17β (E_2). In male animals, exposure to estrogenic compounds can lead, among others, to reduced gonad size, feminization of genetic males, low sperm count and/or quality (10, 11). Due to the lack of understanding of the mechanisms by which these actions are performed, it is important to analyze the role of estrogen in male reproduction.

To better clarify the various functions of estrogen on spermatogenesis, male Japanese eels (*Anguilla japonica*) were used as experimental animals. Under cultivated conditions, male Japanese eel have immature testes containing only non-proliferated spermatogonia (12). It has been proven that complete spermatogenesis in the Japanese eel can be induced by a single injection of human chorionic gonadotropin (hCG) *in vivo* (12), and by supplementation with either hCG (13) or 11-ketotestosterone (11-KT) (14) *in vitro.* In this study, we investigated the role of estrogen in spermatogenesis using the male Japanese eel system described above.

MATERIALS AND METHODS

Animals. Male cultivated Japanese eel (180–200 g in body weight) were purchased from a commercial eel supplier, and kept in circulating fresh-water tanks with a capacity of 500 liters at 23°C.

To 50 male eels, a single injection of hCG dissolved in saline (150 mM NaCl) was given intramuscularly at a dose of 5 IU per gram of body weight. Thirty fish were injected only with saline. Fish were sacrificed at 0, 1, 3, 6, 9, 12, 15, and 18 days after hCG or saline injection, and blood and testes were collected. Serum was immediately separated and E_2 levels were measured by radioimmunoassay according to Kagawa *et al.* (15).

Poly $(A)^+$ RNA was isolated from the testes using the First Track kit (Invitrogen, San Diego, CA).

Northern and in situ hybridization. Poly $(A)^+$ RNA (1 μg) was electrophoresed on a 1% (w/v) agarose denatured gel and blotted onto nylon membranes. The uniformity of sample loading and transfer were monitored by staining the blotted RNA with methylene blue. The eel estrogen receptor β-like cDNA (16) and β-actin cDNA (17)

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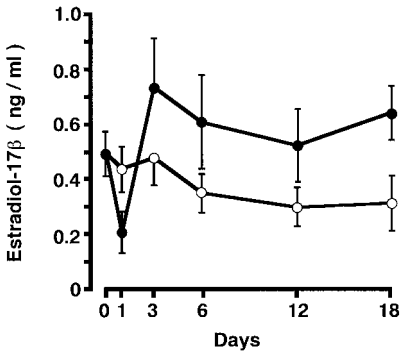

FIG. 1. Changes in serum levels of estradiol-17β following induction of spermatogenesis by hCG injection. Open and closed circles represent control and hCG-injected eel, respectively.

used as probe were randomly labeled, and hybridization and detection were carried out in accordance with a previous report (18).

For *in situ* hybridization, a non radioactive method using a digoxigenin (DIG)-labeled RNA probe was employed (19). Testicular fragments were obtained from non-treated eels. Sense and antisense RNA probes were transcribed *in vitro* from the 286 bp cDNA fragment of the eel estrogen receptor (positions 1607 to 1893) using DIG-labeled UTP and T3 or T7 RNA polymerase. Hybridization and detection were carried out in accordance with a previous report (19).

Implantation of estrogen and its antagonist. E_2 and tamoxifen (a known antagonist of estrogen) were given intraperitoneally to 10 fish each using a silastic capsule (20) at a dose of 1 and 20 μg/g body weight, respectively. Fish were sacrificed 24 days after implantation. Serum E_2 levels in E_2 implanted fish increased after implantation to reach 7 ng/ml within 1 day, then remained constant for 24 days. A vehicle (ethanol:castor oil = 1:9) was implanted in 10 control fish. Ten fish were implanted with E_2 for 48 days to verify whether germ cells advance through spermatogenesis.

Proliferation of germ cells was assayed by the immunocytochemical detection of 5-bromo-2-deoxyuridine (BrdU) incorporated into replicating DNA according to a previous report (21) with minor modifications. Testicular fragments were labeled for 12 h with BrdU and then fixed in Bouin's solution. Fixed samples were embedded in paraffin and prepared for 5-μm histological sections. Incorporation of BrdU was detected by a specific antibody, and the sections were counter stained by hematoxylin in order to count the immunoreacted cells and the total germ cell numbers.

Testicular organ culture. The culture technique employed was the floating method described in Miura *et al.* (14). E_2 and 11-KT were first dissolved in ethanol and then diluted with the medium. Plastic sections for light microscopical observation were prepared in accordance with a previous report (12) and proliferation of germ cells in the cultured testicular fragments was assayed.

Statistics. Results were expressed as means and standard errors (SEM). Differences between means were analyzed by a one-way ANOVA ($p < 0.005$) followed by the Bonferroni multiple-comparison test ($p < 0.005$).

RESULTS

Time course changes in serum estrogen levels after hCG injection. The time course changes in serum E_2 levels after hCG or saline injection is presented in Fig. 1. The average serum level of E_2 on day 0 was 0.49 $\pm$ 0.08 ng/ml, and this level did not show any significant changes during the whole process of hCG induced spermatogenesis.

Expression and localization of eel estrogen receptor mRNA during spermatogenesis. Using eel estrogen receptor cDNA, Northern blot analysis was performed to evaluate the changes in ER transcripts during spermatogenesis. A transcript of approximately 3.8 kb was detected in the testes during the whole process of spermatogenesis. The signals were strongest from 1 to 6 days after hCG injection, coinciding with the early phase of spermatogonial proliferation (Fig. 2A). As control, the same blots were reprobed with a labeled β-actin cDNA. The level of actin transcripts was constant throughout the experimental periods (data not shown), confirming that the observed changes in eel ER mRNA expression are not artificial.

To determine the distribution of eel ER expression in the testis, we performed *in situ* hybridization using DIG-labeled sense and antisense RNA probes (Figs. 2B and 2C). The signal detected with the anti-sense probe was mainly located in Sertoli cells surrounding the spermatogonia, although some positive signals were also observed in interstitial cells. In contrast, the sense probe did not hybridize to any cells.

No signal was observed with the sense probe.

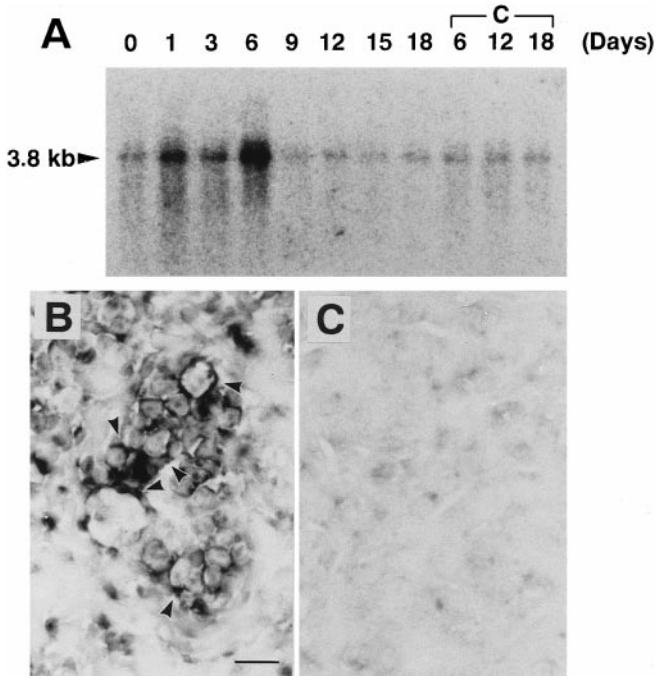

FIG. 2. Eel estrogen receptor mRNA expression in testes. (A) Northern blot analysis was performed using poly $(A)^+$RNA extracted from testes 0, 1, 3, 6, 9, 12, 15, and 18 days after hCG treatment, and 6, 12, and 18 days after saline injection for control (C). *In situ* hybridization was performed using DIG-labeled antisense (B) and sense (C) probes. Testicular samples were obtained from nonhormonal treated eel. Arrowheads indicate Sertoli cells. Bar, 10 μm.

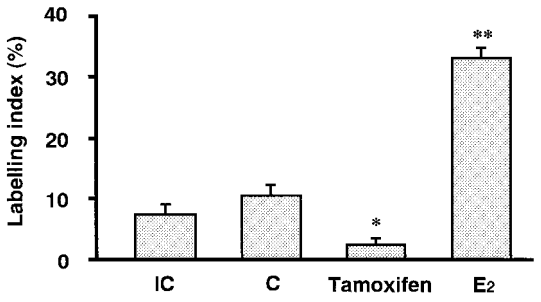

FIG. 3. Effect of estradiol-17β (E_2) implantation on BrdU incorporation in spermatogonial stem cell division. IC, initial controls; C, control groups. *$p < 0.005$; **$p < 0.0001$ (28).

Morphological changes in the testis after implantation of E_2 and tamoxifen. To investigate the functions of estrogen on spermatogenesis, we implanted silastic capsules including E_2 or tamoxifen in the peritoneal cavity of eels. We then assayed the proliferation of spermatogonia by immunoreacting the testicular tissue with 5-bromo-2-deoxyuridine (BrdU) to check for replicating DNA. The BrdU index in control fish was $10.5 \pm 1.7\%$ confirming that spermatogonial stem cells constantly get renewed. After 24 days, the BrdU labeling index showed a remarkable increase in germ cell DNA synthesis in the E_2 implanted specimens, while a significant decrease in the same index was recorded in tamoxifen implanted samples (Fig. 3).

After 48 days of E_2 implantation, the testes possessed only type A and early type B spermatogonia as in the control fish.

Effects of E_2 on spermatogenesis in vitro. We then examined the direct effects of E_2 on spermatogenesis *in vitro.* The testes were cultured for 15 days in media with or without various concentrations of E_2 (10, 100, or 1000 pg/ml) or with 11-KT (10 ng/ml) for positive control. Both E_2 and 11-KT treatments stimulated DNA replication and mitosis of spermatogonial stem cells as in the *in vivo* E_2 implantation (Figs. 4A and 4B). Although E_2 treatment induced spermatogonial mitosis, germ cells did not progress further into spermatogenesis (data not shown).

The next step was to determine whether the mitotic divisions of stem cells induced by E_2 proceeded normally or abnormally by examining if the spermatogonia treated with E_2 were able to advance further into spermatogenesis. Therefore, testicular fragments were cultured with E_2 (1 ng/ml) for 15 days, washed in medium for 6 days, 10 ng/ml of 11-KT supplied on the 21st-day and cultured further for 30 days. Histological observations revealed that the germ cells proceeded normally through all the stages of spermatogenesis (Fig. 5) strongly suggesting that E_2 does not negatively affect germ cells.

DISCUSSION

In the male Japanese eel, E_2 has been detected in the serum and the estrogen receptor mRNA was found to be mainly expressed in Sertoli cells from 1 to 6 days after hCG injection. Thus, estrogen and it's receptor are present in the eel testes as in other vertebrates (1–3). In eel, spermatogonial proliferation begins 3 days after hCG injection, with some germ cells starting meiosis after 12 days (12). Therefore the period of 1 to 6 days after hCG injection corresponds to the early phase of spermatogonial proliferation. These results suggest that estrogen, specially E_2, is involved in the regulation of spermatogenesis through the mediation of Sertoli cells, with the highest activity taking place in the early phases of spermatogonial proliferation.

E_2 implantation significantly increased and tamoxifen, which is antagonist of estrogen, implantation significantly decreased germ cell DNA synthesis compared with control. Furthermore, E_2 treatment also

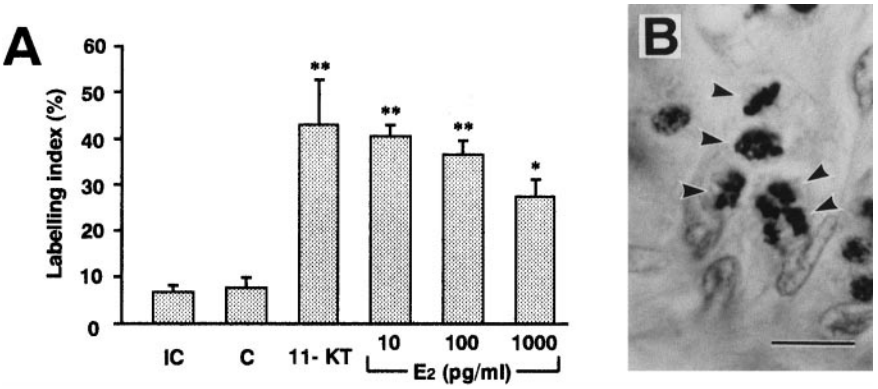

FIG. 4. Effect of estradiol-17β (E_2) on BrdU incorporation in spermatogonia using *in vitro* testicular organ culture. (A) Percentage of BrdU labeling spermatogonia in each group. IC, initial controls; C, control groups; 11-KT, 11-ketotestosterone for positive control. *$p < 0.005$; **$p < 0.0001$ (28). (B) Microphotograph of germ cells undergoing mitotic divisions (arrowheads) in 10 pg/ml of E_2 treated group. Bar, 10 μm.

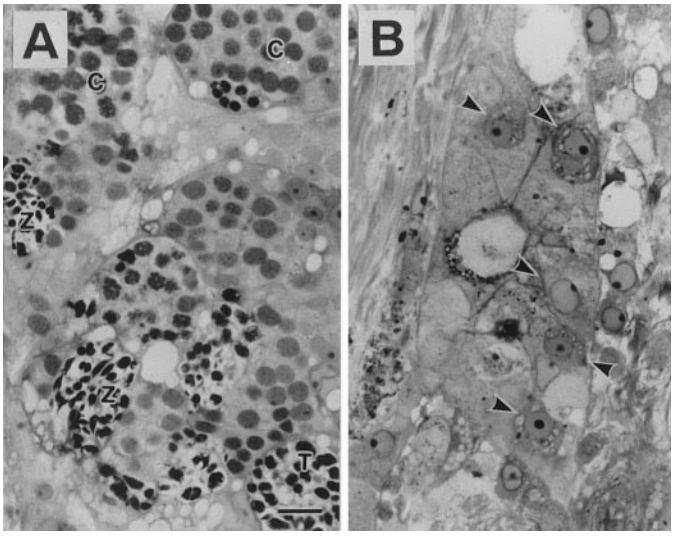

FIG. 5. Light micrograph of cultured testis. (A) Testicular fragments treated with E_2 for 15 days, washed for 6 days, then further cultured with 11-ketotestosterone for 30 days. (B) Control testes treated with E_2 for 15 days and then cultured without hormone for 36 days. Arrowheads, spermatogonial stem cell; C, spermatocytes; T, spermatids; Z, spermatozoa. Bar, 10 μm.

induced DNA synthesis and mitotic division in germ cells in *in vitro* testicular organ culture, indicating that E_2 induces mitotic division of spermatogonia. Even though, E_2 treatment *in vivo* and *in vitro* induced spermatogonial mitosis, germ cells did not progress further into meiosis. Spermatogonial mitosis can be categorized by spermatogonial stem cell renewal and spermatogonial proliferation toward meiosis (cf. 22). In eel, spermatogonial proliferation is controlled by 11-KT, since 11-KT induces the whole process of spermatogenesis from spermatogonial proliferation to spermiogenesis (14). Therefore the spermatogonial mitosis induced by E_2 may not be toward the formation of sperm, but for spermatogonial stem cell renewal.

In vitro testicular organ culture, supplementation of 10, 100, and 1000 pg/ml of E_2 to the culture medium stimulated DNA replication and mitosis of spermatogonial stem cells. The range of the effective E_2 concentration, 10–1000 pg/ml, conforms to the levels found in the male serum. This shows that E_2 is effective at much lower concentrations than 11-KT, since 10 ng/ml of 11-KT are needed to induce full spermatogenesis (14).

In this study, the germ cells that were produced by E_2 stimulation proceeded normally through all the stages of spermatogenesis after treatment with 11-KT. This indicates that these "new" spermatogonia keep the ability to develop into spermatozoa, and suggest that E_2 does not negatively affect the function of germ cells.

Thus it is hypothesized that in the Japanese eel low concentrations of E_2 (10 pg/ml) act on spermatogonial stem cells through Sertoli cells by stimulating and maintaining their proliferation before spermatogenesis is initiated by 11-KT.

Lately, it was reported that environmental estrogens disrupt the endocrine system and damage the male reproductive system in many animals including fish (11, 24, 25). Given that spermatogenesis is a highly regulated process, the presence of xenobiotics in the environment may trigger germ cell renewal at an inappropriate time impairing normal spermatogenesis. Alternatively, environmental estrogens can bind to the estrogen receptors without triggering germ cell renewal and block the beneficial effects of endogenous estrogen allowing for the presence of few stem cells to be transformed into sperm. This might be one of the areas where estrogen action can be responsible for the reported low sperm count in humans. In conclusion, small concentrations of estrogen are necessary for a normal spermatogenesis. On the other hand, environmental estrogen can negatively affect the male reproductive system by disrupting the regulation mechanisms of spermatogonial stem cell proliferation.

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REFERENCES

1. Schlinger, B. A., and Arnold, A. P. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7650–7653.
2. Fasano, S., and Pieratoni, R. (1993) *J. Endocrinol.*
3. Betka, M., and Callard, G. V. (1998) *APMIS* **106,** 252–258.
4. Ciocca, D. R., and Roig, L. M. V. (1995) *Endocrine Rev.* **16,** 35–62.
5. Callard, I. P., and Callard, G. V. (1987) *in* Hormones and Reproduction in Fishes, Amphibians and Reptiles (Norris, D. O., and Jones, R. E., Eds.), pp. 355–384, Plenum Press, New York.
6. Hess, R. A., Bunick, D., Lee, K.-H., Bahr, J., Taylor, J. A., Korach, K. S., and Lubahn, D. B. (1997) *Nature* **390,** 509–512.
7. Wersinger, S. R., Sannen, K., Villalba, C., Lubahn, D. B., Rissman, E. F., and Vires, G. J. D. (1997) *Horm. Behav.* **32,** 176–183.
8. Springer, M. L., and Wade, J. (1997) *Horm. Behav.* **32,** 46–59.
9. Colborn, T., Saal, F. S., and Soto, A. M. (1993) *Environ. Health Perspect.* **101,** 378–384.
10. Sharpe, R. M. (1993) *J. Endocrinol.* **136,** 357–360.
11. Sumpter, J. P. (1995) *Toxicol. Lett.* **82–83,** 737–742.
12. Miura, T., Yamauchi, K., Nagahama, Y., and Takahashi, H. (1991) *Zool. Sci.* **8,** 63–73.
13. Miura, T., Yamauchi, K., Takahashi, H., and Nagahama, Y. (1991) *Dev. Biol.* **146,** 258–262.
14. Miura, T., Yamauchi, K., Takahashi, H., and Nagahama, Y. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5774–5778.
15. Kagawa, K., Takano, K., and Nagahama, Y. (1981) *Cell Tissue Res.* **218,** 315–329.

16. Todo, T., Adachi, S., and Yamauchi, K. (1996) *Mol. Cell. Endocrinol.* **119,** 37–45.
17. Nagae, M., Adachi, S., and Yamauchi, K. (1997) *Fish Physiol. Biochem*. **17,** 179–186.
18. Miura, T., Kudo, N., Miura, C., Yamauchi, K., and Nagahama, Y. (1998) *Mol. Reprod. Dev*. **51,** 235–242.
19. Miura, C., Miura T, Kudo, N., Yamashita, M., and Yamauchi, K. (1999) *Dev. Growth Differ*. **41,** 463–471.
20. Kobayashi, M., Aida, K., and Stacy, N. E. (1991) *Zool. Sci*. **8,** 389–393.
21. Miura, C., Miura, T., Yamashita, M., Yamauchi, K., and Nagahama, Y. (1996) *Dev. Growth Differ*. **38,** 257–262.
22. Clermont, Y. (1972) *Int. Rev. Cytol.* **70,** 27–100.
23. Sumpter, J. P. (1998) **102–103,** 337–342.
24. Sharpe, R. M. (1998) *Biol. Reprod*. **59,** 1084–1094.