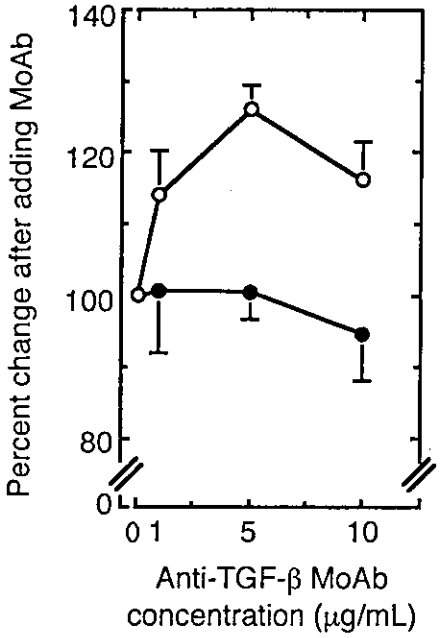


Figure 4. Effect of neutralizing anti–transforming growth factor-β (TGF-β) monoclonal antibody (mAb) on proliferation of pro-B/pre-B cells from young senescence-accelerated mice co-cultured with femoral stromal cells from young mice (open circles) and those from senescent mice (closed circles). The effect of TGF-β on the growth of pro-B/pre-B cells was evaluated on the basis of the number of nonadherent cells after the supplementation of the neutralizing antibody to TGF-β (mouse IgG_1 isotype: R&D Systems, Inc., Minneapolis, MN) at dilutions ranging from 1–10 μg/ml to the coculture system. The mouse IgG_1 isotype (R&D Systems) was used as the mock control. The senescent group shows no substantial rescue effect of mAb, whereas the young group shows significant rescue effect of mAb by repeated-measure analysis of variance testing (P = 0.0111). (Vertical bars indicate SEM of triplicate experiments).

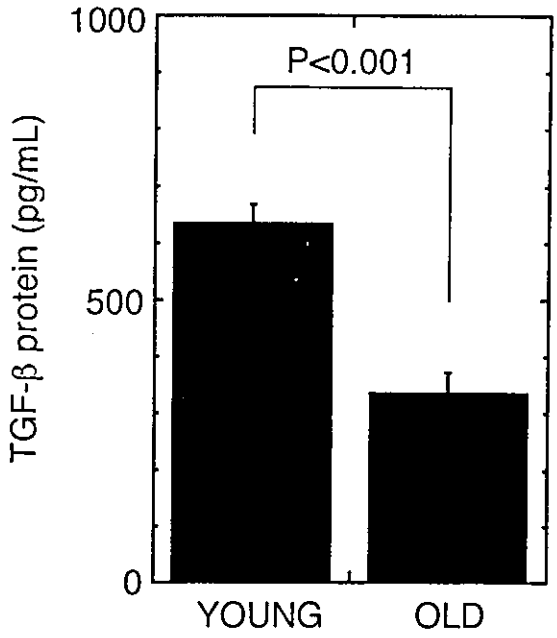


Figure 5. Transforming growth factor-β (TGF-β) protein level in the supernatant of cultured stromal cells from young and senescent senescence-accelerated mice (SAMs) (mean ± SEM of three replicate experiments). Stromal monolayers were prepared by culturing bone marrow (BM) cells from young or senescent SAMs at 1×10^6/ml in 24-well Falcon 3047 flat-bottom plates in 1 ml of the RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS). Confluent adherent layers were formed after 7 days. The supernatant of culture plates was removed, 1 ml of RPMI 1640 was supplemented with 20% FBS, and 2×10^{-5} *M* 2-ME and 1% L-glutamine were added to the culture plates. The culture medium was collected after culture for 7 days and was used for determination of the level of the TGF-β protein produced by stromal cells. The TGF-β concentration in culture medium was determined using a TGF-β–specific enzyme-linked immunoabsorbent assay kit.

group was unexpectedly not recovered by the neutralizing antibody, whereas (although it was not expected) the proliferation of the young group was prominently recovered at 1- and 5-μg/ml doses (statistical significance, $P < 0.001$ in the group of 5 μg/ml). Thus, despite the prominent decrease in IL-7 expression level and in the biological activity of IL-7 in the BM, TGF-β production seemed to have unexpectedly decreased in the senescent group. To confirm the decrease in TGF-β production by stromal cells with age, we directly measured TGF-β protein level in the supernatant of cultured stromal cells derived from young and senescent SAMs using ELISA. Figure 5 shows that the TGF-β protein level in the supernatant of cultured senescent stromal cells is markedly lower than that of cultured young stroma, thus implying that TGF-β production by stromal cells decreases with age (634.0 ± 36.0 vs. 337.0 ± 37.9, young and old, respectively, $P < 0.001$).

Discussion

It has long been questioned whether age-related alterations in B lymphopoiesis are mainly due to a functional impairment of B cell precursor cells or due to that of senescent stromal cells. Several studies showed steady-state B lymphopoiesis and focused on the decrease in B cell production due in part to the decreased IL-7 responsiveness (25, 26). Indeed, our initial experiments demonstrated that the numbers of femoral CFU-pre-B cells (3-6), particularly those forming large colonies, decrease with age, suggesting that age-related alterations of B lymphopoiesis seem to be based on the quality of B cell precursors (Table 1 and Fig. 1).

The expression level of IL-7, a pre-B cell stimulator, was evaluated in BM cells, because IL-7 production decreases during senescence (17–19). Mice in which the *IL-7* gene has been knocked out manifest a prominent decrease in the number of pre-B lymphocytes and a severe impairment in capacity for self-renewal in the pre-B cell compartment, even though the number of pro-B cells appears to be normal. Phenotypically, age-related changes seem closer to the changes in the B lymphopoiesis observed in aged mice (27). Our data showed that the IL-7 mRNA expression level in freshly isolated BM cells decreases with age (Fig. 2). We attempted to measure IL-7 expression level only in freshly isolated BM cells and BM stromal cells, because the expression of IL-7 is easily upregulated

immediately after the start of culture. Because of the limited materials for ELISA, protein expression level was not determined. These findings are consistent with those of Updyke *et al.* (17), who reported that the relative quantity of the IL-7 protein released into the medium for long-term B cell culture decreases with age. A study by Stephan *et al.* (18) suggested that the age-related decrease in the function of BM cells is associated with the impaired release of IL-7. Interestingly, a novel mutant mouse model of aging, *klotho*, was reported to exhibit a similar significant decrease in the level of *IL-7* gene expression in freshly isolated BM cells as determined by RT-PCR analysis (19); the mouse initially exhibits multiple disorders that resemble various aging phenotypes. Based on the findings by other researchers and ourselves, it seems likely that IL-7 production by BM stromal cells decreases with age. As seen in Figures 1–4, comparable results were obtained in reports available in the literature; hence, the present findings obtained through the experiment using SAMs may be applicable to the analysis of natural aging in regular mice.

Next, we demonstrated that the production of TGF-β by marrow stromal cells decreased with age, although the mechanism underlying this phenomenon is not yet known. Young stromal cells inhibited B cell proliferation in the co-culture system, and this inhibition was reversed by treatment with antibodies to TGF-β. The results of the co-culture system demonstrated that significantly fewer lymphocytes could be recovered from the co-culture system with young stromal cells than with senescent stromal cells; conversely, a significantly higher number of CFU-pre-B cells is maintained in the co-culture system with young stromal cells than with senescent stromal cells (Fig. 3A and B). Moreover, the neutralizing antibody to TGF-β restored the proliferative capacity of pro-B/pre-B cells co-cultured with young stromal cells but not that of those co-cultured with senescent stromal cells (Fig. 4). Furthermore, the TGF-β protein level in supernatant of cultured senescent stromal cells is markedly lower than that of young stromal cells (Fig. 5). These results imply that senescent stromal cells are not capable of producing TGF-β. These data agree with those reported by Dubinett *et al.* (28) that IL-7 downregulates both mRNA expression and protein production of TGF-β by murine macrophages. Thus, it seems unlikely that exogenous IL-7 added to our co-culture system would induce TGF-β production by stromal cells derived from a young stroma. Furthermore, Gazit *et al.* (29) have recently reported that fibroblast CFU (CFU-F) isolated from senescent mice produces less TGF-β *in vitro* than CFU-F from young mice and that the matrix of long bones of senescent mice contains less TGF-β than that of young mice. These data suggest that the production of the CFU-pre-B cell regulator TGF-β by stromal cells may decrease with age. Consequently, CFU-pre-B cells co-cultured with senescent stromal cells may proliferate and/or differentiate more rapidly than CFU-pre-B cells co-cultured with young stromal cells in the presence of IL-7.

In the present study, we observed that the CFU-pre-B cell number in the BM decreased with age, whereas, as we have observed previously (13), the total number of splenic B cells remained relatively unchanged. These findings are consistent with those observed in other murine strains by other researchers (3–6) and have been considered to be mediated by a decrease in B lymphocyte production in the BM and increased longevity of mature B cells (30). Furthermore, our data revealed an intrinsic defect in the B-progenitor-cell response to IL-7, as well as an age-related impaired production of not only IL-7 but also TGF-β by stromal cells. In SAMs, the arrest of pro-B cell maturation with advanced aging was evidently associated with the decrease in the number of pre-B cells. This may be explained by the coexistence of an intrinsic defect in the B-progenitor-cell response to IL-7 (i.e., pre-B cells and more immature pro-B cells); this interpretation is in good agreement with previous reports (6). Moreover, a decrease in IL-7 production by stromal cells during aging was confirmed (Fig. 2), which is evident in regular mice (17, 18).

The present study revealed that senescent B lymphopoiesis is suppressed, the background mechanism of which is unlikely different from mechanical B cell damage and its acute responses. Although B cell damage may be based on a positive circuit (i.e., an increase in IL-7 production associated with a decrease in TGF-β production) (28, 31, 32), our present data clearly show that TGF-β production is rather suppressed despite the prominent decrease in IL-7 production. Such homeostatic B lymphopoiesis balanced at the lower level may be a prominent characteristic of the basic mechanism of B lymphopoietic senescence, although the details of this mechanism are as yet unknown.

Another objective of our current study is to address the issue of using SAMs as an experimental mouse model for predicting the possible basic mechanism of senescence and B lymphopoiesis during aging. Aging is a physiological process and is likely controlled by a combination of many different factors. Whether the determinant of accelerated aging in SAMs is the same as that of normal aging in mice remains to be elucidated. However, the determinant factors for aging of an organism are, at present, poorly understood. Thus, different experimental approaches using animal models such as SAMs may provide an insight into such factors, because the study of abnormal systems has often led to the clarification of how a normal system functions.

Our present study, performed using SAMs and focusing on the quantity and quality of B lymphopoietic progenitor cells, suggests age-related alterations in lymphopoietic progenitor cells. Among them, the changes shown in Tables 1 and 2 and Figure 1 are essentially identical to those observed in regular mouse strains as previously reported. Namely, the decreased IL-7 responsiveness of BM cells from aged mice appears to be associated with both the decrease in the number of IL-7–responsive cells and the decrease in colony size and to correlate with findings in other strains (3–6). Furthermore, the number of secondary

colonies generated by the progeny of CFU-pre-B cells derived from large primary colonies was significantly smaller for the BM of senescent mice than for that of young mice (Table 2). Note that there seems to be an almost complete arrest in the production of secondary CFU-pre-B colonies from 36-week-old mice (Table 2), whereas comparable primary BM cells produced the same amount of GM-CFU (109%) from 12-week-old mice, and 65.0% of CFU-pre-B colonies was maintained (Table 1) in 12-week-old mice. The decrease in the number of secondary colonies was prominently observed in the most senescent age group (i.e., 36 weeks old) at the level of "cluster," because there seems to be a split between the time to senescence for spleen atrophy and that for substantial hemopoietic arrest in *in vitro* colonization. Stephan *et al.* (25) reported that a small percentage of BM pro-B cells from aged mice undergo cell cycle and that a large percentage of these cells enter G0/G1 after stimulation with IL-7, suggesting an impairment or delay in their ability to undergo cell division after IL-7 stimulation. The surrogate light chain is a component of the pre-B cell receptor, which is critical for Ig-variable heavy chain selection, cellular proliferation, and survival in the pre-B stage. Sherwood *et al.* (26) and Frasca *et al.* (33) reported that surrogate L chain mRNA and protein levels in IL-7–expanded B cell precursors decrease with age, which is associated with decreased protein levels of the E2A-encoded transcription factors, E47 and E12 (33). Based on these results, impairment in the IL-7 receptor function and its signal transduction in pro-B/pre-B cells may underlie the decrease in B cell production with age. It seems likely that the reduced generation of secondary colonies may be due in part to the deterioration of the proliferative capacity of B-cell progenitors from senescent mice in response to IL-7 rather than to the exclusive differentiation of B-cell progenitors to mature B cells in response to IL-7. In this regard, senescent changes observed in B lymphopoiesis in SAMs may be assumed to be identical to those reported in regular mice.

We thank Dr. Yukio Kodama, Ms. Erika Tachihara, Ms. Yoshiko Usami, and Ms. Yasuko Shinzawa for assistance in the maintenance of SAMs at the experimental animal facilities and Ms. Noriko Katsu and Ms. Chiaki Kimura for secretarial help in the preparation of the manuscript.

1. Hodes RJ. Aging and the immune system. Immunol Rev 160:5–8, 1997.
2. Ghia P, Melchers F, Rolink AG. Age-dependent changes in B lymphocyte development in man and mouse. Exp Gerontol 35:159–165, 2000.
3. Zharhary D. Age-related changes in the capability of the bone marrow to generate B cells. J Immunol 141:1863–1869, 1988.
4. Riley RL, Kruger MG, Elia J. B cell precursors are decreased in senescent BALB/c mice, but retain normal mitotic activity in vivo and in vitro. Clin Immunol Immunopathol 59:301–313, 1991.
5. Jonsson JI, Phillips RA. Interleukin-7 responsiveness of B220+ B cell precursors from bone marrow decreases in aging mice. Cell Immunol 147:267–278, 1993.
6. Stephan RP, Sanders VM, Witte PL. Stage-specific alterations in murine B lymphopoiesis with age. Int Immunol 8:509–518, 1996.
7. Klinman NR, Kline GH. The B-cell biology of aging. Immunol Rev 160:103–114, 1997.
8. Tang J, Nuccie BL, Ritterman I, Liesveld JL, Abboud CN, Ryan DH. TGF-beta down-regulates stromal IL-7 secretion and inhibits proliferation of human B cell precursors. J Immunol 159:117–125, 1997.
9. Takeda T, Hosokawa M, Takeshita S, Irino M, Higuchi K, Matsushita T, Tomita Y, Yasuhira K, Hamamoto H, Shimizu K, Ishii M, Yamamuro T. A new murine model of accelerated senescence. Mech Ageing Dev 17:183–194, 1981.
10. Izumi-Hisha H, Ito Y, Sugimoto K, Oshima H, Mori KJ. Age-related decrease in the number of hemopoietic stem cells and progenitors in senescence accelerated mice. Mech Ageing Dev 56:89–97, 1990.
11. Tsuboi I, Morimoto K, Horie T, Mori KJ. Age-related changes in various hemopoietic progenitor cells in senescence-accelerated (SAM-P) mice. Exp Hematol 19:874–877, 1991.
12. Sakuma A, Tsuboi I, Morimoto K, Horie T, Sugimoto K, Mori KJ. Age-related decreases in the reconstituting ability of hemopoietic cells and the ability of hemopoietic microenvironment to support hemopoietic reconstitution in senescence accelerated (SAM-P) mice. Mech Ageing Dev 73:127–135, 1994.
13. Saitoh T, Morimoto K, Kumagai T, Tsuboi I, Aikawa S, Horie T. Comparison of erythropoietic response to androgen in young and old senescence accelerated mice. Mech Ageing Dev 109:125–139, 1999.
14. Kumagai T, Morimoto K, Saitoh T, Tsuboi I, Aikawa S, Horie T. Age-related changes in myelopoietic response to lipopolysaccharide in senescence-accelerated (SAM) mice. Mech Ageing Dev 112:153–167, 2000.
15. Yamamoto H, Hatano M, Iitsuka Y, Mahyar NS, Yamamoto M, Tokuhisa T. Two forms of Hox11 a T cell leukemia oncogene, are expressed in fetal spleen but not in primary lymphocytes. Mol Immunol 32:1177–1182, 1995.
16. Lawrence DA, Pircher R, Jullien P. Conversion of a high molecular weight latent beta-TGF from chicken embryo fibroblasts into a low molecular weight active beta-TGF under acidic conditions. Biochem Biophys Res Commun 133:1026-10-34, 1985.
17. Updyke LW, Cocke KS, Wierda D. Age-related changes in production of interleukin-7 (IL-7) by murine long-term bone marrow cultures (LTBMC). Mech Ageing Dev 69:109–117, 1993.
18. Stephan RP, Reilly CR, Witte PL. Impaired ability of bone marrow stromal cells to support B-lymphopoiesis with age. Blood 91:75–88, 1998.
19. Okada S, Yoshida T, Hong Z, Ishii G, Hatano M, Kuro OM, Nabeshima Y, Tokuhisa T. Impairment of B lymphopoiesis in precocious aging (klotho) mice. Int Immunol 12:861-871, 2000.
20. Li F, Jin F, Freitas A, Szabo P, Weksler ME. Impaired regeneration of the peripheral B cell repertoire from bone marrow following lymphopenia in old mice. Eur J Immunol 31:500-505, 2001.
21. Nemunaitis J, Tompkins CK, Andrews DF, Singer JW. Transforming growth factor beta expression in human marrow stromal cells. Eur J Haematol 46:140–145, 1991.
22. Lee G, Namen AE, Gillis S, Ellingsworth LR, Kincade PW. Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-beta. J Immunol 142:3875–3883, 1989.
23. Kincade PW. B lymphopoiesis: global factors, local control. Proc Natl Acad Sci U S A 91:2888–2889, 1994.
24. Lebman DA, Edmiston JS. The role of TGF-beta in growth, differentiation, and maturation of B lymphocytes. Microbes Infect 1:1297–1304, 1999.
25. Stephan RP, Lill-Elghanian DA, Witte PL. Development of B cells in aged mice: decline in the ability of pro-B cells to respond to IL-7 but not to other growth factors. J Immunol 158:1598–1609, 1997.
26. Sherwood EM, Xu W, King AM, Blomberg BB, Riley RL. The

reduced expression of surrogate light chains in B cell precursors from senescent BALB/c mice is associated with decreased E2A proteins. Mech Ageing Dev 118:45–59, 2000.

27. von Freeden-Jeffry U, Vieira P, Lucian LA, McNeil T, Burdach SE, Murray R. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J Exp Med 181:1519–1526, 1995.
28. Dubinett SM, Huang M, Dhanani S, Wang J, Beroiza T. Down-regulation of macrophage transforming growth factor-beta messenger RNA expression by IL-7. J Immunol 151:6670–6680, 1993.
29. Gazit D, Zilberman Y, Ebner R, Kahn A. Bone loss (osteopenia) in old male mice results from diminished activity and availability of TGF-beta. J Cell Biochem 70:478–488, 1998.
30. Kline GH, Hayden TA, Klinman NR. B cell maintenance in aged mice reflects both increased B cell longevity and decreased B cell generation. J Immunol 162:3342–3349, 1999.
31. Miller AR, McBride WH, Dubinett SM, Dougherty GJ, Thacker JD, Shau H, Kohn DB, Moen RC, Walker MJ, Chiu R, Schuck BL, Rosenblatt JA, Huang M, Dhanani S, Rhoades K, Economou JS. Transduction of human melanoma cell lines with the human interleukin-7 gene using retroviral-mediated gene transfer: comparison of immunologic properties with interleukin-2. Blood 82:3686–3694, 1993.
32. Fry TJ, Mackall CL. Interleukin-7: from bench to clinic. Blood 99:3892–3904, 2002.
33. Frasca D, Nguyen D, Riley RL, Blomberg BB. Decreased E12 and/or E47 transcription factor activity in the bone marrow as well as in the spleen of aged mice. J Immunol 170:719–726, 2003.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Reproductive Toxicology 18 (2004) 109–120
Reproductive Toxicology
www.elsevier.com/locate/reprotox

Intrauterine position and postnatal growth in Sprague–Dawley rats and ICR mice

Tetsuji Nagao[a,*], Kazuyoshi Wada[b], Makiko Kuwagata[b], Madoka Nakagomi[a], Chiaki Watanabe[b], Shinsuke Yoshimura[b], Yoshiaki Saito[b], Kenji Usumi[b], Jun Kanno[c]

[a] *Department of Life Science, Faculty of Science and Technology, Kinki University, Kowakae 3-4-1, Higashiosaka, Osaka 577-8502, Japan*
[b] *Safety Testing Laboratory, Hatano Research Institute, Food and Drug Safety Center, Hadano, Kanagawa, Japan*
[c] *Division of Cellular and Molecular Toxicology, National Institute of Health Science, Tokyo, Japan*

Received 13 May 2002; received in revised form 10 September 2003; accepted 16 October 2003

Abstract

In rodents, steroid hormones are thought to be transported between adjacent fetuses, and male or female fetuses that develop in utero between female fetuses may have higher serum levels of estradiol, and lower serum levels of testosterone, relative to siblings of the same sex that develop between two male fetuses. The consequence in the variation of postnatal growth, development, and function in the intrauterine position, using various parameters such as anogenital distance, preputial separation and vaginal opening, estrous cycle, locomotor activity, and growth of reproductive organs, were examined in Sprague–Dawley rats. ICR mice were treated with 17β-estradiol before copulation and during pregnancy to address the interaction with endogenous estradiol during pregnancy. In rats, no evidence of effects of prior intrauterine position was observed for any of the parameters examined. Mouse fetal exposure via the mother to low-dose 17β-estradiol revealed no changes in the rate of postnatal growth in males and females that developed in any intrauterine position in utero. The results of this study suggested that the intrauterine position of the embryos/fetuses did not affect the postnatal growth of the reproductive organs, sexual maturation, or behavior in rats and mice.

Keywords: Intrauterine position; Postnatal growth; Sexual maturation; Behavior; Anogenital distance; Rats; Mice

1. Introduction

The development of sexually differentiated phenotypes depends upon the hormonal environment during a critical period of growth [1]. Testosterone secretion by the fetal testis causes a longer anogenital distance (AGD), seen in neonatal males, relative to females. The AGD of newborn rats, mice, and gerbils is longer in males than in females and varies as a function of the intrauterine position of the animals [1–4]. A longer AGD is associated with the presence of males on either side of the developing fetus in utero, and a shorter AGD is associated with the absence of males on either side of the developing female fetus. Females with a male fetus on only one side are immediate [4].

In all litter-bearing species that have been examined to date, the intrauterine position that a fetus occupies relative to fetuses of the same or opposite sex has profound effects on its reproductive, behavioral, and morphological traits measured during adult life [4–7]. Gerbil males and females that developed in utero between two female fetuses or two male fetuses, respectively, did not differ in relative hippocampal size [8].

The effects of intrauterine position are apparently not the result of the position itself, but rather of the movement of steroid hormones between the fetuses, and variations in the hormonal environment relative to the proximity of an individual fetus to other fetuses of the same or opposite sex [9]. Male rats located between two females had elevated serum estradiol and larger prostates than males located between two males, which had elevated serum testosterone and larger seminal vesicles [10]. The effect of intrauterine position in mice has been correlated with concentrations of steroid hormones in amniotic fluid and subsequent sexual activity [11,12].

Recently, intrauterine position has been the focus of discussions in the toxicology community because of its potential to alter the susceptibility of fetuses to endogenous hormones and endocrine disrupting chemicals [13,14]. In this regard, failure to account for intrauterine position in endocrine disrupting chemical toxicology studies could lead to false negative results, especially when adverse alterations

* Corresponding author. Tel.: +81-6-6721-2332; fax: +81-6-6723-2721.
E-mail address: tnagao@msa.kindai.ac.jp (T. Nagao).

0890-6238/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.reprotox.2003.10.009

are produced by low doses in fetuses from only one intrauterine position [14,15]. This possibility has been raised because of investigations into estrogenic compounds in mice. In rats, consistent effects due to intrauterine position on testosterone concentrations, and therefore potential interactions with endocrine disrupting chemicals, have not been found. Howdeshell and vom Saal [16] demonstrated that the greatest response to the estrogenic chemical, bisphenol A, occurred in males and females with the highest background levels of endogenous estradiol during fetal life, due to their intrauterine position, while fetuses with the lowest endogenous levels of estradiol showed no response to maternal bisphenol A within the range of human exposure, suggesting that estrogen-mimicking chemicals interact with endogenous estrogen in altering the course of development. It has been demonstrated that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin interacted with endogenous estradiol to disrupt prostate gland morphogenesis in male rat fetuses [17].

The objectives of this study were to determine the effects of intrauterine position, under normal physiological conditions, on the development of rat offspring, as well as sexual maturation, estrous cycle, behavior, and reproductive organ development. Another objective of this study was to determine whether the intrauterine position of mouse fetuses, which is related to background levels of estradiol and testosterone, would influence the response of the postnatal growth of gonads, including sexual maturation, to low dose 17β-estradiol.

2. Materials and methods

2.1. Animals

Sprague–Dawley rats (Crj:CD, IGS), and ICR mice (Crj:CD-1) were purchased from Charles River, Laboratories, Inc. (Atsugi, Japan). Twenty-seven male rats (9 weeks of age), 84 female rats (8 weeks of age), 130 male mice (9 weeks of age), and 130 female mice (8 weeks of age), were used. The rats and mice arrived with mean weights of 301.1 ± 7.9 g for males and 216.2 ± 8.1 g for females, and 37.2 ± 1.2 g for males and 29.1 ± 0.9 g for females (mean ± S.D.), respectively. The animals were acclimated to the laboratory for 7–14 days prior to the start of the experiments to evaluate weight gain and any gross signs of disease or injury. The animals were housed individually in stainless steel, wire-mesh cages in a room with controlled temperature (22–25 °C) and humidity (50–65%), with lights on from 07:00 to 19:00 h daily. The animals were given access to food (NIH-07-PLD: phytoestrogen low diet, Oriental Yeast Co., Japan) and tap water through metal pipes (distilled water, Wako Pure Chem., Japan) ad libitum. In a few instances, the temperature and humidity were outside the standard ranges, but the magnitude and duration of these incidents were minimal and judged to be of no consequence. The contents of genistein and daidzein in the diet and wood bedding (ALPHA-dri, Shepherd Specialty Paper, USA) used in the present study were determined. Neither genistein nor daidzein were not detected in the diet or wood bedding (detection limit: 0.5 mg/100 g in each individual phytoestrogen, by HPLC).

Animal care and use conformed to published guidelines [18].

2.2. Experiment I (examination of intrauterine position effect on postnatal growth in rats)

2.2.1. Cesarean delivery and fostering

Estrous female rats at 10–11 weeks of age were cohabited overnight with a single male to obtain 66 pregnant females within 4 days. The next morning, females with sperm in their vaginal smears were regarded as pregnant, and this day was designated as day 0 of gestation. Thirty-three pregnant females were killed by CO_2 asphyxiation and cervical dislocation, and subjected to cesarean sectioning on day 21 of gestation. The fetuses were rapidly collected, and their intrauterine position was recorded, identified by tattoo, weighed, and sexed. Anogenital distance (AGD) was measured with a digital micrometer (reproductive precision of 0.01 mm, Digimatic caliper CD-15C, Mitutoyo Co., Kanagawa, Japan) under an Olympus dissecting microscope for each fetus, and the average was taken. The subject was held steady and in the same position during measurement. Measurements were made without knowledge of intrauterine position by one person. The AGD was measured from the center of the phallus to the center of the anus. The fetuses obtained by cesarean delivery were fostered to 33 dams that had just given birth naturally (one litter to each female). The original littermates remained together when cross-fostered. The litter sizes were similar for each cross-fostered dam. The day of cesarean section was considered as postnatal day (PND) 0. Pup body weights were recorded on PND 21 (day of weaning). Following weaning, and until 10 weeks of age, offspring were weighed once a week.

Neonates from 33 pregnant females were categorized as occupying six different intrauterine positions: 2M (male fetus located between two male fetuses; number of pups and litters on PND 0 = 36 and 19); 1M (male fetus that located between a male fetus and a female fetus; $n = 73$ and 27); 0M (male fetus located between two female fetuses; $n = 45$ and 24); 2F (female fetus located between two female fetuses; $n = 38$ and 18); 1F (female fetus located between a female fetus and a male fetus; $n = 83$ and 29); 0F (female fetus located between two male fetuses; $n = 41$ and 27). Fetuses adjacent to dead embryos (resorptions or macerated fetuses), and fetuses that were closest to each ovary or the cervix, were discarded from further analyses.

2.2.2. Observations of postnatal growth

2.2.2.1. Measurement of AGD and reproductive organ weights, and evaluation of sexual maturation. On PND

4, the AGD was measured for pups in each group using calipers with a reproductive precision of 0.01 mm. On PND 21, all pups were weaned and half of the pups in each group (2M = 13, 1M = 37, 0M = 11, 2F = 14, 1F = 43, 0F = 12) were subjected to necropsy, and the testes, epididymides, and prostates with seminal vesicles (fluid was not removed and all lobes were included) in males, and uteri and ovaries in females, were weighed. For the remaining male and female pups in each litter (2M = 21, 1M = 32, 0M = 30, 2F = 23, 1F = 36, 0F = 26), as criteria for sexual maturation, the day of vaginal opening for females (beginning on PND 28), and preputial separation for males (beginning on PND 35), were assessed, and each rat was weighed when these criteria were achieved.

2.2.2.2. Postweaning tests of behavior, evaluation of estrous cycle, and histological observation of reproductive organs. One male and one female were randomly selected from each litter in each group (number of rats examined: 2M = 18; 1M = 27; 0M = 25; 2F = 17; 1F = 27; 0F = 25), and were subjected to an open field test and wheel cage activity test to assess the emotionality and regulatory running activity, respectively. At 4 weeks of age, the rats were placed into a circular area (140 cm in diameter) surrounded by a wall (40 cm in height). The light and noise levels averaged 500 lx and 50 dB, respectively, at the center of the circular area. Rearing, grooming, defecation, and urination were counted, and ambulation was recorded automatically on a computer (Unicom, Inc., Japan), during a 3-min trial between 13:00 and 16:00 h on one day. At 7 weeks of age, the rats were placed into a wheel cage (Nippon Cage, Inc., Japan), 32 cm in diameter and 10 cm in width, as a measure of spontaneous activity. Each rat was kept within the wheel for 24 h with free access to food (NIH-07-PLD) and distilled water in the same animal room. The number of revolutions was automatically recorded with a 20-channel digital counter (Seiko Denki, Inc., Japan).

Each morning (9:00–10:00 h), from 6 to 10 weeks of age, all females in each group were subjected to vaginal lavage. The lavage fluid was applied to a glass slide, air-dried, and stained with Wright–Giemsa stain. Cytology was evaluated and the stage of the estrous cycle was determined using the method of Everett [19].

At 10 weeks of age, 3–5 males in each group were weighed, and anesthetized. Transcardial perfusions were carried out with a mixture of 0.1 M phosphate-buffered 1.25% glutaraldehyde and 2% paraformaldehyde. Following fixation, the prostate gland was sampled, rinsed three times in phosphate buffer, postfixed for 2 h at 4 °C in 2% osmium tetroxide, and dehydrated in alcohol; the prostate gland was embedded in epoxy resin. Ultrathin sections of the prostates were stained with uranyl acetate and lead citrate, and observed with an electron microscope (H-7100, Hitachi, Japan). The remaining males in each group (2M = 18, 1M = 27, 0M = 25) were weighed and subjected to necropsy, and the testes, epididymides, ventral prostate, and dorsal prostates with seminal vesicles, were weighed and fixed in 0.1 M phosphate-buffered 10% formalin solution. All females (2F = 17, 1F = 27, 0F = 25) were weighed and subjected to necropsy when the stage of the estrous cycle was diestrus. The ovaries and uteri were then weighed and fixed in 0.1 M phosphate-buffered 10% formalin solution. These reproductive organs were embedded in paraffin, and tissue sections were stained with H&E for light microscopy.

2.3. Experiment II (examination of low-dose in utero effects of 17β-estradiol in mice)

The objective of this experiment was to determine whether the intrauterine position of male fetuses, which is related to background levels of estradiol (elevated in males located between two female fetuses) and testosterone (elevated in males located between two male fetuses), would influence the response of the developing prostate to low dose 17β-estradiol. In addition, we examined whether the intrauterine position of male and female fetuses would affect the postnatal growth of other reproductive organs and sexual maturation.

2.3.1. Administration, cesarean delivery and fostering

Thirty female mice at 9 weeks of age were administered 17β-estradiol (Sigma Chem. Co., MO, USA) subcutaneously at a dose of 0.05 μg/kg per day for 7 days before mating, during a mating period of 7 days at the longest, and on day 0 through 17 of gestation. In a preliminary study, the offspring of the ICR pregnant females exposed to 17β-estradiol at 0.05 μg/kg per day on day 0 through 17 of gestation showed no changes in weight and histological morphology of reproductive organs in adulthood. However, the offspring of dams exposed to 17β-estradiol at 0.1 μg/kg per day on these gestational days showed changes in the parameters in adulthood (data not shown). In the present study, 30 control females were administered corn oil (Nacalai Tesque, Co., Tokyo). After the administration for 7 days before mating, female mice were caged with untreated males overnight and examined for a vaginal plug the next morning. The day on which a plug was found was termed day 0 of gestation. In this study, 30 female mice in the 17β-estradiol exposed group and the control group copulated and became pregnant. On day 18 of gestation, pregnant females were killed by CO_2 asphyxiation, and subjected to cesarean sectioning. The fetuses were rapidly collected, and their intrauterine position was recorded, identified by tattoo, weighed, and sexed, and then the AGD was measured. The fetuses obtained by cesarean delivery were fostered to 60 dams that had just given birth naturally (one litter to each female). The day of cesarean section was considered as PND 0. Pup body weights were recorded on PND 21 (day of weaning), and at 5, 7, and 10 weeks of age.

Neonates from 30 pregnant females exposed to corn oil and 30 pregnant females exposed to 17β-estradiol were categorized as occupying four different intrauterine positions:

2M (the number of neonates in the 17β-estradiol exposed group and the control group: 38 and 41) and 0M (33 and 32), and 2F (41 and 32) and 0F (28 and 37). Fetuses adjacent to dead embryos, and fetuses that were closest to each ovary or the cervix, were discarded. In this experiment, fetuses of two intrauterine positions, 1M and 1F, were also discarded.

2.3.2. Observations of postnatal growth

2.3.2.1. Evaluation of sexual maturation. On PND 21, all male and female pups (2M, 0M, 2F, 0F) in each litter were weaned. For all male and female mice in each litter, as criteria for sexual maturation, the day of vaginal opening for females (beginning on PND 25), and preputial separation for males (beginning on PND 30), were assessed, and each pup was weighed when these criteria were achieved.

At 10 weeks of age, five males in each group were weighed and processed to the transcardial perfusion to observe the histological alteration of the prostate by electron microscope. The remaining males in each group were weighed and subjected to necropsy, and the testes, epididymides and seminal vesicles, were weighed. All females were weighed and subjected to necropsy. The ovaries were then weighed. These reproductive organs including prostates and uteri were fixed in 0.1 M phosphate-buffered 10% formalin solution and embedded in paraffin, and tissue sections were stained with H&E for light microscopy.

2.4. Data analyses

Statistical analysis of the data for the offspring (AGD, body weight and organ weight, organ/body weight ratios, timing of vaginal opening and preputial separation) was performed using the litter as the unit [20,21]. The AGD, body weight and organ weight, organ/body weight ratios (relative organ weight), timing of vaginal opening and preputial separation, were analyzed using Bartlett's test. When homogeneity of variance was confirmed, one-way analysis of variance was applied to detect the significances among the groups. If a significant difference was detected among the groups, Dunnett's test was applied for multiple comparisons. When variance was not homogeneous, or there was a group whose variance was zero, Kruskal–Wallis analysis of ranks was applied. If a significant effect was detected among the groups, Dunnett's test was applied for multiple comparisons. Comparisons between groups were made using $P \leq 0.05$ as the level of significance.

3. Results

3.1. Experiment 1

3.1.1. AGD and body weights of fetuses at cesarean section and pups at PND 4

Table 1 shows the AGD, body weight, AGD/body weight (AGDI: anogenital distance index), and AGD/$\sqrt[3]{\text{body weight}}$ of fetuses at various intrauterine positions and pups at PND 4. It is reasonable to anticipate that the AGD might vary with body weight of fetus or pup. It has been proposed that the relationship between AGD and body weight should be more properly evaluated using the cube root of the body weight [22–25]. If it is desirable to normalize AGD to body weight, the AGD/$\sqrt[3]{\text{body weight}}$ seems to provide a more appropriate adjustment.

There were no statistically significant differences in any parameter evaluated at cesarean section (PND 0) or PND 4

Table 1
Effects of prior intrauterine position on anogenital distance in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
AGD of fetuses at cesarean section						
No. of litters	19	27	24	18	29	27
No. of pups	36	73	43	38	83	41
Body weight (g)	5.6 ± 0.4[a]	5.6 ± 0.3	5.7 ± 0.4	5.2 ± 0.3	5.4 ± 0.3	5.3 ± 0.4
AGD	2.43 ± 0.22	2.42 ± 0.22	2.42 ± 0.28	1.21 ± 0.20	1.23 ± 0.19	1.22 ± 0.24
AGD/body weight	0.43 ± 0.04	0.42 ± 0.04	0.42 ± 0.05	0.23 ± 0.02	0.22 ± 0.02	0.23 ± 0.02
AGD/$\sqrt[3]{\text{body weight}}$	1.36 ± 0.12	1.36 ± 0.14	1.35 ± 0.18	0.69 ± 0.08	0.70 ± 0.07	0.70 ± 0.08
AGD of pups on PND 4						
No. of litters	19	27	24	18	29	27
No. of pups	34	69	41	37	79	38
Body weight (g)	10.9 ± 1.5	11.2 ± 1.5	10.8 ± 1.1	10.4 ± 1.4	10.3 ± 1.1	10.4 ± 1.3
AGD	4.57 ± 0.54	4.41 ± 0.48	4.43 ± 0.51	2.00 ± 0.22	1.99 ± 0.19	2.00 ± 0.21
AGD/body weight	0.42 ± 0.06	0.40 ± 0.05	0.41 ± 0.03	0.19 ± 0.04	0.19 ± 0.03	0.19 ± 0.04
AGD/$\sqrt[3]{\text{body weight}}$	2.06 ± 0.22	1.99 ± 0.19	1.99 ± 0.19	0.92 ± 0.12	0.91 ± 0.11	0.92 ± 0.13

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.

Table 2
Effects of prior intrauterine position on reproductive organs before maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Organ weight on PND 21						
No. of litters	18	27	24	17	27	25
No. of offspring	13	37	11	14	43	12
Body weight (g)	40.9 ± 6.3[a]	40.5 ± 6.5	40.2 ± 9.6	38.8 ± 6.3	38.2 ± 7.0	40.7 ± 7.0
Testes (mg)[b]	169.3 ± 27.5	172.2 ± 22.2	164.9 ± 26.1			
Testes[c]	416.3 ± 48.4	429.2 ± 40.3	418.2 ± 46.7			
Epididymides (mg)[b]	23.3 ± 3.1	23.5 ± 4.8	21.9 ± 4.4			
Epididymides[c]	60.5 ± 10.8	58.2 ± 7.8	55.2 ± 7.0			
Prostate + SV (mg)[b,d]	47.2 ± 9.9	46.7 ± 10.3	45.9 ± 7.9			
Prostate + SV[c,d]	115.6 ± 18.0	115.7 ± 19.0	117.2 ± 19.5			
Ovaries (mg)[b]				24.3 ± 4.0	22.9 ± 3.9	24.8 ± 3.6
Ovaries[c]				63.5 ± 10.1	60.8 ± 9.5	61.5 ± 7.3
Uterus (mg)[b]				10.2 ± 2.0	11.2 ± 3.7	11.8 ± 2.9
Uterus[c]				26.4 ± 4.5	28.8 ± 6.8	29.1 ± 6.0

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.
[b] Absolute weight.
[c] Relative weight (g or mg per 100 g body weight).
[d] Seminal vesicle.

between groups 2M, 1M and 0M in males, or groups 2F, 1F and 0F in females.

No significant differences in viability of fetuses at cesarean section (PND 0), or that from PND 0 to PND 4 (the number of pups died; 2M = 2, 1M = 4, 0M = 2, 2F = 1, 1F = 4, 0F = 3), were detected between the groups. In addition, there were no statistically significant differences in body weight at PND 0 and 4.

3.1.2. *Body weight and reproductive organ weight of offspring at PND 21*

The absolute and relative weights of testes, epididymides, and prostates with seminal vesicles in males, and ovaries and uteri in females, as well as body weight of offspring at PND 21 are shown in Table 2. Irrespective of the intrauterine position, no significant differences were detected between the groups in absolute or relative reproductive organ weights, or body weights of male and female weanlings, suggesting that the intrauterine position did not affect postnatal growth before weaning in rats.

3.1.3. *Sexual maturation and estrous cycle of offspring*

Table 3 shows the days of preputial separation in males, and of vaginal opening in females. There were no significant differences in these endpoints of sexual maturation or body weight at which these criteria were achieved between the groups. The estrous cycle of female offspring from 6 to 10 weeks of age is shown in Table 4. No significant differences were detected between the groups in mean estrous cycle length, or the frequency of females showing each stage of estrous cycle.

Table 3
Effects of prior intrauterine position on sexual maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
No. of litters	18	27	24	17	27	25
No. of offspring	21	32	30	23	36	26
Day of preputial separation	43.3 ± 1.3[a]	43.4 ± 1.2	44.0 ± 1.8			
Body weight (g)[b]	211.8 ± 5.5	212.1 ± 4.3	212.9 ± 5.2			
Day of vaginal opening				33.8 ± 2.2	33.8 ± 1.8	34.1 ± 1.7
Body weight (g)[b]				125.6 ± 4.1	124.6 ± 4.4	126.1 ± 3.9

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.
[b] Body weight when the criterion was achieved.

Table 4
Effects of prior intrauterine position on estrous cycle in Sprague–Dawley rats

	Group		
	2F	1F	0F
No. of litters	17	27	25
No. of female offspring	23	36	26
Mean estrous cycle length (day)	4.16 ± 0.29[a]	4.08 ± 0.30	4.20 ± 0.42
No. of females showing Regular cycle (%)	18 (78.3)	28 (77.8)	21 (80.8)
No. of females showing Irregular cycle (%)	5 (21.7)	8 (22.2)	5 (19.2)

2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses. No significant differences were observed between groups.
[a] Mean ± S.D.

3.1.4. Behavior and locomotor activity of offspring

Table 5 shows the results of an open field test at 4 weeks of age, and spontaneous activity within the wheel for 24 h at 7 weeks of age, for male and female offspring. There were no significant differences between groups 2M, 1M and 0M in latency, ambulation, rearing, grooming, defecation and urination, or number of revolutions for 24 h in a wheel cage. In the females, urination in group 0F was significantly increased as compared with that in group 2F, whereas other behavioral parameters, including the number of revolutions in a wheel cage were comparable between groups 2F, 1F and 0F.

3.1.5. Weights and histology of reproductive organs of offspring in adulthood

Table 6 shows the terminal body weights and reproductive organ weights of male and female offspring at 10 weeks of age. No significant differences were observed in the body weights, or the absolute and relative organ weights, between the groups. In the histological observation of the prostates by electron microscope, and reproductive organs of males and females by light microscope, no changes were observed in any of the reproductive organs, including the prostates, of the offspring.

3.2. Experiment II

3.2.1. AGD and body weights of fetuses at cesarean section

Table 7 shows the body weight, AGD, AGD/body weight, and AGD/$\sqrt[3]{\text{body weight}}$, of embryonic day 18 (PND 0) fetuses exposed to corn oil or 17β-estradiol. There were no significant differences in any of the parameters between the groups. No significant differences in viability of fetuses at cesarean section, or that from PND 0 to PND 21 were detected between the groups (the number of pups died from PND 0 to PND 21: see Tables 7 and 8).

3.2.2. Sexual maturation of offspring

Table 8 shows the days of preputial separation in males, and of vaginal opening in females. There were no significant differences in these endpoints of sexual maturation or body weight at which these criteria were achieved between the groups.

Table 5
Effects of prior intrauterine position on postnatal behavior in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Open field						
No. of litters	18	27	24	17	27	25
No. of offspring	18	27	25	17	27	25
Latency (s)	20.4 ± 40.8[a]	17.9 ± 16.9	15.3 ± 16.2	12.0 ± 9.4	13.8 ± 12.3	16.9 ± 36.1
Ambulation (cm)	676.3 ± 411.3	627.1 ± 417.2	659.0 ± 501.9	940.6 ± 538.1	1039.8 ± 436.3	970.7 ± 449.8
No. of rearing	2.3 ± 3.1	3.0 ± 3.2	1.5 ± 1.4	3.5 ± 2.1	4.5 ± 3.4	3.8 ± 2.3
No. of grooming	0.6 ± 0.9	0.7 ± 0.7	1.1 ± 1.2	0.8 ± 0.9	0.4 ± 0.5	0.8 ± 0.8
No. of defecation	2.8 ± 1.9	2.1 ± 1.5	3.3 ± 2.2	1.9 ± 1.9	1.7 ± 1.8	1.8 ± 1.9
No. of urination	0.4 ± 0.6	0.4 ± 0.6	0.5 ± 0.5	0.2 ± 0.4	0.5 ± 0.5	0.7 ± 0.6**
Spontaneous activity						
Count/24 h	1547 ± 467	1789 ± 697	1559 ± 638	4107 ± 1140	4429 ± 1501	4746 ± 1831

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.
**Significantly different from group 2F, $P < 0.01$ (by multiple comparison and Student t-test).
[a] Mean ± S.D.

Table 6
Effects of prior intrauterine position on reproductive organs after maturation in Sprague–Dawley rats

	Group					
	2M	1M	0M	2F	1F	0F
Organ weight at 10 weeks old						
No. of litters	18	27	24	17	27	25
No. of offspring	18	27	25	17	27	25
Body weight (g)	417.2 ± 31.6[a]	416.1 ± 34.4	413.6 ± 36.9	270.0 ± 23.2	271.8 ± 28.9	273.6 ± 29.1
Testes (mg)[b]	3.00 ± 0.20	2.98 ± 0.15	3.00 ± 0.17			–
Testes[c]	0.72 ± 0.05	0.72 ± 0.06	0.73 ± 0.07			–
Epididymides (mg)[b]	0.77 ± 0.05	0.78 ± 0.07	0.76 ± 0.06			–
Epididymides[c]	0.19 ± 0.01	0.19 ± 0.02	0.18 ± 0.02			–
Ventral prostate (g)[b]	0.46 ± 0.08	0.44 ± 0.08	0.43 ± 0.10			–
Ventral prostate[c]	0.11 ± 0.02	0.11 ± 0.02	0.11 ± 0.03			–
Dorsal prostate (g) + SV[b,d]	1.53 ± 0.28	1.56 ± 0.24	1.52 ± 0.27			–
Dorsal prostate + SV[c,d]	0.37 ± 0.07	0.38 ± 0.05	0.37 ± 0.07			–
Ovaries (mg)[b]				92.6 ± 13.3	91.8 ± 13.7	95.4 ± 16.9
Ovaries[c]				34.3 ± 3.6	33.8 ± 3.4	35.0 ± 5.8
Uterus (g)[b]				0.36 ± 0.06	0.38 ± 0.06	0.38 ± 0.05
Uterus[c]				0.13 ± 0.02	0.14 ± 0.03	0.14 ± 0.02

2M, male fetus between two male fetuses; 1M, male fetus between a male fetus and a female fetus; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 1F, female fetus between a male fetus and a female fetus; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.
[b] Absolute weight.
[c] Relative weight (g or mg per 100 g body weight).
[d] Seminal vesicle.

Table 7
Effects of prior intrauterine position on anogenital distance in ICR mice exposed to 17β-estradiol

Treatment and intrauterine position	Corn oil				17β-Estradiol			
	2M	0M	2F	0F	2M	0M	2F	0F
No. of litters	28	30	29	27	24	28	30	27
No. of pups	41	32	32	37	38	33	41	28
Body weight (g)	1.41 ± 0.08[a]	1.42 ± 0.05	1.32 ± 0.05	1.33 ± 0.04	1.42 ± 0.09	1.41 ± 0.10	1.32 ± 0.07	1.30 ± 0.11
AGD	1.92 ± 0.07	1.90 ± 0.06	0.95 ± 0.02	0.95 ± 0.03	1.92 ± 0.08	1.93 ± 0.06	0.93 ± 0.09	0.95 ± 0.05
AGD/body weight	1.36 ± 0.09	1.35 ± 0.10	0.75 ± 0.03	0.73 ± 0.05	1.40 ± 0.09	1.38 ± 0.10	0.75 ± 0.05	0.74 ± 0.07
AGD/$\sqrt[3]{\text{body weight}}$	1.71 ± 0.07	1.70 ± 0.10	0.88 ± 0.03	0.89 ± 0.05	1.70 ± 0.09	1.72 ± 0.11	0.89 ± 0.07	0.88 ± 0.08

2M, male fetus between two male fetuses; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.

Table 8
Effects of prior intrauterine position on sexual maturation in ICR mice exposed to 17β-estradiol

Treatment and intrauterine position	Corn oil				17β-Estradiol			
	2M	0M	2F	0F	2M	0M	2F	0F
No. of litters	28	30	29	27	24	28	30	27
No. of pups	39	30	31	35	37	31	39	28
Day of preputial separation	27.2 ± 1.5[a]	27.3 ± 1.3			27.0 ± 1.8	26.9 ± 2.0		
Body weight (g)	30.3 ± 1.9	31.1 ± 1.5			30.0 ± 2.1	31.3 ± 1.8		
Day of vaginal opening			24.5 ± 1.6	25.1 ± 1.5			24.4 ± 1.7	24.9 ± 1.6
Body weight (g)			21.5 ± 0.9	21.6 ± 1.2			21.6 ± 1.1	22.0 ± 1.5

2M, male fetus between two male fetuses; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 0F, female fetus between two male fetuses.
No significant differences were observed between groups.
[a] Mean ± S.D.

Table 9
Effects of prior intrauterine position on reproductive organs after maturation in ICR mice exposed to 17β-estradiol

Treatment and intrauterine position	Corn oil				17β-Estradiol			
	2M	0M	2F	0F	2M	0M	2F	0F
No. of litters	28	30	29	27	24	28	30	27
No. of pups	34	25	26	30	32	26	34	23
Terminal body weight (g)	51.5 ± 4.2[a]	53.6 ± 4.4	40.3 ± 2.7	41.2 ± 3.4	55.1 ± 5.1	53.1 ± 6.2	41.8 ± 2.1	42.1 ± 3.1
Testes (g)	257.9 ± 28.4[b]	266.5 ± 23.5			259.3 ± 25.5	260.3 ± 19.9		
	486.3 ± 76.3[c]	484.2 ± 71.3			488.3 ± 62.3	479.1 ± 60.9		
Epididymides (mg)	89.5 ± 8.7	92.2 ± 9.3			94.6 ± 7.1	93.1 ± 7.1		
	175.2 ± 21.5	161.9 ± 16.9			161.2 ± 13.6	173.6 ± 4.9		
Seminal vesicle (mg)	413.9 ± 30.6	452.2 ± 13.9			431.3 ± 18.2	454.8 ± 21.0		
	812.8 ± 56.9	802.6 ± 44.2			811.5 ± 42.3	809.3 ± 33.5		
Ovary (mg)			15.3 ± 3.3	14.6 ± 4.2			15.5 ± 3.9	15.3 ± 4.5
			35.6 ± 7.5	33.2 ± 4.6			34.1 ± 6.9	33.6 ± 5.1

2M, male fetus between two male fetuses; 0M, male fetus between two female fetuses; 2F, female fetus between two female fetuses; 0F, female fetus between two male fetuses.
Five males in each group were processed to the transcardial perfusion. Male pups shown here were subjected to necropsy.
No significant differences were observed between groups.
[a] Mean ± S.D.
[b] Absolute weight.
[c] Relative weight (mg per 100 g body weight).

3.2.3. *Weights and histology of reproductive organs of offspring in adulthood*

Table 9 shows the terminal body weights and reproductive organ weights of male and female offspring at 10 weeks of age. No significant differences were observed in the body weights, or the absolute and relative organ weights, between the groups. In the histological observation of the prostates by electron microscope, and reproductive organs of males and females by light microscope, no changes were observed.

4. Discussion

4.1. *Anogenital distance*

The AGD of newborn rats and mice is longer in males than in females, and it has been demonstrated that the AGD varies as a function of the intrauterine position of the animals [1–4]. The AGD is commonly regarded as a hormonally sensitive developmental measure in rodents [26], and it has been reported that a longer AGD is associated with the presence of males on either side of the developing fetus in utero, and a shorter AGD is associated with the absence of males on either side of the developing female fetus [27,28]. Evidence supports the hypothesis that exposure to testosterone and estrogen in utero are critical components of the intrauterine position effect [29]. Female mouse fetuses located between two males have significantly higher serum testosterone levels and lower estradiol levels than their sisters that were located between two females. Male mice located between two females have significantly higher levels of estradiol and lower levels of testosterone than males located between two males [4,12]. The mechanism for these intrauterine position effects can be traced to amniotic fluid transport between adjacent fetuses in uterus [30,31]. However, our data were not consistent with previous reports showing a significant effect of intrauterine position on AGD in rats and mice [2,32–34].

A failure to replicate the effects of intrauterine position on AGD may have potentially arisen for a number of methodological reasons. A set of potential problems revolves around possible errors in the measurement of the AGD. One possibility was that our calipers were not accurate enough to detect small mean differences between females located in various positions in the uterus, found by other investigators [2,32–34]. However, as the calipers could be read to an accuracy of 0.01 mm, they were clearly accurate enough to detect differences of this magnitude. Another possibility is that of human error. Given the short distances being measured, it was absolutely essential that all fetuses or pups be oriented in exactly the same fashion, as even a slight arching of the animal's back could significantly distort the AGD measurements. Two attempts were made to minimize these sorts of errors: (i) efforts were made to orient all fetuses or pups in exactly the same fashion when measuring, and (ii) two independent measurements were taken for each fetus or pup and averaged to obtain the value used. In most cases, the different measurements were highly similar for the same animal.

Simon and Cologer-Clifford [35] reported an absence of an intrauterine position effect on AGD in CF-1 mice. Their finding is only the second study to examine AGD in CF-1 mice, and the original report was more than 10 years old [2]. Therefore, it is possible that either genetic drift, or differences in the source of the CF-1 breeding stock, may

underlie the discrepant findings. In this context, Jubilan and Nyby [6] also found no effect of intrauterine position on the AGD/body weight (AGDI) in CF-1 offspring, using stock from the same supplier employed by the Simon and Cologer-Clifford [35] report.

4.2. Sexual maturation and estrous cycle

Since prenatal exposure of females to testosterone delays vaginal opening [36,37], it was predicted that females situated proximate to males in utero would display vaginal opening later than females not proximate to males during gestation. However, in the present study in rats and mice, there were no significant differences in days of vaginal opening or preputial separation between the groups (see Tables 3 and 8), suggesting that intrauterine position did not influence the sexual maturation in males and females. vom Saal [4] reported that 2F and 0F mice did not differ significantly in the age at vaginal opening, although 2F tended to exhibit vaginal opening at a slightly younger age than 0F (see categorization of the different intrauterine positions shown in Section 2).

Female mouse fetuses occupying an intrauterine position between male fetuses exhibit longer estrous cycles in adulthood than females formerly residing in utero next to other female fetuses [11,27]. Prior intrauterine position is therefore a source of individual variation in the production of, and sensitivity to, cues that modulate the timing of puberty and the length of subsequent estrous cycles in female mice, suggesting that prenatally androgenized females occupying an intrauterine position between male fetuses may have a reproductive advantage over other females at high population densities [4]. In the present study of rats, however, 0F and 2F did not differ significantly in the estrous cycle length, although the estrous cycle length of 2F (4.16 ± 0.29) tended to be shorter than that of 0F (4.20 ± 0.42). Prior studies have shown that, in the absence of males, vaginal estrus does not correlate with ovulation in peripubertal CF-1 female mice [38,39]. Further studies in which ovulation is confirmed by the presence of corpora lutea and tubal ova is thus required.

4.3. Behavior

Kinsley et al. [40] demonstrated that female mice located in utero between two female fetuses exhibited higher levels of regulatory running activity (locomotor activity) in adulthood than females located between two male fetuses. Male mice, which were less active than females, were also influenced by intrauterine contiguity, indicating that intrauterine position influences the behaviors involved in the maintenance of metabolic homeostasis. Previous work has shown that female rats and mice display higher levels of regulatory running activity than males, and that perinatal testosterone is responsible for this sex difference [41–43]. The present study also showed female rats displayed higher levels of running activity than males.

In the present study of rats, however, there were no significant differences in spontaneous activity in the wheel cage, or in ambulation in the circular area, as well as the frequency of rearing, grooming and defecation between the groups in both sexes, suggesting no intrauterine position effects on locomotor activity in rats. Interestingly, the frequency of urination for females that developed in utero between male fetuses was significantly increased, more than in females that developed in utero between female fetuses (see Table 5). Females that were located between female fetuses in utero were found to urine mark at higher rates than females that were located between male fetuses, in adulthood in CF-1 mice [2]. The frequency of urination in the circular area, observed in the present study, would relate to the emotionality of the animals when placed in a novel environment, and differ from urine marking. Female urine marking may play an important role in communication between female mice, as well as in inter-sexual communication. It has been suggested that in natural populations of mice, females urine mark to advertise their dominant breeding status to other females; urine-marking appears to be dependent on female social/reproductive status [44]. Taken together, these observations suggest that the intrauterine position did not affect behavior as evaluated by the open field test and the wheel cage.

4.4. Prostate development

Growth and differentiation of the prostate is primarily under the control of androgen. Expression of the androgen metabolizing enzyme, 5α-reductase, within prostatic mesenchyme cells is also necessary for normal development of the prostate [45]. The possibility that estrogen might be involved in modulating the effects of androgen on prostatic development during early life has been the subject of speculation for over 60 years [46–49]. Timms et al. [50] demonstrated that development of the urogenital system in male and female rat fetuses is influenced by their intrauterine proximity to fetuses of the same or opposite sex, and suggested that exposure to supplemental estradiol (due to being positioned between two female fetuses) induces prostatic bud development in females, and enhances the growth of prostatic buds in both males and females. An enlarged prostate in males located between two female fetuses was hypothesized to be mediated by an elevated level of serum estradiol, relative to males located between two males, due to the transport of estradiol from adjacent female fetuses [4,30]. This hypothesis was confirmed in a study in which estradiol was experimentally elevated by 50% in male mouse fetuses (via maternal administration), and the estrogen-treated males showed both a significant increase in prostatic glandular buds and significantly larger buds during fetal life, as well as enlarged prostates in adulthood [46].

In the present study, however, the weights of the prostates (with seminal vesicles) of the rats at PND 21, and the ventral and dorsal prostates (with seminal vesicles) of the rats at 10

weeks of age, were not significantly different between the groups. In addition, morphological observation of prostates in the rats and mice, in weanlings or adulthood, by light and electron microscope revealed no alteration in males located in any uterine position.

4.5. Developmental exposure to 17β-estradiol: interaction with endogenous estradiol during pregnancy in mice

In the present study we examined the effect of 17β-estradiol administration to pregnant mice on the early development of the prostate in male mouse fetuses, with attention being paid to the intrauterine position of the males. Timms et al. [17] reported that exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) significantly reduced serum estradiol in males located between two females, but not males located between two males, and also significantly interfered with initial budding and subsequent growth of the prostate in males located between two females or two males. In sharp contrast, the seminal vesicles were larger in the control males located between two males than in control males located between two females, similar to prior findings in mice [29], and TCDD only decreased the size of the seminal vesicles in males located between two males. Taken together, the findings of Timms et al. [17] demonstrate that in utero exposure to TCDD disrupts the development of the prostate, but this disruption depends on an interaction with background levels of estradiol. Howdeshell and vom Saal [16] reported that fetal mouse exposure via the mother to an estrogen-mimicking chemical, bisphenol A, increased the rate of postnatal growth in males and females, and also advanced the timing of puberty in females. They also demonstrated that the greatest response to bisphenol A occurred in males and females with the highest background levels of endogenous estradiol during fetal life, due to their intrauterine position, while fetuses with the lowest endogenous levels of estradiol showed no response to maternal bisphenol A treatment, suggesting that estrogen-mimicking chemicals interact with endogenous estrogen in altering the course of development.

In the present study, however, mouse fetal exposure via the mother to low-dose 17β-estradiol revealed no changes in the rate of postnatal growth in males and females that developed in any intrauterine position in utero. Therefore, we concluded that exposure to low-dose estrogenic endocrine disrupting chemicals during fetal life does not contribute to the intrauterine position.

5. General discussion

We are at a loss to explain why we were unable to replicate the effects of intrauterine position on AGD, or to find intrauterine position effects upon sexual maturation, and the estrous cycle. However, we know the difficulty in demonstrating intrauterine position effects upon morphology and behavior [35]. In addition, in contrast to earlier work [11] which examined blood androgen titers in mouse fetuses, Baum et al. [51] reported that whole-body androgen levels in female rat fetuses did not vary as a function of intrauterine position, and suggested that intrauterine position effects upon rodent morphology and behavior may not have the robust generality that is generally assumed.

Howdeshell and vom Saal [16] demonstrated that one source of variability in the response of both male and female mouse fetuses to an estrogen-mimicking chemical, bisphenol A, is their background levels of endogenous sex hormones. They suggested that a very small increase in the level of endogenous estradiol may substantially increase the susceptibility of fetuses to endocrine disrupting chemicals consumed or absorbed through the skin or lungs by pregnant animals and humans.

Contiguous [1,52], caudal [53,54], and no effect [51,55–57], due to intrauterine position, have been reported. Hotchkiss et al. [55] in a study with Sprague–Dawley rats examined the effect of intrauterine position on concentrations of testosterone in several different tissues. No effect of either contiguous or caudal intrauterine position on testosterone concentration was detected in fetal carcasses, reproductive tracts, or amniotic fluid. Furthermore, no correlation was found between masculinization due to intrauterine position and increasing anogenital distance. It is unclear at this time why there is such a discrepancy between the previous findings and the present results in rats and mice. However, varied strains of rats and mice, multiple uncontrolled variables, and different criteria for defining the effects of intrauterine positioning, may all contribute to this uncertainty. In addition, the discrepancies in the data may be attributed to such factors as the dietary influences (such as background levels of phytoestrogens and caloric intake), caging (steel versus polycarbonate), bedding, housing (group versus individual), and seasonal variation, as well as differences among the studies in control body and prostate weights [58,59].

The results of the present study clearly showed that intrauterine position of embryos/fetuses did not influence postnatal development, including sexual maturation and behavior.

Acknowledgments

The technical support provided by the reproductive and developmental biology group was essential to this study and was greatly appreciated. This study was supported by grants from the Ministry of Health, Labor and Welfare of Japan.

References

[1] Clemens LG, Gladue BA, Coniglio LP. Prenatal endogenous influences on masculine sexual behavior and genital morphology in male and female rats. Horm Behav 1978;10:40–53.

[2] vom Saal FS, Bronson FH. In utero proximity to female mouse fetuses to males: effects on reproductive performance during later life. Biol Reprod 1978;19:843–53.
[3] Clark MM, Galef Jr BG. Effect of uterine position on rate of sexual development in female Mongolian gerbils. Physiol Behav 1988;42:15–8.
[4] vom Saal FS. Sexual differentiation in litter bearing mammals: influence of sex of adjacent fetuses in utero. J Anim Sci 1989;67: 1824–40.
[5] Clark MM, Galef Jr BG. Prenatal influences on reproductive life history strategies. Trends Ecol Evol 1995;10:151–3.
[6] Jubilan BM, Nyby JG. The intrauterine position phenomenon and precopulatory behaviors of house mice. Physiol Behav 1992;51:857–72.
[7] Hernandes-Tristan R, Arevalo C, Canals S. Effect of prenatal uterine position on male and female rats sexual behavior. Physiol Behav 1999;67:401–8.
[8] Sherry DF, Galef BG, Clark MM. Sex and intrauterine position influence the size of the gerbil hippocampus. Physiol Behav 1996;60: 1491–4.
[9] Tarraf CG, Knight JW. Effect of intrauterine position on conceptus development, placental and endometrial release of progesterone and estrone in vitro, and concentration of steroid hormones in fetal fluids throughout gestation in swine. Domest Anim Endocrinol 1995;12:179–87.
[10] vom Saal FS, Timms BG. The role of natural and manmade estrogens in prostate development. In: Naz RK, editor. Endocrine disruptors: effects on male and female reproductive systems. Boca Raton, FL: CRC Press; 1999. p. 307–27.
[11] vom Saal FS, Bronson FH. Variation in length of the estrous cycle in mice due to former intrauterine proximity to male fetuses. Biol Reprod 1980;22:777–80.
[12] vom Saal FS. Variation in infanticide and parental behavior in male mice due to prior intrauterine proximity to female fetuses. Elimination by prenatal stress. Physiol Behav 1983;30:675–81.
[13] Clark MM, Karpiuk P, Galef BG. Hormonally mediated inheritance of acquired characteristics in Mongolian gerbils. Nature (London) 1993;364:712.
[14] Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenberg JG, vom Saal FS. Exposure to bisphenol A advances puberty. Nature (London) 1999;401:763–4.
[15] Welshons WV, Nagel SC, Thayer KA, Judy BM, vom Saal FS. Low-dose bioactivity of xeno17β estradiols in animals: fetal exposure to low doses of methoxychlor and other xeno17β estradiols increases adult prostate size in mice. Toxicol Ind Health 1999;15:12–25.
[16] Howdeshell KL, vom Saal FS. Developmental exposure to bisphenol A: interaction with endogenous estradiol during pregnancy in mice. Am Zool 2000;40:429–37.
[17] Timms BG, Peterson RE, vom Saal FS. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin interacts with endogenous estradiol to disrupt prostate gland morphogenesis in male rat fetuses. Toxicol Sci 2002;67:264–74.
[18] Guide for the care and use of laboratory animals. NIH Publication 86–23, Bethesda, MD: National Institutes of Health; 1985.
[19] Everett JW. Neurobiology of reproduction in the female rat: a fifty-year perspective. New York: Springer-Verlag; 1989.
[20] Haseman JK, Hogan MD. Selection of the experimental unit in teratology studies. Teratology 1975;12:165–72.
[21] Holson RR, Pearce B. Principels and pitfalls in the analysis of prenatal treatment effects in multiparous species. Neurotoxicol Teratol 1992;14:221–8.
[22] Bliss CI. Statistics in biology, vol. I. New York: McGraw-Hill; 1967. p. 116–7.
[23] Wise LD, Vetter CM, Anderson CA, Antonello JM, Clark RL. Reversible effects of triamcinolone and lack of effects with aspirin or L-656224 on external genitalia of male Sprague–Dawley rats exposed in utero. Teratology 1991;44:507–20.
[24] Clark RL. Endpoints of reproductive system development. An evaluation and interpretation of reproductive endpoints for human health risk assessment. Washington, DC: ILSI Press; 1999. p. 10–27.
[25] Gallavan RH, Holson JF, Stump DG, Knapp JF, Reynolds VL. Interpreting the toxicologic significance of alterations in anogenital distance: potential for confounding effects of progeny body weights. Reprod Toxicol 1999;13:383–90.
[26] Heinrichs WL. Current laboratory approaches for assessing female reproductive toxicity. In: Dixon RL, editor. Reproductive toxicology. New York: Raven Press; 1985. p. 95–108.
[27] vom Saal FS. Variation in phenotype due to random intrauterine positioning of male and female fetuses in rodents. J Reprod Fertil 1981;62:633–50.
[28] Vandenbergh JG, Huggett CL. The anogenital distance index, a predictor of the intrauterine position effects on reproduction in female house mice. Lab Anim Sci 1995;45:567–73.
[29] Nonneman DJ, Ganjam VK, Welshons WV, vom Saal FS. Intrauterine position effects on steroid metabolism and steroid receptors of reproductive organs in male mice. Biol Reprod 1992;47:723–9.
[30] Even MD, Dhar MG, vom Saal FS. Transport of steroids between fetuses via amniotic fluid in relation to the intrauterine position phenomenon in rats. J Reprod Fertil 1992;96:709–16.
[31] vom Saal FS, Dhar MG. Blood flow in the uterine loop artery and loop vein is bidirectional in the mouse: implications for transport of steroids between fetuses. Physiol Behav 1992;52:163–71.
[32] Gandelman R, vom Saal FS, Reinisch JM. Contiguity to male fetuses affects morphology and behavior of female mice. Nature (London) 1977;266:722–4.
[33] McDermott NJ, Gandelman R, Reinisch JM. Contiguity to male fetuses influences ano-genital distance and time of vaginal opening in mice. Physiol Behav 1978;20:661–3.
[34] Zielinski WJ, Vandenbergh JG, Montano MM. Effects of social stress and intrauterine position on sexual phenotype in wild-type house mice (*Mus musculus*). Physiol Behav 1991;49:117–23.
[35] Simon NG, Cologer-Clifford A. In utero contiguity to males does not influence morphology, behavioral sensitivity to testosterone, or hypothalamic androgen binding in CF-1 female mice. Horm Behav 1991;25:518–30.
[36] Brown-Grant K, Sherwood MR. "The early androgen syndrome" in the guinea pig. J Endcrinol 1971;49:277–91.
[37] Goy RW. Experimental control of psychosexuality. Phil Trans R Soc London 1970;259:149–62.
[38] Stiff ME, Bronson FH, Stetson MH. Plasma gonadotropins in prenatal and prepubertal female mice: disorganization of pubertal cycles in the absence of a male. Endocrinology 1974;94:492–6.
[39] Perrigo GH, Bronson FH. Foraging effort, food intake, fat deposition and puberty in female mice. Biol Reprod 1992;29:455–63.
[40] Kinsley C, Miele J, Konen C, Ghiraldi L, Svare B. Intrauterine contiguity influences regulatory activity in adult female and male mice. Horm Behav 1986;20:7–12.
[41] Hitchcock FA. Studies in vigor: V. The comparative activity of male and female albino rats. Am J Physiol 1925;75:205–10.
[42] Gentry RT, Wade GN. Sex differences in sensitivity of food intake body weight and running wheel activity to ovarian steroids in rats. J Comp Physiol Psychol 1976;90:747–54.
[43] Broida J, Svare B. Sex differences in the activity of mice: modulation by postnatal gonadal hormones. Horm Behav 1984;18:65–78.
[44] Hurst JL. Urine marking in populations of wild house mice (*Mus domesticus* Rutty). II. Communications between females. Anim Behav 1990;40:223–32.
[45] Berman DM, Russell DW. Cell-type-specific expression of rat steroid 5α-reductase isozymes. Proc Natl Acad Sci USA 1993;90:9359–63.
[46] vom Saal FS, Timms BG, Montano MM, Palanza P, Thayer KA, Nagel SC, et al. Prostate enlargement in mice due to fetal exposure

to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. Proc Natl Acad Sci USA 1997;94:2056–61.
[47] Zuckerman S. The endocrine control of the prostate. Proc R Soc Med 1936;29:1557–68.
[48] Glenister TW. The development of the utricle and of the so-called 'middle' or 'median' lobe of the human prostate. J Anat 1962;96:443–55.
[49] Blacklock NJ. The development and morphology of the prostate. In: Ghanadian R, editor. The endocrinology of prostate tumours. Lancaster, England: MTP Press; 1983. p. 1–13.
[50] Timms BG, Petersen SL, vom Saal FS. Prostate gland growth during development is stimulated in both male and female rat fetuses by intrauterine proximity to female fetuses. J Urol 1999;161:1694–701.
[51] Baum MJ, Woutersen PJA, Slob K. Sex difference in whole-body androgen content in rats on fetal days 18 and 19 without evidence that androgen passes from males to females. Biol Reprod 1991;44:747–51.
[52] Tobet SA, Dunlap JL, Gerall AA. Influence of fetal position on neonatal androgen-induced sterility and sexual behavior in female rats. Horm Behav 1982;16:251–8.
[53] Houtsmuller EJ, de Jong FH, Rowland DL, Slob AK. Plasma testosterone in fetal rats and their mothers on day 19 of gestation. Physiol Behav 1995;57:495–9.
[54] Richmond G, Sachs BD. Further evidence for masculinization of female rats by males located caudally in utero. Horm Behav 1984;18:484–90.
[55] Hotchkiss A, Parks LG, Ostby J, Lambright C, Wolf C, Wilson VS. A quantitative determination of the environmental sources of variability in androgens of fetal Sprague–Dawley rats. Biol Reprod (Abstract) 2000;62:183.
[56] Meisel RL, Ward IL. Fetal female rats are masculinized by male littermates located caudally in the uterus. Science 1981;213:239–42.
[57] Slob AK, Van der Schoot P. Testosterone induced mounting behavior in adult female rats born in litters of different female to male ratios. Physiol Behav 1982;28:1007–10.
[58] NIEHS, National Toxicology Program (NTP). Endocrine disruptors low dose peer review report; 2001. p. 467.
[59] Witorsch RJ. Low-dose in utero effects of xenoestrogens in mice and their relevance to humans: an analytical review of the literature. Food Chem Toxicol 2002;40:905–12.

ANALYTICAL SCIENCES APRIL 2004, VOL. 20

The Best Paper in Bunseki Kagaku, 2002

Screening of Endocrine Disrupting Chemicals Using a Surface Plasmon Resonance Sensor

Kazunobu ASANO,† Atsushi ONO, Setsuko HASHIMOTO, Tohru INOUE, and Jun KANNO

New Business Development, Biacore K.K., Koyo Bldg. 5F, 2-9-5 Shiba Koen, Minato-ku, Tokyo 105-0011, Japan

Because concern over endocrine disrupting reactions caused by chemicals to humans and animals is growing, a rapid and reliable screening assay for endocrine disrupting chemicals is required. We have developed an *in vitro* screening assay based on a hormone receptor mechanism using a surface plasmon resonance (SPR) sensor. The interaction between an estrogen receptor α (ER) and an estrogen response element (ERE) is monitored in real time, when ER is injected over the SPR sensor chip on which a DNA fragment containing ERE is immobilized. In the presence of a chemical with estrogenic activity, the ER-ERE interaction is enhanced and the kinetic parameters are altered. We have validated the assay in terms of its specificity, dose dependency, optimal reaction conditions and reproducibility. It has been shown that the assay is very reliable as a rapid and quantitative screening method to judge the estrogenic activities of chemicals.

Introduction

Recently, concern has grown that some chemicals, such as organic chloride insecticides, plasticizers and detergents can cause endocrine disrupting effects to wild animals and humans.[1] Many of them are supposed to pose endocrine disrupting activities through direct interaction with the hormone receptors, such as estrogen receptor, thus modifying or inhibiting the physiological hormonal activities.[2] Chemical safety is evaluated by a set of the toxicological tests, such as carcinogenicity, teratogenicity, mutagenicity, reproduction tests. However, they have limitation to evaluate the chronic toxicity of chemicals. Moreover, the mechanisms of the endocrine disrupting activities are yet to be elucidated and the test methods to evaluate the effects are not well established.[3]

Several test methods have been reported to detect the endocrine disrupting activities caused by hormone receptors, *i.e.* competitive receptor binding assay, a cell growth assay using breast cancer cells expressing the estrogen receptors (MCF7),[4] cell-based reporter assay,[5] an *in vivo* rodent uterotrophic test,[6,7] and a vitellogenin assay using medaka fish (Oryzias latipes).[8] Many of these methods require a long time to obtain results. Furthermore, the endocrine disrupting activities can not always be detected when the chemicals are administered to the animals due to physiological regulations concerning the animal bodies. It is not easy to detect the hormonal effects of chemicals. The existing toxicological methods are not always the best way to detect the effects which have the feedback mechanism or the effects *via* the receptors. Therefore, a novel approach is sought for the rapid assessment of the endocrine disrupting activities of the chemicals.

The hormone receptors are the ligand dependent transcription factors. For example, estrogen receptor (ER) changes its conformation upon binding of the endogenous ligand, estrogen, and binds to the specific sequence of the DNA located upstream of the target genes and activates transcription of the genes (Fig. 1). Many chemicals with diverse structures have been reported to have estrogenic activities. Due to the variety of the structure, it is unlikely that all the chemicals act with the same mechanism. Each chemical may pose a different regulatory effect on the gene expression.[9]

Recently, a surface plasmon resonance (SPR) sensor is emerging as a novel analytical instrument.[10] The SPR sensor has features that it can monitor molecular interaction without labeling the molecules in real time. It is, therefore, suitable for high throughput screening assays. Compared to the existing technologies which monitor the binding amounts at the end of the interactions, the SPR sensor is unique to be able to detect the processes throughout the association and the dissociation of the interaction. This feature enables detailed analyses of chemical effects to receptors.

We have established a cell free screening assay focusing on the hormone receptor mechanism as a high throughput screening method for the endocrine disrupting chemicals. In order to measure the interaction of the biological molecules using the SPR sensor, one of the test molecules is immobilized

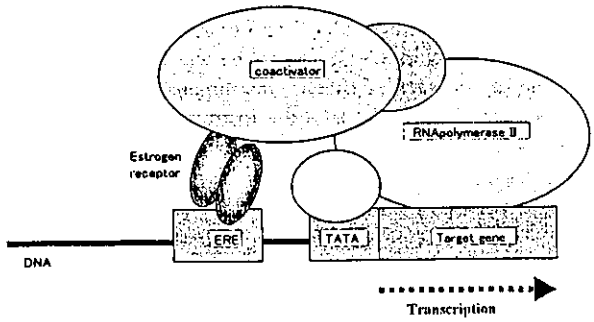


Fig. 1 Functional mechanism of the estrogen receptor in gene regulation.

† To whom correspondence should be addressed.

This is an English edition of the paper which won the Best Paper Award in Bunseki Kagaku, 2002 [*Bunseki Kagaku*, **2002**, *51(6)*, 389].

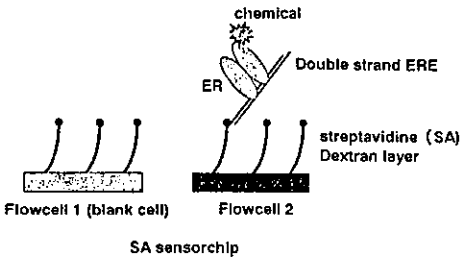


Fig. 2 Scheme of the ER-ERE assay with an SPR sensor.

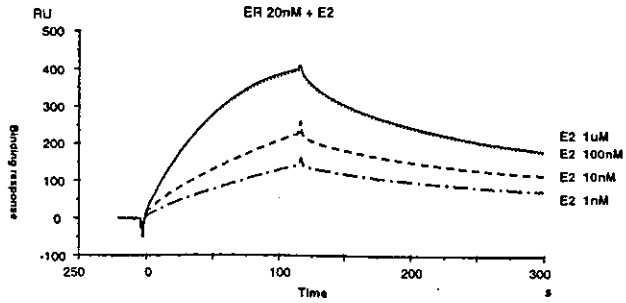


Fig. 3 Dose-dependent responses of E2.

on the sensor chip surface and a solution containing the other molecule is passed over the sensor surface at a constant flow rate through the microfluidics. A small mass changes, resulting from the binding and the dissociation of the two molecules on the sensor surface is monitored as SPR signals. The time course of the changes in the SPR signals is displayed as a curve called a sensorgram. Unlike from the conventional technologies, the SPR sensor can measure the interactions using a small amount of non-labeled samples within a short time. With regard to the interaction of the molecules, the SPR sensor can give not only the affinity of the two molecules at the equilibrium (as the dissociation constant, K_D or the affinity constant, K_A) but also the information on two molecules binding or dissociation velocity, namely the association rate constant (k_a) and the dissociation rate constant (k_d).

We have designed an assay to monitor the interaction of estrogen receptor α (ER) and the estrogen response element (ERE)[11] that is located in the promoter region of the estrogen target genes by immobilizing the DNA fragment containing the ERE sequence on the sensor chip and injecting purified ER over the sensor chip (Fig. 2). Thirty chemicals were tested for the estrogenic activities.

Experiments

Reagents and instruments

Reagent. Tricine, $CaCl_2$, $MgCl_2$, KOH, Tween 20, NaOH and HCl were purchased from Nacalai Tesque and DMSO from Sigma. Estrogen receptor (ER) was purchased from PanVera. ER was aliquoted into 5 µl and stored at –80°C. Biotinylated estrogen response element (ERE) DNA (5′-biotin-tcgagcaaagtcaggtcacagtgacctgatcaat-3′) of viterogenin gene and the anti-strand DNA have been synthesized by Nisshinbo. The synthesized oligomers were diluted with MilliQ water to 1 mg/ml and stored at –20°C. The running buffer for Biacore 3000 was prepared by filtering a solution of 25 mM Tricine, 160 mM KCl, 5 mM $MgCl_2$ (pH 7.8), 0.05% Tween 20.

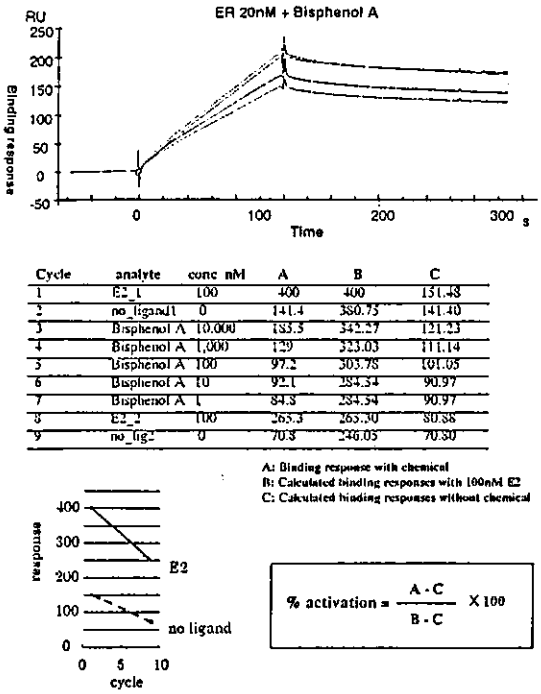
Instrument. The assay was performed using Biacore 3000 (Biacore AB), the heat block (EYELA) and the circulator (Asone). Sensor chip SA (Biacore AB) was used. Through the assay, the sample rack of Biacore instrument was cooled to 4°C by connecting the circulator to the instrument and the reaction was run at 25°C.

Operation

Immobilization of biotinylated ERE. For the immobilization of biotinylated ERE to the sensor chip, a streptoavidin preimmobilized sensor chip (Sensor chip SA) was set to the Biacore 3000 instrument and the instrument was equilibrated with running buffer. In order to stabilize the sensor surfaces, 100 mM NaOH and 50 mM HCl were injected for 30, 5 times. After checking the baseline stability, we performed immobilization of the biotinylated ERE. Biotinylated ERE (1 mg/ml) was diluted hundred thousand times with the running buffer. Then, 100 µl of the ERE solution was heated in boiling water for 5 min and chilled rapidly to denature the biotinylated ERE. The solution was injected over the sensor surface to immobilize approximately 60 RU onto the SA sensor chip surface. Then, the complementary ERE (1 mg/ml) was diluted to 100 times with the running buffer and denatured by the same method. This solution was injected for 2 min over the sensor surface where the biotinylated ERE was immobilized to form double stranded ERE on the surface. Biotin (1 µg/ml) was injected to block free SA on the sensor surface. A separate flow cell was used as a blank cell on which only biotin was immobilized.

Preparation of the test chemicals. Each chemical was dissolved with 100% DMSO to make 0.1 M stock solution, and stored at –80°C. Immediately before the assay, 1 µl of the stock solution was diluted 500 times using the chilled running buffer. Also ER stock solution was diluted to 40 nM using a chilled running buffer. A 50-µl volume of the ER solution and 50 µl of the chemical solution of each concentration were mixed to give final concentrations of 20 nM ER and 10 µM to 1 nM of the chemical. The samples were kept at 4°C in a sample rack to maintain the ER activity. 17β-Estradiol was used as a positive control. First, we prepared the various concentration of 17β-estradiol (1 µM to 1 nM) and measured the binding of ER to ERE (Fig. 3). As the binding activity of ER to ERE was plataued over 100 nM 17β-estradiol, we decided to use 100 nM 17β-estradiol as a positive control in the following experiments. We also prepared a negative control solution which did not contain any chemicals. After the preparation of samples, the samples were treated at 37°C, 5 min and rapidly cooled. The samples were then set on the sample rack for measurements.

Assay of ER and ERE. The prepared samples were injected for 2 min at a flow rate of 20 µl/min over the immobilized ERE and the blank flowcell. Injection command of "kinject" was used and the dissociation phase was monitored for 2 min. The "kinject" command is one of the injection commands specially designed for the kinetic analysis in the Biacore instrument. Upon injecting the samples using "kinject" command, the sample solution was clearly separated by two air plugs at the both ends of the sample solution from the running buffer in order to prevent the sample solution from being diluted by the running buffer. The command is also designed to monitor dissociation of the bound molecule without being disturbed by the movement of the injection needle for the set period of time. After monitoring the binding and dissociation, 100 mM NaOH and 50 mM HCl were injected 30 s each for regeneration of the sensor surfaces. All the measurements were run automatically.



Cycle	analyte	conc nM	A	B	C
1	E2_1	100	400	400	151.48
2	no_ligand1	0	141.4	380.75	141.40
3	Bisphenol A	10,000	133.5	342.27	121.23
4	Bisphenol A	1,000	129	323.03	111.14
5	Bisphenol A	100	97.2	303.78	101.05
6	Bisphenol A	10	92.1	284.54	90.97
7	Bisphenol A	1	84.3	284.54	90.97
8	E2_2	100	265.3	265.30	80.88
9	no_lig2	0	70.8	246.05	70.80

A: Binding response with chemical
B: Calculated binding responses with 100nM E2
C: Calculated binding responses without chemical

$$\% \text{ activation} = \frac{A - C}{B - C} \times 100$$

Fig. 4 Data evaluation of chemical screening.

Data evaluation

A set of the assay consisted of 5 concentrations of the test chemical, a negative control (no chemical) and a positive control (100 nM 17β-estradiol). The results were compared as the ratio to the positive control (% activation). ER was unstable and lost its binding activity to ERE during the assay period in spite of optimizing the assay conditions. We have developed an assay design to correct for any loss of the binding activity of ER over time. A positive control cycle and a negative control cycle were run at the beginning and the end of the assay. The binding responses were recorded. Based on the rate of loss in the positive and negative control samples, the binding responses of the positive and negative controls for each cycle were calculated. The enhancement of ER binding by the test chemical was expressed as the ratio to those by the positive control of 100 nM 17β-estradiol, namely as a relative activation (% activation) using the formula and the corrected binding signals, as shown in Fig. 4.

Results

Validation of the ER-ERE assay using Biacore

In order to confirm the significance of the ER assay, the binding of ER to ERE was tested with a varying concentration of ER. The binding signals increased in relation to the increasing concentrations of ER. ER did not bind to the sensor surface where no ERE was immobilized (Fig. 5). A 1 μM volume of BSA did not show any significant binding to ERE surfaces (Fig. 6). These observations indicate that the assay monitors the specific binding of ER to ERE. Comparing the results with 10, 20 and 40 nM ER, we often observed relatively low binding signals with 10 nM ER. Higher binding signals were obtained by adding a final concentration of 1 μM BSA to 10 nM ER. Due to the low protein concentration, ER was lost by absorption to the surfaces of the plastic vials and tips and the actual concentration of ER became lower than 10 nM. Based on

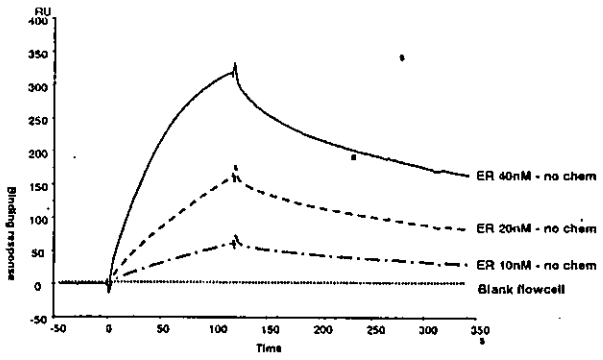


Fig. 5 Dose-dependent responses of ER-1.

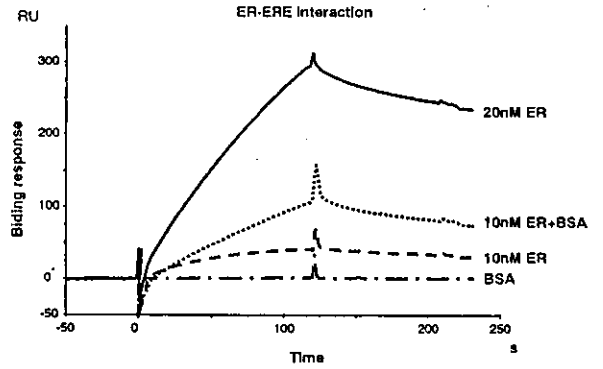


Fig. 6 Dose-dependent responses of ER-2.

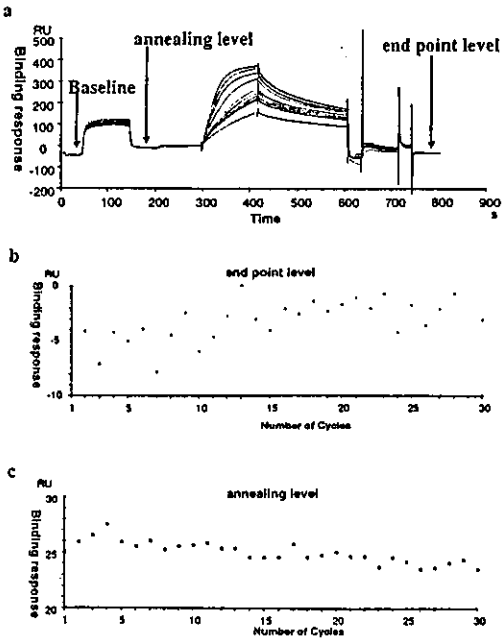


Fig. 7 Reproducibility of the ER-ERE assay.

those results, we decided to run the following assays with the final ER concentration of 20 nM.

It is important to regenerate the sensor surfaces to achieve reproducible results in the Biacore assay. We have repeated 30 cycles of the assay (Fig. 7a) and monitor the end point levels (Fig. 7b). It was confirmed that the sensor surfaces were properly regenerated and the assay showed high reproducibility.

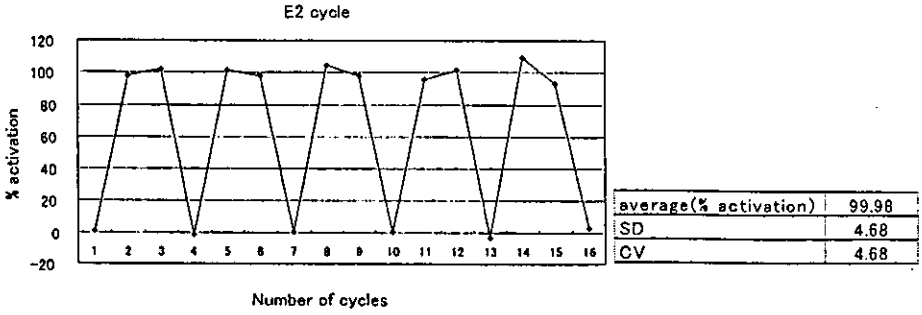


Fig. 8 Precision of the ER-ERE assay.

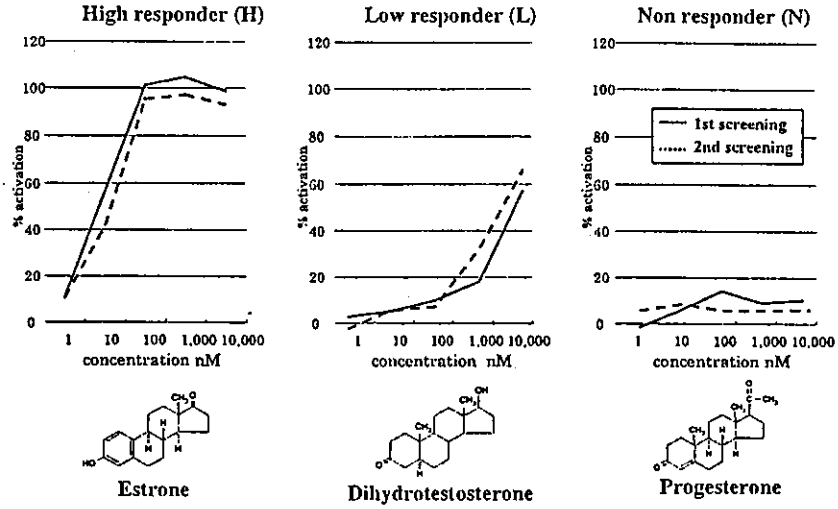


Fig. 9 Three types of chemical responses.

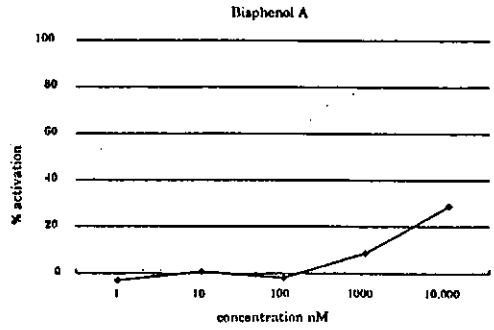


Fig. 10 Results of Bisphenol A.

The difference in the end point level was within 4 RU and the annealing level of anti-ERE was kept constant at around 25 RU throughout 30 cycles (Fig. 7c). We tested the reproducibility by repeating positive and negative controls for 16 cycles. It was shown that the results were with high precision with a CV% value of 4.68%, as shown in Fig. 8.

Screening results of 30 chemicals

We tested 30 chemicals to check the dose-dependent activation of the ER binding. One cycle of the assay took 15 min and the screening of one chemical was completed with 9 cycles in 2.5 h, including 5 different concentrations of the test chemicals, the positive and negative controls repeated twice for each control. We calculated the relative activation (% activation) using the formula shown in Fig. 4 for 30 chemicals. Based on the values of % activation at 100 nM of each chemical, chemicals could be classified into three groups (Fig. 9): the chemicals that showed more than 50% of the activation as "high responders", those with 20 - 50% as "low responders" and those less than 20% as "non-responders". The results obtained with two independent sets of screening were summarized in Table 1. 28 out of 30 chemicals showed the same results in the first and the second screening. 17β-Estradiol and its derivatives were classified to "high responders", while male hormones (progesterone) were "non-responders". Bisphenol A which is regarded as one of the endocrine disruptors, was classified among "low responders" (Fig. 10).

Furthermore, the differences in the effect of the chemicals on the ER binding activities were observed in the different shapes of the sensorgrams among those of 17β-estradiol, bisphenol A, 17α-estradiol, diethylstilbestrol (DES), tamoxifen and progesterone (Fig. 11). We have plotted the binding level at the end of the injection of ER in the presence of 1 μM of the test chemical (Y axis) *versus* the binding stability 2 min after the end of the ER injection (X axis), as shown in Fig. 12. We found that the agonists and the antagonists had significantly different patterns. The antagonists (such as tamoxifen) had a tendency to stabilize the binding of ER to ERE. The assay using Biacore indicated the possibility not only to detect the estrogenic activities of the chemicals, but to distinguish the antagonists from the agonists.