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Senescent B Lymphopoiesis Is Balanced in Suppressible Homeostasis: Decrease in Interleukin-7 and Transforming Growth Factor- β Levels in Stromal Cells of Senescence-Accelerated Mice

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The suppression of the B cell population during senescence has been considered to be due to the suppression of interleukin-7 (IL-7) production and responsiveness to IL-7; however, the upregulation of transforming growth factor- β (TGF- β) was found to contribute to B cell suppression. To investigate the mechanism of this suppression based on the interrelationship between IL-7 and TGF- β during senescence, senescence-accelerated mice (SAMs), the mouse model of aging, were used in this study to elucidate the mechanisms of B lymphopoietic suppression during aging. Similar to regular senescent mice, SAMs showed a decrease in the number of IL-7-responding B cell progenitors (i.e., colony-forming unit pre-B [CFU-pre-B] cells in the femoral bone marrow [BM]). A co-culture system of B lymphocytes and stromal cells that the authors established showed a significantly lower number of CFU-pre-B cells harvested when BM cells were co-cultured with senescent stromal cells than when they were co-cultured with young stromal cells. Interestingly, cells harvested from a senescent stroma and those from the control culture without stromal cells were higher in number than those harvested from a young stroma, thereby implying that an altered senescent stromal cell is unable to maintain self-renewal of the stem cell compartment. Because TGF- β is supposed to suppress the proliferative

capacity of pro-B/pre-B cells, we added a neutralizing anti-TGF- β antibody to the co-culture system with a pro-B/pre-B cell-rich population to determine whether such suppression may be rescued. However, unexpectedly, any rescue was not observed and the number of CFU-pre-B cells remained unchanged when BM cells were co-cultured with senescent stromal cells compared with the co-culture with young stromal cells, which essentially showed an increase in the number of CFU-pre-B cells ($P < 0.001$ in 5 $\mu\text{g/ml}$). Furthermore, TGF- β protein level in the supernatant of cultured senescent stroma cells was evaluated by enzyme-linked immunosorbent assay, but surprisingly, it was found that TGF- β concentration was significantly lower than that of cultured young stromal cells. Thus, TGF- β activity was assumed to decline particularly in a senescent stroma, which means a distinct difference between the senescent suppression of B lymphopoiesis and secondary B lymphocytopenia. Concerning proliferative signaling, on the other hand, the level of IL-7 gene expression in cells from freshly isolated BM decreased significantly with age. Therefore, the acceleration of proliferative signaling and the deceleration of suppressive signaling may both be altered and weakened in a senescent stroma (i.e., homeosuppression). *Exp Biol Med* 229:494-502, 2004

Key words: aging; B-lymphopoiesis; interleukin-7; transforming growth factor- β ; senescence-accelerated mice; homeosuppression

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Introduction

Aging is accompanied by changes in the immune system, leading to a decrease in the overall cellular and humoral responsiveness (1). Because the most marked age-associated change in the immune system is the rapid involution of the thymus after puberty, most of the decline in humoral immunity has been attributed to changes in the T-cell compartment rather than to an intrinsic primary B cell

deficit. Consequently, attention has been focused on age-associated changes in T lymphocytes and their functions (2). However, it has recently been clarified that there are also deficiencies in B cell development in the bone marrow (BM) of aged animals (3–6). Alterations in B cell development may include both the skewing of V-gene utilization, particularly in cells responsive to phosphoryl choline, and the decrease in the generation of various developmental B cell subsets. The altered representation of these subsets appears to be a consequence of a developmental arrest of the maturation of pro-B cells and the earliest stage of surface Ig-positive cells (7). Age-related changes in the B cell development may account for the deterioration of the immune system in senescent mice.

B lymphopoiesis is suppressed during senescence not only in mice but also in humans. A decrease in interleukin-7 (IL-7) production by stromal cells and a simultaneous reduction in B lymphocyte reactivity to IL-7 are considered as a possible background for this negative senescent regulation. Furthermore, in addition to senescence, B cells were noted to be regulated by two pathways not only for IL-7 but also for transforming growth factor- β (TGF- β) in regular mice; that is, not only the downregulation of the former but also the upregulation of the latter simultaneously play a role in suppression regulation (8). This is in good agreement with the observation that the supplementation of IL-7 could not compensate for the B cell suppression. In this study, possible senescence-associated alterations in the productions of IL-7 and TGF- β are examined in senescence-accelerated mice (SAMs).

Senescence-accelerated mice provide a unique model system for studying senescence or aging in higher organisms, because they exhibit a marked acceleration of aging, which has been confirmed to be the same manner as that observed in the regular mice. Senescence-accelerated mice are characterized by the early onset of aging (mean life span of 40 weeks under conventional conditions), loss of general behavioral activity, increased skin coarseness, and spinal lordokyphosis (9). Because the number of splenic cells starts to decrease at approximately 30 weeks old, the SAMs used were, in general, 30 weeks old or slightly older. Although one must carefully interpret the results of studies using SAMs because the mechanism of "accelerated aging" may not be associated with that of "normal aging," results of previous studies conducted by other researchers and ourselves indicate that SAMs are a suitable model for predicting the possible mechanism of aging in hematopoietic systems (10–14).

The purposes of this study are to confirm the status of B lymphopoiesis in SAMs compared with that in other regular strains and to elucidate the mechanism of age-related changes in B lymphopoiesis in SAMs. Here, we examined age-related changes in the number and function of B cell progenitors in the BM and their supportive microenvironment.

Materials and Methods

Mice. A senescence-prone substrain of the AKR/J mouse, SAMs/P-1 (9), from The Jackson Laboratory in Bar Harbor, ME, was kindly provided by Dr. Toshio Takeda, Emeritus, the Chest Disease Research Institute, Kyoto University. The mice were bred and maintained at the experimental animal facility of the National Institute of Health Sciences under pathogen-free conditions. Male SAMs designated as "young (8–12 weeks old)" or "senescent (30–36 weeks old)" were used in the present study; these ages were selected because the number of splenic cells and/or hemopoietic progenitor cells start to decrease at approximately 30 weeks of age (11).

Preparation of BM Cells. The BM cell suspensions were prepared by repeatedly flushing the cells from femurs and dispersing them by trituration through a 23-gauge hypodermic needle with the Iscove-modified Dulbecco medium (IMDM; Invitrogen Corp., Carlsbad, CA) or RPMI 1640 medium (Invitrogen).

In Vitro Colony Assays. Colony-forming unit pre-B (CFU-pre-B) cells were assayed by suspending mononuclear cells in 1-ml aliquots of the recombinant IL-7 (rIL-7)-supplemented MethoCult M 3630 medium (Stem Cell Technologies Inc., Vancouver, Canada) in 35-mm, plastic petri dishes. Femoral BM cells from three mice per group were pooled and assayed. A MethoCult M 3630 medium consisting of 1 ml of the semisolid IMDM medium containing 1% methylcellulose, 30% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT), 0.1 mM 2-mercaptoethanol (2-ME), 2 mM L-glutamine, and 10 ng/ml of rIL-7 (R&D Systems, Inc., Minneapolis, MN) was used. Granulocyte-macrophage colony-forming units (GM-CFUs) were assayed by suspending mononuclear cells in the alpha medium containing 1% methyl cellulose, 30% FBS, 1% bovine serum albumin, 1 mM 2-ME, and 10 ng/ml of granulocyte-macrophage colony-stimulating factor (Genzyme, Cambridge, MA) and plating 1-ml aliquots in 35-mm, plastic dishes. Both CFU-pre-B cells and GM-CFUs in culture plates in triplicate were incubated at 37°C in a fully humidified atmosphere of 5% carbon dioxide in air. Aggregates of 50 or more cells in 7-day cultures were counted as colonies. Aggregates ranging from 10 to 49 cells were counted as clusters.

Co-culture of Stromal Monolayers and Pro-B/Pre-B Cell-Rich Populations. Stromal monolayers were prepared by culturing BM cells derived from young or senescent SAMs at 1×10^6 /ml in 96-well Coster 3596 or 24-well Falcon 3047 flat-bottomed plates in 0.2 or 1 ml of the RPMI 1640 medium supplemented with 20% FBS. Confluent adherent layers were formed after 7 days. To obtain pro-B/pre-B cell-rich populations, the bulk culture of pooled BM cells from young SAMs stimulated with rIL-7 was performed as described previously (4). Briefly, BM cells from young SAMs were cultured at 1×10^6 cells/ml in RPMI 1640 supplemented with 20% FBS, 2×10^{-5} M

2-ME, 1% L-glutamine, and 2 ng/ml of murine rIL-7 (Genzyme) and plated in six-well Coster 3516 culture trays. Nonadherent cells were harvested after 4 days of culture. This bulk culture provided a highly rich (>10-fold) source of IL-7-responsive B220⁺, CD43⁺, IgM⁻, pro-B/pre-B cells (data not shown). Pro-B/pre-B cell-rich populations were suspended at 5×10^4 /ml in RPMI 1640 supplemented with 20% FBS, 2×10^{-5} M 2-ME, 1% L-glutamine, and 1 ng/ml of murine rIL-7. Aliquots (0.1 or 1.0 ml) of this cell suspension were added to established stromal cell monolayers in 96- and 24-well flat-bottomed trays, respectively, and co-cultured at 37°C in a fully humidified atmosphere of 5% carbon dioxide in air. Nonadherent cells were harvested after 3 days, counted, and cloned using the CFU-pre-B colony assay system.

Extraction of Total RNA and Polymerase Chain Reaction (PCR). Total RNA was extracted from BM cells using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First, messenger RNA (mRNA) was reverse transcribed using superscript (Life Technologies, Grand Island, NY) and random hexamers. Next, PCR amplification of complementary DNA (cDNA) was performed with the graded dilution of cDNA for semi-quantitative evaluation of IL-7 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression under the following conditions: IL-7 cDNA, 95 °C for 1 min, 55°C for 2 mins, and 72°C for 3 mins for 35 cycles; and GAPDH cDNA, 94°C for 30 secs, 60°C for 30 secs, and 72°C for 1 min for 20 cycles. Murine IL-7 and GAPDH primers were synthesized based on a published cDNA sequence (15):

IL-7 (sense) 5'-GCCTGTCACATCTGAGTGGC-3'

IL-7 (antisense) 5'-CAGGAGGCATCCAG-GAACTTCTG-3'

GAPDH (sense) 5'-TGAAGGTCGGTGTGAACG-GATTGGC-3'

GAPDH (antisense) 5'-CATGTAGGCCATGAGGTC-CACCAC-3'

The expected amplified PCR products were 496 and 982 base pairs long for IL-7 and GAPDH, respectively. The PCR products were photographed using the Bio-Rad 2000 gel documentation system (Bio-Rad Laboratories, Hercules, CA), and intensities of expressions were evaluated by ImageGauge version 3.11 (Science Lab 98 for Windows; Fuji Film, Tokyo, Japan). In this experiment, GAPDH expressions were not altered among the experimental groups.

Effect of Anti-TGF- β Antibody on Growth of Pro-B/Pre-B Cell-Rich Population Co-Cultured with Stromal Cells. To examine the effect of TGF- β produced by stromal cells on the growth of pro-B/pre-B cells, a neutralizing monoclonal antibody (mAb) to TGF- β (mouse IgG₁ isotype, R&D Systems) at dilutions ranging from 1–10 μ g/ml was added to the co-culture system. The mouse IgG₁ isotype (R&D Systems) was used as mock control. The

number of nonadherent cells in the co-cultures was determined 3 days later.

Determination of Level of TGF- β Protein Produced by Cultured Stromal Cells. Stromal monolayers were prepared by culturing BM cells from young and senescent SAMs at 1×10^6 /ml in 24-well Falcon 3047 flat-bottomed plates in 1 ml of the RPMI 1640 medium supplemented with 20% FBS. Confluent adherent layers were obtained after 7 days. The supernatant in the culture plates was removed; and then 1 ml of RPMI 1640, supplemented with 20% FBS, 2×10^{-5} M 2-ME, and 1% L-glutamine were added to the culture plates. The culture medium was collected after 7 days of culture and was used for the determination of the level of the TGF- β protein produced by stromal cells. The TGF- β concentration in the culture medium was determined using a TGF- β -specific enzyme-linked immunoabsorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's instructions. All the samples were assayed in triplicate. The samples were acid activated (16) by adding 1/5 vol of 1 N hydrochloride at room temperature and neutralized after 10 mins by adding of 1/5 vol of 1.2 N NaOH in 0.5 M HEPES, and the mixture was diluted with the same volume of calibrator diluents in the ELISA kit.

Statistical Analysis. Data were analyzed using the analysis of variance (ANOVA). Values were considered significantly different at $P < 0.05$.

Results

Decrease in Number of B Cell Progenitors (Pre-B Cells). Age-related changes in the numbers of B lymphocytes and hematopoietic progenitors differ from each other. Table 1 summarizes the results of the triplicate experiments. The number of femoral GM-CFU cells from 30-week-old and 36-week-old senescent mice assayed on the basis of their colony-forming ability increased to 112% and 109%, respectively, that of GM-CFU from 12-week-old mice. In contrast, the CFU-pre-B colony assay, using Day 7 B cell colonies as the end point, was used to determine the number of IL-7-responsive B cell progenitors in young and senescent BM cells. The numbers of femoral CFU-pre-B cells from 30-week-old and 36-week-old mice decreased to 75.7% and 65.0%, respectively, that from 12-week-old mice. Furthermore, the decrease in the number of CFU-pre-B cells from femoral BM in senescent mice could not be counteracted by increasing IL-7 concentration in the culture medium 4-fold or by extending the culture period (data not shown).

Significant Decrease in Number of Large Pre-B Colonies. Among B cell colonies of various sizes, we noted that the number of relatively larger B cell colonies decreased significantly with aging (Fig. 1). The number of cells per colony ranged from 50–5000. Therefore, CFU-pre-B cell colonies in Table 1 were categorized according to their size, namely, small (50–200 cells), intermediate (201–

Table 1. Age-Related Changes in Number of Hematopoietic Progenitor Cells in Senescence-Accelerated Mice^a

| | Mean \pm SEM of triplicate experiments (%) | | |
|-------------------------|--|----------------------------|--------------------------|
| | 12-week-old mice | 30-week-old mice | 36-week-old mice |
| Femoral GM-CFU cells | 72,762 \pm 672 | 81,250 \pm 2811 (112%) | 79,058 \pm 4763 (109%) |
| Femoral CFU-pre-B cells | 13,868 \pm 516 | 10,505 \pm 1083* (75.7%) | 9017 \pm 220** (65.0%) |

^a GM-CFU, granulocyte-macrophage colony-forming unit; CFU-pre-B, colony-forming unit pre-B.

* $P < 0.05$; ** $P < 0.001$.

3000), and large (>3000 cells). As shown in Figure 1, the numbers of large, intermediate, and small B cell colonies for all groups decreased with age (58.3% for large colonies, 75.8% for intermediate colonies, 78.4% for small colonies in 30-week-old mice relative to those in 12-week-old mice; 22.1% for large colonies, 52.0% for intermediate colonies, and 76.5% for small colonies in 36-week-old mice relative to those in 12-week-old mice). The decrease in the numbers was statistically most significant for large colonies in 30- and 36-week-old mice relative to those in 12-week-old control ($P < 0.001$ and $P < 0.005$, respectively) and also for intermediate colonies in 36-week-old mice relative to those in 12-week-old control ($P < 0.05$).

Decrease in IL-7 Expression Level in BM. As observed in the regular senescent mice, the number of pre-B cell progenitors also decreased in SAMs. Therefore, the expression level of IL-7, which is known to be a pre-B cell stimulator, was evaluated in BM cells. IL-7 expression level decrease during senescence (17–19). In this study, IL-7 mRNA expression level was evaluated by reverse tran-

scriptase (RT)-PCR. The BM stromal cell-derived IL-7 is a positive regulator of *in vivo* B lymphopoiesis. As shown in Figure 2, the IL-7 mRNA expression level in BM cells from senescent mice was 6.2% that from young mice. These findings are comparable to those in the literature (17–19). Further experiments using SAMs were designed.

Decrease in Proliferative Capacity of B Cell Progenitors, Pre-B Cell Response to IL-7. The decrease in IL-7 expression level is also associated with the decrease in the responsiveness of pre-B cells to IL-7. To evaluate such responsiveness, we used a recloning assay to determine the proliferative capacity of the progeny of CFU-pre-B cells. Cells derived from 36 large colonies were pooled and recloned for 7 days in a semisolid medium supplemented with rIL-7. Table 2 shows the results of the recloning study. The numbers of secondary colonies, which included small (50–200 cells) and intermediate (201–3000) colonies and clusters (10–49 cells) generated from individual large primary colonies and derived from 30-week-old and 36-week-old femoral BMs, decreased significantly to 69.0% and 2.7%, respectively, that of secondary colonies grown from large primary colonies derived from 12-week-old femoral BM. Furthermore, cells from small primary colonies derived from either young or senescent mouse BM formed no secondary colonies. In B lymphopoiesis, unlike in the case of myelogenous progenitors, the results indicate that the responsiveness of CFU-pre-B cells to IL-7 decreases with age.

Decrease in Maintenance Capacity of Stromal Cells for B Lymphopoiesis. Although the pre-B progenitor cells were altered during senescence, a decrease in the maintenance capacity of stromal cells for B cell lineages may also be of importance in association with hematopoietic senescence (17, 18, 20). Using a co-culture system in 24-well flat-bottomed trays, we determined whether the capacity of stromal cells to support B lymphopoiesis is altered with age. Interestingly and unexpectedly, the number of lymphocytes recovered from the coculture of pro-B/pre-B cells with young stromal cells was significantly lower than that recovered from senescent stromal cells (Fig. 3A). In contrast, the total number of CFU-pre-B cells recovered from the co-culture with young stromal cells was significantly higher than that recovered from the co-culture with senescent stromal cells (Fig. 3B).

Decrease in TGF- β Production by Senescent Stromal Cells. On the basis of the above-mentioned co-

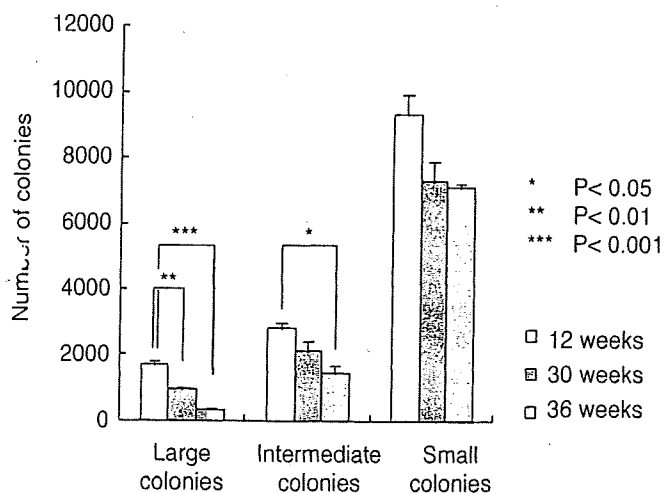


Figure 1. Age-related changes in number of B cell colonies of large, intermediate, and small sizes (mean \pm SEM of three replicate experiments). Femoral bone marrow (BM) cells from three mice per group were harvested and pooled. The preparation of BM cell suspensions is described in the "Materials and Methods" section. Colony-forming unit pre-B (CFU-pre-B) cells were assayed by suspending mononuclear cells in 1-ml aliquots of the recombinant interleukin-7 (rIL-7)-supplemented MethoCult M 3630 medium (Stem Cell Technologies Inc., Vancouver, Canada) in 35-mm, plastic petri dishes. Culture plates in triplicate for CFU-pre-B cells were incubated at 37 °C in a fully humidified atmosphere of 5% carbon dioxide in air. According to the size of CFU-pre-B cell colonies shown in Table 1, colonies were categorized as follows: small (50–200 cells), intermediate (201–3000), and large (>3000 cells).

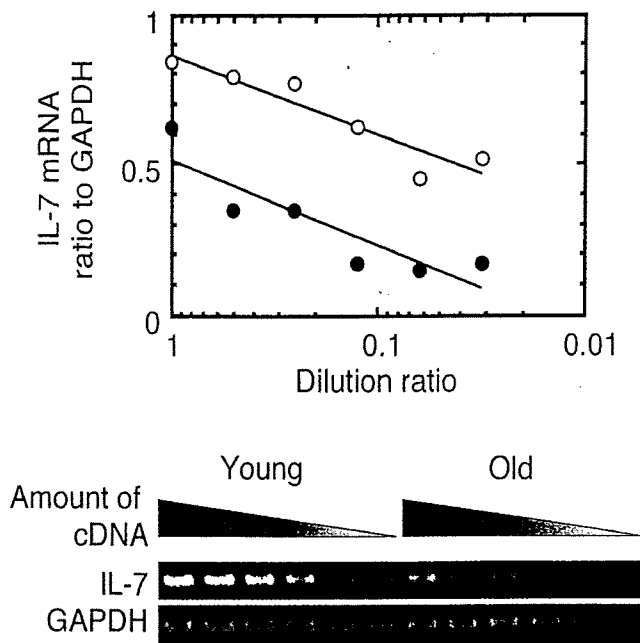


Figure 2. Expression level of interleukin-7 (IL-7) messenger RNA (mRNA) in bone marrow (BM) cells freshly isolated from young and old senescence-accelerated mice (mean \pm SEM of three replicate experiments). Vertical bars for SEM are within the symbols. Total RNA was extracted from BM cells using the TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The extracted mRNA was reverse transcribed using Superscript (Life Technologies, Grand Island, NY) and random hexamers. The reverse-transcribed complementary DNAs (cDNAs) are then amplified by polymerase chain reaction (PCR) using specific primers for murine IL-7 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The conditions and primer sequences used for the PCR amplification of cDNAs are shown in the "Materials and Methods" section of reference 15. The expected amplified PCR products were 496 and 982 base pairs long for IL-7 and GAPDH, respectively. In this experiment, GAPDH expressions were not altered among the experimental groups.

culture data, we propose a hypothesis that the CFU-pre-B inhibitory activity of BM cells may reside predominantly in young stromal cells rather than in senescent stromal cells. Because stromal cells produce BM-derived TGF- β (21) and are also a negative regulator of B lymphopoiesis (22–24), we investigated whether TGF- β production by stromal cells is reduced with age. Figure 4 shows percent changes in the number of the same seeded BM cells cocultured with young stromal cells (open circles) or senescent stromal cells (closed circles) in 96-well flat-bottomed trays after adding a graded dose of a neutralizing mAb to TGF- β . The high-

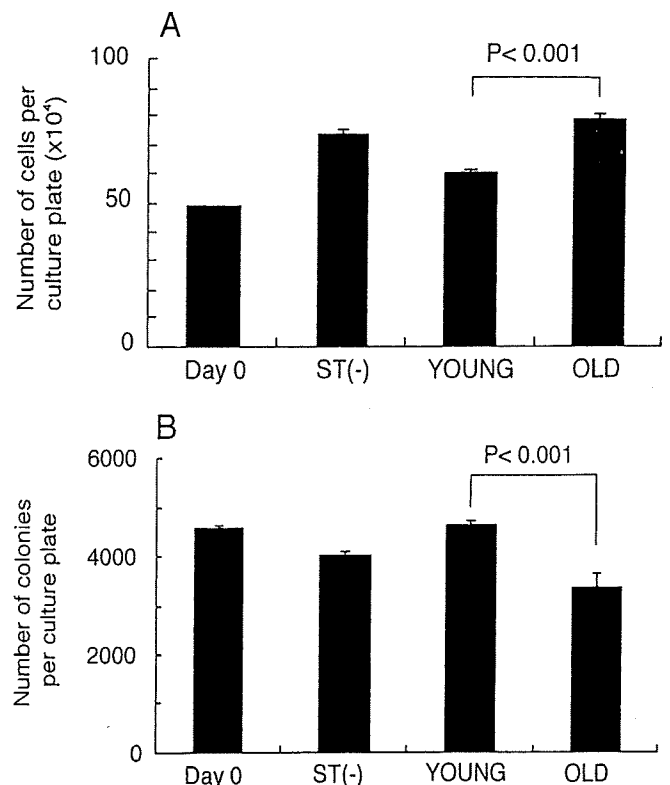


Figure 3. (A) Co-culture of pro-B/pre-B cell-enriched populations with stromal cells: change in the number of nonadherent cells (mean \pm SEM of three replicate experiments). (B) Co-culture of pro-B/pre-B cell-rich populations with stromal cells: change in total number of CFU-pre-B cells (mean \pm SEM of three replicate experiments). Stromal monolayers were prepared by culturing bone marrow (BM) cells from young or senescent senescence-accelerated mice (SAMs) at 1×10^6 /ml in 96-well Coster 3596 or 24-well Falcon 3047 flat-bottom plates in 0.2 or 1 ml of the RPMI 1640 medium supplemented with 20% fetal bovine serum. Confluent adherent layers were formed after 7 days. To obtain Pro-B/pre-B cell-rich populations (>10-fold) (i.e., interleukin-7 [IL-7]-responsive B220⁺, CD43⁺, IgM⁺, pro-B/pre-B cells), pooled BM cells from young SAMs stimulated with recombinant IL-7 were cultured, as described in the "Materials and Methods" section (4). Nonadherent cells were harvested and counted. Day 0 indicates nonadherent cell number at the beginning of co-culture; ST (-), nonadherent cell number after culture with IL-7 alone; YOUNG, nonadherent cell number after co-culture with young stromal cells in the presence of IL-7; OLD, nonadherent cell number after co-culture with senescent stromal cells in the presence of IL-7.

dose group (10 μ g/ml and more; data not shown) exhibited a toxic effect, but the lower-dose group showed a significant difference of responses between the young and senescent groups, implying that the proliferation of the senescent

Table 2. Secondary B Cell Colonies Derived From One Large Colony-Forming Unit B Cell Colony^a

| Donor mouse age (week) | Mean \pm SEM of triplicate experiments | | | | | |
|------------------------|--|---------------|---------------|---------------|--|---------------|
| | Large | Intermediate | Small | Cluster | No. of total colonies ^b (with cluster) ^c | % to a/% to b |
| 12 | ND | 1.4 \pm 0.6 | 5.8 \pm 0.7 | 7.2 \pm 0.6 | 7.2 \pm 0.6 (14.4 \pm 1.0) | 100%/100% |
| 30 | ND | ND | 2.3 \pm 0.5 | 7.4 \pm 1.7 | 2.3 \pm 0.5* (9.7 \pm 1.4)** | 24%/69% |
| 36 | ND | ND | ND | 0.4 \pm 0.2 | ND (0.4 \pm 0.2)** | —/2.7% |

^a Percentages for 30-week-old or 36-week-old mice compared with 12-week-old mice are shown in parentheses. ND, not detected.

^b Number of total colonies by large, intermediate, and small B cell colonies without clusters.

^c Number of total colonies with clusters.

* $P < 0.005$; ** $P < 0.01$.

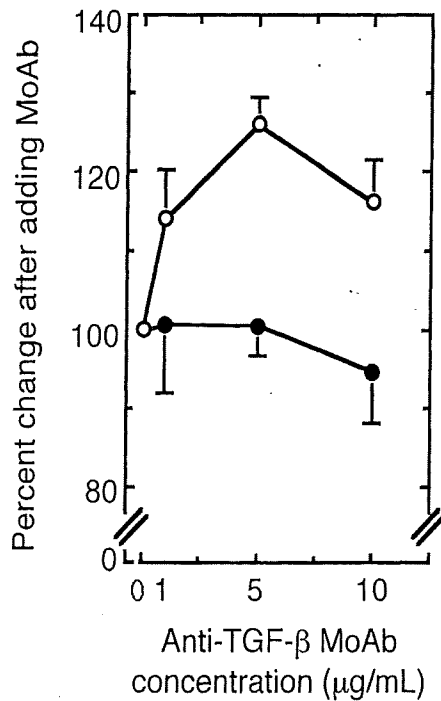


Figure 4. Effect of neutralizing anti-transforming growth factor- β (TGF- β) monoclonal antibody (mAb) on proliferation of pro-B/pre-B cells from young senescence-accelerated mice co-cultured with femoral stromal cells from young mice (open circles) and those from senescent mice (closed circles). The 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 IgG₁ isotype: R&D Systems, Inc., Minneapolis, MN) at dilutions ranging from 1–10 μ g/ml to the coculture system. The mouse IgG₁ 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).

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- μ g/ml doses (statistical significance, $P < 0.001$ in the group of 5 μ g/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

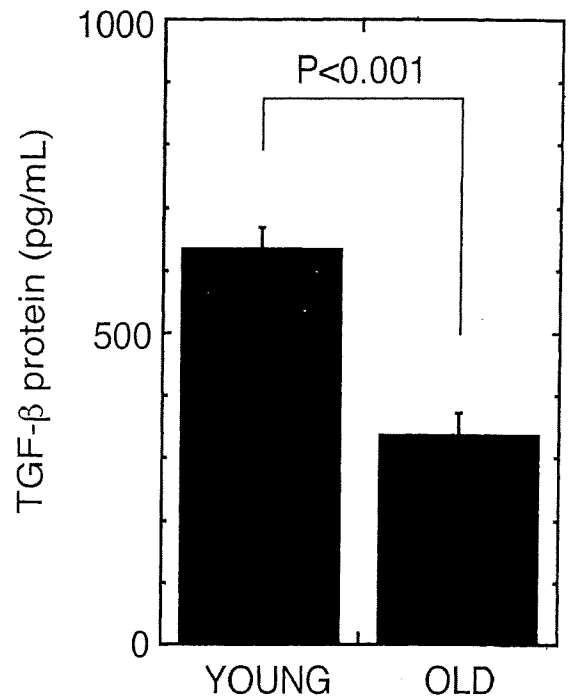


Figure 5. Transforming growth factor- β (TGF- β) protein level in the supernatant of cultured stromal cells from young and senescent senescence-accelerated mice (SAMs) (mean \pm SEM of three replicate experiments). Stromal monolayers were prepared by culturing bone marrow (BM) cells from young or senescent 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.

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.

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

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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 17 β -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 17 β -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.

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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 intermediate [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

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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 17 β -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 \pm 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 1 (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 cohoused 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 CO₂ 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 17 β -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 17 β -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 17 β -estradiol (Sigma Chem. Co., MO, USA) subcutaneously at a dose of 0.05 μ g/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 17 β -estradiol at 0.05 μ g/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 17 β -estradiol at 0.1 μ g/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 17 β -estradiol exposed group and the control group copulated and became pregnant. On day 18 of gestation, pregnant females were killed by CO₂ 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 17 β -estradiol were categorized as occupying four different intrauterine positions:

2M (the number of neonates in the 17 β -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 transcatheter 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 per-

formed 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 I

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 \pm 0.4 ^a | 5.6 \pm 0.3 | 5.7 \pm 0.4 | 5.2 \pm 0.3 | 5.4 \pm 0.3 | 5.3 \pm 0.4 |
| AGD | 2.43 \pm 0.22 | 2.42 \pm 0.22 | 2.42 \pm 0.28 | 1.21 \pm 0.20 | 1.23 \pm 0.19 | 1.22 \pm 0.24 |
| AGD/body weight | 0.43 \pm 0.04 | 0.42 \pm 0.04 | 0.42 \pm 0.05 | 0.23 \pm 0.02 | 0.22 \pm 0.02 | 0.23 \pm 0.02 |
| AGD/ $\sqrt[3]{\text{body weight}}$ | 1.36 \pm 0.12 | 1.36 \pm 0.14 | 1.35 \pm 0.18 | 0.69 \pm 0.08 | 0.70 \pm 0.07 | 0.70 \pm 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 \pm 1.5 | 11.2 \pm 1.5 | 10.8 \pm 1.1 | 10.4 \pm 1.4 | 10.3 \pm 1.1 | 10.4 \pm 1.3 |
| AGD | 4.57 \pm 0.54 | 4.41 \pm 0.48 | 4.43 \pm 0.51 | 2.00 \pm 0.22 | 1.99 \pm 0.19 | 2.00 \pm 0.21 |
| AGD/body weight | 0.42 \pm 0.06 | 0.40 \pm 0.05 | 0.41 \pm 0.03 | 0.19 \pm 0.04 | 0.19 \pm 0.03 | 0.19 \pm 0.04 |
| AGD/ $\sqrt[3]{\text{body weight}}$ | 2.06 \pm 0.22 | 1.99 \pm 0.19 | 1.99 \pm 0.19 | 0.92 \pm 0.12 | 0.91 \pm 0.11 | 0.92 \pm 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 \pm 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) ^a | 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 17 β -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 17 β -estradiol

| Treatment and intrauterine position | Corn oil | | | | 17 β -Estradiol | | | |
|-------------------------------------|-------------------------------|------------------|----------------|----------------|-----------------------|------------------|----------------|----------------|
| | 2M | OM | 2F | OF | 2M | OM | 2F | OF |
| 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 \pm 4.2 ^a | 53.6 \pm 4.4 | 40.3 \pm 2.7 | 41.2 \pm 3.4 | 55.1 \pm 5.1 | 53.1 \pm 6.2 | 41.8 \pm 2.1 | 42.1 \pm 3.1 |
| Testes (g) | 257.9 \pm 28.4 ^b | 266.5 \pm 23.5 | | | 259.3 \pm 25.5 | 260.3 \pm 19.9 | | |
| | 486.3 \pm 76.3 ^c | 484.2 \pm 71.3 | | | 488.3 \pm 62.3 | 479.1 \pm 60.9 | | |
| Epididymides (mg) | 89.5 \pm 8.7 | 92.2 \pm 9.3 | | | 94.6 \pm 7.1 | 93.1 \pm 7.1 | | |
| | 175.2 \pm 21.5 | 161.9 \pm 16.9 | | | 161.2 \pm 13.6 | 173.6 \pm 4.9 | | |
| Seminal vesicle (mg) | 413.9 \pm 30.6 | 452.2 \pm 13.9 | | | 431.3 \pm 18.2 | 454.8 \pm 21.0 | | |
| | 812.8 \pm 56.9 | 802.6 \pm 44.2 | | | 811.5 \pm 42.3 | 809.3 \pm 33.5 | | |
| Ovary (mg) | | | 15.3 \pm 3.3 | 14.6 \pm 4.2 | | | 15.5 \pm 3.9 | 15.3 \pm 4.5 |
| | | | 35.6 \pm 7.5 | 33.2 \pm 4.6 | | | 34.1 \pm 6.9 | 33.6 \pm 5.1 |

2M, male fetus between two male fetuses; OM, male fetus between two female fetuses; 2F, female fetus between two female fetuses; OF, 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 \pm 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 OF mice did not differ significantly in the age at vaginal opening, although 2F tended to exhibit vaginal opening at a slightly younger age than OF (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, OF 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 OF (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 17 β -estradiol: interaction with endogenous estradiol during pregnancy in mice

In the present study we examined the effect of 17 β -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 17 β -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.

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