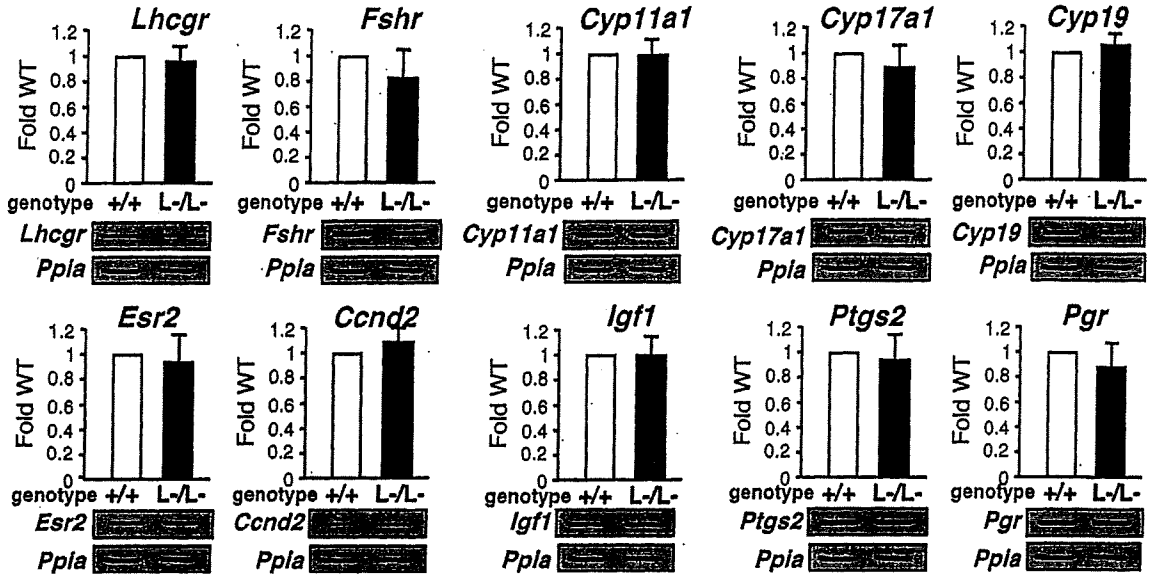

Fig. 3. No significant alterations in mRNA levels of several major regulators in folliculogenesis. Shown is semiquantitative RT-PCR of LH receptor (*Lhr*), FSH receptor (*Fshr*), p450 side chain cleavage enzyme (*Cyp11a1*), 17-α-hydroxylase (*Cyp17a1*), Aromatase (*Cyp19*), estrogen receptor-β (*Esr2*), cyclin D2 (*Ccnd2*), insulin-like growth factor 1 (*Igf1*), cyclooxygenase 2 (*Ptgs2*), or progesterone receptor (*Pgr*) gene expression in $AR^{+/+}$ and $AR^{-/-}$ ovaries. Results shown were representative (using one ovary per genotype in each experiment) of five independent experiments.

plasmid (Promega) using Lipofectamine reagent (GIBCO/BRL, Grand Island, NY) to normalize transfection. Results shown are representative of five independent experiments.

Results and Discussion

Subfertility of $AR^{-/-}$ Female Mice at 8 Weeks of Age. The *Ar* gene located on the X chromosome was disrupted in mice by using the Cre/Lox P system (6) (Fig. 1 *a–c*). Female $AR^{-/-}$ mice showed normal growth compared with the wild-type littermates (Fig. 1*d*), with no detectable bone loss (Fig. 1*e*) or obesity common for male $AR^{-/Y}$ mice (8, 9). Young (8-week-old) $AR^{-/-}$ females appeared indistinguishable from the wild-type littermates, displayed normal sexual behavior (7), and produced the first offspring of normal body size at the expected age. Macroscopic appearance of their reproductive organs, including uteri, oviducts, and ovaries, also appeared normal (Fig. 1*f*). Histological analysis showed no significant abnormality in the uterus or pituitary (Fig. 1*e*), whereas mammary ductal branching and elongation were substantially reduced, as revealed by whole-mount analysis (Fig. 1*h*). Serum levels of 17β-estradiol, progesterone, testosterone, luteinizing hormone, and follicle-stimulating hormone were also within normal range in 8-week-old mutant females at the proestrus stage (Fig. 1*g*), suggesting that the two-cell two-gonadotrophin system in female reproductive and endocrine organs (18) was intact in $AR^{-/-}$ mice at 8 weeks of age. The most obvious early sign of abnormal reproductive function in the $AR^{-/-}$ females was that their average numbers of pups per litter were only about half of those of the wild-type littermates, ($AR^{+/+}$, 8.3 ± 0.4 pups per litter; $AR^{-/-}$, 4.5 ± 0.5 pups per litter) (Fig. 1*i*).

$AR^{-/-}$ Female Mice Developed POF Phenotypes. Histological analysis of 8-week-old $AR^{-/-}$ ovaries clearly showed that numbers of atretic follicles were significantly increased, with decreased numbers of corpora lutea (Fig. 2 *b* and *f*). This finding suggests that the reduced pup numbers were due to impaired folliculogenesis in AR-deficient ovaries. Indeed, AR protein expression was readily detectable in the wild-type 8-week-old ovaries (Fig. 1*j*), with AR expressed at the highest levels in growing follicle granulosa cells at all developmental stages and at relatively low levels in corpora lutea. Thus, AR appears to play a regulatory role in granulosa cells during their maturation to the luteal phase.

To investigate this possibility, we examined the ovarian phenotype of female $AR^{-/-}$ mice at different ages. At 3 weeks, ovaries contain various stages of follicles, including primary, secondary, and antral follicles in wild-type animals (Fig. 2*a*) (19). In $AR^{-/-}$ ovaries at 3 weeks of age, the folliculogenesis appeared to be unaltered, with normal numbers and localization of primary and secondary follicles (Fig. 2 *a* and *e*). However, degenerated folliculogenesis became evident with further aging. Although follicles and corpora lutea at all developmental stages were still present, corpora lutea numbers were clearly reduced in 8-week-old $AR^{-/-}$ mutants (Fig. 2 *b* and *f*), similar to that observed in another mouse line (20). Expected apoptosis was seen in atretic follicles by activated caspase-3 immunohistochemistry assays (Fig. 2*i*). But, by 32 weeks of age, defects in folliculogenesis in $AR^{-/-}$ ovaries became profound, with fewer follicles observed and increased atretic follicles (Fig. 2 *c* and *g*), and >40% (5 of 12 mice) of the $AR^{-/-}$ females were already infertile. By 40 weeks, all $AR^{-/-}$ females became infertile, with no follicles remaining (Fig. 2 *d* and *h*); at the same age, $AR^{+/+}$ females were fertile and had normal follicle numbers. Consistent with progressive deficiency in folliculogenesis, the pup number per litter steadily decreased in aging $AR^{-/-}$ females (Fig. 2*i*). These data indicate that AR plays an important physiological role at the preluteal phase of folliculogenesis.

Alteration in Gene Expressions of Several Major Regulators Involved in the Oocyte–Granulosa Cell Regulatory Loop. To explore the molecular basis underlying the impaired folliculogenesis in $AR^{-/-}$ ovaries, we analyzed expression of several major known regulators and markers of folliculogenesis (21–23). Surprisingly, no significant alterations in mRNA levels of LH receptor (*Lhr*), FSH receptor (*Fshr*), p450 side chain cleavage enzyme (*Cyp11a1*), 17-α-hydroxylase (*Cyp17a1*), aromatase (*Cyp19*), estrogen receptor-β (*Esr2*), cyclin D2 (*Ccnd2*), or insulin-like growth factor 1 (*Igf1*) of 8-week-old $AR^{-/-}$ ovaries at the proestrus stage, and further cyclooxygenase 2 (*Ptgs2*) or progesterone receptor (*Pgr*) at the estrus stage, were detected by

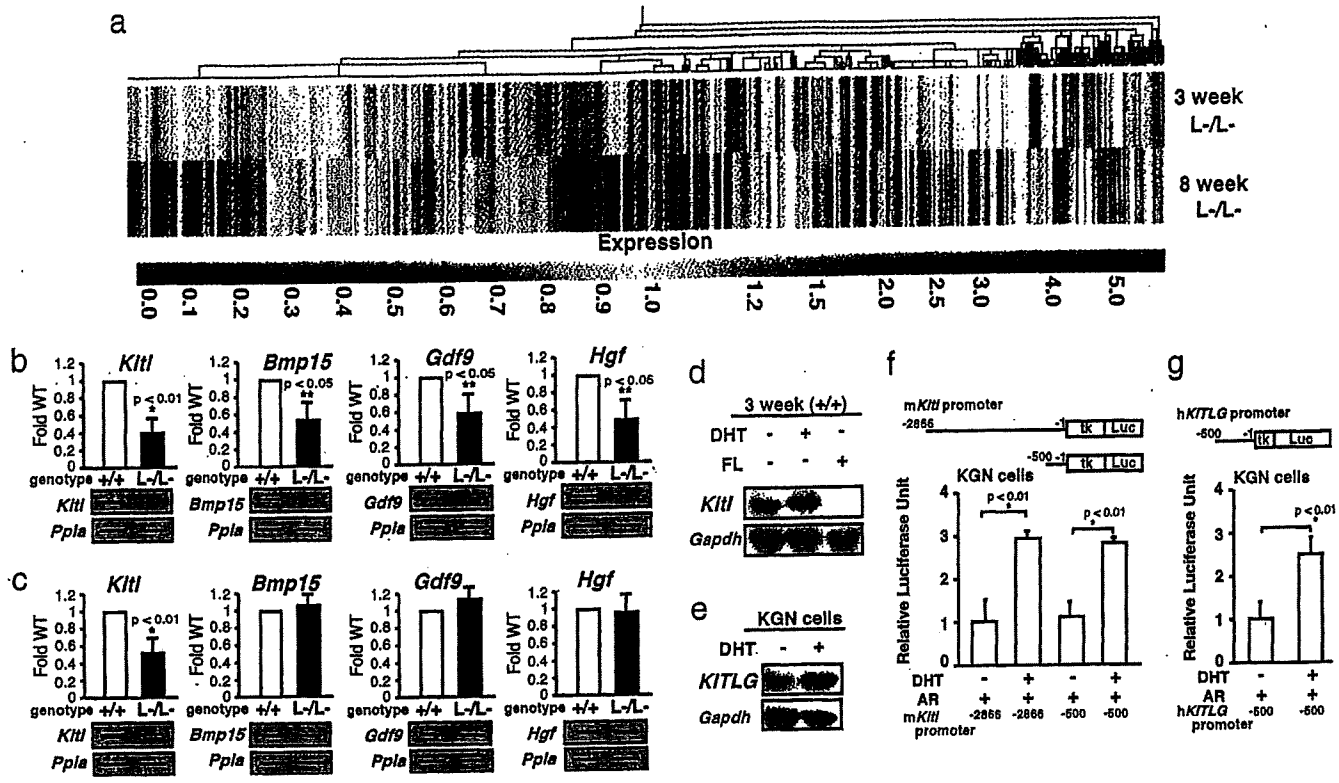

Fig. 4. Genome-wide microarray analysis and semiquantitative RT-PCR revealed that expression of the oocyte–granulosa cell regulator loop was down-regulated in $AR^{-/-}$ ovaries. (a) Microarray analysis of $AR^{-/-}$ compared with $AR^{+/+}$ ovaries at 3 and 8 weeks of age. Data obtained from microarray analysis as described in *Materials and Methods* were used to generate a cluster analysis. Each vertical line represents a single gene. The ratios of gene expression levels in $AR^{-/-}$ ovaries compared with wild type are presented. (*b* and *c*) Semiquantitative RT-PCR analysis of AR-regulated genes identified from the microarray study. Results shown are representative (using one ovary per genotype in each experiment) of five independent experiments. Data are shown as mean ± SEM and were analyzed by using Student's *t* test. (*d*) Comparison of *Kitl* gene expression by Northern blot analysis among placebo-, DHT-, and flutamide (FL)-treated $AR^{+/+}$ mouse ovaries. (*e*) Induction of *KITLG* gene expression by DHT treatment in KGN cells. (*f* and *g*) Androgen responsiveness in the mouse and human *kit ligand* promoters by a luciferase assay performed by using KGN cells. Data are shown as mean ± SEM and were analyzed by using Student's *t* test.

semiquantitative RT-PCR analysis (Fig. 3). Genome-wide microarray analysis (17) of RNA from 8-week-old $AR^{-/-}$ ovaries at the proestrus stage has been undertaken to identify AR-regulated genes. In comparison with $AR^{+/+}$ ovaries, expressions of 772 genes were down-regulated, whereas 351 genes were up-regulated in $AR^{-/-}$ ovaries (Fig. 4*a*; see also Tables 1 and 2, which are published as supporting information on the PNAS web site). Several genes known to be involved in the oocyte–granulosa cell regulatory loop (24) were identified as candidate AR target genes, including KIT ligand (*Kitl*) (25), morphogenetic protein 15 (*Bmp15*) (26), growth differentiation factor-9 (*Gdf9*) (27), and hepatocyte growth factor (*Hgf*) (28). Impaired folliculogenesis had been reported in mice deficient in each of these three regulators (26, 27, 29). To validate the microarray data, we performed semiquantitative RT-PCR analysis of 8-week-old $AR^{-/-}$ ovary RNA and confirmed that expression of these factors was down-regulated (Fig. 4*b*). To identify a regulator downstream of the AR signaling at an earlier stage of folliculogenesis, 3-week-old $AR^{-/-}$ ovaries that, as pointed out earlier, display no apparent phenotypic abnormality were examined. Fewer genes had altered expression levels (519 genes up-regulated; 326 genes down-regulated) (Fig. 4*a*; see also Tables 3 and 4, which are published as supporting information on the PNAS web site), and, of the four regulators tested by RT-PCR, only *Kitl* was found to be down-regulated at this age (Fig. 4*c*). Because *Kitl* is a granulosa cell-derived factor and stimulates oocyte growth and maturation (29–31), down-regulation of the *Kitl* expression in 3-week-old or even younger $AR^{-/-}$ ovaries may trigger impairment in folliculogenesis at a later age. To test for possible *Kitl* gene regulation by AR, 3-week-old wild-type females were treated with 5α-dihydrotestosterone (DHT). At 4 h after hormone injection, a clear induction of *Kitl* expression was observed in the ovaries, whereas a known antiandrogen flutamide attenuated the induction by DHT (Fig. 4*d*). The induction of endogenous human *kit ligand* (*KITLG*) gene by DHT was also observed in human granulosa-like tumor cells (KGN) in culture (Fig. 4*e*). Furthermore, androgen-induced transactivation of mouse and human *kit ligand* promoters (32) was observed by a luciferase reporter assay (33) in KGN (Fig. 4 *f* and *g*), 293T, and HeLa (data not shown) cells. However, no response to DHT was detected in the similar assay using promoters of the *Bmp15*, *Gdf9*, and *Hgf* genes (data not shown). Thus, we have shown that, in a regulatory cascade controlling folliculogenesis, *Kitl* represents a direct downstream target of androgen signaling.

As an upstream regulator, AR may also be indirectly involved in control of expression of other genes critical for folliculogenesis, because an age-dependent down-regulation of *Bmp15*, *Gdf9*, and *Hgf* gene expression was also observed in $AR^{-/-}$ ovaries. *Bmp15* and *Gdf9* are oocyte-derived factors that promote the development of surrounding granulosa cells in growing follicles (34, 35), whereas *Hgf* is secreted by theca cells and acts as a granulosa cell growth factor (36). Down-regulation of these factors, presumably due to decreased *Kitl* expression, may lead to impaired bidirectional communication between oocyte and granulosa cells (24) and, eventually, to early termination of folliculogenesis, as in POF syndrome.

Thus, we have identified AR as a novel regulator of follicu-

logenesis that apparently acts in the regulatory cascade upstream of the major factors controlling ovarian function, confirming the previous findings of the AR expression in granulose cells of growing follicles (3). Although not immediately relevant to the ovarian physiology, abnormal development of the mammary glands observed in our AR-deficient mice adds further strong evidence of an essential role of the AR not only in male, but also in female, reproductive function.

With increasing age of the first childbirth by women in the modern society, POF syndrome has become an important social and medical problem. Our findings suggest that POF syndrome may be caused by an impairment in androgen signaling and that X chromosomal mutations affecting the *AR* gene function may play a key role in hereditary POF. From clinical perspective, the present study provides evidence that AR can be a beneficial therapeutic target in treatment of POF syndrome patients.

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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*

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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 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.
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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 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).

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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 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 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 ± 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.

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Special Review

ゲノム毒性学
形質非依存型トキシコゲノミクスの導入

Toxicology in Genome Age: Introduction of Phenotype-independent Toxicogenomics

菅野 純　相﨑健一　五十嵐勝秀　小野 敦　中津則之
Jun Kanno, Ken-ichi Aisaki, Katsuhide Igarashi, Atsushi Ono, Noriyuki Nakatsu

形質非依存型トキシコゲノミクスに適用するため，マイクロアレイから細胞1個当たりのmRNA絶対量を得る方法（percellome）を開発した．これにより遺伝子発現量を，ゼロを起点とする均等目盛で表示し直接比較することができるようになった．そのため，今まで用いられてきたコントロールに対する比率表示と違って，割り算をする必要がなく，発現値ゼロの表示が自由に行え，コントロール群も処置群も同列に表示することが可能となった．また，さらなる標準化操作が原則的に不必要なため，測定したすべての遺伝子についてマイクロアレイ間はもとより，実験間での直接比較が行える．この特長は，生物学者が内容を直感的に把握しやすいようなデータの可視化にも役立ち，その後のデータ解析とインフォマティクス形成を促進することが示されつつある．異なったプラットフォーム間でのデータ互換にも拡張可能であり，トキシコゲノミクスに必要な大型データベースやコンソーシアム構築にも貢献する可能性が高い．

key words
マイクロアレイ技術，トキシコゲノミクス，分子毒性学，創薬支援，化学物質安全性評価

[i] 菅野 純　国立医薬品食品衛生研究所 安全性生物試験研究センター 毒性部　E-mail：kanno@nihs.go.jp
1985年東京医科歯科大学大学院医学研究科博士課程修了，医学博士（人体病理学，実験病理学専攻）．国立医薬品食品衛生研究所毒性部室長を経て，2002年より同部長．内分泌かく乱関連などの分子毒性学研究，トキシコゲノミクスプロジェクトなどを所掌業務との有機的連携のもとに推進．
相﨑健一，五十嵐勝秀，小野 敦，中津則之　国立医薬品食品衛生研究所 安全性生物試験研究センター 毒性部

はじめに

生物界（biosphere）は化学物質界（chemosphere）との相互作用の中で存在している．食べ物も薬も毒も，経口，吸入，あるいは皮下や血管内へ進入してきて生体分子と相互作用を起こす．この関係は，食物を選ぶ過程で例えば毒のあるものを避け，薬草を見いだすなど太古の昔から存在しているが，近代生活においてその複雑さが急速に増してきた．体内への直接的な摂取を目的としたものに加え，生活の利便性のために開発・利用される物質の増加が国民の安全と安心に係る問題として注目されている．しかし，それらの規制決定に係る毒性評価を生体側から見ると，身体に入るまでの“物質の分類”はもはや重要ではなく，むしろ身体に入った後にどのような反応がいかに惹起されるかが問題となる．

創薬の世界では，薬効のある物質を見つけ出すことが重要であり，次いで，その毒性が検討される．つい最近までは，“アマゾンに新しい植物を探しに行く”ことが主流であったが，近年は“何十万ものリード化合物のライブラリー”を充実させ，目的とする薬効を発揮する物質を，目的に合った方法でスクリーニングする方策がとられる．“薬”の毒性は，概念上，薬効の延長線上の毒性（例えば過剰インシュリンによる低血糖）と予期せぬ副作用（まさしく“side effect”）とがある．いずれにせよ，“薬”についてはcost-benefit（費用便益・費用対効果）の概念が強く働き，多少の毒性があっても使用することが少なくないし，患者側からの要望があればなおさらである．

本稿では，cost-benefitの対象を人（ヒト）に限定して話を進める．ヒトにおける毒性を検討するためには，ヒトからの情報が一番正確なことは言うまでもない．薬の開発の過程では，“臨床試験”なる“人体実験”が可能である．もちろん，ヒトに使っても薬効のもたらす利益よりも副作用たる毒性が十分に小さいであろうことを，各種の動物実験によって確かめてから，厳重な管理体制のもとで，かつ本人の了解を得たうえで“人体実験”に入るわけである．この場合の毒性には，用量作用関係の概念が乏しい．すなわち，実際に薬として投与するときの薬用量において，どのような毒性（副作用）が現れるかが，最大の焦点なのである．この貴重な人体実験でわざわざ生死に関わるような大量投与を行うことはない．また，薬効が期待できないような微量の投与も当然行わないわけである．

これに対して，いわゆる化学物質，例えば，家庭用品，工業製品，食品添加物などの現代生活の利便性に欠かせない物質に由来する化学成分の体内への侵入に対しては，一般

的にcost-benefitの概念が弱く働き，可能ならばゼロにしたいという傾向がある．しかし，“完全ゼロ”は使用する限り基本的には不可能であるので，どのくらいの量までなら安全と見なせるかを検討することが行われてきている．

これらの物質については，人体実験が倫理的にも現実的にもできないと考えるのが通常である（ボランティアを募ることができれば，それは可能かもしれないが，発癌性が疑われたり，蓄積性が高いもの，例えばダイオキシンやPCBのようなものは，いくらボランティアが名乗り出てくれても投与させてもらう気にはならないものである）．なお，薬でも“人体実験”が事実上できない対象がある．それは，胎児と子どもである．いずれの場合も，現在のところ，ヒトの身代わりとしてモデル動物を用いることになる．

Ⅰ．毒性における量と質の問題

では，どのくらいの量までなら安全と見なせるか．多量に摂取すれば毒性は強く，少量になれば毒性は弱まるという大原則（毒性は用量に関して単調増加する）のもとでは，“毒性に閾値がある”と考えられる場合と，“閾値が存在しない”と考えられる場合とで，扱いを分けている．前者の場合は無毒性量あるいは無作用量をラットなどの実験動物で求め，種差や個体差を勘案した係数（不確実係数あるいは安全係数と呼ぶ）で割って，安全の目安となる基準値とする．後者の場合は，無毒性量の代わりに，俗に“運悪く雷に打たれて死ぬ確率”を目安とする実質安全量（virtually safe dose，通常10^{-5}ないし10^{-6}の危険率を適用）を採用し，同様の手続きを経てヒトへの外挿を行っている．これらの判断が正しいか否かを検討する材料としてはヒトでの中毒事例，自殺事例，事故事例やそれらに関する疫学調査が活用され，それに基づく基準設定法の修正が折に触れて加えられてきた歴史がある（他方，化学物質の輸送や取り扱いに際した注意度を決めるために，毒物・劇物の指定が行われているが，これは，ラットなど単回曝露時のLD_{50}（半数致死量；動物の半数が14日以内に死亡する量）が低値のものを“危険度が高い”として規制するものである）．

それでは，毒性の質的な問題はどのように取り扱われてきたか．生物学が現象の記述学に基礎を置いていた段階での毒性学は，創薬の場にしろ，一般的な化学物質の毒性評価の場にしろ，その要求される役割を果たすために，投与された化学物質と症状との連関性に基づいた化学物質の体系化を基盤として発達してきた．その過程での様々な経験を取り入れる形で，前述の“不確実係数”や“LD_{50}”の概念が利用され，現在まで，非常に有効に機能してきている．ここまでの毒性学は，化学物質の投与とそれによる症状発現（毒性）の関連性をブラックボックスを介して分類し体系化するものであり，回帰モデル（regression model）の概念に根差した後向きの検討が行われることが多かった．しかし，サリドマイド禍（奇形発生）に代表されるようにげっ歯類の実験動物では毒性が確認されず，ヒトに使用して初めて催奇形性が明らかになった事例の存在は，この方法の限界を示している．

近年，科学の進歩により，毒性学は生体内で引き起こされる反応の分子レベルから形態レベルまでメカニズム記述を基礎とするものへと変貌しつつある．ここで，活躍するのがハイスループット性の高いマイクロアレイ技術である．しかし，マイクロアレイから得られた遺伝子発現プロファイルによる検討も，依然としてブラックボックスが介在している場合は，その時に観測される毒性形質と関連付け，いわゆる化学物質のフィンガープリント（指紋）として毒性反応の類型化を行うことが多い．このような関連付けを“phenotypic anchoring”と呼ぶことがある[1]．

Ⅱ．形質非依存型トキシコゲノミクス（phenotype-independent toxicogenomics）

これに対して，分子毒性学の立場から一番知りたいことは，生体内で実際に起こっている一連の事象であり，トランスクリプトーム（transcriptome）の場合にはすべての遺伝子の情報をもとにした遺伝子カスケードの全容解明である（図1）．これがわかれば，膨大な時間と費用の掛かる長期毒性試験（ラットなどを用いる）の代替として，より早く，安く，正確な評価，種差や個人差を勘案した正確なヒト毒性予測が可能となることが強く期待される．特に胎児，新生児，小児，成人，老人の各発達段階における生体側の反応様式・感受性の変化や，複数の物質の進入による複合作用なども包括的に扱えるようになると考えられる．すなわち，実験動物で得た所見をヒトに外挿する際に，実験動物のブラックボックスとヒトのブラックボックスを繋げる経験則が“不確実係数”であるが，これを責任遺伝子カスケードの解明によってバイパスする方策を得ることになる（図2）．これを実現させるためには，例えば，マウスにおいては遺伝子ノックアウト手法により遺伝子ごとの機能解析が可能であり，ヒトではSNPs解析が同様に利用できる．しかし，形質発現が伴わない場合には解析が行き詰まることが多い点で，これらは形質発現に依存的な手法である．全ゲノムが明らかになった現在，この目的のためには形質発現の有無にかかわらずすべての遺伝子の発現をモニターすることを目的としたアプローチを考慮せざるをえない．

さらに，創薬における毒性分野への要求の1つに，副作用による臨床段階での開発中止例，あるいは市販後の販売中止例の減少が挙げられる．すなわち，“動物実験では毒性が

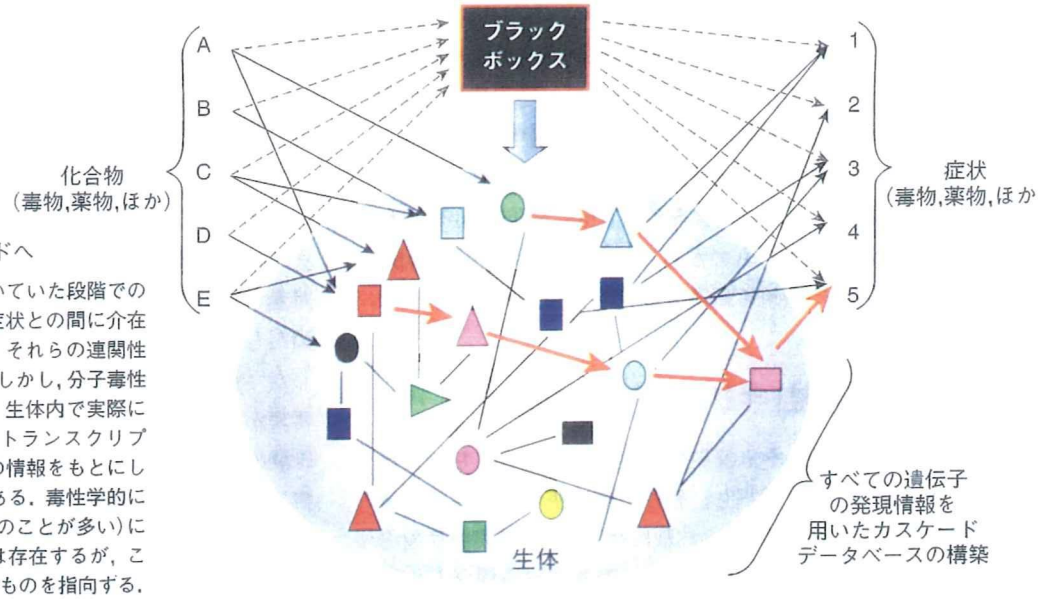

図1. リンケージからカスケードへ

生物学が現象の記述学に基礎を置いていた段階での毒性学は，投与された化学物質と症状との間に介在するブラックボックスを挟んでの，それらの連関性に基づいた体系化が行われてきた．しかし，分子毒性学の立場から一番知りたいことは，生体内で実際に起こっている一連の事象であり，トランスクリプトームの場合にはすべての遺伝子の情報をもとにした遺伝子カスケードの全容解明である．毒性学的に重要なマーカー遺伝子(数十～数百のことが多い)についてのこのようなデータベースは存在するが，ここではすべての遺伝子を対象としたものを指向する．

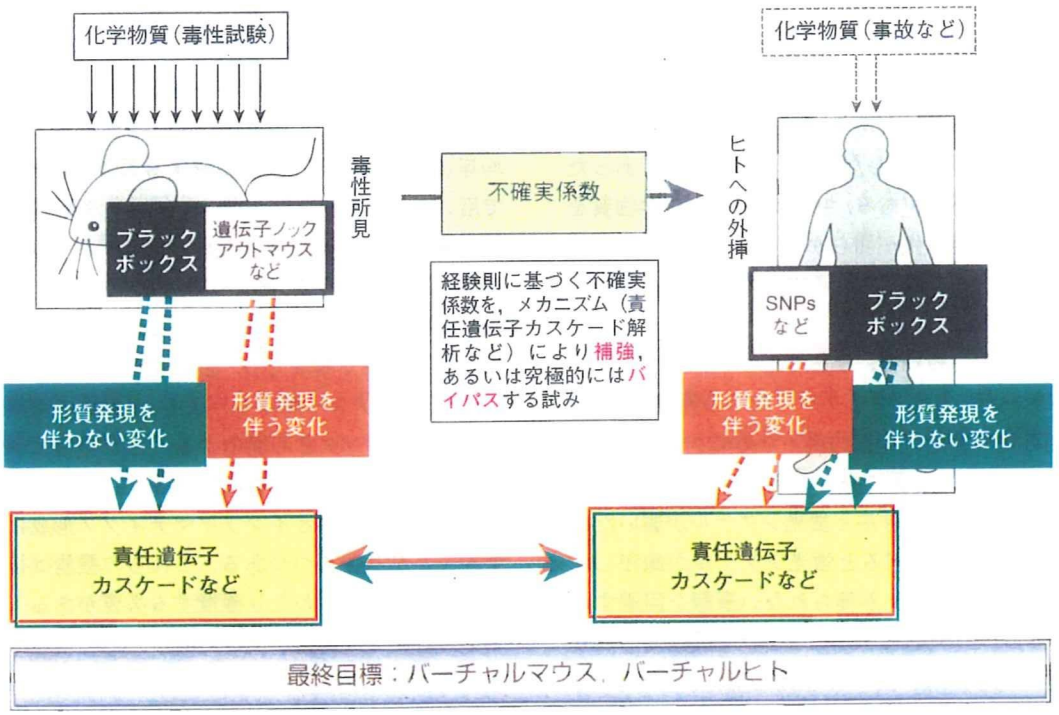

図2. 毒性評価法への分子毒性学の導入

毒性学の近代化のための分子毒性学の導入は，実験動物のブラックボックスとヒトのブラックボックスを繋げるために採用されている経験則ベースの“不確実係数”を，何らかのメカニズム解析により補強，あるいは究極的にバイパスすることを目的とする．その際，ブラックボックスの解明手段には例えば遺伝子ノックアウトマウスやヒトにおけるSNPs情報が活用できるが，いずれも形質発現を伴う場合にのみ有効に働く．形質発現と結び付かない部分については，網羅的な情報収集を行わざるをえないと考える．

なかったことから臨床試験に進んだところ，ヒトで毒性が現れ，開発中止となった．そのために何億円もの経費が無駄になった．このような事態を回避せよ”，言い換えると，動物で所見がなくともヒトでの毒性を予測することが要求されているわけである．サリドマイドはマウスには目に見える変化を起こさないかもしれないが，血管新生や免疫修飾など，種々の作用が誘導されることが報告されている．これは，マウスでは形質発現が明らかでなくとも，ヒトへの影響を予測する方策の存在の可能性を示している．

また，恒常性維持機構に深く関わる内分泌かく乱化学物質

の問題など，外界からの影響が効率良く中和されてしまい，形質変化がモニターしにくい対象を扱う場合にも，形質発現の有無にかかわらずmRNAやタンパク質の発現修飾を観測することが有効な影響解析手段となることが考えられる．

このように，今後の毒性学におけるトランスクリプトーム解析，すなわちトキシコゲノミクス（toxicogenomics）は，従来の“形質依存型”のものから“形質非依存型（phenotype-independent）”に発想を転換する時期に来ていると言えよう．

Ⅲ．形質非依存型トキシコゲノミクスの条件

形質依存型では，ある特定の毒性所見にリンクした遺伝子をマーカーとして選択し，それが毒性発現に重要であると認定することから始まる．これに対して，形質非依存型トキシコゲノミクスの特徴は，まずは形質発現情報などの情報を用いずに，自らの遺伝子発現プロファイル情報のみを頼りに遺伝子発現変化の解析を開始しようとする点にある．すなわち，ある毒性所見にリンクしたマーカー遺伝子を認定できないので，測定するすべての遺伝子はどれも平等に重要であると仮定する必要がある．そして，そのすべてがどれだけ発現増加したか，減少したか，あるいは不変であったかを正確に観測する必要がある．さらに，幾多の化学物質を検討した結果初めて全体像が明らかになるため，複数の実験の結果を長きにわたり集積し，それらのデータを縦横に解析する必要がある．

この条件を満たすためには，今までのマイクロアレイ手法には問題があった．まず，マイクロアレイの性能として，mRNAの測定可能な範囲が比較的狭いために1枚当たりに用いる総mRNA量を一定量に揃える必要があった点である．これはmRNAが少な過ぎると蛍光シグナルが弱いためにデータが得られず，多過ぎると蛍光シグナルが飽和してしまって定量性の良いデータが得られない事態を回避するための措置である．この場合，サンプル中の細胞1個当たりのmRNAの絶対的な多寡に関する情報は消失してしまう．このような相対的な情報でのサンプル間のmRNA発現の比較のために，種々の標準化手法が編み出されている[2)~10)]．原則的には，統計学的な有意差検定をもとにした変動遺伝子の抽出が行われる．このような計算に際しては，大半の遺伝子はサンプル間で不変であるとの前提が必要であり，その結果，多数の遺伝子が“変動したとは言えない”と位置付けられることとなる．また，変動の大きさを表現するためにコントロール群のサンプルに対して何倍変化したかを比率表示することが多い．この場合，コントロール群のサンプルでほとんど発現していない遺伝子は表示が困難となるばかりでなく，異なる時期に実施した複数の実験を比較する際に，コントロール群の実験間変動を吟味する情報が消失してしまうという問題が加わる．

Ⅳ．Percellomeとミルフィーユ・データ（millefeuille data）

このような問題を解決し，形質非依存型トキシコゲノミクスに適用するため，筆者らは，細胞1個当たりのmRNA絶対量を得る方法（Percellome）を，当時それに必要な条件を満たしていたアフィメトリクス社のGeneChipを対象に開発した（特許出願中，投稿中）．このシステムは大きく4つの要素から成っている．第1にRNA用に準備したサンプル破砕液のごく一部からそのDNA濃度を簡便に測定する方法，第2に用量関係を考慮し工夫されたスパイクRNA液の調製と，それの破砕液への添加法，第3にHill式に基づいた絶対化アルゴリズム，そして第4に，マイクロアレイの用量相関性能の検証や，バージョンが異なるマイクロアレイ間のデータ変換，ひいては，異なったメーカーのマイクロアレイ間のデータ変換に用いる標準サンプルセットとデータ変換アルゴリズムである．Percellomeデータは細胞1個当たりの絶対量であるので，各遺伝子の発現量をゼロを起点とする均等目盛りで表示し直接比較することが可能である．今まで用いられてきたコントロールに対する比率表示と違って，割り算をする必要がないため，発現値がゼロの場合の表示が自由に行えるうえ，何よりも，コントロール群も処置群も同列に表示することが可能となった．また，さらなる標準化操作が不必要であるため，測定したすべての遺伝子について，マイクロアレイ間はもとより，実験間の直接比較が可能となった．データを可視化することが非常に容易になったため，生物学者がその内容を直感的に把握しやすくなり，その後のデータ解析とインフォマティクス形成に大きく貢献することが示されつつある．これらの機能は複数の実験からの結果を長きにわたり蓄積する必要があるトキシコゲノミクス研究には重要なことである．また，後述するように異なったプラットフォーム間でのデータ互換にも拡張可能であり，共通の大型データベースやコンソーシアム構築にも貢献する可能性が高い．

1．方法の概略

(1) DNA測定

細胞1個当たりのmRNA情報を得るために，サンプルを構成する総細胞数を測定する．実際に細胞数を計測することは特に実質臓器の場合には困難なため，その代替指標として，細胞核内のゲノムDNA量を用いる．サンプルをDNA測定専用に消費することを避けるため，RNA調製用の組織破砕液のごく一部（通常，10 μl）をDNA測定に用いるプロ

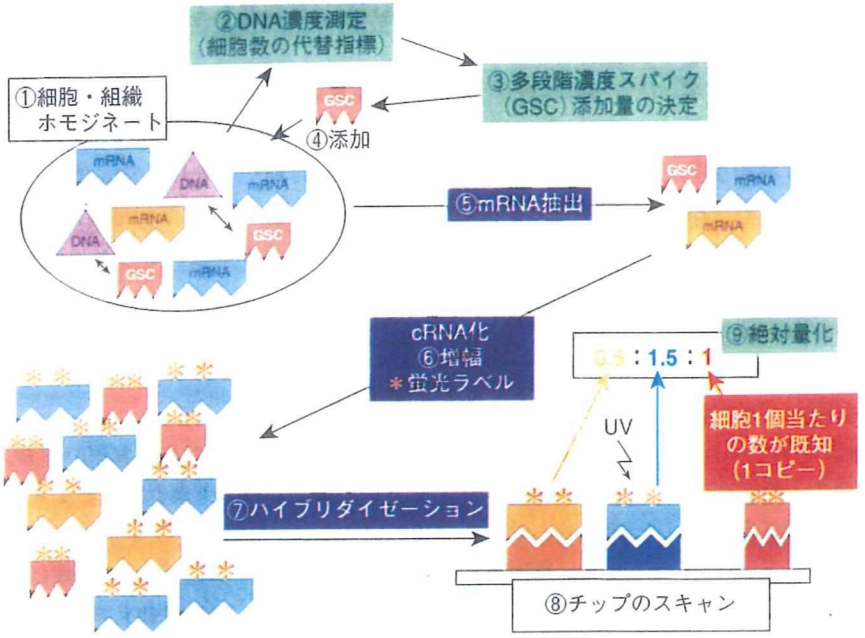

図3. 絶対量化の概略

絶対量化の原理は，①のサンプル・ホモジネートの細胞数をDNA量としてとらえ，細胞個数に比例した量の多段階濃度スパイクRNAカクテル（dose-graded spike cocktail；GSC）を添加する（②～④）．その後の⑤～⑧は通常の手順を踏む．⑧におけるGSCのシグナルが細胞1個当たりの既知コピー数を示している．測定したいサンプル中のmRNAの細胞1個当たりのコピー数はGSCのシグナル強度との比較から求めることができる（⑨）．GeneChipにおいては④のRNA濃度と⑧の蛍光強度の関係がHill式で記述できることを確認しており，それを用いた変換式により測定されたすべての遺伝子についてサンプルの細胞1個当たりの絶対量が導き出される．緑の囲み：本法で追加された手順．青の囲み：アフィメトリクスのプロトコール手順．

トコールを確立した．

(2) 多段階濃度スパイクカクテル（dose-graded spike cocktail；GSC）

細胞1個当たりのmRNAの標準として，組織破砕液に添加するスパイクRNAには，アフィメトリクス社のGeneChipが使用者のために用意していた5種類の枯草菌由来遺伝子のRNAを用いた．5種類の枯草菌RNAを各々約2,000塩基の長さに合成し，5段階の用量に配合したカクテルを作製した．これにより，広い濃度範囲をカバーする標準用量作用曲線をすべてのサンプルに導入することが可能となった．

(3) 絶対量化プログラム

アフィメトリクスGeneChipにより，蛍光シグナルとmRNA量との間にHill式に従う関係が成立することを後述のLBM標準サンプルなどにより確認した．その結果から，Hill式の直線化式によりGSCを直線化して絶対量化を行う変換アルゴリズムを開発し，それを自動実行するプログラムを独自に開発した．

(4) GeneChipの用量相関性確認およびバージョン間・プラットフォーム間データ変換対応のためのLBM (liver-brain mix) 標準サンプルおよびデータ変換アルゴリズム

遺伝子発現プロファイルが大きく異なる一対の組織を一定の比率で相互に希釈し合ったサンプルセットを表記の目的のために用意した．具体的には，肝と脳を用い，100：0，75：25，50：50，25：75，および0：100の混合比の5サンプルから成るセットを用意した．

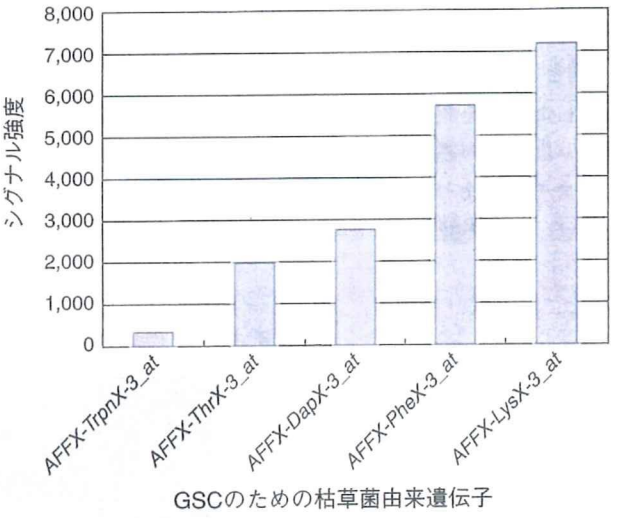

図4. 多段階濃度スパイクRNAカクテル（GSC）

GSCはグラフに示すように，低い値から高い値まで幅広い領域をカバーするように5種類の枯草菌mRNAを合成して5段階の濃度に混合したものである．これを適切に添加することによりすべてのサンプル中に細胞1個当たりの指標とmRNA検量線を導入している．その結果GeneChip 1枚ごとのデータの歪みを検出することが可能となり絶対量化の精度を格段に高める結果となっている．また，同様の理由で新旧のバージョン間や異なったプラットフォーム間のデータ変換の際にも，標準曲線として有効に機能する．

2. 絶対量化の原理

基本的原理は，サンプルの細胞数（ゲノムDNA濃度で代