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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.

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 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 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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AGE-RELATED HYPERMETHYLATION OF THE *hMLH1* PROMOTER IN GASTRIC CANCERS

Tomoko NAKAJIMA[1,2], Yoshimitsu AKIYAMA[1], Junichi SHIRAISHI[3], Tomio ARAI[4], Yuka YANAGISAWA[1], Miyuki ARA[1], Yoshiharu FUKUDA[5], Motoji SAWABE[4], Kiyoshi SAITOH[6], Ryuichi KAMIYAMA[2], Katsuiku HIROKAWA[3] and Yasuhito YUASA[1]*

[1]*Department of Molecular Oncology, Tokyo Medical and Dental University, Tokyo, Japan*
[2]*School of Allied Health Sciences, Tokyo Medical and Dental University Faculty of Medicine, Tokyo, Japan*
[3]*Department of Pathology and Immunology, Tokyo Medical and Dental University, Tokyo, Japan*
[4]*Department of Pathology, Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan*
[5]*Department of Health Promotion, Tokyo Medical and Dental University, Tokyo, Japan*
[6]*Department of Pathology, International Medical Center of Japan, Tokyo, Japan*

To determine whether methylation of the *hMLH1* promoter is related to increasing age and gastric carcinogenesis, we examined *hMLH1* methylation and expression in 100 gastric cancers. *hMLH1* methylation and aberrant protein expression were observed in 9 and 13 cancers, respectively. Normal and intestinal metaplastic tissues adjacent to cancers with hypermethylation did not exhibit any *hMLH1* methylation, indicating that it may be specific to gastric cancers. The frequency of *hMLH1* methylation significantly increased with age. These results suggest that *hMLH1* methylation plays an important role in gastric carcinogenesis in old people.

Key words: *aging; methylation,* hMLH1*; gastric cancer*

Gastric cancer is 1 of the most common malignancies worldwide and the leading cancer in several countries. Gastric cancer is generally classified into 2 histological types, differentiated (intestinal) and undifferentiated (diffuse).[1] The incidence of gastric cancers in old people has been increasing in some countries including Japan because of extension of the life span in the general population.[2] Many gastric cancers in old people are of the differentiated type.[3] The molecular mechanisms underlying gastric carcinogenesis including those in old people have not been clarified yet.

Aberrant CpG island methylation is a powerful mechanism for the inactivation of gene activity, and is observed in various cancers.[4] A correlation between aberrant DNA methylation of the *hMLH1* promoter and its reduced protein expression has been reported in colorectal and gastric cancers with microsatellite instability (MSI).[5–7] Several other gene promoters, such as *estrogen receptor* and *MyoD*, were hypermethylated in normal colonic mucosae, which has been linked to aging.[8, 9]

To determine whether or not methylation of *hMLH1* is related to increasing age and gastric carcinogenesis, we examined *hMLH1* methylation and expression in gastric cancers.

MATERIAL AND METHODS

Tissue samples

A total of 100 human primary gastric cancers was collected at Tokyo Metropolitan Geriatric Hospital and the International Medical Center of Japan. Patients were 63 male and 37 female with a mean age of 69.5 ± 11.9 (mean ± SD) years (range 43–99 years). Genomic DNAs of primary cancers were extracted from frozen or paraffin-embedded tissues as described previously.[10, 11]

Immunohistochemistry

Sections of formalin-fixed and paraffin-embedded primary cancer tissues were de-paraffinized in xylene and then re-hydrated in graded ethanol solutions. Immunohistochemistry (IHC) was carried out with an ENVISION system (DAKO Japan Co., Ltd., Kyoto, Japan).[12] Endogenous peroxidase activity was blocked by incubation with 0.03% hydrogen peroxide in methanol for 20 min. Antigen retrieval was accomplished by microwave irradiation. Slides were placed in 10 mM citric acid buffer and then in a microwave oven for 10 min. To block non-specific protein binding, sections were treated with 10% normal goat serum for 15 min. Monoclonal anti-hMLH1 antibody (clone G168-15; PharMingen, San Diego, CA) was diluted at 1: 75 and incubated for 12 hr at 4°C. Sections were treated with ENVISION labeled polymer reagent (DAKO) for 1 hr, and then incubated with 0.02% hydrogen peroxide and 0.6 mM 3, 3′-diaminobenzidine in phosphate-buffered saline. Sections were counterstained with hematoxylin and dehydrated in graded ethanol solutions, then cleared in xylene. Adjacent normal and intestinal metaplastic tissues were used as internal controls.

Combined bisulfite restriction analysis

Bisulfite treatment was performed using a CpGenome DNA Modification Kit (Oncor, Gaithersburg, MD) according to the manufacturer's instructions. The Combined bisulfite restriction analysis (COBRA) protocol was performed as described.[13] Bisulfite-modified DNA was amplified by nested PCR with specific primers for the *hMLH1* promoter. The primer sequences used for primary and secondary PCR amplification of *hMLH1* were as follows: primary PCR forward, 5′-AGTCGTTTTAGGGAGGGA(C/T)GAAG-3′; primary PCR reverse, 5′-CCGAATAACCCCTACCAC(A/G)AAC-3′; secondary PCR forward, 5′-TGT(C/T)GTTGAAGGGTGGGGTTGG-3′; and secondary PCR reverse, 5′-ACCTTCAACCAATCACCTCAATAC-3′.[14] Primary PCR was performed in 25 μl reaction mixtures, as follows: 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, 55°C for 2 min and 72°C for 1 min, and then a final 10 min extension at 72°C. Secondary PCR was performed in 25 μl reaction mixtures, as follows: 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, 60°C for 2 min and 72°C for 1 min, and then a final 10 min extension at 72°C. The PCR products were then digested with a specific restriction enzyme, *Rsa* I, for at least 2 hr and then electrophoresed on 15% polyacrylamide gels.[15,16]

Statistical analysis

Comparisons among continuous and categorical variables in hMLH1 expression and methylation status were made using the Mann-Whitney test, and the chi-square or Fisher's exact probabil-

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*Correspondence to: Department of Molecular Oncology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Fax: +81-3-5803-0125. E-mail: yuasa.monc@tmd.ac.jp

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FIGURE 1 – Immunohistochemical staining of hMLH1 in a representative cancer (PG17). The poorly differentiated adenocarcinoma portion (left side) did not express the hMLH1 protein, whereas the adjacent noncancerous cells (right side) showed nuclear hMLH1 staining. Original magnification ×50. Scale bar = 50 μm.

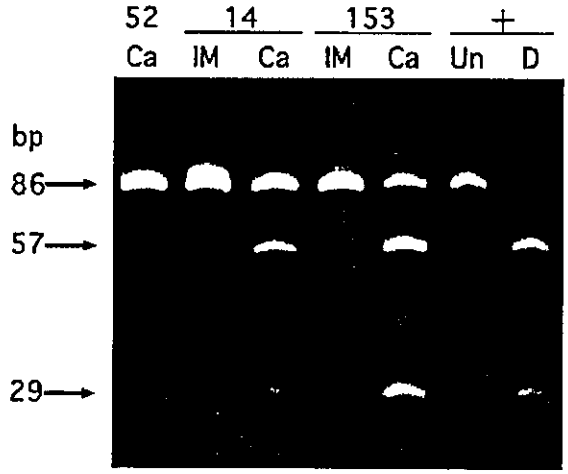

FIGURE 2 – COBRA of *hMLH1* promoter methylation in 3 representative gastric cancers. *Rsa* I only cleaves the methylated alleles, yielding 57- and 29-bp bands. IM, intestinal metaplasia; Ca, cancer; Un, undigested; D, digested. +denotes a positive control DNA methylated with *Sss* I methylase.

ity test. Trends in proportions as to age were assessed using the Cochran-Armitage test. Then, multiple logistic regression models were used to assess the independent association with parameters that exhibited significant differences in univariate analyses. Analyses were performed using SPSS version 7.5.1J ($p < 0.05$ was considered significant).

RESULTS

Expression of the hMLH1 protein

We examined hMLH1 protein expression in the 100 primary gastric cancers by staining paraffin sections with anti-hMLH1 antibody. Thirteen cancers (13%) showed no staining for the hMLH1 protein (Fig. 1), whereas the remaining 87 showed nuclear hMLH1 staining. All the adjacent normal and/or intestinal metaplastic tissues, including from 13 cases without hMLH1 expression, exhibited nuclear hMLH1 expression.

Methylation status of the hMLH1 *promoter*

To determine whether the absence of hMLH1 expression in cancers is due to methylation of the CpG sequence within the promoter or not, the methylation status of the *hMLH1* promoter was analyzed by means of the COBRA procedure. The region amplified on the secondary PCR corresponds to −133 to −48 in relation to the translational start site and contains 1 methylation-specific *Rsa* I site. Of the 100 gastric cancers investigated, 8 (8%) exhibited *hMLH1* methylation (Fig. 2).

To further analyze other CpG sites in cases without *hMLH1* methylation by the COBRA procedure, we examined the methylation status by sequencing the secondary PCR product, which contains 2 more CpG sites other than the *Rsa* I site.[14] Because the cancer DNAs were treated with sodium bisulfite, unmethylated cytosine was converted to thymine, whereas the methylated cytosine remained unchanged. As a result, only 1 cancer (PG 66) exhibiting aberrant hMLH1 expression showed a distinct methylation pattern in the 2 CpG sites (data not shown). However, the cytosine band at the *Rsa* I site in this tumor was very faint, which is consistent with the negative result by the COBRA procedure. Totally, *hMLH1* methylation was observed in the 9 cancers. *hMLH1* methylation was only found in cancers without protein expression (9/13, 69.2%), *i.e.*, not in cancers with normal expression (87 cases), demonstrating a significant correlation of transcriptional loss with *hMLH1* methylation ($p < 0.001$).

When we analyzed the 6 intestinal metaplastic tissues adjacent to the methylation-positive cancers, we did not detect any *hMLH1* methylation (Fig. 2). Neither of the 2 normal gastric tissue samples examined exhibited *hMLH1* methylation either.

Associations between hMLH1 *expression and methylation status, and clinicopathologic characteristics*

Table I illustrates the clinicopathologic characteristics of the gastric cancers[1] according to hMLH1 expression and the methylation status. Patients with cancers showing aberrant hMLH1 expression and methylation were significantly older than ones with cancers showing no aberrant phenotypes. When the ages of patients were stratified, the prevalence of aberrant hMLH1 expression and methylation increased significantly with age (Cochran-Armitage test for trend, $p = 0.015$ and $p = 0.028$, respectively) (Fig. 3).

A frequent location in the lower third of the stomach was significantly associated with aberrant hMLH1 expression, but marginally with methylation (Table I). The sizes of cancers without hMLH1 expression were significantly larger compared with ones showing normal expression. Because there was a large cancer without hMLH1 expression (19 cm × 19 cm), larger cancers without expression may be significant. This cancer, however, did not exhibit *hMLH1* methylation, indicating no size difference between the methylation-positive and -negative cancers (Table I).

Multiple logistic regression analysis revealed that age, size and location in the lower third were independently associated with aberrant hMLH1 expression (Table II). *hMLH1* hypermethylation was only found to be significantly associated with increasing age on multivariate analysis involving logistic regression.

DISCUSSION

Our study showed that aberrant hMLH1 protein expression and *hMLH1* methylation were observed in 13 and 9, respectively, of the 100 gastric cancers. Because *hMLH1* methylation was only detected in cancers exhibiting aberrant protein expression and all of the 9 cancers with hypermethylation exhibited strong MSI using 5 microsatellite markers (unpublished observation), transcriptional silencing through DNA methylation of the *hMLH1* promoter is the underlying cause of mismatch repair defects in some gastric cancers.

TABLE I - CLINICOPATHOLOGIC CHARACTERISTICS OF THE GASTRIC CANCER PATIENTS WITH REFERENCE TO hMLH1 IHC AND METHYLATION

Characteristics[1]	hMLH1 IHC			*hMLH1* methylation		
	Abnormal	Normal	*p*-value	Abnormal	Normal	*p*-value
Gender (male/female)	7/6	56/31	0.543[2]	5/4	58/33	0.722[2]
Age (years, mean ± SD)	78.0 ± 12.4	68.2 ± 11.4	0.010[3]	80.6 ± 11.8	68.4 ± 11.4	0.009[3]
Size (cm, mean ± SD)	9.2 ± 4.2	6.8 ± 4.1	0.015[3]	8.5 ± 3.5	7.0 ± 4.2	0.094[3]
Tumor location						
Upper third	2	20		1	21	
Middle third	1	37	0.011[2]	1	37	0.051[2]
Lower third	10	30		7	33	
Macroscopic type						
Type 1	0	3		0	3	
Type 2	8	27		5	30	
Type 3	2	30	0.251[2]	2	30	0.416[2]
Type 4	1	13		0	14	
Type 5	1	2		1	2	
Depth of tumor invasion						
SM	1	7		1	7	
MP	3	13		2	14	
SS	0	18	0.201[3]	0	18	0.579[3]
SE	5	44		4	45	
SI	4	5		2	7	
Lymph node metastasis (+/−)	10/3	57/29	0.317[2]	7/2	60/30	0.394[2]
Liver or peritoneal metastasis (+/−)	0/13	4/83	0.568[2]	0/9	4/87	0.682[2]
Histological type						
Differentiated	5	46		3	48	
Undifferentiated	8	41	0.251[2]	6	43	0.224[2]
Tumor infiltration						
INF α	2	9		1	10	
INF β	8	43	0.148[2]	6	45	0.120[2]
INF γ	1	33		0	34	
Lymphatic invasion (+/−)	12/1	71/16	0.306[2]	8/1	75/16	0.525[2]
Venous invasion (+/−)	9/4	66/21	0.416[2]	6/3	69/22	0.399[2]

[1]The clinicopathologic characteristics of gastic cancers were classified according to the Japanese classification.–[2]Chi-square test or Fisher's exact probability test.–[3]Mann-Whitney test.

All the adjacent normal tissues examined revealed normal hMLH1 expression and no *hMLH1* methylation, which is consistent with previous reports.[16,17] Moreover, all the examined intestinal metaplastic tissues adjacent to cancers showing aberrant hMLH1 expression exhibited normal protein expression and an unmethylated status. Therefore, hypermethylation of *hMLH1* may be specific to gastric cancer but not to intestinal metaplasia. Nevertheless, these results do not necessarily exclude the possibility that the methylated state of *hMLH1* might be present in the noncancerous tissue. The methylation, however, may occur in only a minor fraction of cells, which is not detectable by the assays used.

The frequencies of aberrant hMLH1 expression and hypermethylation increased with age. The cancer patients of 81 years of age or more exhibited higher frequencies of aberrant hMLH1 expression (6/22, 27.3%) and hypermethylation (4/22, 18.2%), whereas ones of 60 years of age or less exhibited frequencies of 4.2% (1/24) and nil, respectively. Multivariate analysis involving logistic regression revealed that age was independently associated with *hMLH1* hypermethylation. These results suggest a strong relationship of *hMLH1* methylation to increasing age. It was reported that *hMLH1* belonged to type C (cancer-specific) of CpG island methylator phenotype.[15] Our results, however, suggest that *hMLH1* methylation is not only cancer-specific but also age-related. This may be explained by the fact that we analyzed more cancers in old people. It is likely that *hMLH1* methylation plays an important role in gastric carcinogenesis in old people.

Gastric cancers in very old people mostly develop as the differentiated type and are frequently located in the lower third of the stomach.[3] It has also been reported that gastric cancers with the

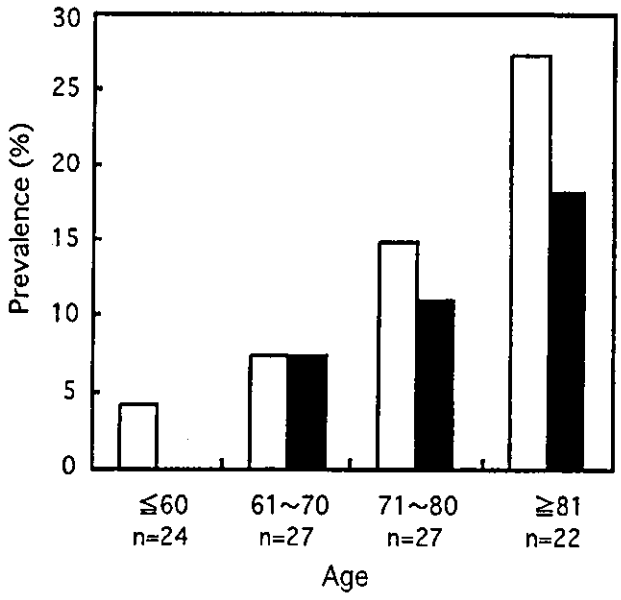

FIGURE 3 – Frequencies of the absence of hMLH1 protein expression (open bars) and hypermethylation (closed bars) in gastric cancers stratified as to age (years).

TABLE II – LOGISTIC REGRESSION ANALYSIS FOR FACTORS ASSOCIATED WITH hMLH1 IHC AND METHYLATION IN GASTRIC CANCER PATIENTS

	hMLH1 IHC			*hMLH1* methylation		
	OR[1]	95% CI[2]	*p*-value	OR	95% CI	*p*-value
Age	1.08	1.01–1.15	0.026	1.10	1.02–1.20	0.019
Size	1.23	1.03–1.47	0.023			
Location[3]	9.01	1.67–48.6	0.011	4.78	0.88–25.9	0.070

[1]Odds ratio.–[2]Confidence interval.–[3]The lower third/not lower.

severe MSI phenotype showed higher frequencies of the differentiated type and lower third location.[18, 19] These reports coincide with our present data as to a frequent lower third location of cancers with aberrant hMLH1 phenotypes. The frequencies of aberrant hMLH1 expression and hypermethylation, however, were low in differentiated type cancers in this study. Thus, there was no significant difference in the frequencies of hMLH1 aberrations between differentiated type and undifferentiated type cancers. Pathological analysis suggested that gastric cancers in very old people principally develop as the differentiated type, and then progress to the undifferentiated type with time.[3] Because most of our cases with aberrant hMLH1 expression contained portions of the differentiated type, some of these cancers might have also started as the differentiated type.

Our results suggest that there may be a distinct type of gastric cancer in old people, which exhibits higher frequencies of aberrant *hMLH1* methylation and expression. It remains unknown why CpG island methylation of *hMLH1* occurs in some gastric cancers and increases with age. Recently, late onset of cancers with hypermethylated *hMLH1* gene has been also reported in the colorectum,[20,21] but the mechanism has not been explained, either. It has been reported that the DNA methylation of several genes including *hMLH1* was not associated with overexpression of 1 of 3 DNA methyltransferases, *DNMT1*, *DNMT3A* and *DNMT3B*.[22] An imbalance between DNA methyltransferase and demethylase activities may be responsible for *hMLH1* hypermethylation.[23] Further studies are necessary to clarify the mechanism underlying *hMLH1* hypermethylation and its role in gastric cancer development in old people.

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Estrogen receptors in atherosclerotic human aorta: inhibition of human vascular smooth muscle cell proliferation by estrogens

Yasuhiro Nakamura [a,b,*], Takashi Suzuki [a], Yasuhiro Miki [a], Chika Tazawa [a], Kumiko Senzaki [a], Takuya Moriya [a], Haruo Saito [b], Tadashi Ishibashi [b], Shoki Takahashi [b], Shogo Yamada [b], Hironobu Sasano [a]

[a] *Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan*
[b] *Departments of Radiology, Tohoku University School of Medicine, Sendai, Japan*

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Abstract

Estrogen has been postulated to exert direct anti-atherogenic effects via binding to estrogen receptors (ERs) in vascular smooth muscle cells (VSMCs). Therefore, we believe it is important to examine the status of ER expression in the human cardiovascular system and its disorders. In this study, we first evaluated the relative abundance of messenger RNA (mRNA) of both ER subtypes (ERα and ERβ) in the human aorta using reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR). We then examined the immunolocalization of both ERs in VSMCs of human atherosclerotic lesions. In order to examine which ER subtype was associated with the anti-atherogenic effects of estrogen, we examined the effects of estrogen in two VSMC cell lines, one positive only for ERα and the other positive only for ERβ. The relative abundance of mRNAs for both ERs was higher in female aorta with a mild degree of atherosclerosis than in female aorta with a severe degree of atherosclerosis ($P < 0.05$). In addition, the number of ERα and/or ERβ double positive cells in the neointima was higher in female aorta with a mild degree of atherosclerosis than in female aorta with severe atherosclerosis ($P < 0.05$). Our in vitro study found that estradiol was able to significantly inhibit the proliferation of ERα positive VSMCs but not ERβ positive VSMCs ($P < 0.05$). Moreover, estradiol was found to significantly suppress proliferating cell nuclear antigen (PCNA) mRNA levels in ERα positive VSMCs compared to that of ERβ positive VSMCs, consistent with the findings of cell proliferation. Results from this study suggest that estrogens can inhibit the proliferation of VSMCs through ERα, especially in pre-menopausal women. Our study also indicates that decreased levels of ER, especially ERα, in the female atherosclerotic neointima may be associated with progression of atherosclerotic changes.

Keywords: Atherosclerosis; Estrogen receptor; Vascular smooth muscle cells

1. Introduction

Various epidemiological studies have reported a relatively low incidence of cardiovascular disease (CVD) in pre-menopausal women whereas in the post-menopausal period women have been reported to have a marked increase in CVD (Glendy et al., 1937). Estrogen has, therefore, been proposed as a cardioprotective agent, especially in women (Stampfer et al., 1991). On the other hand, the significance of hormone replacement therapy (HRT) has remained controversial because recent randomized controlled trials failed to show a protective effect of HRT in reducing the risk of coronary artery disease and instead revealed an increased risk of heart disease, stroke, invasive breast cancer and venous thrombo-embolism (Hulley et al., 2002; Miller et al., 2002; Simon et al., 2001; Viscoli et al., 2001). However, results of several experimental, and clinical and epidemiological studies have also demonstrated that estrogen is predominantly involved in the suppression of development of atherosclerosis in various animal models, suggesting that estrogens may have direct anti-atherogenic effects on the cardiovascular system (Barrett-Conner and Bush, 1991; Hodgin et al., 2001; Sullivan et al., 1995). In addition to various systemic effects such as changes in plasma lipid profiles, estrogens have been suggested to exert direct anti-atherogenic effects through an initial interaction with

* Corresponding author. Tel.: +81-22-717-8050; fax: +81-22-717-8051.
E-mail address: nakamura@patholo2.med.tohoku.ac.jp (Y. Nakamura).

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the estrogen receptor (ER) in VSMCs (Song et al., 1998). Therefore, it is considered important to examine the status of ER expression in the human cardiovascular system and its disorders.

Results of recent studies have demonstrated that there are two subtypes of ER, ERα and β (Kuiper et al., 1996; Kuiper et al., 1997). Both receptor isoforms possess high conservation of amino acid sequences in the region of the hormone binding domain, an area known to be important in binding with its ligand (Kuiper et al., 1996; Kuiper et al., 1997). Several groups of investigators have reported the presence of ERα and β in the vascular wall, especially in VSMCs, of the aorta and other arteries in humans and various experimental animals (Brouchet et al., 2001; Linder et al., 1998; Sudhir et al., 1997). Expression of ER subtypes, however, has not been examined in detail in the human vascular system associated with atherosclerotic changes, especially in the neointima. Therefore, in this study, we first examined the relative level of ER mRNA expression using reverse transcription followed by real-time quantitative polymerase chain reaction (RT/real-time qPCR) and ER protein localization by immunoreactivity in the human aorta, and then correlated these findings with the degree of atherosclerosis, sex, and other features of the patient history. Several studies have been published that indicate important physiological roles for ERα and ERβ in the cardiovascular system (Farhat et al., 1996; Rubanyi et al., 2002). The present study examined whether ERα and/or ERβ may have been involved in the vascular protective effects of estrogen in human VSMCs. In this study, we subsequently examined the effects of estrogens on two cell lines derived from human VSMCs, one positive only for ERα and the other positive only for ERβ in order to study the importance of ligands binding to ERα and/or ERβ and their potential role in protecting the human vascular system from CVDs, such as atherosclerosis.

2. Materials and methods

2.1. Specimens

Human abdominal aortas were collected at the time of autopsy from deceased patients who had not received any prior hormone therapy. All autopsies were performed at Tohoku University Hospital, Sendai, Japan, within 3 h post-mortem from 39 subjects (17 male, 22 female, mean 57.7 ± 3.8 years old). The Ethics Committee at Tohoku University School of Medicine approved the research protocol for this study. The classification of atherosclerosis defined by the American Heart Association in 1995 (Stary et al., 1995) was employed in this study in order to quantify the degree of atherosclerotic change in each specimen. We evaluated immunohistochemical findings of the neointima and media separately because of differences in the biological features between VSMCs present in the neointima and media, as was proposed by previous reports (Ross, 1993; Villaschi et al., 1994). For RT/real-time qPCR studies, we could examine only whole aortas due to the difficulty incurred in trying to separate the neointima from the media by macroscopic examination. Aortic specimens were tentatively classified into the following five groups, A_1, A_2, A_3, A_4, and A_5 based on the sex of the deceased patient, degree of atherosclerosis, and status of menstruation (Group A_1: male, normal or mild atherosclerosis, corresponding to group I to III in the AHA classification; Group A_2: male, advanced atherosclerosis, corresponding to group IV to VI in the AHA classification, Group A_3: pre-menopausal female, normal or mild atherosclerosis, Group A_4: post-menopausal female, advanced atherosclerosis, and Group A_5: post-menopausal female, mild atherosclerosis). The distribution of the cases among these groups is summarized as follows: A_1, 9 cases; A_2, 8 cases; A_3, 6 cases; A_4, 9 cases; and A_5, 7 cases (Table 1). In addition, 28 fresh tissue specimens (8 male, 20 female, mean 55.1 ± 4.7 years old) were available for RT/real-time PCR studies (A_1, 4 cases; A_2, 4 cases; A_3, 6 cases; A_4, 8 cases; and A_5, 6 cases) (Table 1). These specimens were immediately frozen in liquid nitrogen and stored at −80 °C until further use.

2.2. Immunohistochemical staining

Antibodies used in this study were as follows: monoclonal antibody for ERα was purchased from Novocastra Laboratories (Newcastle, UK), and rabbit polyclonal antibody for ERβ was obtained from Upstate Biotechnology (Lake Placed, NY, USA). The dilutions of primary antibodies were 1:50 for both ERα and ERβ.

Table 1
Subjects for immunohistopathology and RT/real-time PCR analysis

Group	Sex	Atherosclerosis	Mense	*n*	Age	*n*	Age
A_1	Male	Mild[a]	−	9	41.2 ± 8.1	4	42.5 ± 15.6
A_2	Male	Severe[b]	−	8	69.4 ± 2.4	4	67.5 ± 7.0
A_3	Female	Mild	+	6	25.0 ± 5.6	6	25.2 ± 7.2
A_4	Female	Severe	−	9	74.7 ± 2.9	8	71.0 ± 4.6
A_5	Female	Mild	−	7	71.9 ± 4.0	6	64.2 ± 6.3
Total				39	57.7 ± 3.8	28	55.1 ± 4.7

[a] Mild atherosclerosis means the samples which belonged to group I to III in the AHA classification.
[b] Advanced atherosclerosis means the subjects which belonged to group IV to VI in the AHA classification.

Details of immunohistochemical procedures have been previously described by Sasano et al. (Sasano et al., 1996). All thin sliced sections were immunostained by a biotin-streptavidin method. As a positive control, breast carcinoma tissue was used for ERα (Linder et al., 1998) and normal mammary gland tissue was used for ERβ immuno-staining, respectively (Linder et al., 1998). Furthermore, as a negative control, primary antibody was replaced with 0.01 M PBS. No specific immunoreactivity was detected in negative control samples. We utilized double immunostaining with DAB and Vector-blue for ERs and α-smooth muscle actin (α-SMA) (Data Corporation, Carpinteria, CA), respectively, to further characterize ER positive cells in the human aorta of immunopositive cases (Mori et al., 1997). In addition, we used a monoclonal antibody against CD34 antigen for endothelial cells (Nichirei, Tokyo, Japan) and CD68 antigen for macrophages (Dako Corporation) in adjacent tissue sections to examine whether these proteins were expressed in these cells. We performed H-score, a quantitative value that evaluates the intensity as well as the number of immunoreactive ERα and ERβ cells present in VSMCs of the aortic neointima and media (McCarty et al., 1985). After determining the areas of evaluation by simultaneous observation using a multi-headed light microscopy, three authors (T.S., Y.M., and H.S.) independently evaluated 100 VSMCs. When inter-observer differences were <5%, the mean value was determined as the H-score. When inter-observer differences were greater than 5%, the three aforementioned authors above re-evaluated the discrepant immunostained slides simultaneously using a multi-headed light microscope, after which the mean value was obtained.

2.3. cDNA synthesis and real-time PCR

Total RNA was extracted by homogenizing frozen tissue samples in 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA) followed by a phenol-chloroform phase extraction and isopropanol precipitation. All RNA samples were quantified by spectrophotometry and stored at −80 °C until processed for reverse transcription (RT). The SUPERSCRIPT Preamplification system RT kit (Gibco-BRL, Grand Island, NY) was employed in the synthesis and amplification of complementary DNA (cDNA). cDNA was synthesized from total RNA (2 μg) using 25 ng/μl oligo $(dT)_{12-18}$ primer (Life Technologies Inc., Gaitherburg, ND) on a PTC-200 Peltier Thermal Cycler DNA Engine (MJ Research Inc., Watertown, MA). To test for the presence of genomic DNA contamination, we performed the RT step in the absence of SUPERSCRIPT™ II RNase H⁻ reverse transcriptase (Gibco-BRL) followed by PCR. RT-PCR products lacking reverse transcriptase in the initial RT step were run on an ethidium-bromide stained 2% agarose gel. No band was observed in these samples (data not shown). The resulting cDNA was used as a template for real-time PCR. Real-time PCR was carried out with the Light Cycler System (Roche Diagnostics GmbH, Mannheim, Germany) using the DNA binding dye SYBER Green I (Roche Diagnostics GmbH) for the detection of PCR products. Primers are summarized in Table 2. Details of the protocol used to quantify PCR products with the LightCycler real-time thermal cycler have been previously described by Nakamura et al. (Nakamura et al., 2003). As a positive control, T-47D human breast cancer cells were used for ERα and ERβ (Vladusic et al., 2000). Negative control experiments lacked cDNA substrate to check for the presence of exogenous contaminant DNA. No amplified products were observed under these conditions. The mRNA levels for both Ers in each case are summarized as a ratio of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and evaluated as a ratio (%) compared with that of each plasmid cDNA, described later.

2.4. Synthesis of both ERs and GAPDH plasmids

To determine the quantity of target cDNA transcripts, cloned plasmid DNAs for both ERs and GAPDH were used to generate standard curves for real-time quantitative PCR. The cDNAs for both ERs and GAPDH were amplified by conventional PCR using reverse transcribed total RNA from T-47D cells. PCR products were cloned into the pGEM-T Easy vector. The plasmids were purified by a standard miniprep, and the respective inserts released from the vector with a single *EcoRI* digest and subjected to direct sequencing to verify amplification of the correct sequences.

Table 2
Primer sequences used in RT/real-time PCR analysis

cDNA	Sequence	Size (bp)	Reference
ERα	Forward 5′-AAGAGCTGCCAGGCCTGCC-3′ Reverse 5′-TTGGCAGCTCTCATGTCTCC-3′	167	Pujol et al. (1998)
ERβ	Forward 5′-GCTCAATTCCAGTATGTA-3′ Reverse 5′-CCTGGTGTAAAAACGTGA-3′	241	Moore et al. (1998)
GAPDH	Forward 5′-TGAACGGGAAGCTCACTGG-3′ Reverse 5′-TCCACCACCCTGTTGCTGTA-3′	307	Tokunaga et al. (1987)
PCNA	Forward 5′-TCTCAGCCATATTGGAGATG-3′ Reverse 5′-CAGGTACCTCAGTGCAAAAG-3′	186	Sasaki et al. (2002)