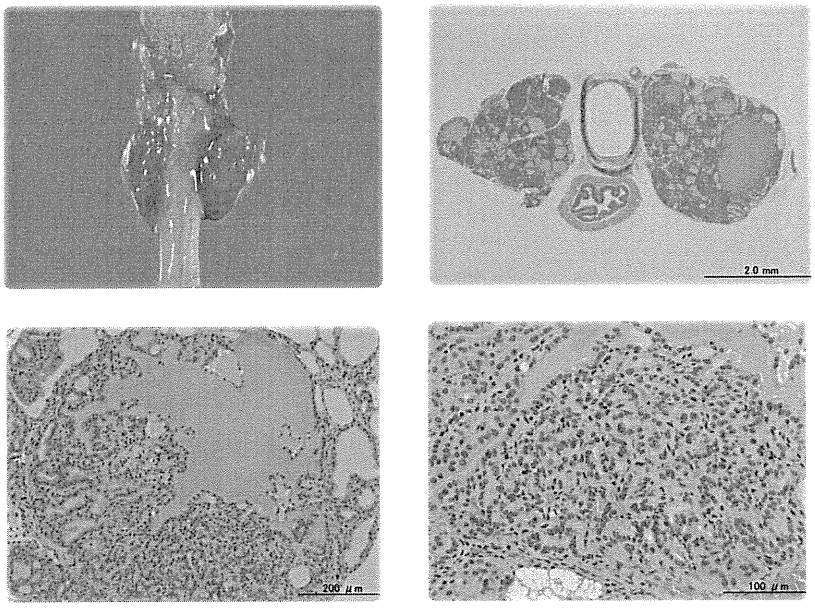
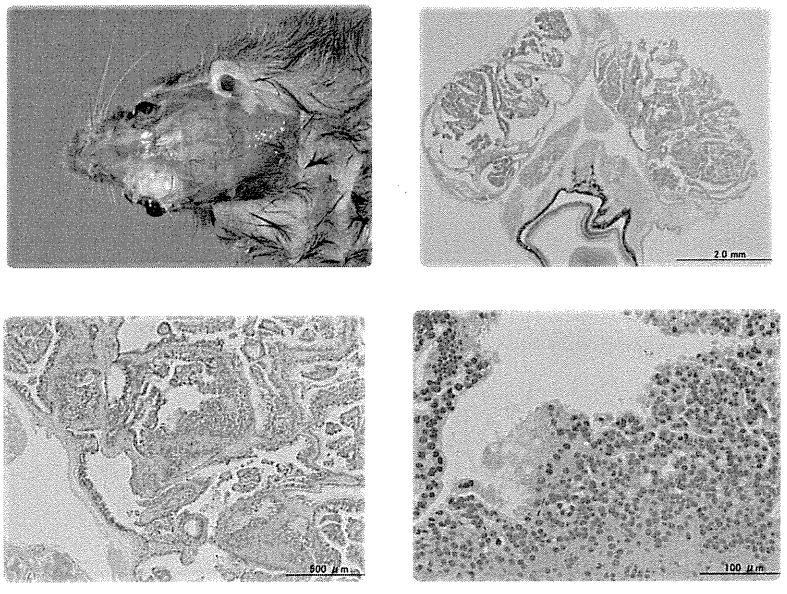
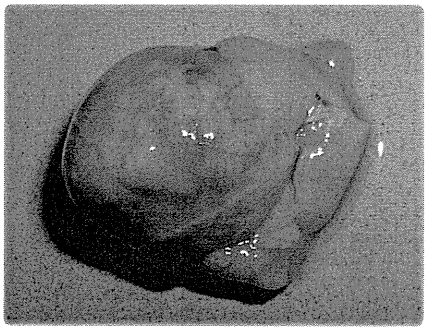
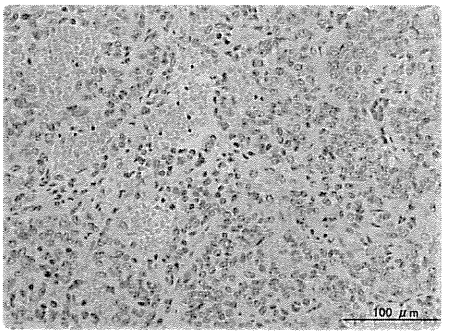


甲状腺：濾胞細胞腺癌(2-108F)

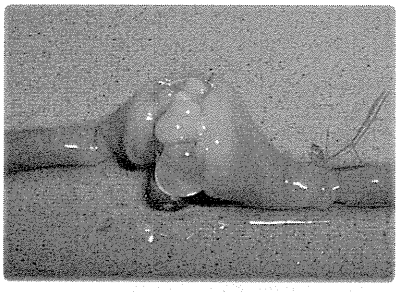


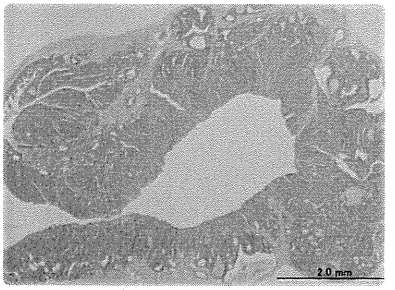
ハーダー腺：腺癌(3-105F)

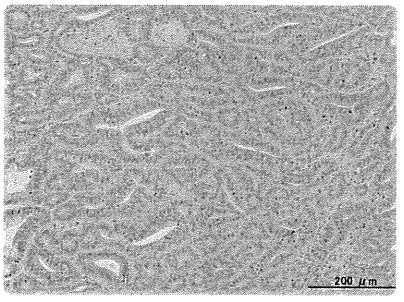


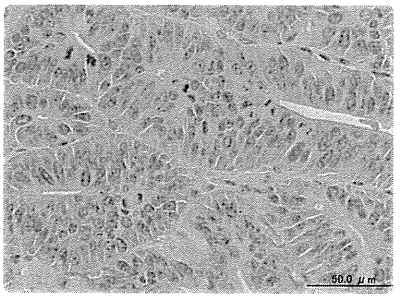


4-007 肝臓嚢胞

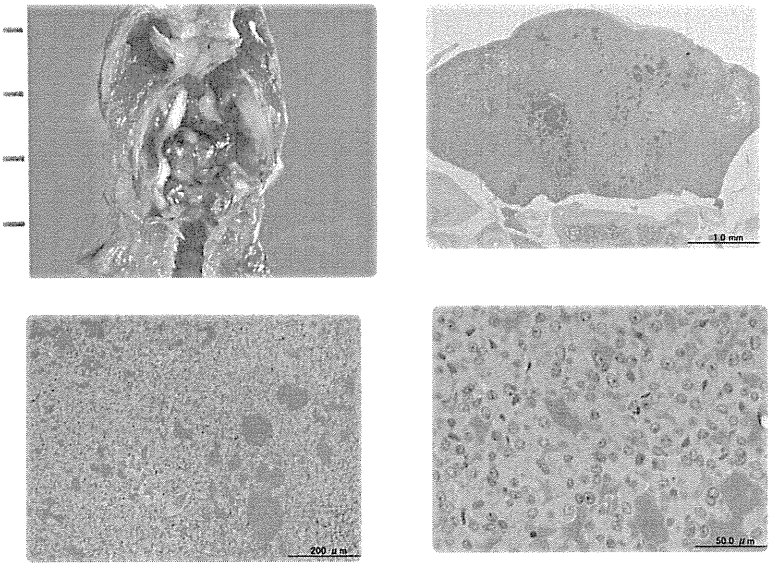




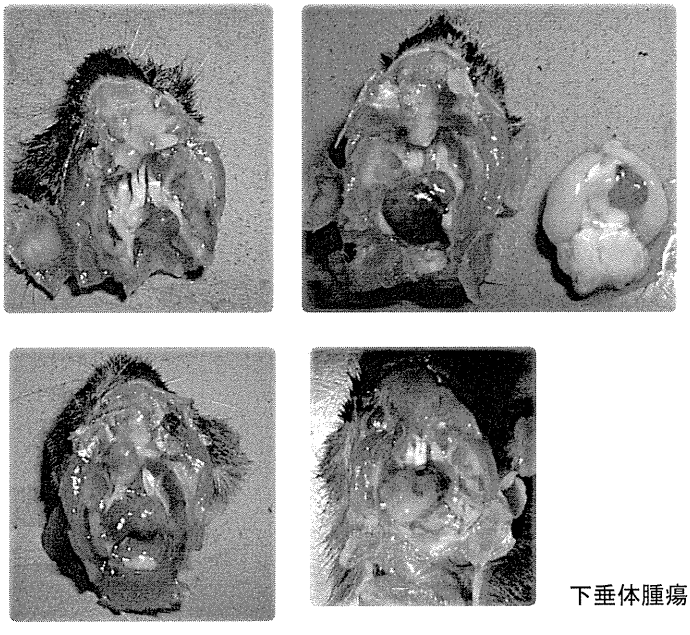




3-005 回腸腺癌

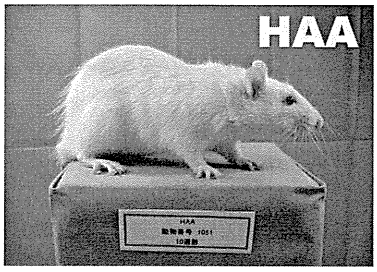


下垂体：前葉腺腫(3-105F)



下垂体腫瘍

3. 近交系ラットによる一生涯試験の検証(提案)



NBRP Rat No: 0242

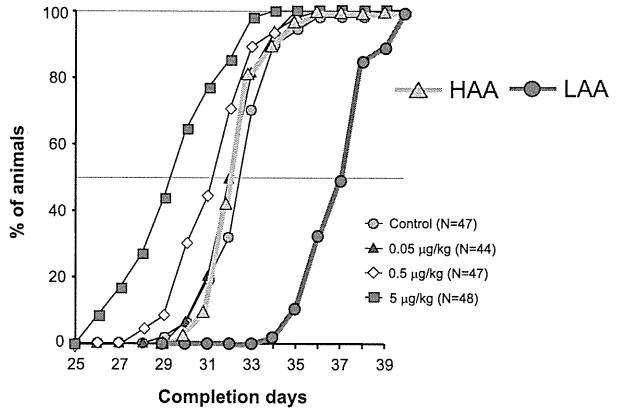
NBRP Rat No: 0243

(Photos were downloaded from NBRP-Rat Homepage.)

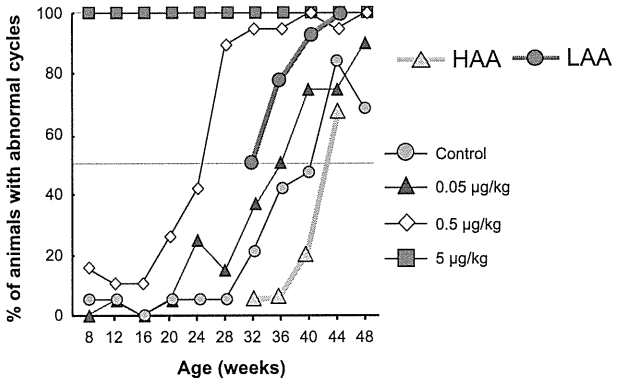
Hatano rats: Hatano rats have been bred from Sprague-Dawley rats on the basis of their avoidance learning in the shuttle-box task. High active avoidance (**HAA**) rats show good avoidance learning. Low active avoidance (**LAA**) rats show poor avoidance learning

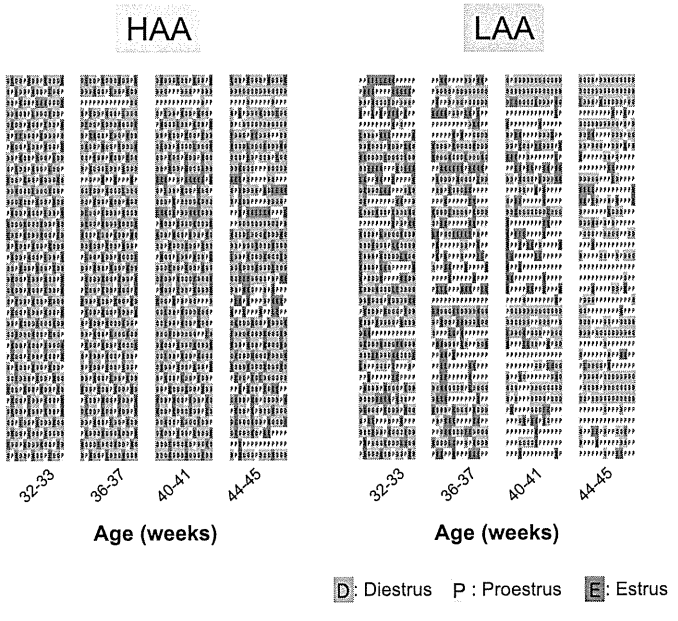
Effects of neonatal DES exposure on sexual maturation in rats

A. Vaginal opening

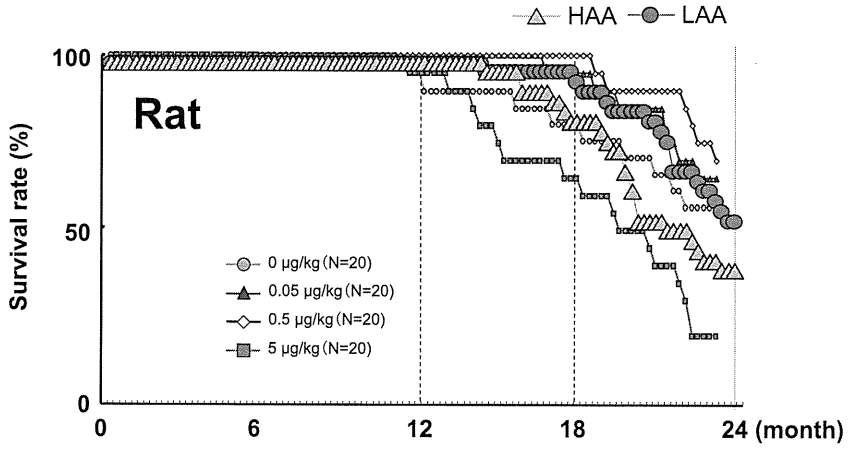


Effects of neonatal DES exposure on estrous cycles in rats

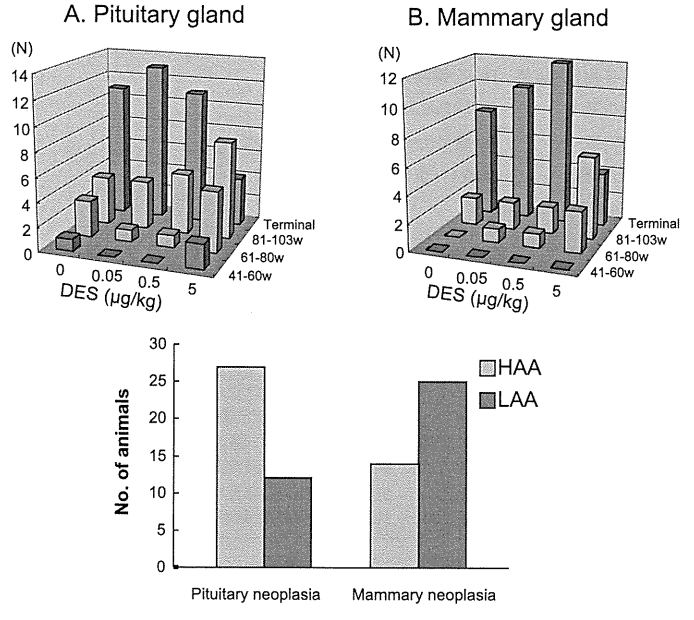




Effects of neonatal DES exposure on survival curve in rats



Effects of neonatal DES exposure on tumorigenesis in female rats



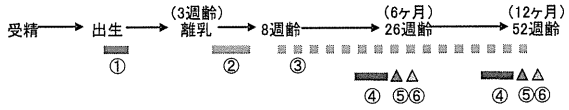
HAAラット＆LAAラット

- 膣開口の時期が異なる
- 性周期異常の発現時期が異なる
- 生存日数が異なる
- 自然発生腫瘍の種類が異なる

化学物質の視床下部・下垂体・性腺軸への遅発性影響を解析する良いモデルである。

化学物質の内分泌かく乱性を確認する一生涯試験（案）

1) 動物種、系統　HAAラット、LAAラット・・・・SD ラットから分離された近交系
2) 投与物質、経路　DES、強制経口
3) 投与量　0、0.05、0.5 μg/kg/day
4) 動物数　10 腹以上/群（生児出産雌として）
5) 投与時期　生後1～生後5日
6) end point



項目	時期	方法
① ■ 投与	1～5日齢	新生児に強制経口投与する。
② ■ 性成熟	3～6週齢	膣開口、陰茎包皮分離の完成時期を観察する。
③ ■ 性周期	8～52週齢	2週間ごとに毎日、膣スメアを採取し、観察する。
④ ■ 行動試験	24,48週齢	条件回避学習試験を実施する。
⑤ △ 剖検、器官重量	26,52週齢	生殖器系の器官重量を測定する。
⑥ △ 免疫機能検査	26,52週齢	抗ヒツジ赤血球抗体を測定する。

別添５

Ⅲ. 研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
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Nagao T, Takada N, Onoda N.	Transgenerational teratogenesis by prenatal exposure to endocrine disrupting chemicals.	Genes and Environ	33	50-60	2011
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Kawakami E, Kinouchi N, Adachi T, Ohsawa Y, Ishimaru N, Ohuchi H, Sunada Y, Hayashi Y, Tanaka E and Noji S.	Atelocollagen-mediated systemic administration of myostatin-targeting siRNA improves muscular atrophy in caveolin-3-deficient mice.	Dev Growth Differ	53	48-54	2011
Lei Y, Ripen AM, Ishimaru N, Ohigashi I, Nagasawa T, Jeker LT, Bösl MR, Holländer GA, Hayashi Y, Malefyt RW, Nitta T, Takahama Y.	XCL1-mediated medullary accumulation of thymic dendritic cells contributes to thymic development of regulatory T cells.	J Exp Med	208	383-394	2011
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林良夫、新垣理恵子、石丸直澄	Sjögren症候群	日本内科学会雑誌	100	1262-1268	2011
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Fujimoto N, Kitamura S, Kanno J.	Androgen dependent transcription of a mouse prostatic protein gene, PSP94: Involvement of estrogen receptors.	J Steroid Biochem Mol Biol	127	301-306	2011
Matsukura H, Aisaki K, Igarashi K, Matsushima Y, Kanno J, Muramatsu M, Sudo K, Sato N.	Genistein promotes DNA demethylation of the steroidogenic factor 1 (SF-1) promoter in endometrial stromal cells.	Biochem Biophys Res Commun	412(2)	366-372	2011

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Baba A, Ohtake F, Okuno Y, Yokota K, Okada M, Imai Y, Ni M, Meyer CA, Igarashi K, Kanno J, Brown M, Kato S.	PKA-dependent regulation of the histone lysine demethylase complex PHF2-ARID5B.	Nat Cell Biol	13(6)	668-645	2011
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太田亮、大沢基保	周産期のアレルギー Developmental Immunotoxicology (DIT)	周産期医学	41(5)	609-613	2011

Ⅳ. 研究成果の刊行物・別刷り

Genes and Environment, Vol. 33, No. 2 pp. 50–60 (2011)

Review

Transgenerational Teratogenesis by Prenatal Exposure to Endocrine Disrupting Chemicals[1]

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(Received March 30, 2011; Revised April 6, 2011; Accepted April 6, 2011)

Congenital malformations can be induced in the offspring of laboratory animals treated with the mutagens, ethylnitrosourea, methylnitrosourea, mitomycin C, triethylenemelamine or procarbazine before copulation. The spectra of malformations in the offspring classified as male-mediated malformations after exposure of paternal mice to mutagens showed no evidence of mutagen-specificity or germ-cell stage-dependent variations. Recently, we demonstrated the increased incidence of congenital malformations in the offspring of male mice exposed *in utero* to synthetic estrogens such as diethylstilbestrol (DES), 17β-estradiol (E_2) or ethynyl estradiol (EE), and that the induction of male-mediated malformations by DES, E_2 or EE showed a clear threshold effect. Developmental exposure to DES, E_2 or EE caused partial atrophy and feminization in the genital tract. They also showed transgenerational effects when applied prenatally at a dose which caused histopathological changes in the testes. Germ-cell series in normal testis have mechanisms to select against spontaneously arising mutation; but these selection mechanisms may not function efficiently in chemically-damaged testes. Based on these results and considerations, we propose as a hypothesis that transgenerational teratogenesis by prenatal exposure to synthetic estrogens may occur as a consequence of testicular toxicity. Moreover, since DES has been reported to be non-genotoxic, epigenetic mechanisms such as DNA methylation may be involved in the transgenerational teratogenesis induced by estrogenic drugs. The expression patterns of DNA methyltransferases (Dnmts) mRNA, global DNA methylation levels in testicular cells of embryos exposed to estrogenic drugs or in epididymal sperm of mature male mice exposed prenatally to estrogenic drugs were different from those in the controls. Results shown in this review support the proposal that, when evaluating the toxicities of environmental chemicals including endocrine disruptors, epigenetic effects such as DNA methylation should be taken into account.

Key words: transgenerational effects, congenital malformations, male-mediated teratogenesis, endocrine disruptor, synthetic estrogens, epigenetics, developmental exposure

Introduction

Although induction of germinal mutations by chemicals is well documented in experimental animals, there is no firm evidence that any agent has induced germinal mutations in men. Direct study of chemically-induced transmitted genetic effects in humans is virtually impossible, so the genetic risk must be estimated from animal experiments. Congenital malformations can be induced in the offspring of laboratory animals exposed to environmental mutagens before copulation (1–6). The spectra of congenital malformations in the offspring classified as male-mediated malformations after exposure of paternal mice to mutagens showed no evidence of mutagen-specificity or a germ-cell stage-dependent variation. More importantly, none of the spectra differed significantly from the type distribution of spontaneous malformations. In addition, when a teratogen was applied at the organogenic stage, embryos whose sires were preconceptually exposed to a mutagen suffered malformations with a higher frequency than those derived from untreated males. Thus, paternal exposure to mutagens can enhance susceptibility of F_1 embryos not only to "spontaneous teratogenesis", but also "induced-malformations" (7).

Estrogenic drugs may have transgenerational toxicity. Exploration of this possibility in animal models is justified due to the increasing concern about the health effects of endocrine disruptors in the environment. However, little is still known about such effects, except for the potential of a transgenerational carcinogenic effect of diethylstilbestrol (DES) reported in mice by Walker (8) and others. DES has been shown to have reproductive tract teratogenicity and carcinogenicity in both women and laboratory animals, and a practical concern was whether children born to women prenatally exposed to DES would have an increased risk of cancer

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(8). The studies carried out using CD-1 mice by Walker (8), Walker and Kurth (9) and Newbold *et al.* (10) showed that prenatal or neonatal exposure of females to DES can increase the incidence of reproductive tract tumors in female offspring. In a study carried out by Walker and Kurth (9) using the embryo-transfer technique, both maternal and germ cell-related pathways were identified for their transmission of carcinogenic risk of DES through females. These results imply that estrogenic drugs may cause persistent modification of germ cells when applied developmentally, and therefore they may exert a transgenerational effect.

We have demonstrated a significant increase in the incidence of congenital malformations in the offspring of male mice prenatally treated with DES, ethynyl estradiol (EE) or 17β-estradiol (E_2) (11). In our study, male mice were transplacentally exposed to estrogenic drugs on days 9 through 16 of gestation, and their fetal offspring were examined for evidence of transgenerational teratogenic effects due to the drugs. The teratogenic effect on external characteristics was examined because it is expressed early in life and can be monitored easily either phenotypically or quantitatively. Outbred ICR mice were used for the treatment and as untreated mating partners for the treated males because teratogenesis mediated by mutagenized germ cells has been studied extensively using this strain. With the treatment schedule used in our study, primordial germ cells (PGCs) were exposed to estrogenic drugs. Critical evidence for the susceptibility of mouse PGCs to genetic damage was reported by Shibuya and co-workers (12,13). They treated transplacentally C3H/He mice with varying doses of ethylnitrosourea (ENU) on gestational day 8.5, 10.5 or 13.5 and examined the incidence of recessive visible mutations at six specific-loci in F_1 of a cross of the treated males × tester females from the PW strain. The results led them to conclude that mutations at specific-loci can be induced in the PGCs by ENU at a several fold higher rate than in stem cell spermatogonia. Vulnerability of PGCs to ENU was further confirmed by Wada *et al.* (14), who examined skeletal malformations in the offspring of male mice transplacentally treated with this agent on gestational day 10.5. In this review, we show the characteristics of male-mediated teratogenesis of environmental mutagens, and demonstrate that the treatment of male mice at the PGC stage with estrogenic drugs caused a clear increase in the incidence of congenital malformations in the subsequent generation.

Congenital Malformations in the Offspring of Male Mice Exposed to Potent Mutagens

Transgenerational teratogenesis experiments (male-mediated teratogenesis): In the experiments on male-mediated congenital malformations, male mice were exposed to mutagens. The treated males were individually caged with untreated virgin females of the same strain (15). The mating intervals were days 1–21 or days 64–80 after the last dosing. Copulations during these periods involved, respectively, treated post-meiotic cells (spermatozoa and spermatids) and pre-meiotic cells (spermatogonial stem cells) at the time of treatment. Presence of a vaginal plug defined day 0 of pregnancy and pregnant females copulated with treated males were killed on day 18 of gestation to observe the fetal morphology. Fetal malformations inspected were gross external and skeletal abnormalities. Significantly enhanced frequency of malformed fetuses per live fetus in the treated series over the control level was taken as evidence of male-mediated teratogenesis, and the abnormalities detected were collectively referred to as F_1 malformations.

Germ cell stages at risk for male-mediated teratogenesis: Male germ cells from primordial to post-meiotic stages are continuously at risk of the induction of F_1 malformations by exogenously applied agents, as evidenced by the data shown in Table 1 for urethane (15,16) and ENU (2,14). It is also clear from Table 1 that all the agents that were effective in inducing F_1 malformations are known mutagens in mice and other *in vivo* systems. Taken together, there is no doubt that genetic damage to the male-germ-line is the mechanism of male-mediated teratogenesis induced by exposure to environmental mutagens.

Possible nature of germ-line mutations responsible for transgenerational teratogenesis induced by mutagens: With a specific-locus test for seven visible markers, six compounds were identified as effective inducers of heritable point mutations in spermatogonial stem cells; i.e., triethylenemelamine (TEM) (23), mitomycin C (MMC) (24), ENU (25), methylnitrosourea (MNU) (26), procarbazine (PCZ) (27), propyl methanesulfonate (PMS) (28). As shown in Fig. 1, all these agents were effective in producing male-mediated congenital malformations when applied at the spermatogonial stem cell stage in ICR mice (4). From the dose-response curves shown in Fig. 1, we estimated the genotoxically effective dose FD_2 in mmole/kg, the dose required to produce externally malformed fetuses with a frequency of 2%, to be 0.07 for TEM and MMC, 0.6 for ENU and MNU, 1.8 for PCZ, and 3.0 for PMS (Table 2). These dose values demonstrate that TEM and MMC are the strongest mutagens for producing male-mediated malformations, followed by (ENU, MNU), PCZ and PMS in that order. From dose-response data on specific-locus mutations, we estimated $MD_{0.02}$ in mmole/kg, the dose required to produce visible mutations at the seven loci with an average frequency of 0.02%, to be 0.006 for TEM, 0.01 for MMC, 0.3 for ENU, 0.2 for MNU, 1.3 for PCZ and 4.6 for PMS.

Table 1. Mutagens tested for induction of F_1 malformations in mouse and rat

Mutagen	Germ cell stage (Species)	Effect	Reference
TEM	SG (Mouse)	+	4
MMC	SG (Mouse)	+	4
ENU	PGC (Mouse)	+	14
	PM (Mouse)	+	2,17
	SG (Mouse)	+	2,17
MNU	PM (Mouse)	+	1
	SG (Mouse)	+	1
PCZ	SG (Mouse)	+	4
PMS	SG (Mouse)	+	4
X-rays	PM (Mouse)	+	15,17,18
	SG (Mouse)	+	15,17,18
Urethane	PGC (Mouse)	+	16
	PM (Mouse)	+	15,17
	SG (Mouse)	+	15,17
CPA	PM (Mouse)	+	19
EMS	PM (Mouse)	+	20
DMBA	PM (Mouse)	+	17
	SG (Mouse)	−	17
AA	SG (Mouse)	−	Nagao (unpublished)
AF-2	PM (Mouse)	−	17
4NQO	PM (Mouse)	−	17
EGM	SG (Mouse, Rat)	−	21
TCDD	SG (Rat)	−	22

+, positive; −, negative

Abbreviations used for mutagens: TEM, triethylenemelamine; MMC, mitomycin C; ENU, ethylnitrosourea; MNU, methylnitrosourea; PCZ, procarbazine; PMS, propyl methanesulfonate; CPA, cyclophosphamide; EMS, ethyl methanesulfonate; DMBA, 7,12-dimethylbenz (a) anthracene; AA, acrylamide; AF-2, 2-(furyl)-3-(5-nitro-2-furyl)-acrylic acid amide (furylfuramide); 4NQO, 4-nitroquinoline l-oxide; EGM,ethylene glycol monomethyl ether; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Abbreviations used for germ cell stage: SG, spermatogonial stem cells; PGC, primordial germ cells; PM: post-meiotic germ cells.

Again, the ranking of the genotoxic potency was TEM = MMC > ENU = MNU > PCZ > PMS. Furthermore, the FD_2 value was very close to the $MD_{0.02}$ value for all mutagens used. We thus concluded that, in mutagenized spermatogonial stem cells, genetic changes responsible for male-mediated malformations are produced in a manner similar to that involved in the production of specific-locus mutations. This means that they probably represent point mutations, namely, genetic changes not associated with gross rearrangements.

The excellent correlation between FD_2 and $MD_{0.02}$ further implies that the number of target loci per genome for the production for male-mediated F_1 malformations is two orders of magnitude higher (i.e., 2/0.02) than that for the specific-locus mutations (4). A similar conclusion was derived from the X-ray results of studies by Nomura (17).

A high percentage of malformations in fetuses from mutagenized paternal germ cells are the result of increased yields of spontaneously occurring mal-

Table 2. Genotoxic potency of the 5 mutagens to induce F_1 fetal abnormalities and specific-locus mutations in spermatogonial stem cells

Mutagen	F_1 fetal abnormalities	Specific-locus mutations
	FD_2^* (mmole/kg)	$MD_{0.02}^\dagger$ (mmole/kg)
TEM	0.007	0.006
MMC	0.007	0.01
ENU	0.6	0.3
PCZ	1.8	1.3
PMS	3.0	4.6

*Effective dose for producing F_1 fetal abnormalities with a frequency of 2%. Data are from references (2,4).

†Effective dose for producing specific-locus mutations with frequency of 0.02%. Data are from references (23–25,27,28) See footnote of Table 1 for the abbreviations used for mutagens.

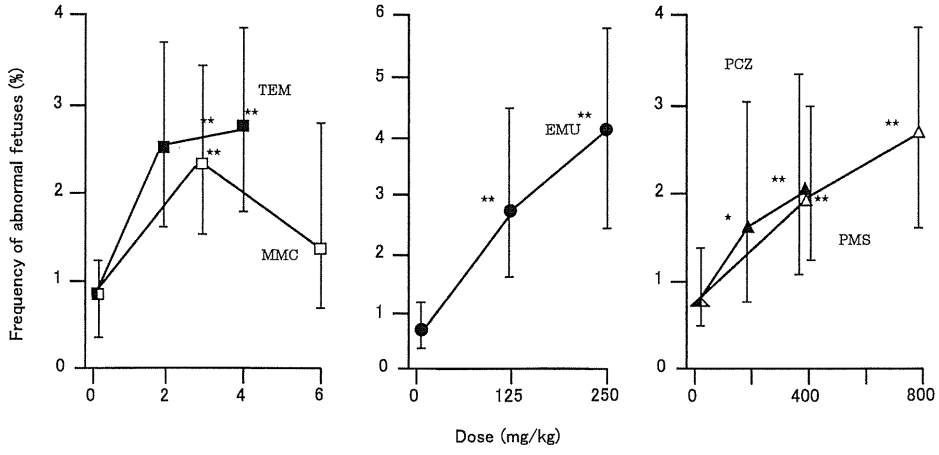

Fig. 1. Dose-response relations of malformed fetuses observed after exposure of ICR mice to chemical mutagens at spermatogonial stem cell stage. Data are from references (2,4). The vertical lines represent 95% confidence intervals of the frequencies. See footnote of Table 1 for the abbreviations used for mutagens. *Significantly different from control, $p=0.05$. **Significantly different from control, $p=0.01$.

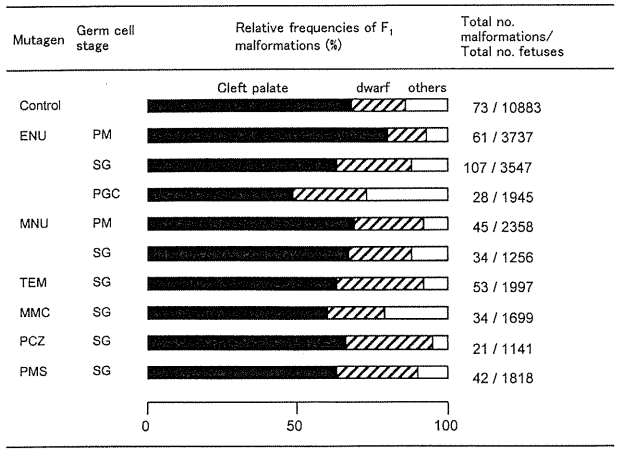

Fig. 2. Relative frequencies of external malformations detected in fetal offspring of ICR males exposed to various kind of mutagens at various germ-cell stages. Data are from references (1,2,4,14). See footnote of Table 1 for the abbreviations used for germ cell stages.

formations: Figure 2 shows the type-distribution of external malformations observed after treatment with various mutagens at the spermatogonial stem cell stage. In order to compare with type-distribution of spontaneously induced abnormalities, the data pooled for the concurrent and historical controls are also shown in Fig. 2. Among external malformations induced after treatment of spermatogonial stem cells, cleft palate was the most common, followed by dwarfism, and others (exencephaly and abnormal limbs such as polydactyly and syndactyly) in any mutagen-treated groups. Spectra of external abnormalities recorded as male-mediated congenital defects in any mutagen-treated groups were indistinguishable from each other, showing no evidence of a mutagen-specificity. More importantly, spectra determined after exposure of paternal germ cells to mutagens did not differ from the spectrum of spontaneously induced malformations. These results clearly characterize male-mediated teratogenesis, as the spectra of fetal malformations induced after treatment of embryos at the organogenic stage with these mutagens showed marked differences from the control spectrum. Among external abnormalities in embryos of ICR mice treated with ENU on day 8 of gestation, microphthalmia (27%) was the most common, followed by exencephaly (1.1%), cleft palate (0.8%) and hydrocephaly (0.8%) (3). Thus, we are inclined to hypothesize that the high percentage of external malformations in fetuses from mutagenized paternal germ cells is the result of increased yields of spontaneously occurring malformations. In other words, spontaneous fetal malformations may also arise, at least partly, as a genetic disease.

Figure 2 shows the type-distribution of external abnormalities detected in fetuses derived from post-meiotic germ cells and spermatogonial stem cells treated with ENU and MNU. In both mutagens, the main types of fetal abnormalities were cleft palate, dwarfism, exencephaly and abnormal limbs in treatments with both post-meiotic germ cells and spermatogonial stem cells. The spectra in different stages of paternal germ cells were indistinguishable, irrespective of the kind of mutagens used, showing no evidence of a germ-cell stage dependent variation. In summing up all the data on external malformations, it seems that the spectrum of external abnormalities in fetuses derived from mutagen-treated paternal germ cells mimics the spectrum of spontaneously occurring malformations and, as far as external abnormalities are concerned, evidence for male-mediated teratogenesis can be obtained only from a quantitative comparison of the incidence of malformed fetuses between the exposed and the control groups. Spontaneous spectrum of male-mediated malformations often showed a strain-dependent variation. The predominant types of external malformations were cleft palate and dwarfism in the ICR strain, microphthalmia and micrognathia in C57BL/6N (5), and cleft palate and open eyelids in A/Jax (6). In our studies with the ICR strain, the average frequency (cleft palate and dwarfism) was 2:1 in the ENU experiment. In the experiments reported by Kirk and Lyon (18), who used (C3H/HeHx101/H) F_1 hybrids, the ratio was 1:25. Thus, it is reasonable to suggest that the spectrum of induced male-mediated malformations also depends on the strain of mice used.

Modified susceptibility to induced-teratogenesis in the offspring of males exposed to environmental mutagens: Fetuses derived from males exposed to mutagens may also have enhanced susceptibility to induced-teratogenesis. "Induced-teratogenesis" means teratogenesis induced in the fetuses of mothers exposed to teratogens. This possibility was tested in an experiment where ICR females mated with ENU-exposed males of the same strain, and those mated with untreated males, were treated with ENU in the organogenic period, and the fetuses were inspected for external and skeletal malformations at term. As exemplified by the data in Fig. 3, there was a lot of evidence of increased sensitivity to microphthalmia as induced-malformations in the offspring of males exposed to mutagens. Thus, paternal exposure to mutagens can enhance susceptibility of F_1 embryos not only to spontaneous teratogenesis, but also to induced-malformations. Paternal exposure may increase the risk of fetal malformations due to the mother's exposure to drugs during pregnancy (7).

Transgenerational Adverse Events Induced by Endocrine Disruptors

The synthetic estrogen DES is a potent perinatal endocrine disruptor. In humans and experimental animals, exposure to DES during critical periods of

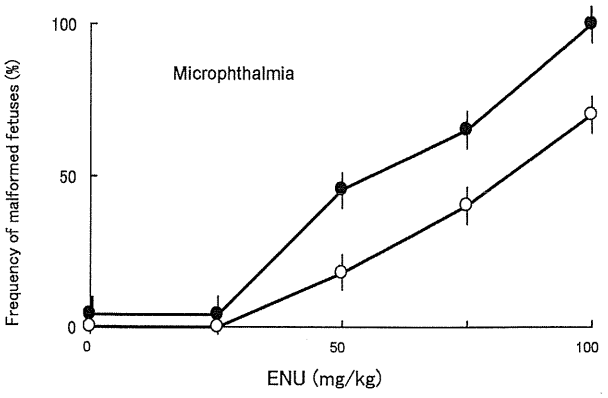

Fig. 3. Frequencies of fetuses with microphthalmia observed 10 days later after ENU exposure on day 8 of gestation to pregnant ICR females that had been conceived by ICR males exposed to ENU at spermatogonial stem cell stage (closed circles) or by untreated ICR males (open circles). Vertical lines represent standard errors of the frequencies. Data are from reference (7).

reproductive tract differentiation permanently alters estrogen target tissues and results in long-term abnormalities such as uterine neoplasia that are not manifested until later in life. Using mice exposed to DES developmentally, multiple mechanisms have been identified that play a role in its carcinogenic and toxic effects. Since mechanistic studies provided support that environmental estrogens cause both genetic and epigenetic alterations in developing target tissues (29–32), the possibility was raised that abnormalities seen following developmental exposure to DES could be transmitted to subsequent generations.

Exploration of this possibility in animal models is justified due to the increasing concern about the health effects of endocrine disruptors in the environment. However, little is still known about such effects, except for the potential for a transgenerational carcinogenic effect of DES reported in mice by Walker (8) and others. DES had been shown to have reproductive tract teratogenicity and carcinogenicity in both women and laboratory animals, and a practical concern was whether children born to women prenatally exposed to DES would have an increased risk of cancer (8). The studies carried out using CD-1 mice by Walker (8), and Walker and Kurth (9) showed that prenatal or neonatal exposure of female mice to DES can increase the incidence of reproductive tract tumors in female offspring. In a study carried out by Walker and Kurth (9) using the embryo-transfer technique, both maternal and germ-cell related pathways were identified for the transmission of carcinogenic risk of DES through females. Studies by Newbold *et al.* (10,33) showed that prenatal or neonatal exposure to DES led to tumors in the female and male genital tract, and in addition, the susceptibility for tumors was transmitted to the descendants through the maternal germ cell lineage. Mice were exposed to DES prenatally on days 9–16 of gestation, or neonatally on days 1–5. When female mice (F_1) reached sexual maturity, they were bred to untreated males. Female and male offspring (DES-lineage or F_2) from these matings were ages to 17–24 months and examined for genital tract abnormalities. An increased incidence of proliferative lesions of the rete testis, as well as tumors of the reproductive tract, was observed in DES-lineage males (33). Further, in DES-lineage females, an increased incidence of uterine adenocarcinoma was seen (10). These results suggest that this increased susceptibility to tumors is passed on from the maternal lineage to subsequent generations of male and female descendants; the mechanisms involved in these transgenerational events include genetic and epigenetic effects. Together, the data of Newbold *et al.* indicate the unique sensitivity of the developing organism to endocrine-disrupting chemicals, the occurrence of long-term effects following developmental exposure, and the possibility of adverse effects to be transmitted to subsequent generations (34). Multigenerational effects of DES have been reported by other laboratories and some of these reported transmission through the paternal lineage (35,36).

Increased incidence of congenital malformations in the offspring of male mice exposed to estrogenic drugs at the embryonic stage: We demonstrated the increased incidence of congenital malformations in the offspring of male mice exposed prenatally to a synthetic estrogen such as DES, EE, estradiol benzoate (EB), or E_2 (11,37). The results have shown that prenatal exposure of males to estrogenic compounds is hazardous not only for the development of the reproductive tract in the exposed mice, but also for embryonal development in the subsequent generation. In the groups showing male-mediated teratogenesis after transplacental exposure to an estrogenic compound on days 9 through 16 of gestation, anatomical or histopathological changes (unilateral atrophy, elongated configulation) were induced in testes, epididymides, or seminal vesicles of the males at the mature stage. Some males after prenatal exposure to estrogen at fairly high dose level had markedly dilated Müllerian duct remnants, which resembled the uteri. In the mature males of the group showing no male-mediated teratogenesis, no testicular damage was observed even by ultrastructural observation. Thus, it is reasonable to suggest that transgenerational teratogenesis by prenatal exposure to estrogenic compounds may occur as a consequence of their anatomical or histopathological testicular damages. As pointed out earlier, germ-cell series in normal testes have mechanisms to select against spontaneously arising mutations, but these selection mechanisms may not function efficiently in chemically-damaged testes (37).

In contrast to the reproductive tract abnormalities, all

of the external malformations detected in the fetal descendants of males exposed prenatally to estrogenic compounds were types known to occur in the ICR mice used in our studies. Namely, with regard to the types of malformations, the transgenerational effects observed in our studies were neither atypical for ICR mice nor specific to the drugs tested or the stage of the germ-cell development at the time of exposure. The relative ratios of cleft palate, dwarfism, and exencephaly in the groups exposed to EB-treated, EE-treated and control series were 1:0.3:0.07, 1:0.4:0.07 and 1:0.2:0.07, respectively. Similarly, cleft palate was the most common, followed by dwarfism and exencephaly in this order among external malformations produced in the offspring of ICR males treated with a mutagen at the spermatogonial stem cell or post-meiotic cell stage (1,2,4,7). Taken together, it seems that heritable damage was induced in embryonic germ cells (i.e., PGCs) after exposure to EB or EE, and the offspring inherited the damage and were sensitized to spontaneous teratogenesis. PGCs are at risk of chemical induction of transmissible changes, as shown by the induction of specific-locus mutations and dominant skeletal mutations with ENU (12,14). However, it is not clear whether the germ-cell damage responsible for the effects observed in our transgenerational teratogenic studies with synthetic estrogens was genetic or "epigenetic" in nature. Epigenetics is typically defined as the study of heritable changes in gene expression that are not due to changes in DNA sequences.

Of particular interest in our studies is that the induction of male-mediated malformations induced by the exposure to estrogenic drugs showed a clear threshold effect (11), and estrogenic drugs such as DES have been reported to be non-genotoxic (38), suggesting the non-genotoxic action of estrogenic drugs for transgenerational teratogenesis. Despite such uncertainty, the results of our studies on male-mediated teratogenesis of estrogenic drugs agree with those of previous reports on transgenerational carcinogenicity of DES (9,35) in suggesting vulnerability of developing germ cells to estrogenic drugs. Thus, the transgenerational effects of estrogenic drugs do not seem to be a rare phenomenon in mice. Studies of the molecular mechanisms of the effects are required to evaluate the implications of these observations in humans.

Epigenetic transgenerational inheritance of endocrine disruptors: A wide variety of chemical and physical agents have the potential to cause adverse effects by causing heritable changes to the genome, resulting in heritable alterations in phenotype. This is often thought, indeed assumed, to be a consequence of mutations, which may occur through either a genotoxic mechanism, or indirectly as a result of a non-genotoxic mechanism. A genotoxic mechanism involves either the agent itself or a metabolite of it interacting directly with DNA (39), thus, providing a substrate for mutagenesis. Alternatively, a non-genotoxic compound (i.e., the compound and its metabolites do not bind to DNA) may cause mutations through an indirect, secondary mechanism. For example, hyperplasia can occur in response to necrosis (40), and this may result in mutagenesis as a consequence of the less than perfect fidelity of DNA polymerase (41). However, mutagenesis is not the mechanism underlying heritable alterations to the genome (42). Indeed, it is appropriate to consider that heritable alterations in phenotype may also have an epigenetic basis (43–46).

Examples of probable epigenetic transgenerational effects are known. Mice treated with ionizing radiation or urethane produce offspring in which the frequency of tumors is greatly increased (15). It is very unlikely that this result could be due to the induction and transmission of new mutations in tumor suppressor genes. Instead, it may well be that epigenetic defects are being transmitted, which predispose cells to produce tumors (47). Another study has shown that irradiated mice produce offspring that are unusually sensitive to carcinogens, in comparison to animals with untreated parents (48). This again argues in favor of epigenetic transmission. There are other examples in the literature of such transmission (49).

Newbold *et al.* showed altered methylation patterns in several uterine genes that were permanently dysregulated following developmental DES treatment (50,51). The estrogen-responsive proteins lactoferrin (LF) and c-fos were permanently up-regulated in the uterus following developmental exposure to DES and the promoter regions of these genes were shown to be hypomethylated (50,51). Although the consequences of these types of alterations are unclear, studies suggested that methylation patterns can be passed to subsequent generations (52). A recent report by Skinner and his colleagues supports this theory since prenatal exposure to vinclozolin (an antiandrogenic compound) or methoxychlor (an estrogenic compound) caused adverse effects on testes morphology and male fertility, and that these effects were transmitted to subsequent generations (53). In addition, this report showed that these two chemical compounds caused epigenetic alterations in the DNA, specifically hyper- and hypo-methylation, and that these alterations were also observed in subsequent generations (53,54). Since the responses of estrogen regulated genes are set during development, altered hormone responses may be transmitted to subsequent generations.

In natural populations, both sexes may encounter affected, as well as unaffected, individuals during the breeding season, and any diminution in attractiveness could compromise reproductive success. Crews *et al.* (55) examined mate preference in male and female rats whose progenitors had been treated with vinclozolin.

This effect was sex-specific, and they demonstrated that females three generations removed from the exposure discriminate and prefer males who do not have a history of exposure, whereas similarly epigenetically imprinted males do not exhibit such a preference. These observations suggest that the consequences of endocrine disruptors are not just transgenerational, but can be transpopulational, because in many mammalian species, males are the dispersing sex. The results indicate that epigenetic transgenerational inheritance of endocrine disruptor action represents an unappreciated force in sexual selection (55).

The study of Dolinoy's group shows that maternal exposure to the endocrine disrupting chemical bisphenol A shifted the coat color distribution of viable yellow agouti (A^{vy}) mouse offspring toward yellow by decreasing CpG (cytosine-guanine dinucleotide) methylation in an intracisternal A particle retrotransposon upstream of the *Agouti* gene. DNA methylation at the A^{vy} locus was similar in tissues from the three germ layers, providing evidence that epigenetic patterning during early stem cell development is sensitive to bisphenol A exposure (56).

In women, prenatal exposure to DES is associated with adult reproductive dysfunction. The menstrual and reproductive characteristics in a unique cohort comprising daughters of women exposed prenatally to DES were reported (57). Their data provide evidence of menstrual irregularity and delayed menstrual regularization in the daughters of women exposed *in utero* to DES. The findings were the first documented example of transplacental carcinogenesis in humans, and are compatible with speculation regarding transgenerational transmission of DES-related epigenetic alterations in humans. Most of the outcomes seen in the women have been replicated in prenatally exposed mice (58–61); thus, the mouse model is useful for investigating DES-related mechanisms. Studies of developmentally exposed rodents indicate that DES exerts its influence on reproductive tissues through epigenetic mechanisms involving disrupted estrogen signaling and permanent changes in gene expression, probably due to altered DNA methylation (62–64). Rodent studies have identified altered expression in multiple genes, including those inducible by estrogen, such as LF, estrogen receptor, epidermal growth factor, and specific proto-oncogenes, as well as genes involved in the structural development of the reproductive tract, and those governing embryonic development (65–75).

Altered expression of DNA methyltransferases and genomic DNA methylation in testes of embryos exposed to estrogen in mice: As mentioned earlier, induction of male-mediated congenital malformations by estrogenic drugs in our studies showed a clear threshold effect. In addition, since DES has been reported to be non-genotoxic (38), epigenetic mechanisms such as DNA methylation may be involved in the transgenerational teratogenesis induced by estrogenic drugs.

DNA methylation is catalyzed by a family of DNA methyltransferases (Dnmts) that is composed mainly of Dnmt1, Dnmt3a, Dnmt3b and Dnmt3L. Dnmts are mainly involved in the maintenance of DNA methylation patterns following replication. Dnmt3a and Dnmt3b are involved in the establishment of *de novo* methylation (76). Expression patterns of Dnmts mRNA in the normal developing testes of mice have already been reported (77,78). The results of Sakai *et al.* (77,79) strongly suggest that it is not Dnmt1, but some other type of Dnmts that contributes to the creation of DNA methylation patterns in male germ cells, while Dnmt3a2 and Dnmt3L are responsible for the global DNA methylation in mouse male germ cells.

In our studies, C57BL/6N mice, an estrogen-sensitive strain (80), were treated with DES during pregnancy. Expression of Dnmt1, 3a, 3a2, 3b, and 3L mRNA in embryonic testes or epididymides of mature males were analyzed by real-time PCR. The expression patterns of Dnmts in testicular cells of embryos exposed to DES were different from those in controls (Fig. 4), suggesting that exposure of embryos to synthetic estrogens during the early developmental stage of gonads affects the Dnmts expression profile in germ cells. The alterations in the expression levels of Dnmts may be involved in the formation of aberrant DNA methylation. In addition, changes in global DNA methylation levels in the testes of embryos exposed to EE were found, as well as influence of the mRNA expression of imprinted genes *H19* and *Igf2* (insulin-like growth factor 2 gene) (Fig. 5) and altered genomic DNA methylation status of imprinted genes.

Recently, adverse effects induced by chemicals and current changes in DNA methylation have been reported in several laboratories. For example, exposure of mouse preimplantation embryos to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which binds to the aryl hydrocarbon receptor, reduced the fetal body weight and increased the methylation levels of the imprinted genes *H19* and *Igf2*, as well as Dnmt activity (81). Another report indicated that exposure of pregnant female rats during the developmental period of gonadal sex determination to vinclozolin or methoxychlor induced transgenerational adverse effects on male fertility and altered the DNA methylation patterns in the germline (53). In these cases, changes in DNA methylation may also be involved in the adverse processes that occur. Taken together, these results support the proposal that, when evaluating the toxicities of environmental chemicals including endocrine disruptors, epigenetic effects, such as DNA methylation, should be taken into account (42,82).

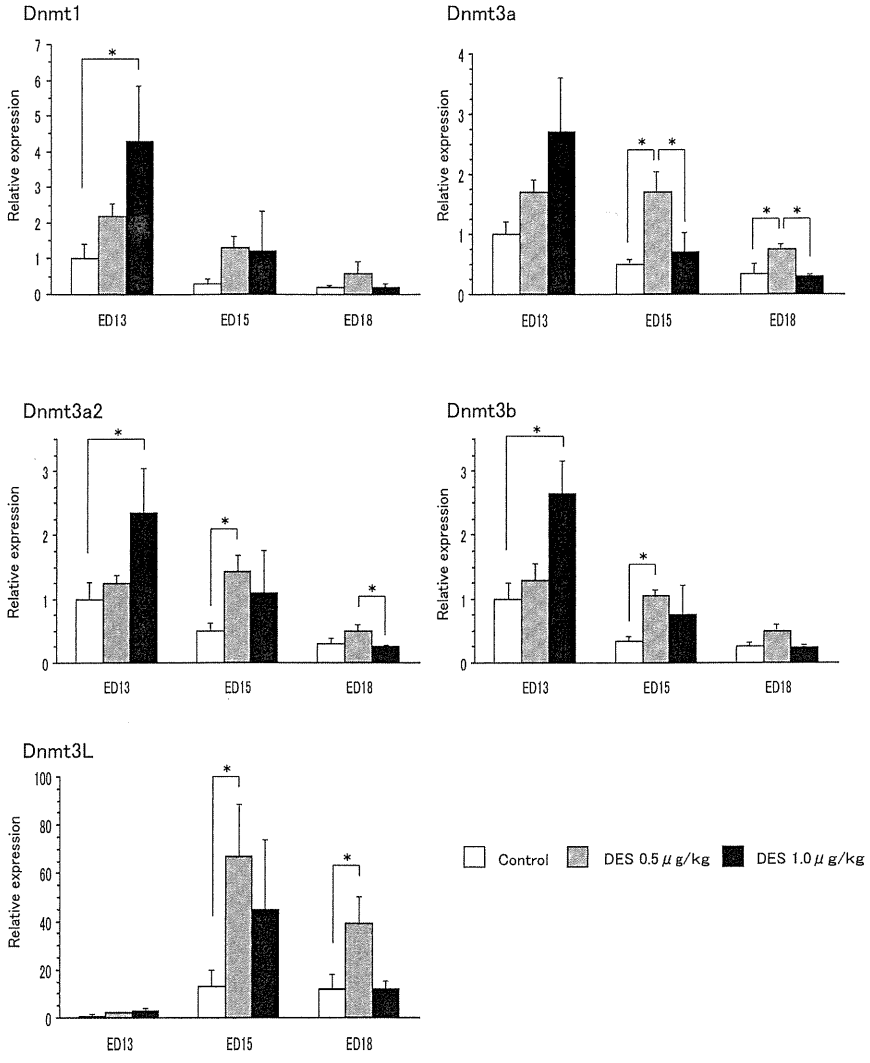

Fig. 4. Expression of Dnmts mRNA in the testes of ICR mouse embryos exposed to DES on days 8 through 11 of gestation. Real-time PCR was done. Vertical lines represent standard errors of the means. *Significantly different from control, $p=0.05$.

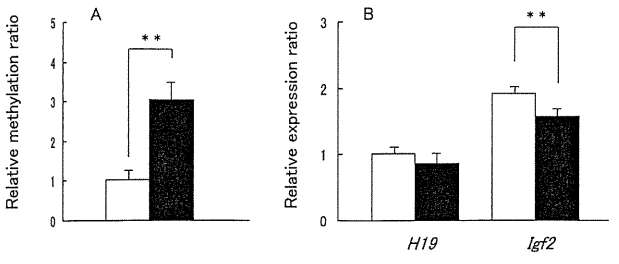

Fig. 5. Global DNA methylation (A) and real-time PCR analysis of mRNA expression of imprinted mouse *H19* and *Igf2* genes (B) in embryonic day 13 embryos exposed to EE. Expression ratios of targeted genes relative to the control. Open column and closed column represent control group and EE-exposed group, respectively. **Significantly different from control, $p=0.01$.

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