benzene-induced apoptosis. Recently, Yu et al. (2007) induced bone marrow cell apoptosis via oral treatment of benzene in mice. However, they applied a very high dose of benzene, about 2,000 mg/kg, which was more than ten times greater than the dose used in the present study. Therefore, in an in vivo system, benzene-induced apoptosis may occur only at a certain high dose. Indeed, we failed to find apoptotic evidence in the mouse bone marrow cells even when WT mice were treated with 300 mg/kg b.w. benzene solution (data not shown). The fact that no difference was found in the results between the gavage doses of 150 mg/kg b.w. and 300 mg/kg b.w. was not surprising since transformation of benzene to toxic metabolites has some limitations due to the saturation of benzene metabolism at certain high gavage doses (approximately >50 mg/kg gavage dose) (Sabourin et al. 1987). Considering that appropriate doses of benzene can induce leukemia but higher doses only induce aplastic anemia without induction of hemopoietic malignancies, such unique properties of benzene in association with apoptosis should be considered to clarify the mechanisms of benzene-induced leukemia and aplastic anemia. In summary, caspase-4 and -12 genes, which are inflammatory caspases, were differentially activated in bone marrow cells via benzene treatment in the mice lacking the p53 gene, suggesting that the p53 gene may partially participate in regulating the activation of the caspase genes in benzene-exposed mouse bone marrow. However, benzene-induced activation of those inflammatory caspase genes was not likely to be associated with cellular apoptosis in the mouse bone marrow. The transcriptional changes in caspase-4 and -12 may simply be due to gene regulation in response to the effect of benzene exposure on bone marrow cells.

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Hematopoietic neoplastic diseases develop in C3H/He and C57BL/6 mice after benzene exposure: Strain differences in bone marrow tissue responses observed using microarrays

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ABSTRACT

In this study, *Trp*53-deficient and wild-type mice of both C57BL/6 and C3H/He strains were exposed to benzene (33, 100, and 300 ppm; 6 h/day, 5 days/week for 26 weeks) and then observed for lifetime. As results, first, the incidence of nonthymic lymphomas in C57BL/6 mice and acute myeloid leukemias (AMLs) in C3H/He mice showed linear responses at the lower exposure level in *Trp*53-deficient mice; second, the incidence of thymic lymphomas in C57BL/6 mice and nonthymic lymphomas in C3H/He mice increased without a plateau-like ceiling; thus, the former equivocal induction of hematopoietic neoplasms (HPNs) in the case of low-dose benzene exposure was assumed to be based on the DNA repair potential in wild-type mice, and the latter limited increase in HPNs in the case of high-dose benzene exposure was considered to be due to excessive apoptosis in wild-type mice. Concerning the incidence of AMLs, though a dose of 300 ppm benzene inhalation induced 9% AMLs in wild-type C3H/He mice-AML-prone, it induced AMLs in 38% of *Trp*53-deficient C3H/He mice. Because AMLs were also observed in *Trp*53-deficient mice, including in the C57BL/6 mice, benzene exposure may also be a potent inducer of AMLs in mice with some strain differences. In the present study, to elucidate the hematopoietic stem cell-specific, aryl hydrocarbon-receptor-related low-dose adverse effect, global gene expression in the bone marrow was analyzed at 28 days after 2-week-intermittent exposure to 150 mg/kg b.w. benzene, by gavage, i.e., equivalent to the above inhalation protocol with 300 ppm. We observed two conceptually different gene expression profiles; "common gene profiles" (CGPs) shared among mice in each group, and "stochastic gene profiles" (SGPs), i.e., unique union genes from one individual mouse to another. The CGPs of the experimental group and the SGPs of each individual mouse were separately characterized by individual assay. Concerning the CGPs, reciprocal strain differences between C3H/He and C57BL/6 mice in expression gene profiles, both plausible for leukemogenesis, were identified; namely, dominant downmodulations of *Sltm* and *Cryl1*, related to suppression of apoptosis and genomic instability in C3H/He mice, respectively, and dominant downmodulations of *Atrx/rad54* and *Kdm2a*, related to a decrease in DNA repair and genomic instability, respectively, in C57BL/6 mice. These findings imply that these reciprocal gene expression differences induced by benzene exposure may lead each strain to undergo different hematopoietic neoplastic pathways. In contrast, each individual mouse often shows a unique SGP. SGPs often include transcription factors, which regulate reciprocal signaling pathways including further SGPs. Among them, apoptosis-related genes expressed in C57BL/6 mice and those in C3H/He mice were attributable to different combinations of SGPs. Such stochastic case-by-case gene expression may be in good agreement with the individual and strain differences observed following benzene exposure. Because gene chip microarray techniques can elucidate stochastic changes in gene expression profiles, possible stochastic toxicology and its future role are discussed.

1. Introduction

Benzene-induced hematotoxic signaling is via aryl hydrocarbon-receptors (AhRs) [1] and specifically exhibited in hematopoietic progenitor cells [2,3]. The three major questions regarding benzene-induced hematopoietic neoplasms (HPNs)

Abbreviations: AML, acute myeloid leukemia; AhR, aryl hydrocarbon-receptor; HPNs, hematopoietic neoplasms.

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addressed are as follows: first, why is the incidence of HPNs equivocal in the case of low-dose benzene exposure despite the significant genotoxicity of benzene even at low doses [4]; second, why is there a plateau-like ceiling in the increase in the incidence of HPNs following high-dose exposure despite a low acute toxicity [5]? Third, why are acute myeloid leukemias (AMLs) *not* commonly observed in mice following benzene exposure even though they are frequently observed in humans after occupational benzene exposure [6–8]?

Since C3H/He mice are known to be AML-prone and to produce AMLs following radiation exposure [9,10], and C57BL/6 mice are known to be lymphoma-prone [11–13], these two strains were used for comparison. Owing the possible higher incidence of leukemias observed in *Trp*53-deficient mice [11,14,15], these mice of both C57BL/6 and C3H/He strains, were exposed to benzene (6 h/day, 5 days/week, for 26 weeks) and their leukemogenicities throughout their lifetimes were compared to determine whether *Trp*53-deficient C3H/He AML-prone mice, answer the above questions [11]. The followings are the results. For the first query, the apparently linear induction of HPNs following low-dose benzene exposure in *Trp*53-deficient mice was considered to be based on the DNA repair potential at low-dose level in wild-type mice. For the second query, the limited increase in the incidence of HPNs following high-dose benzene exposure was found to be due to excessive apoptosis in wild-type mice. For the last query, development of AML was observed in 38% of *Trp*53-deficient C3H/He mice [11].

Global gene expression analysis performed 28 days after a 2-week-intermittent benzene exposure at a dose of 150 mg/kg b.w./day for elucidation of the hemopoietic stem cell-specific, AhR-related low-dose adverse effect revealed reciprocal strain differences in relation to plausible hematopoietic leukemia/lymphomagenesis between C3H/He and C57BL/6 mice. We observed dominant downregulations of *Sltm* (scaffold attachment factor [SAF]-like transcription modulator) and *Cryl1* (crystallin, lambda 1), known to induce the suppression of apoptosis [16] and genomic instability, respectively [17,18] in C3H/He mice, and dominant downmodulations of *Atrx* (alpha thalassemia/mental retardation syndrome X-linked homolog)/*rad54* and *Kdm2a* (dimethyl Lys36 histone H3 [H3K36me2] histone demethylase), known to induce the suppression of DNA repair [19] and increase genomic instability [20], respectively, in C57BL/6 mice, implying that these reciprocal gene expression changes induced by benzene may lead to development of HPNs in both strains via different plausible neoplastic pathways toward the hematopoietic neoplasms.

2. Exposure doses and hematopoietic neoplasms

The hematotoxic signaling induced by benzene exposure is via AhRs [1]; thus, toxicity is specifically induced in hematopoietic progenitor cells of the bone marrow because they only specifically express AhRs during the steady-state [21–24]. Intermittent exposure of benzene at doses below 300 ppm by inhalation, 6 h/day, 5 days/week, for 26 weeks, was carried out so as not to induce aplastic anemia. Benzene exposure doses of 33, 100 and 300 ppm were therefore used along with sham exposure for comparison. Using the intermittent inhalation protocol with 300 ppm exposure dose, the incidence of leukemias induced was the maximum, which results in 30–50% of HPNs in general, depending on the mouse strain used [11,12,25–27]. Exposure concentrations of over 300 ppm tend to result in aplastic anemia and death in mice [25].

As results, first, the incidences of nonthymic lymphoma and AMLs in C3H/He mice showed apparently linear responses at the lower exposure dose in *Trp*53-deficient mice (Fig. 1A); second, the incidences of thymic lymphoma in C57BL/6 and nonthymic lymphoma in C3H/He mice increased without a plateau-like ceiling (Fig. 1B); thus, the former threshold-like equivocal induction of HPNs following low-dose benzene exposure is assumed to be based on the DNA repair potential in wild-type mice, and the latter limited increase in the incidence of HPNs following high-dose benzene exposure is due to excessive apoptosis owing to *Trp*53 in wild-type mice [11].

3. Incidence of myeloid leukemia in C3H/He mice

Concerning the incidence of AMLs, following exposure to 300 ppm benzene, 9% of the wild-type C3H/He AML-prone mice developed AMLs, whereas 38% of the *Trp*53-deficient mice developed AMLs (Fig. 2). Because AMLs were also observed in including *Trp*53-deficient C57BL/6, the lymphoma-prone mice, the induction of AMLs by benzene exposure was considered to be plausible not only in humans but also in mice with some strain differences. Further detailed incidences and histopathological findings observed in the study are described elsewhere [11].

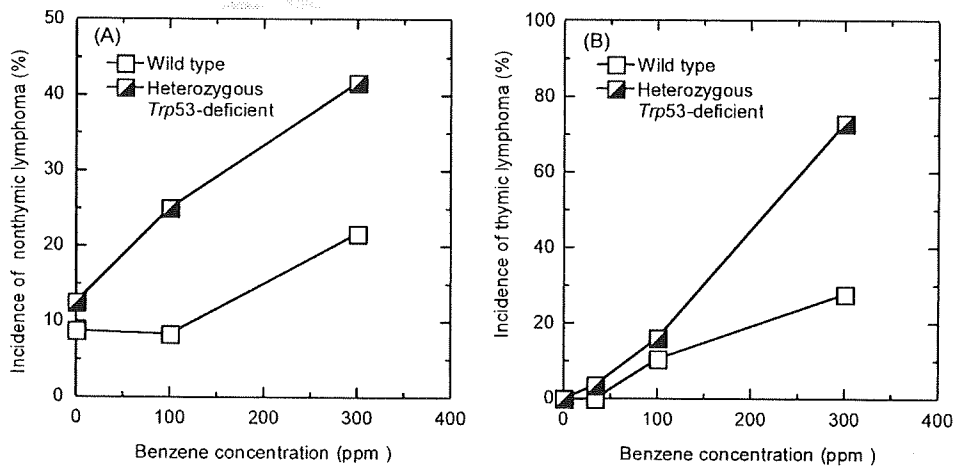

Fig. 1. Incidence of HPNs (percent) (ordinate) vs. dose of benzene exposure (abscissa): nonthymic lymphoma in C3H/He mice (A) and thymic lymphoma in C57BL/6 mice (B). Open square symbols represent wild-type mice, whereas half-closed square symbols represent heterozygous *Trp*53-deficient mice. Number of mice per group (%) of each data point is 2/23 (8.7), 2/24 (8.3), and 7/23 (30.4) for wild-type mice, and 5/24 (12.5%), 6/24 (25.0), and 10/24 (41.7) for heterozygous *Trp*-53-deficient mice for benzene exposure doses of 0, 100 and 300 ppm, respectively. The data are obtained from reference originally from [11].

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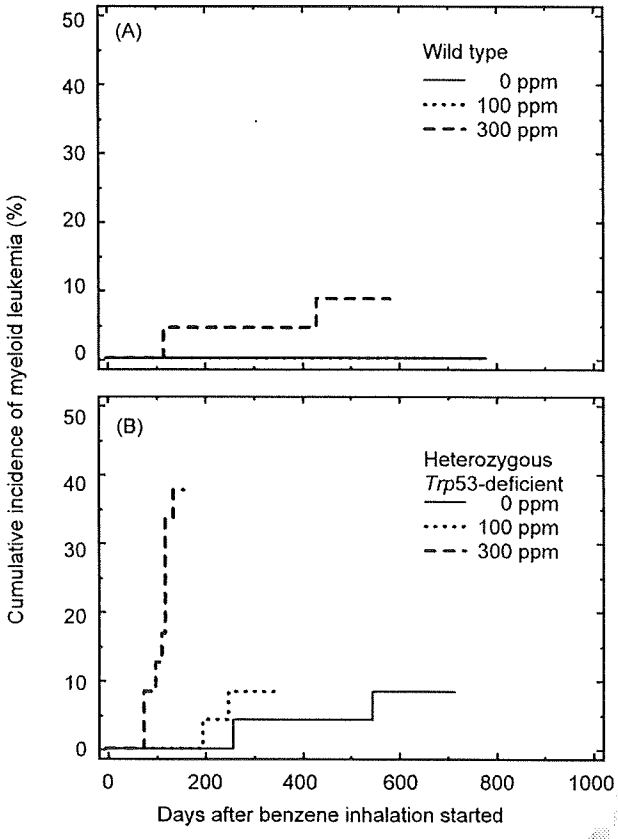

Fig. 2. Cumulative incidences of AMLs during the lifetime of C3H/He mice; wild-type mice (A) and heterozygous *Trp*53-deficient mice (B). Bold dotted lines indicate 300 ppm exposure dose, regular dotted lines indicate 100 ppm exposure dose, and solid lines indicate sham exposure controls. Statistical significance determined by log rank test: (A) no significant difference between groups; (B) 0 vs. 300 ppm, $p = 1.5 \times 10^{-4}$; 100 vs. 300 ppm, $p = 1.8 \times 10^{-4}$. Number of mice per group (%) at each data point is 0/23 (0), 0/24 (0), and 2/23 (8.7) for wild-type mice, and 2/24 (8.3%), and 2/24 (8.3), and 9/24 (37.5) for heterozygous *Trp*53-deficient mice for benzene exposure doses of 0, 100, and 300 ppm, respectively. The figures are taken from reference from [11].

4. Strain differences, reciprocal increase in genomic instability, and commonality-stochasticity relationship: microarray study

The dose of 150 mg/kg b.w./day for benzene exposure by gavage is the leukemogenic dose equivalent to the above-mentioned inhalation exposure dose, which induce similar incidences of HPNs through a lifetime [28,29]. Microarray analysis was performed after 2 weeks of intermittent gavage exposure to the equivalent dose of benzene. The 45,101 probe sets obtained were applied to an Affymetrix GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA). We observed two conceptually different gene expression profiles; "common gene expression profiles" (CGPs) and "stochastic gene expression profiles" (SGPs).

Benzene exposure-specific gene expression intensities analyzed by two-way analysis of variance (ANOVA) with a *p*-value of less than 0.05 were considered statistically significant and a total of 258 probe sets were obtained (CGPs). Among them, five categories were identified by dendrogram analysis without any supervising information, consisting of four different reciprocal components, i.e., two strains with or without benzene exposure (data not shown). Category #1 consists of genes with upregulation during the steady-state and downregulation after benzene exposure predominantly in the C3H/He strain; Category #2 consists of genes with upregulation during the steady-state and downregulation after benzene exposure predominantly in the C57BL/6 strain; Category #4 consists of genes with upregulation during the steady-state as well as after benzene exposure predominantly in the C3H/He strain; and Category #5 consists of genes with upregulation during the steady-state as well as after benzene exposure predominantly in the C57BL/6 strain. Category #3 consists of genes with downregulation, but shows no expression difference between both strains, either during the steady-state or after benzene exposure. The characteristics of each category and sample representative common genes are shown in Table 1. Namely, Category #1 consists of *Cryl1* and *Sltm*; the former is known to be related to genomic instability [17,18], whereas the latter is known to be related to suppression of apoptosis [16]; thus, this category suggests plausible benzene-induced gene expression for C3H/He-dominant induction of hematopoietic disorders. Other C3H/He-dominated changes in Category #4 involve *WSTF/Baz1b* (Williams–Beuren syn-

Table 1
Sample representative common genes in each category.

Affymetrix system name	Common name	Genbank ID	Description
Category #1: steady-state upregulation (B6 < C3) and Bz-induced downregulation (B6 < C3)			
1447112_s_at	Cryl1	C85932	Crystallin, lambda 1
1424452_at	Sltm	BC019992	SAFB-like, transcription modulator
Category #2: steady-state upregulation (C3 < B6) and Bz-induced downregulation (C3 < B6)			
1435329_at	Kdm2a/Fbxl11	BE690994	Lysine (K)-specific demethylase 2A/F-box and leucine-rich repeat protein 11
1420947_at	Atrx/rad 54	BB825830	Alpha thalassemia/mental retardation syndrome X-linked homolog (human)
1423521_at	Lmnb1	AA270173	Lamin B1
1429658_a_at	Smc2	BI684556	Structural maintenance of chromosome 2
1450051_at	Atrx/rad 54	BB825830	Alpha thalassemia/mental retardation syndrome X-linked homolog (human)
1420946_at	Atrx/rad 54	BB825830	Alpha thalassemia/mental retardation syndrome X-linked homolog (human)
1449292_at	Rb1cc1	BE570980	RB1-inducible coiled-coil 1
1434045_at	Cdkn1b ($p27^{kip1}$)	BB354528	Cyclin-dependent kinase inhibitor 1B (P27)
Category #3: steady-state expression (C3 ≈ B6) and Bz-induced downregulation (C3 ≈ B6)			
Category #4: steady-state downregulation (B6 < C3) and Bz-induced upregulation (B6 < C3)			
1420975_at	Baz1b/WSTF	BB253608	Bromodomain adjacent to zinc finger domain, 1B/Williams syndrome transcription factor (WSTF)
1424875_at	Spg20	BB040507	Spastic paraplegia 20, spartin (Troyer syndrome) homolog (human)
1420849_at	Crnkl1	AV143435	Crn, crooked neck-like 1 (Drosophila)
Category #5: steady-state downregulation (C3 < B6) and Bz-induced upregulation (C3 < B6)			
1435054_at	Eme1	BG064903	Essential meiotic endonuclease 1 homolog 1 (*S. pombe*)

C3: C3H/He, B6: C57BL/6, Bz: benzene.

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drome transcription factor, also known as *BAZ1B*; a component of the WICH complex [WSTF-ISWI ATP-dependent chromatin-remodeling complex]), *Crnkl1* (Crn, crooked neck-like 1), and *Spg20* (spastic paraplegia 20, spartin [Troyer syndrome] homolog), the functions of which are related to enhancement of double-strand break repair [30], the cell cycle suppression [31], and upregulation of a potential suppressive factor [32], respectively, suggesting C3H/He-dominant reciprocal genomic stabilization against the above-mentioned function in Category #1.

C57BL/6-dominated CGPs for genomic stabilization are identified solely on the basis of the upregulation of *Eme1* (essential meiotic endonuclease 1 homolog 1, a component of the Mus81-Eme1 structure-specific endonuclease) in Category #5 and downregulation of *Lmnb1* (lamin B1) in Category #2, which repair DNA damages [33] associated with cell cycle suppression [34] after benzene exposure. The remaining genes identified in Category #2 are plausible genes that respond to possible neoplastic changes; such as downregulation of $p27^{kip1}$, which results in release from cell cycle suppression [35–37], downmodulation of *Kdm2a*, which increases genomic instability [20], downregulation of *Atrx/Rad54*, which suppresses DNA repair [19], downregulation of *Smc2* (structural maintenance of chromosomes 2), which suppresses double-strand break repair [38], and suppression of *Rb1cc1* (RB1-inducible coiled-coil 1), which increases in genomic instability owing to the suppression of Rb1-inducible genomic stabilization [39]. These are common trends observed in gene expression profiles for each strain or, in some cases for both strains, which are plausible gene expression changes in mice with benzene-induced HPNs.

In contrast to the above CGPs in the two strains, or either strain, each individual mouse often shows unique SGPs, as shown in Fig. 3 ("*stochastic gene expression*"). Such SGPs often include transcription factors, which regulate reciprocal signaling pathways including further SGPs. Each mouse among the five mice in the group shows expression intensities of each gene different from those of the others. The two panels on the top of Fig. 3 showing the apoptosis-related genes of C57BL/6 (left) and C3H/He (right) mice reveal the different SGP combinations for each animal (numbers 01–05 in C57BL/6 mice, and 06–10 in C3H/He) mice. Huntingtin interacting protein 1 (*HIP-1*), known to be an apoptosis inducer [40,41], is expressed in three out of five C57BL/6 mice but is not overexpressed in any C3H/He mice. Investigation of such stochastic, case-by-case gene expression requires a prohibitive number of cases to clusterize each signal statistically; however, this may eventually support the observed strain differences after benzene exposure.

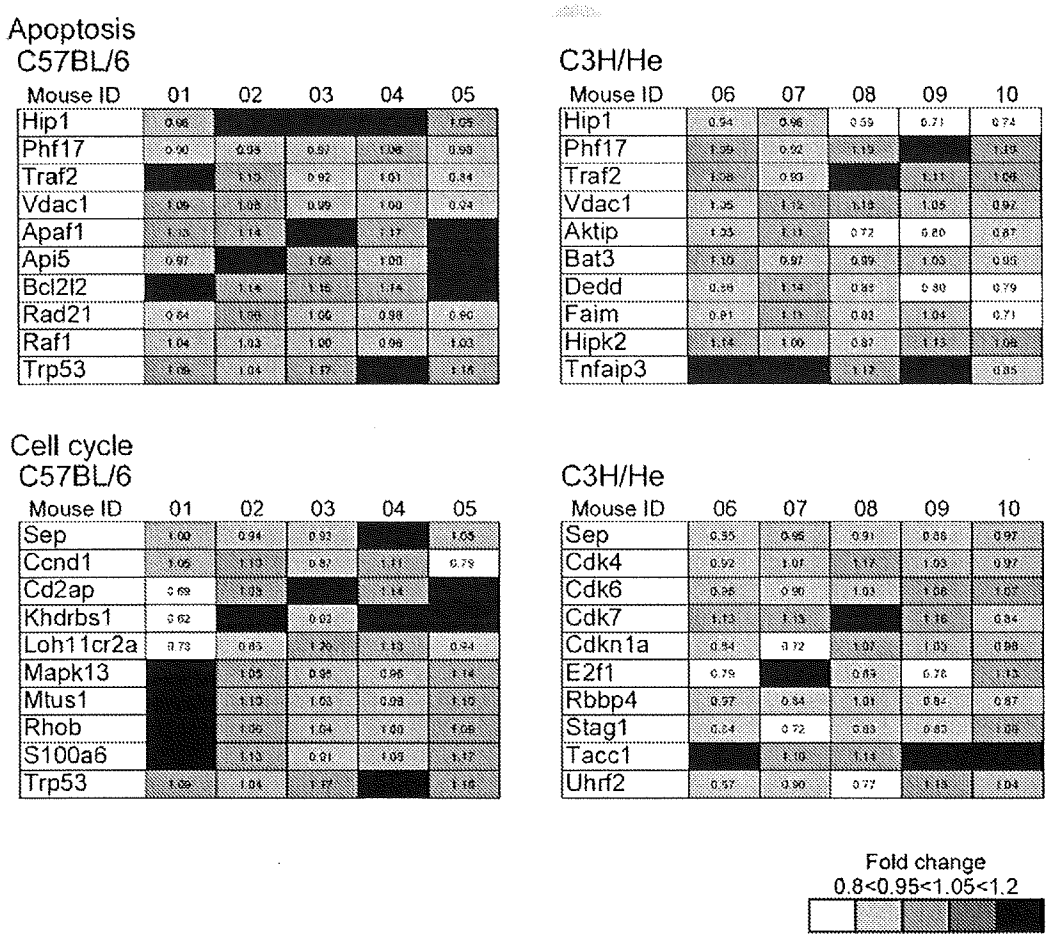

Fig. 3. Stochastic gene expression intensities in five mice of each strain: C57BL/6, 01–05 on the left; and C3H/He, 06–10 on the right. Ten representative genes for apoptosis, shown in the upper panels, and cell cycle-related genes, shown in the lower two panels are provided. Expression intensities are shown along the fold change scale in the bottom. Stochastic union genes were selected from each principal component analysis between a profile from one individual mouse and five other profiles from mice without benzene exposure, and total union genes with contribution scores above 0.9 or below −0.9 were collected; 1519 probe sets were obtained from C57BL/6 mice, and 1174 probe sets were obtained from C3H/He mice. Properties of selected union genes in each strain were analyzed by gene ontology (GO) and classified into categories, such as apoptosis-related or cell cycle-related.

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When one looks at the relationship between CGPs and SGPs in available gene expression signaling networks, possible bilateral relationships, i.e., centralizing signals from stochastic genes toward a commonly expressed gene, and diffusing signals from a commonly expressed gene to stochastic genes, are unsupervisedly identified. The CGPs are commonly shared among mice in each group, whereas the SGPs are unique union genes from one individual mouse to another. While the former profiles are definitive, and thus possibly diagnostic, the latter profiles are considered to be probabilistic and predictable if a number of cases can be clusterized statistically. Previous toxicological concept focused on the former definitive profiles simply owing to a lack of methodology for the latter. Because gene chip microarray techniques can now elucidate the above-mentioned stochastic changes, the newly developing field of stochastic toxicology may open up a world of new xenobiotic responses, which were ignored in previous toxicological studies as data of random dispersion [42,43].

Conflicts of interest statement

There are no conflicts of interest.

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内分泌攪乱化学物質の低用量作用と毒性学のあたらしい課題

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内分泌攪乱化学物質(いわゆる環境ホルモン)がヒトや野生生物の生殖に影響を与えるという危惧が新聞などで取り上げられてから10年以上が過ぎた．先頃は哺乳瓶に由来するビスフェノールAや玩具に関するフタル酸エステル類の扱いが取り上げられたが，ひと頃のように大きな話題にはならなかった．けだし内分泌攪乱問題は過去の出来事のように感じている人は少なくないかもしれない．しかしそうした人びとにも，その後この問題がどうなっているのか訝(いぶか)しく感じている向きはこれまた少なくないであろう．それだけに，内分泌攪乱化学物質問題とは，いったい何だったのか，いまそれはどんな意味をもって人々に関わっているのか．そんな点を中心に，これまでにわかってきたことを整理してみたいと思う．

* *

毒性学では，比較的高用量域における実験的生体反応の用量相関直線を，実験データ以下の低用量域へ外挿して無反応域[*1]を求め，それら無反応域よりも低い用量での生体影響を“無影響”と推定している．無反応域以下では，障害性はないと考えるわけである．平易な論理であるが，或る事柄が“ナイ”ということを予測し，保証するということは，科学の方法では大いに稀少なことである．必然的にこの論理には，用量相関が線形であるべきことなど様々の前提条件が求められ，これが破れるとその保証が効かなくなる．

しかし線形の用量相関を自明とする安易な認識とも相俟って，危惧された物質の環境中での曝露量は充分に微量と考えられたから，既知の無影響量以下の内分泌攪乱化学物質の曝露が，環境生物やヒトに生体影響を惹き起こす可能性は想定外であった．取り上げられた例もフロリダのアポプカ湖や五大湖での事故のように極端なケースにならざるを得なかった[*2]．だから低用量の内分泌攪乱化学物質にヒトや野生生物の生存を危うくする様々の危惧があるとして，これらが社会問題化したとき，環境生物やヒトで危惧される事象の事実関係もさることながら，もし事実とすれば“なぜ見落とされたのか”，あるいは，実験動物の観察でなぜ見いだされなかったのか，という疑問がまず取り上げられた．確かに，内分泌ホルモンに類する受容体を通じた低用量の影響が関与する可能

[*1] 無作用量(NOEL: no observed effect level)とか，無影響量(NOAEL: no observed adverse effect level)と呼ばれる．

[*2] たとえば，フロリダ州のアポプカ湖では，1980年，dicofolおよびDDTとその代謝物の汚染によりワニの棲息数が減少した．ミシガン湖周辺のカモメでは，DDT/DDEによるとされる雌化現象が観察された．

性などは，レイチェル・カーソンの『沈黙の春』[1]を思い起こした少なからぬ研究者が指摘した．経済協力開発機構(OECD)の試験開発部門が，従前，ホルモン剤の開発などに用いて知られていた“子宮腫大試験”を，環境中の化学物質の調査用にまったく新しく取り上げて調べ直すことになった経緯も，生体のホルモン受容体を介した影響への視点が働いていたし，世界保健機関(WHO)の化学物質安全計画部門がさらなる新たな試験法の開発の重要性を強調した所以でもあった．

こうした中で，高用量域から低用量域への直線外挿の如何を調べてゆくうちに，内分泌ホルモン影響には，それまで認識されていなかった「低用量作用」があることがわかってきた．たとえば，機構の異なるいくつかの複合影響をもたらす物質で生体影響について用量相関を見ると，しばしば非線形反応を呈し，低用量域ではじめて浮かび上がってくる性質が見いだされる．内分泌攪乱化学物質の低用量作用は，追試につぐ追試をうみ，にわかに注目を集めることとなったが，当時，メカニズムなど原理的な説明がつかなかったこととも相俟って，内分泌攪乱現象の真偽の決着はつかなかった*3．

かくして，内分泌攪乱化学物質に関する最初の国際ワークショップ，ウェイブリッジ会議から10年を経た2007年，その10周年のワークショップが，フィンランド科学アカデミーと欧州委員会(EU)の主催によりヘルシンキで開催された．そこでは，相加的で，無作用量(閾値)の認められない反応や，感染・免疫機構ないし神経・行動を中心に，これまで知られていなかった低用量影響がつぎつぎと紹介された．内分泌攪乱化学物質の生体影響の詳細には，依然として未知の要素が含まれているものの，ようやく，その片鱗が明らかになってきたのを感じた．

それらを取り入れてこれまでの経過を振り返れば，冒頭で述べた毒性学の論理の前提条件が，どこかで破れていたといわざるを得ない．しかもそれは，想定外の非線形反応にもとづく作用にとどまらず，未知の事柄を含む生物学の認識そのものに関わっていたため，条件の破綻として気付かれなかったものである．近代毒性学の中でいまだ理論化されていない論理の破れ，それは何だったのか．なぜ旧来の毒性学はこれを明らかにできなかったのか．いま低用量問題の本質のありかを解く意義は，ここにある．

「低用量問題」とは*4

内分泌攪乱化学物質の性質として，低用量作用が取り上げられたのは，化学物質のホルモン受容体を介した影響による極微量作動性の有無への疑問が発端であった．それはやがて「反応閾値の有無」への疑問へとつながり，それらの「相乗性・相加性の有無」，あるいは「高用量から低用量への外挿的推定の妥当性」や「反応の線形／非線形用量相関問題」などの諸問題に連関していった．やがて低用量問題として取り上げられた諸点が，相互に密接な関連をもったいわば“ひとつの問題”であることがわかってきた．だから解明の戦略は，その一角を取り崩すことであった．

2002年，ロンドン大学のコーテンカンプ(A. Kortenkamp)らは，内分泌攪乱性を有すると考えられる微量の物質のいくつかを一括して作用させたところ，個別に作用させたときには何らの影

*3 内分泌攪乱化学物質の疑いがあって作用機序の明らかでなかったものとしては，PCBsやPBBs，あるいは殺虫剤のうち塩素基や臭素基に置き換えたハロゲングループをもつような化学物質については，それらのフェノールの官能基の一部がステロイドホルモン受容体アゴニスト(作用物質)もしくはアンタゴニスト(競合物質)として働く性質があることがわかってきた．

*4 2000年10月，米国環境保護庁(EPA)は，いわゆる内分泌攪乱問題で対象となっているような物質影響が，通常の試験法で従来求められてきた無作用量(NOEL)や無毒性量よりも低い用量域で観察され得るかに焦点をあて，「低用量問題に関するワークショップ」をノースカロライナで開催した．そこでは，ビスフェノールA(BPA)の低用量データ報告の認否について，確認されたとする報告と認められなかったとする報告の双方に信頼性(credibility)を確認する結果となった．さらに低用量作用を示す試験の再現性や，長期試験がジエチルスチルベストロール(DES)にもBPAにも作用を示さなかった事実に言及し，低用量問題の不確実性を結論した．(http://www.epa.gov/scipoly/oscpendo/pubs/edmvs/lowdosepeerfinalrpt.pdf)

響を見せなかった用量の物質が，混合によって女性ホルモン(エストロゲン)にも匹敵する高いホルモン様活性を起こし得ることを試験管内反応で明らかにした*5[2][3]．個々ではわずかな影響に留まったとしても，複合した場合にこのような加算効果があるとすると，その影響はもはや無視できなくなると考えられる．これを相加性複合効果と呼んでいるが，かれらの実験のもうひとつのポイントは，限りなく閾値に近い低用量でこの相加性が認められたということである．続いて，個体レベルの試験系でも，ふたつのグループから顕著な相加効果が報告された[4][5]．すなわちデンマークにおける2007年のワークショップ*6での発表で，クリスチャンセン(S. Christiansen)らは，抗アンドロゲン作用をもつビンクロゾリン，フルタミド，およびプロシミドンなどの農薬の複合投与で，尿道下裂や肛門生殖突起間長の短縮などが，混合体の投与動物群のみに見られたこと[6]を，ライダー(C. V. Rider)らは，そのビンクロゾリン，プロシミドンの他，リヌロンなどの農薬と，BBP, DBP, DEHPなどのフタレート類，あわせて7種の混合投与*7で，同様の指標による複合効果が観察された[7][8]，と報告した．閾値付近での相加性の観察そのものが，従前では想定外な試験である．また，この結果は，フタレート類という類似の生体作用を有する物質の組合せゆえに認められた特異な現象とする考え方もあろうが，少なくとも類似のホルモン様作用シグナルに関する限り，複合効果を否定できないことが確定した点では，この結果のもつ意味は重く，今後のリスクアセスメント上，大きな検討課題を負うこととなった[9][10][11]．

種々のホルモンとホルモン類似物質による障害

以上のような認識に立って，身の回りの物質に目を向けてみると，われわれは，内分泌攪乱性が危惧される化学物質もさることながら，多くのホルモン物質，ホルモン類似物質そのものに取り囲まれて暮らしていることに気づく．もとより，生体ホルモンは，本来，低用量で作用し，また，用量，投与方法によっては有害になり得る性質のものである．したがって生体にはそのような影響を避けるためのより緻密な防護システムが備わっており，これが順調に機能していることが，外界から過度な影響を受けないようにする要件と考えられる．胎児の血清中に含まれる高濃度のα-フェトプロテインは，母胎間での女性ホルモンの影響を吸収する役割をもっているし，更年期女性に対するホルモン補充療法が，乳がんへのリスクなど様々の副作用を念頭において，ガイドラインに沿って慎重に行われることもそうした事情にもとづいている[12]．

これに対して，胚細胞期や胎児期・新生児期のように，まだ機能発達が安定する前の時点では，ホルモン様物質が生体ホルモンに置き換わって不可逆的な影響を及ぼすことが無視できない，とするデータが集積してきた[13]．この点は，思春期も同様と考えられる*8．こうした置き換え効果による障害や不全の可能性には，もとより注意が求められていた．ちなみに，ヒトでの発がん性に予防効果が期待され，ほぼ無制限に健康に良いかのごとくに理解されてきたいわゆる植物ホルモン(phytoestrogens)のひとつ，大豆イソフラボン*9

*5 これはかつてタフト大学のソト(A. Soto)が試験管内の複合アッセイ系確立の可能性を論じた報告にならって，個体レベルでの影響を見たものである(A. M. Soto et al.: Environ. Health Perspect., **105**(3), 647(1997))．実際のデータは，著者らの文中にあるような相乗性(synergy)は意味せず，相加性(additive)に相当する．

*6 第4回内分泌攪乱物質ワークショップ(2007年5月28～31日)．デンマーク環境省の後援で，コペンハーゲンにて開催された．

*7 フタル酸エステル類は，フタル酸とアルコールのエステル体で，ポリ塩化ビニルを主成分としたプラスチックの可塑剤として汎用されている．発生期の動物への曝露で，毒性，とくに生殖発生毒性が認められるため，フタル酸ビス(2-エチルヘキシル)(EDHP)をはじめとするフタル酸エステル類の玩具への使用は禁止されている．フタル酸エステル類の内分泌攪乱性は，女性ホルモン受容体への親和性が弱く確定していないが，何らかの複合的な影響の可能性を疑う研究者もあり，ここに示されるような複合影響の検討が行われてきた．最近，シャープ(R. M. Sharpe)らは周産期にフタル酸を曝露したラットにおける性分化の変調を報告し，注目されている(文献(8))．

*8 EPAは思春期アッセイ試験の採用を重視しており，またこの点は，WHOのグローバルアセスメントでも，巻頭の要旨で取り上げられるべきであったと考えられる．

*9 その功罪とも，糖質のはずれた大豆イソフラボン・アグ

の場合も，それまでの想定に反してその取り過ぎには，障害が惹き起こされる可能性が喚起されるようになっている*10．また，牛乳由来の調製乳を与えられた乳幼児と，豆乳由来の調製乳を与えられた乳幼児とでは，尿中のゲニスタインやダイゼインなどの植物ホルモン濃度は，後者は前者の500倍高いという報告[14]をあげて米国内分泌学会では注意を促している．こうした認識は，内分泌攪乱化学物質への理解の中で深まったもので，ホルモン様作用をもつ化学物質への注視は，もはやリスクアセスメントの必須要件となりつつある．

あらたに見いだされる低用量での生体影響

内分泌攪乱化学物質が，線形の用量反応関係をとらず，U字型や逆U字型の反応曲線をとったり，非常に低用量域で特異的反応を示すことについて，当初，そのこと自体に疑念を投げかける声も少なくなかった[15][16]．それはひとつに，当時その原理的説明がなし得ずいわば現象論に留まったこと，そしてなによりも安全性試験の領域で，非線形反応の想定される例を取り上げなかったからである．しかし様々の核内受容体やDNAの転写因子群の交差反応ネットワークを形成する受容体群や共役して働く補助因子での，至適の用量作動域がしばしば相互にずれていたりすることや，用量の増加とともに受容体反応が飽和に達し不応答状態になるといった現象が明らかにされるにつれて，問題点が整理され，急速に理解が進んでいる[17]．こうして理解のギャップの埋められた事柄は少なくないが，他方，まだ未知の領域にとどまるといわざるを得ない事柄も多い．米国の国立環境影響研究所(NIEERL)ではそうした点を重視して，これまでの研究計画のタイムラインを，より長期的展望をもって設定し，今後の検討に入っている[18]．

(1) 低用量域で観察される確率論的な生体反応

低用量影響の中には，種々の試験法を適用すると，しばしば意味のあるデータとは認識されず，いわばノイズのような結果として見られることが稀ではない．低用量での変化は頻度も低く，平均値をとるとしばしば測定のバラツキの中に隠れてしまうからである．内分泌攪乱現象が，特異な高感受性の遺伝的体質によって特定の個体に起こるものと考えて，たとえばエストロゲン受容体遺伝子多型を探索する研究も進められた．すると確かに先駆的な研究の中には，そうした原因形質が見いだされてきた[19]．しかし他方，非遺伝的に，確率論的に惹き起こされる可能性も見いだされている．この面からの研究は充分には進展していないが，ミシガン州立大学のグッドマン(J. Goodman)は，発がんプロモータの研究の中でつぎのような観察をしている．それは，フェノバルビタールによるDNAのメチル化という修飾の形成確率は，平均すれば実験群は対照群と差異が認められなかったが，ネズミ1匹ごとに検出してみると，対照群とは異なって，個体ごとに大きく変動した値が観察されたというものである[20]．DNAのメチル化は，エピジェネティックな変化と呼ばれるが，ここで認められた低用量におけるメチル化は確率論的に形成され，純系動物でも個体ごとに異なり同じ結果にはならない．このフェノバルビタールによるDNAのメチル化には，発がんのプロモータ作用が知られており，結果として，個体ごとに発がんの臓器分布や頻度が異なってくるという症状のランダムさを惹き起こすことになる．エピジェネティックな変化としては，他に，クロマチン凝縮，ヒストン修飾などがあるが，内分泌攪乱化学物質における低用量反応に対しても，こうしたエピジェネティックな現象として理解する考え方が急速に進展している[21]．これこそ従来の毒性学が想定してこなかった現象で，この領域での今後の進展に注視する必要がある．なお，こうしたエピジェネティックな変化がゲノムに刷り込まれて(＝ゲノムインプリンティング)，世代を超えて伝達され，固定されてゆく可能性も現実の問

リコンのエストロゲン類似作用に関連するものと考えられている．

*10 厚生労働省：大豆及び大豆イソフラボンに関するQ&Aを参照．(http://www.mhlw.go.jp/houdou/2006/02/h0202-1a.html)

題となっている[22][23].

(2) 高感受性期: 胎生期・新生児期・思春期の問題

機能的に安定する前の胎生期での影響に関して，無視できない不可逆的な事象が指摘されていることは前述した[24]．胎生期・新生児期・思春期問題には，低用量問題との関連を示すデータが少なからず見いだされており，WHOの報告書「グローバルアセスメント」[25]でも指摘された通り，胎児や新生児では，ウィンドウ効果*11と呼ばれるわずかな期間での投与が特異的な不可逆反応を惹き起こす現象が知られている[26][27]．また，野生型の成体では検知されない用量レベルだが，遺伝子改変動物などを用いた過剰反応系の動物を用いると検出される，“新しい概念の影響”の観点から，①閾値問題，②非線形の用量相関，あるいは③相加反応などの問題を見直す試みも進んでいる．内分泌攪乱化学物質として危惧される物質の生体影響研究では，影響メカニズムが未解明である一方，確認や追試が必要となることも少なくない．とくに時を経て遅れて現れる成長後の行動にかかる影響については，解析法そのものに未知の点が少なくない．系統的な実験的情報収集が求められる所以である．

WHOの「グローバルアセスメント」では触れられなかったが，性ホルモンのバランスの不安定な“思春期”に関する研究も，胎生期・新生児期と同様に注意が払われるべきと考えられる．胎生期や思春期などの性成熟の臨界期への曝露が与える影響の評価基準は，いまだ定まっていない．疫学的に尿道下裂の発症に関与する遺伝子 *CXorf6* がクローニングされ[28][29]，実験的には胎生期へのビスフェノールAの投与が思春期の早発傾向とつながるとの報告もなされている[30]．*CXorf6* のような遺伝子と化学物質との持続的な相互作用など，今後の研究が求められている．

*11 形態形成期である胎生期の狭い胎齢期間に，異物投与期特異的に生体影響が観察されること．

(3) 内分泌器官の拡張や，内分泌機能の概念の拡張

この10年余りの研究により，従来，性ホルモン受容体では想定されていなかった細胞内器官や組織に，内分泌器官の役割が，見いだされてきた．トマス(P. Thomas)らによる膜受容体*12の同定は，そのカテゴリーに含まれる発見であった[31][32]．この発見は，オルファニデス(G. Orphanides)らによって指摘されていた，従来の性ホルモン受容体機能に一般的であった核内受容体で説明の困難だった即時型反応を，遺伝子発現を介さないノンゲノミック(non-genomic)な機構にもとづくホルモン様作用[33]で理解するうえで決定的な役割を果した．やがて，細胞小器官である小胞体の膜にもエストロゲン受容体(ER)の局在が見いだされ[34]，急峻な反応への対応機構が明らかになっていった．これらの発見は，内分泌攪乱問題には，多くの未知の要因が関与していることをあらためて喚起した．肝臓や，脂肪細胞など，これまで内分泌器官とは考えられてこなかった臓器が，内分泌器官としての役割を果たしていることも明らかになってきた．たとえばノニルフェノールという物質は，通常の方法で見るとごく弱い女性ホルモン様の作用をもっているにとどまるが，肝臓に注目すると，生殖器よりもずっと強い活性を示すことが，岡崎国立基礎生物学研究機構の井口泰泉らによって明らかにされている[35]．これは，“内分泌器官としての肝臓”という見方につながる結果である．脂肪組織についても同様のことが指摘されている[36]．イボニシでの内分泌攪乱の知られる有機スズが脂肪組織の増殖を惹き起こすことや，それらの機構に核内受容体が関与していることなどは，これに関連するかもしれない．かくして，同じ受容体結合能を有するリガンド物質が，広範な標的受容体シグナル機構，さらにはまったく異なった表現型(フェノタイプ)の発現に関わる共働

*12 核内受容体と区別する．核内受容体が細胞内で，細胞質や核内にあって，特異的リガンド物質をDNAと特異的に相互作用を促し，転写に寄与するのに対して，細胞膜上に分布し，核酸と直接的相互作用を介さずにホルモン受容体影響を惹起する．

補助因子などとの相互作用を惹き起こすことなど，つい先頃までの認識を書き換える驚くべき関係が浮かび上がっている．

内分泌機能をもつ新しい器官の発見に加えて，内分泌器官そのものの概念を変える事象も見いだされている．異物受容体と呼ばれているダイオキシン受容体は，エストラジオールが存在しない状態では，P300と名付けられているタンパク分子の助けで転写活性化を担って，女性ホルモン様の作用をもつことが東京大学分子細胞生物学研究所の加藤茂明のグループによって発見された[37]．しかもここでは，エストラジオールがあるときは，この分子は，反対にユビキチン・リガーゼ(Ubiquitin ligase)と呼ばれる複合体を形成し，エストロゲン受容体を壊して，抗女性ホルモン様の役割を発揮する[38]．これは，ホルモン受容体でもない，異物受容体と呼ばれる生体内分子が，種々のホルモン様の作用を時に機能を変化させつつ発揮するということである．内分泌攪乱問題の分子的基盤が，概念的に大きく拡大しているものと考える所以である．内分泌系の拡がりを認識すれば，この発見の示唆する重みに改めて驚かされるであろう．

「生体調節障害の毒性学」を確立するために

内分泌攪乱化学物質問題が取り上げられるきっかけになったことそのものは，ヒトや野生生物の生殖や内分泌機能に関する危惧にあった．やがてその可能性の原点がホルモン作動性の化学物質の低用量での影響にあるものとの認識に近づいた．しかしこれは従来の試験法では有害性が観察されないなど，その背景となるメカニズムがなかなか明らかにならなかった．

すでに見たように，従来の試験法で観察されなかった背景は，この低用量作用が，従来の試験法が対象としていた毒性とは異なった生体障害機構にもとづくものであったためだった．内分泌攪乱化学物質では，生体内分子の壊変や変質などといった構造異常の前に，むしろ曝露影響は，低用量であるがゆえに通常の生体の生理的調節水準下で目に見えない形で微視的機能不全へと進行し，エピジェネティックに，次第に持続的調節不全に陥る生体異物相互作用の調節異常にもとづくのである．これまで注目されてこなかった事柄であり，事実，受容体を介した諸々の影響に焦点は収束しつつあったが，それらがどのように障害に結びつき得るかは，想定にとどまっていた．それは明らかに従来型の，生体分子の酸化や還元，DNAや脂質などの高分子への付加体形成や架橋形成などの化学的修飾，主として生体物質の壊変，変質といった化学反応を基礎とした直接的構造変化とは異なっており，それらに主眼をおいて作られてきた試験法では評価できなかったということであったと考えられる．

この問題の発端の頃，頻度の低い，胎生期のような形態形成期の事象や，小児の生殖器系に限局した内分泌攪乱現象を，稀な確率論的現象と解釈する報告がなされたことがあった．これも以上のような背景と結びついていたものと考えられる．裏返せば，フィードバック機構や“可逆性”の背後で，むしろ低用量曝露にあってはじめて惹き起こされるこの種の毒性は，それまでの毒性試験では検討されず，想定されていなかった．事実，先頃出版されたタイル(R. W. Tyl)らによるビスフェノールAの2世代試験でも，従来の毒性試験にない幅広の用量点をとった試験であったにもかかわらず，何らの影響も認められなかった[39]．タイルらの試験法と，この間工夫を重ねて行われた一連の研究における実験条件との違いには，前者における，持続投与によって惹き起こされるウィンドウ効果の棄却や，被験動物に不応性が導き出されることなどがあるものと推定されるが，この試験法の無力については，背景の解明，両者の相違の可視化のために，胎生期の狭い期間に限局した特異的な遺伝子発現と，投与異物との相互作用の詳細などが明らかにされる必要がある．

低用量における生体異物相互作用の調節障害は，内分泌攪乱化学物質問題を契機として見いだされた，これまでの毒性学の標的に含まれない生体障害性を基礎としたあたらしい概念の毒性現象であ

る．だから従来の毒性学の方法論に加えて，独自に進めるべき研究課題を含んでいる．たとえばこれらの調節障害を原理とした有害性では，通常の調節が生理的範囲から異常状態に移行する過程が，従来の表現型による線引きの困難ないわば振幅の変化のような，境界を含んでいる．そうした境界には直接的な構造異常が伴っていないようであり，これを裏付ける生体分子シグナル機構のより詳細な研究が必要である．こうした事柄は，あたらしい毒性学で求められるこれまでにない課題である．内分泌攪乱化学物質問題によってはじめて見いだされたこのあたらしい生体障害の概念は，当初の想定を超えて，一般論として生体調節障害の全域に及ぶ課題の拡がりを内包している．この点は，米国内分泌学会の内分泌攪乱物質に関する最近発表されたはじめての公式声明の冒頭でも指摘されている[40]．“化学物質の生体調節障害”という課題を対象としたあたらしい毒性学の確立のためには，さらなる概念の構築・整理と，対応する試験法の樹立を一層確かなものとする必要がある．

後記　本稿をまとめるにあたって戴いた，国立基礎生物学研究所・井口泰泉教授の助言に深謝する．

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Effects of diethylstilbestrol on ovarian follicle development in neonatal mice

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ABSTRACT

Previous results show that diethylstilbestrol (DES) causes polyovular follicles through estrogen receptor (ER) β and increases the number of follicles, suggesting that DES might affect follicular growth and development. Effects of neonatal DES exposure on follicle development were precisely examined in the ovaries of C57BL/6J and ERβ knockout (βERKO) mice. In the DES-exposed C57BL/6J mice, both primary follicle (PmF) progression from primordial follicles at 5 days of age and secondary follicle (SF) progression from PmFs at 10 days of age were delayed as compared with those in the oil-exposed controls. These results indicate that DES may suppress follicle development in neonatal mouse ovaries. DES exposure also decreased the number of follicles in 5-day-old C57BL/6J, WT and βERKO mice, suggesting that DES inhibits follicle formation and development through ERα in the neonatal mouse ovaries.

1. Introduction

In mice, neonatal exposure to diethylstilbestrol (DES) causes various abnormalities in the female reproductive organs, skeletal tissues and muscles [1–5]. In the ovary, several morphological changes are detected including absence of corpora lutea, hypertrophy of interstitial tissue and hemorrhagic cysts [6]. Polyovular follicles (PFs), which contain two or more oocytes per follicle, are also induced in the ovaries of mice exposed to DES perinatally [7]. DES induces polyovular follicles in the ovary directly *in vitro*, so pituitary gonadotropins may not be essential for the occurrence of polyovular follicles [8].

The actions of estrogen are mediated by estrogen receptors (ER), ERα and ERβ. ERα is a predominant form of ER in the uterus, vagina, ovary, testis, pituitary, mammary glands, kidney and adrenal glands, while ERβ expresses potently in the prostate, epididymis and ovary [9]. In the ovary, ERα is localized in interstitial and thecal cells, whereas ERβ is localized in granulosa cells [10]. DES can bind to both ERα and ERβ with higher affinity than that of 17β-estradiol (E2) [9]. In a study of ERα knockout (αERKO) and ERβ knockout (βERKO) mice, it is shown that effects of neonatal DES treatment on the uterus, oviduct, vagina, seminal vesicle and prostate are mediated through ERα [11]. On the other hand, our results have shown that DES induces polyovular follicles through ERβ [12]. In addition, an increase in the number of follicles larger than 50 μm in diameter is induced in the DES-exposed mice compared with that in the oil-exposed mice [12]. This result suggests that neonatal DES exposure might affect follicular growth and development, however, which ER subtypes are involved in the DES signaling is not clear.

Folliculogenesis is a sequence of events which commences after birth and continues through adulthood in rodents. Female germ cells proliferate and form cell clusters called germ cell cysts in the embryonic period [13]. Soon after birth, programmed cyst breakdown results in germ cell loss and follicle formation. Approximately one-third of oocytes survive to form primordial follicles (PrFs), while the others are lost via apoptosis. At 22.5 days post-coitus, most germ cells are separated. Then an oocyte begins to be surrounded by a monolayer of flat pregranulosa cells. Pregranulosa cells might be associated with the cyst breakdown and PrF formation [14,15]. Some portion of PrFs at the inner region of the ovary (inner cortex) develop to primary follicles (PmFs), which consist of an oocyte larger than 20 μm surrounded by cuboidal granulosa cells. Then, PmFs grow to secondly follicles (SFs), which

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consist of an oocyte surrounded by multiple granulosa cell layers [16].

Various intraovarian factors such as transcription factors and growth factors are essential for the growth and development in follicles [15]. Interaction between oocytes and surrounding somatic cells, which are sources of intraovarian factors and their targets, is also important for folliculogenesis. Factor in germline α (FIGα), newborn ovary homeobox encoding gene (Nobox) and spermatogenesis- and oogenesis-specific basic helix–loop–helix (bHLH) transcription factor 1 (Sohlh1) are transcription factors which express in oocytes and have crucial roles in the PrF development [17–19]. In granulosa cells, Foxl2, a member of the forkhead (Foxo)/hepatocyte nuclear factor 3 gene family, and Foxo3a are important to PmF development [20–22]. Nerve growth factor (NGF) and TrkB receptor have also important roles in PmF progression from PrF [23–25]. Kit ligand (KL), secreted from granulosa cells, binds to its receptor (Kit), which is present in the oocyte membrane. Parrott and Skinner [26] show that KL stimulates PrF to develop into PmF and promotes thecal cell recruitment from stromal cells in rats. Thus, folliculogenesis is a very comprehensive event, however, its mechanism is not completely understood. If DES affects folliculogenesis, DES may alter the expression of these intraovarian factors.

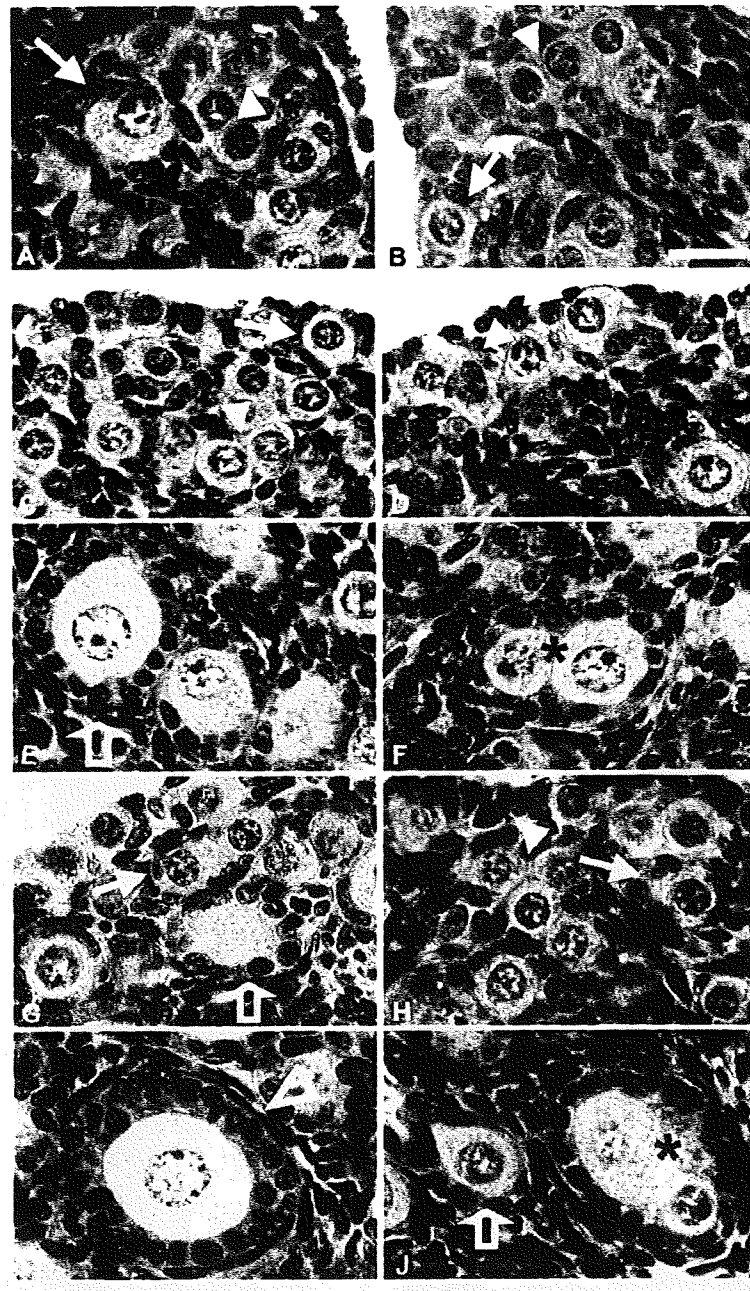

Fig. 1. Histology of ovaries in oil- (A, C, E, G and I) or neonatally DES-exposed (B, D, F, H and J) mice. Ovaries of 3- (A and B), 5- (C–F) and 7-day-old mice (G-J). In 5- and 7-day-old mice, ovaries show the outer cortex (C, D, G and H) and the inner cortex (E, F, I and J), respectively. Both oil- and DES-exposed mouse ovaries contain germ cell cysts (arrowheads) in the outer cortex (A–D and H), and primordial follicles (PrFs) (arrows) (C, D, G and H), primary follicles (PmFs) (open arrows) (E, F, G and J) and secondary follicle (SF) (open arrowhead) (I). Polyovular follicles (asterisks) were also seen in DES-exposed mice (F and J). Scale bar = 25 μm.

Table 1
Sequences of oligonucleotides used as primers for RT-PCRor real-time quantitative PCR.

Gene	Forward sequence (5′ → 3′)	Reverse sequence (5′ → 3′)
Figα	CCAAAGAGCGTGAACGGATAA	AGAGCCTTCAGCTTGGCAAAG
Nobox	TGCCGCTGGAGCTAAAGAGTA	CAACATAGCAGGCCAGTCCAT
Sohlh1	CCTGGCGAATCAGATTGCA	CCGAGACACAGCAGATGGTTT
Foxl2	AGCCAAGTTCCCGTTCTACGA	AGGTTGTGGCGGATGCTATTC
Foxo3a	AAGAACTCCATCCGGCACAA	CCCGTGCCTTCATTCTGAA
NGF	GGCCGAGGTGAACATTAACAA	CGGCACTTGGTCTCAAAAAAG
TrkB	CCTGCGGCACATAAATTTCA	GAACGGATTACCCGTCAGGAT
Kit	TACACGT GCAGCAACAGCAA	GAAGGCCAACCAGGAAAAGTT
KL	GGA AAAT AGT GGATGACC ACGT GT	TGGCCTCTTCGGAGATTCTTT
Cyclophilin	AGGTCCTGGCATCTTGTCCAT	CCATCCAGCCATTCAGTCTTG

This study examined the effects of neonatal DES exposure on the cyst breakdown and formation of PrFs. In order to study the effects of DES on follicle growth, we examined histology and the mRNA expression using real-time PCR in neonatal mouse ovaries. Furthermore, to elucidate the involvement of ER subtypes in the DES signaling, histological analysis was performed in βERKO mouse ovaries.

2. Materials and methods

2.1. Animals

Adult C57BL/6J mice (CLEA Japan Inc., Tokyo, Japan) were kept under 12 h light/12 h dark by artificial illumination (lights on 0800-2000) at 23–25 °C. They were fed a commercial diet (MF, Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water *ad libitum*. All animals were maintained in accordance with the NIH guide for the care and use of laboratory animals, and all experiments were approved by the institutional animal care committee of the Yokohama City University. βERKO mice from a C57BL6/129sv background, were obtained by mating females heterozygous and males homozygous for ERβ gene disruption, as described previously [27]. The day of birth was regarded as day 0 of age. Female pups of C57BL/6J, wild-type (WT) and βERKO mice were injected subcutaneously with 3 μg DES (Sigma Chemical, St Louis, MO, U.S.A.) dissolved in 0.02 ml sesame oil or the vehicle alone from days 0 to 4 for 5 days.

2.2. Histological analysis

Ovaries of 5-day-old C57BL/6J, WT or βERKO mice treated neonatally with oil or DES were fixed overnight in Bouin's solution at room temperature for Hämatoxylin and Eosin (HE) stain. Ovaries were embedded in paraffin, serially sectioned at 8 μm and stained with HE. The number of primordial follicles, primary follicles, secondary follicles and polyovular follicles in a section of ovaries was counted. Five different sections in the mid-portion of each ovary at least 50 μm apart were selected for counting the number of PmFs, SFs, polyovular follicles and all follicles. Five animals in WT or βERKO mice exposed to oil or DES were used for counting the follicles.

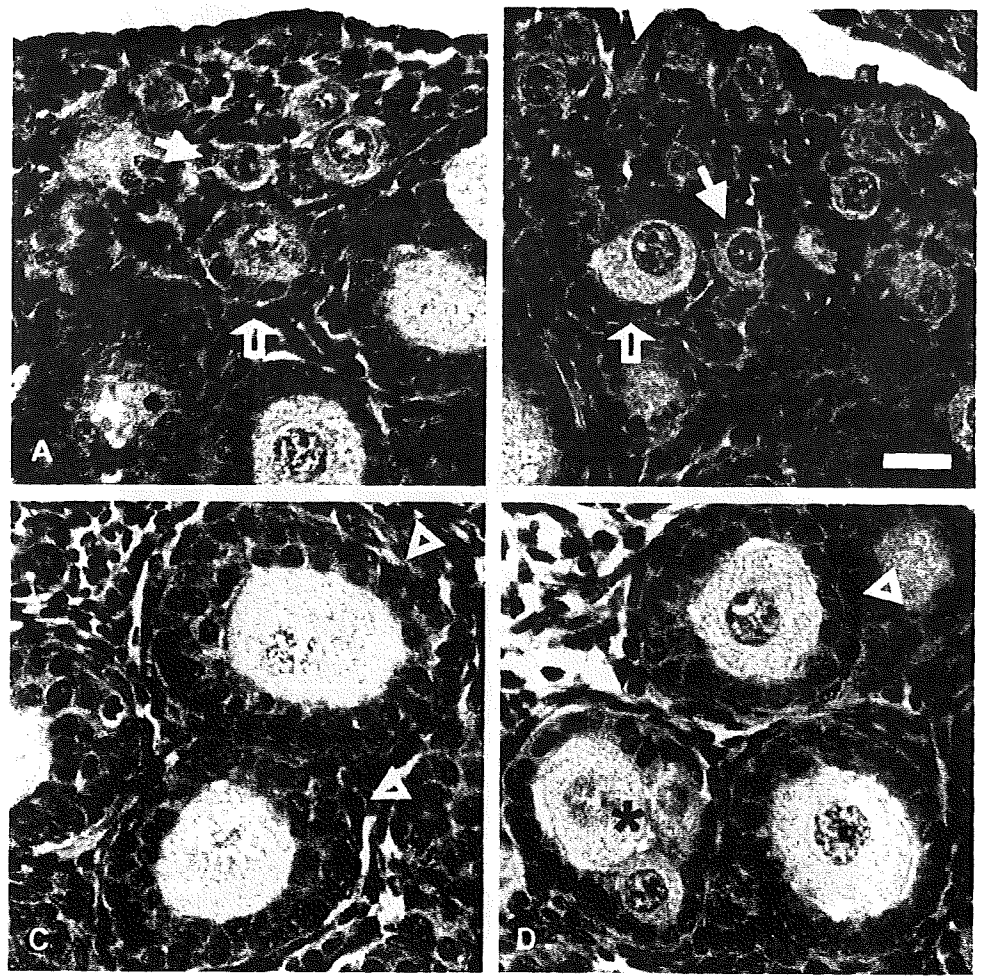

Fig. 2. Histology of ovaries in 10-day-old, oil- (A and C) or neonatally DES-exposed (B and D) mice. In the outer cortex of the ovary, PrFs (arrows) and PmFs (open arrows) were observed (A and B). SFs (open arrowheads) are seen in inner cortex (C and D). A polyovular follicle (asterisk) is also seen in DES-exposed mice. Scale bar = 25 μm.

2.3. Real-time quantitative PCR

Total RNA was isolated from the ovaries of 2-day-old C57BL/6J mice treated neonatally with oil or DES, and reverse transcribed into cDNA by the Super Script II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, U.S.A.) using 0.05 mM oilgo dT primer (Invitrogen). Real-time PCR was carried out by a Smart Cycler II System (Takara Bio Inc., Otsu, Japan) with SYBR Premix Ex Taq™ (Takara). Relative mRNA expression of Figα, Nobox, Sohlh1, Foxl2, Foxo3a, NGF, TrkB, Kit and KL (Table 1) was determined by the second derivative method. Cyclophilin was chosen as an internal standard to control variability in amplification due to differences in starting mRNA concentration. Melt curve analysis showed a single sharp peak for all samples. Five to ten mice were used for each group and three independent experiments were carried out for each study. Data analysis was performed followed by a paper previously described [28].

2.4. Statistical analysis

Parametric variables were analyzed by two-way analysis of variables with Student's *t*-test, Dunnett or Fisher's exact probability tests. Data were expressed as the mean ± standard error. $p < 0.05$ was considered significantly different.

3. Results

3.1. Histological analysis

In 3-day-old mice, germ cell cysts were observed in the outer cortex of both oil- and DES-exposed mouse ovaries (Fig. 1A and B). Primordial follicles, follicles that were an oocyte surrounded by a squamous granulosa cell layer, were observed both in the outer and inner cortex (Fig. 1A and B; arrows). Germ cell cysts (arrowheads) were observed in the ovaries of 3- and 5-day-old control mice (Fig. 1A and C) and 3-, 5- and 7-day-old DES-exposed mice (Fig. 1B, D and H). In 5- and 7-day-old mice, primary follicles, follicles that were an oocyte surrounded by a monolayer of cuboidal granulosa cells, were formed in the inner cortex of ovaries both oil- and DES-exposed mice (Fig. 1E, F, I and J). Secondary follicles, follicles including two or more layers of granulosa cells, have begun to appear in 7-day-old control mice, however, no SF was detected in DES-exposed mice (Fig. 1I and J). SFs were observed in both ovaries of 10-day-old oil- and DES-exposed mice (Fig. 2B and D). Polyovular follicles were frequently observed in ovaries of 5-, 7- and 10-day-old DES-exposed mice (Fig. 1F and J and Fig. 2D).

3.2. Ontogenic changes in the percentages of PrFs, PmFs and SFs

The number of follicles per section did not change from 5- to 10-day-old oil control mice (Fig. 3A). In contrast, the number of follicles in 5-day-old DES-exposed mice was significantly less than that of oil controls. PrFs decreased significantly with age in both oil control and DES-exposed mice and the percentage of PrFs per section in DES-exposed mice was significantly higher than that in oil control mice (Fig. 3B).

The percentages of PmFs and SFs per section increased significantly in both oil control and DES-exposed mouse ovaries from 7 to 10 days of age compared with those in 5 days (Fig. 3C). However, in DES-exposed mice, the percentages of PmFs at 5 days and SFs at 7 and 10 days were significantly lower than those in age-matched oil controls (Fig. 3C).

3.3. Polyovular follicles in the ovary of DES exposed mice

In 5-, 7- and 10-day-old mice treated with DES neonatally, the percentage of polyovular follicles was significantly higher as compared with that in oil controls (Fig. 4). No age related change was found in the percentage of polyovular follicles in either oil- or DES-exposed mice between 5 and 10 days of age.

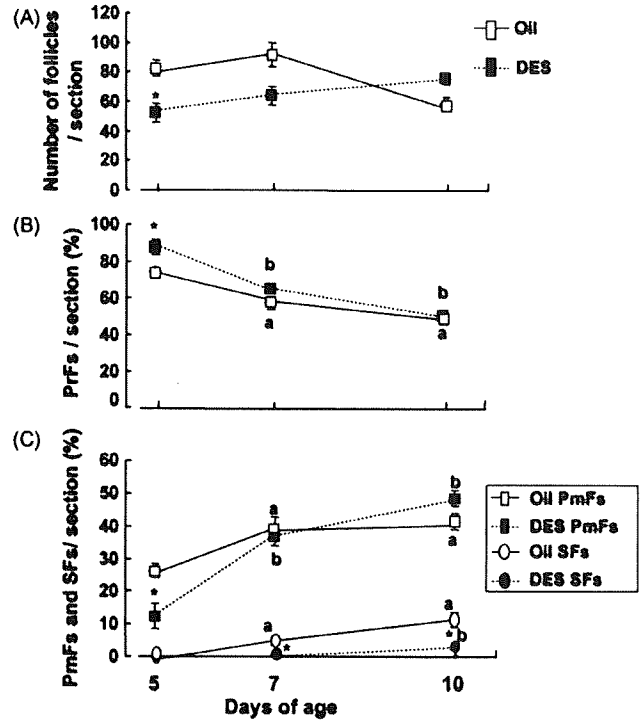

Fig. 3. The number of follicles per section of oil- or DES-treated mouse ovaries (A). Percentages of PrFs (B), PmFs and SFs (C) per section of oil- or DES-treated mouse ovaries. *$p < 0.05$, compared with age-matched oil control mice. (a and b) $p < 0.05$, compared with 5-day-old mice exposed to oil (a) or DES (b). Squares indicate PrFs and circle indicate SFs.

3.4. Histological analysis of βERKO mice treated neonatally with DES

Germ cell cysts, PrFs and PmFs were observed in the ovaries of 5-day-old WT mice exposed to oil or DES neonatally (Fig. 5A and B). However, no SF was found in both oil- or DES-exposed WT mice. In βERKO mice, germ cell cysts, PrFs and PmFs were observed in ovaries of 5-day-old mice exposed to oil or DES neonatally (Fig. 5C and D). Interestingly, SFs have begun to appear in 5-day-old oil-injected βERKO mice, however, no SF was detected in 5-day-old DES-exposed βERKO mice (Fig. 5D and E). While PFs were frequently observed in ovaries of 5-day-old DES-exposed WT mice (Fig. 5F), PFs were not found in DES-exposed βERKO mice (Fig. 5D).

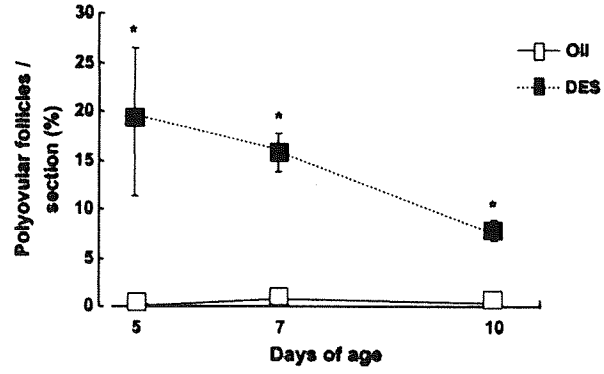

Fig. 4. Percentage of polyovular follicles per section in ovaries from oil- or neonatally DES-treated mice. *$p < 0.05$, compared with age-matched oil controls.

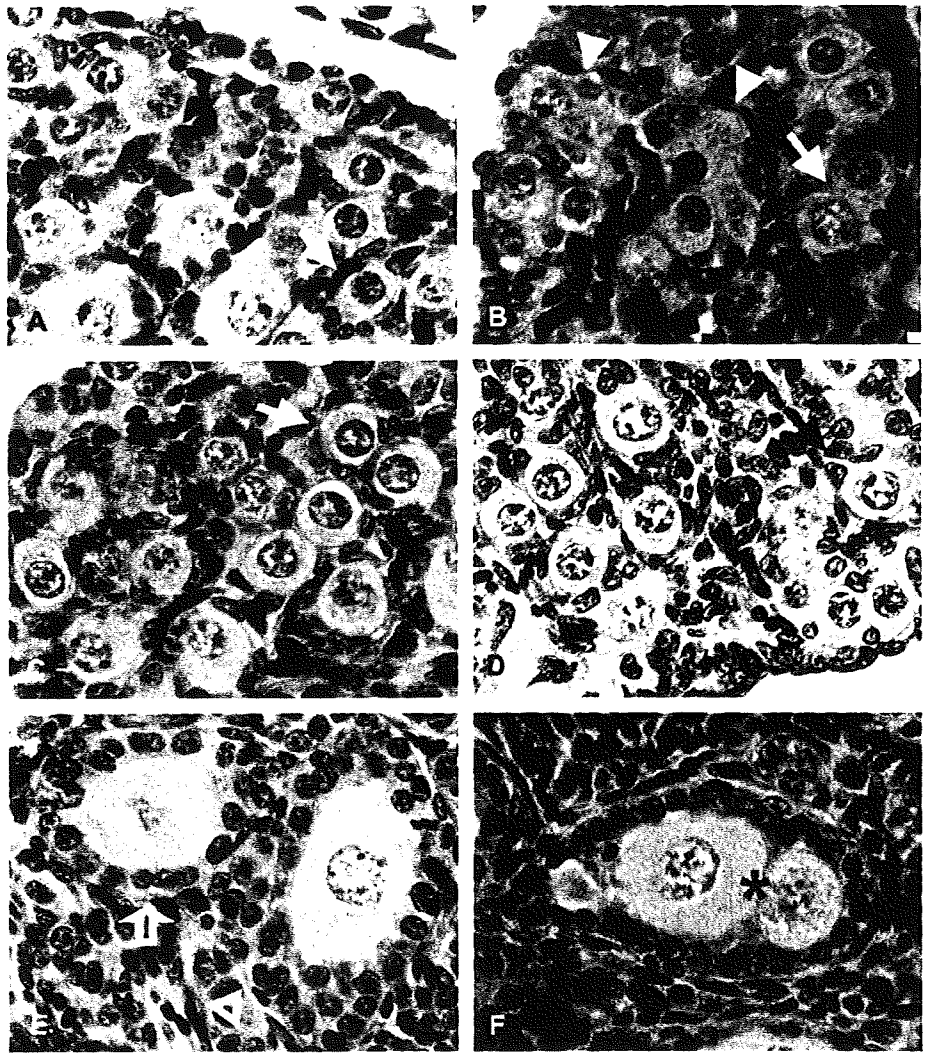

Fig. 5. Histology of ovaries in 5-day-old WT (A, B and F) or βERKO (C–E) mice exposed to oil (A, C and E) or DES (B, D and F) neonatally. E shows SF in the inner cortex of oil treated βERKO mice (open arrowhead). F shows a polyovular follicle in DES treated WT mouse ovaries (asterisk). Arrowheads indicate germ cell cysts and arrows indicate PrFs. Open arrow indicate PmF. Scale bar = 25 μm.

3.5. Changes in the number of follicles from oil- or DES-exposed WT or βERKO mice

In DES-exposed 5-day-old WT mice, the number of follicles per section was significantly less than that in oil-exposed mice similar to the results in C57BL/6J mice (Fig. 6A). A significant decrease in the follicle number caused by neonatal DES treatment was also recognized in βERKO mice (Fig. 6A). In WT mice, the percentages of PrFs and PmFs were not changed by DES treatment (Fig. 6B). Moreover, in βERKO mice, the percentages of PrFs and PmFs were not changed by neonatal DES treatment similar to the results in WT mice (Fig. 6B). As described above, the percentage of SFs significantly decreased in DES-exposed βERKO mice compared with that in oil-exposed βERKO mice (Fig. 6B).

DES treatment increased number of mice with polyovular follicles significantly both in WT and βERKO mice at 5 days of age (Fisher's exact probability test). The percentage of polyovular follicles per section in DES-exposed WT mice was significantly higher than that in oil controls (Fig. 6B). However, the percentage of polyovular follicles per section did not change in oil controls and DES-exposed βERKO mice.

3.6. Changes in the mRNA expression in 2-day-old mouse ovaries

The mRNA expression of Figα, Nobox, Sohlh1, Foxl2, Foxo3a, NGF, TrkB, Kit and KL, genes which are associated with follicular growth, was not changed by neonatal DES treatment in 2-day-old mouse ovaries (Fig. 7).

4. Discussion

This study shows that neonatal DES exposure delayed follicle development in C57BL/6J mouse ovaries. In the ovary of 5-day-old DES-exposed mice, the percentage of PrFs was significantly higher than that in oil-exposed mice, whereas the percentage of PmFs was significantly lower. The number of follicles was also decreased by DES exposure in 5-day-old mice. These results suggest that DES suppresses PrF formation and PmF progression from PrFs in 5-day-old mouse ovaries. In 10-day-old DES-exposed mice, the percentage of PrFs was not altered, however, the percentage of SFs was significantly lower than that in oil controls. These results suggest that neonatal DES exposure also suppresses SF progression from PmFs in 10-day-old mice.