Fig. 4

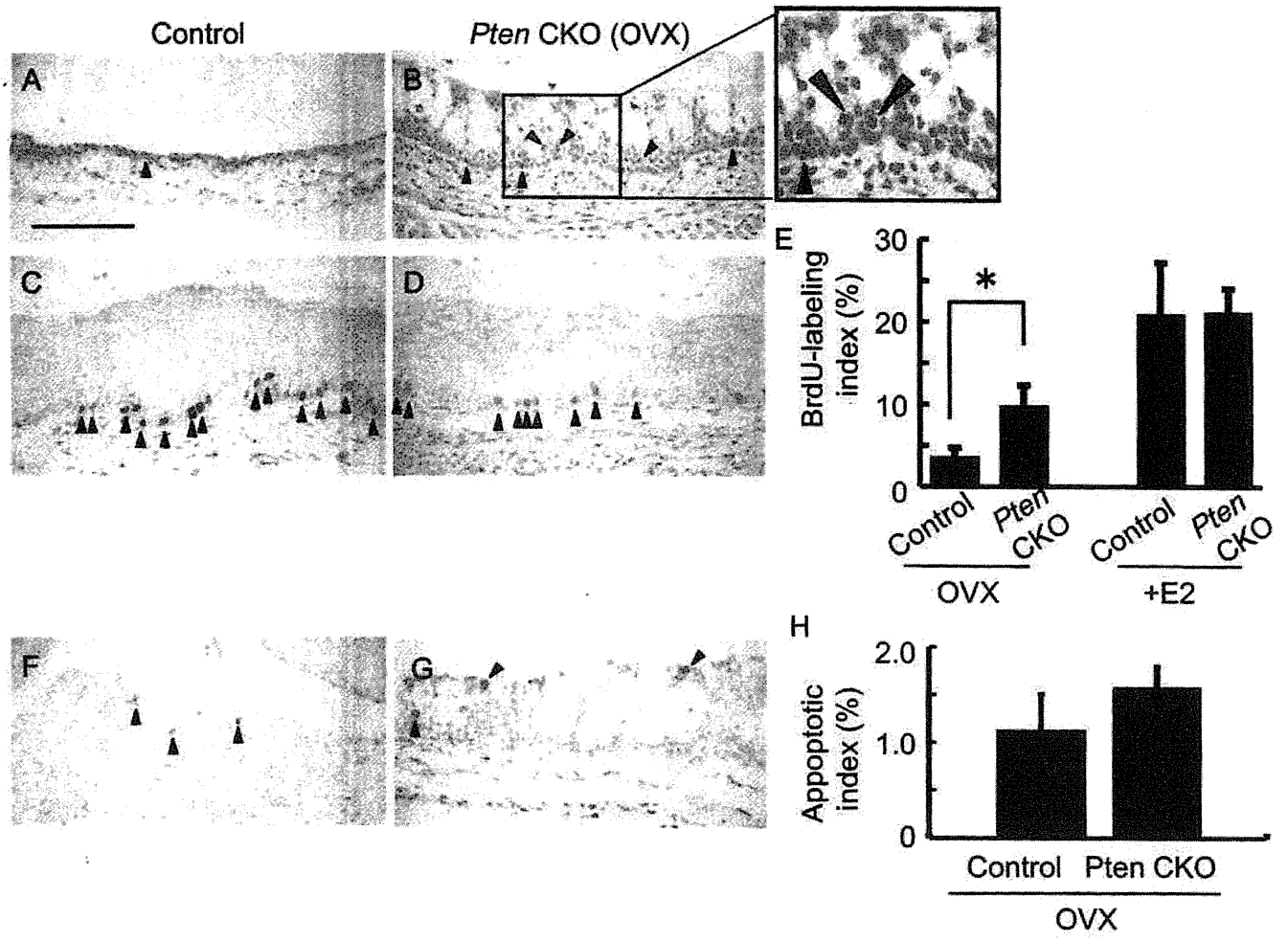

Control
Pten CKO (OVX)
A
B
C
D
E
BrdU-labeling index (%)
30
20
10
0
*
Control
Pten CKO
Control
Pten CKO
OVX
+E2
F
G
H
Appoptotic index (%)
2.0
1.0
0
Control
Pten CKO
OVX

Fig. 5

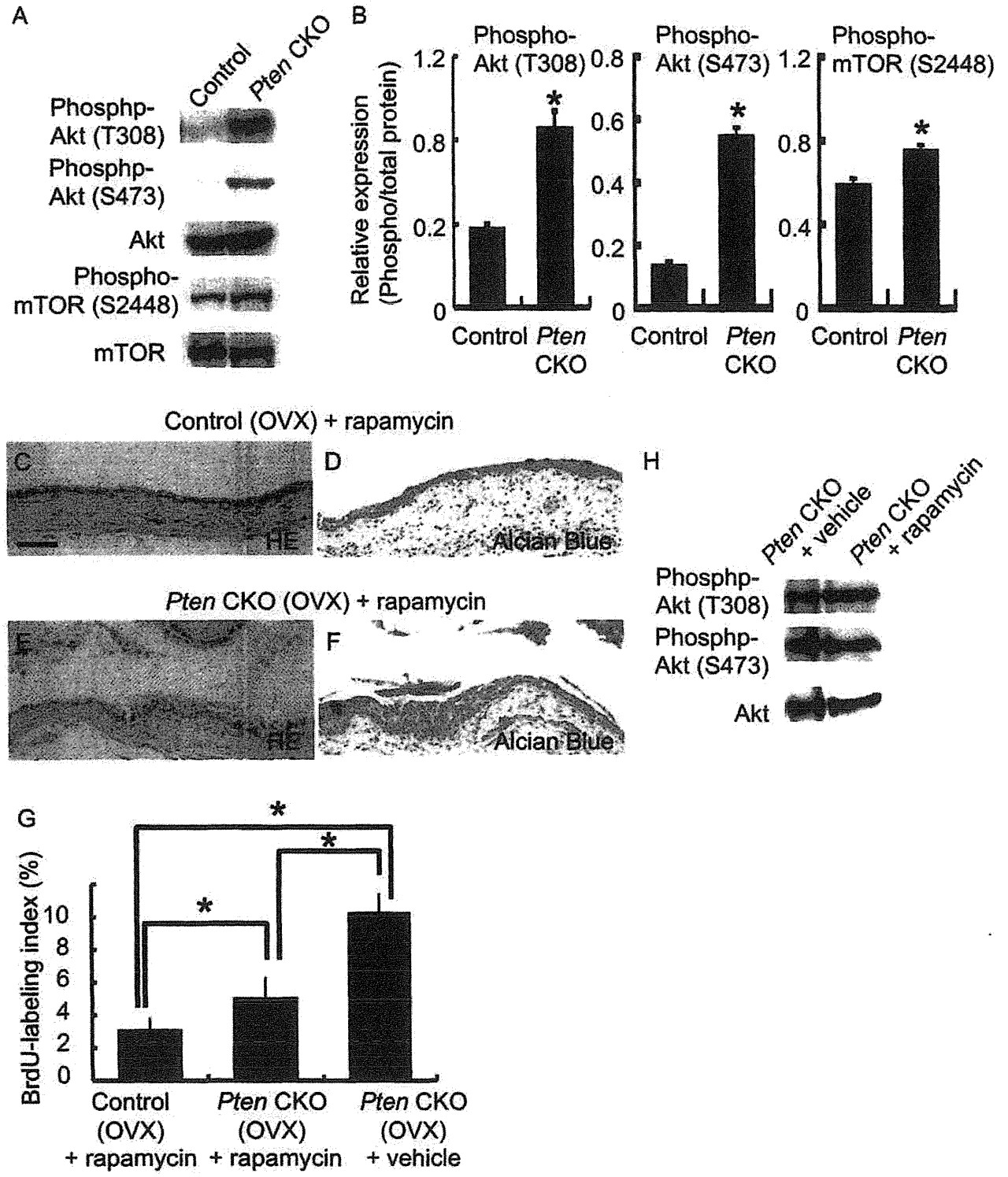

A
Control
Pten CKO
Phosphp-Akt (T308)
Phosphp-Akt (S473)
Akt
Phospho-mTOR (S2448)
mTOR
B
Phospho-Akt (T308)
Phospho-Akt (S473)
Phospho-mTOR (S2448)
Relative expression (Phospho/total protein)
Control
Pten CKO
Control (OVX) + rapamycin
C
HE
D
Alcian Blue
Pten CKO (OVX) + rapamycin
E
HE
F
Alcian Blue
H
Pten CKO + vehicle
Pten CKO + rapamycin
Phosphp-Akt (T308)
Phosphp-Akt (S473)
Akt
G
BrdU-labeling index (%)
Control (OVX) + rapamycin
Pten CKO (OVX) + rapamycin
Pten CKO (OVX) + vehicle

Fig. 6

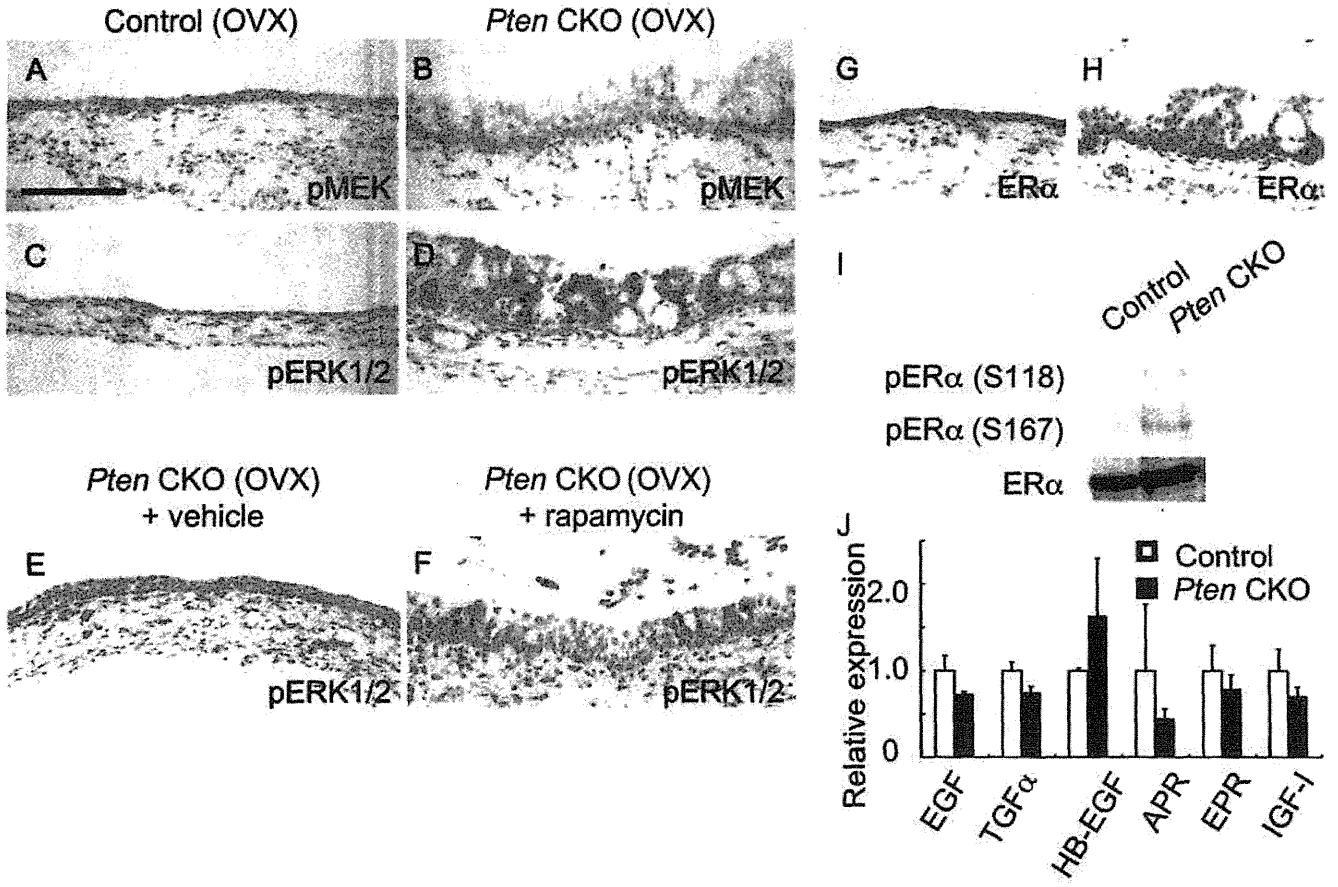

Control (OVX)
Pten CKO (OVX)
A
pMEK
B
pMEK
C
pERK1/2
D
pERK1/2
G
ERα
H
ERα
I
Control
Pten CKO
pERα (S118)
pERα (S167)
ERα
Pten CKO (OVX)
+ vehicle
Pten CKO (OVX)
+ rapamycin
E
pERK1/2
F
pERK1/2
J
Relative expression
2.0
1.0
0
Control
Pten CKO
EGF
TGFα
HB-EGF
APR
EPR
IGF-I

Fig. 7

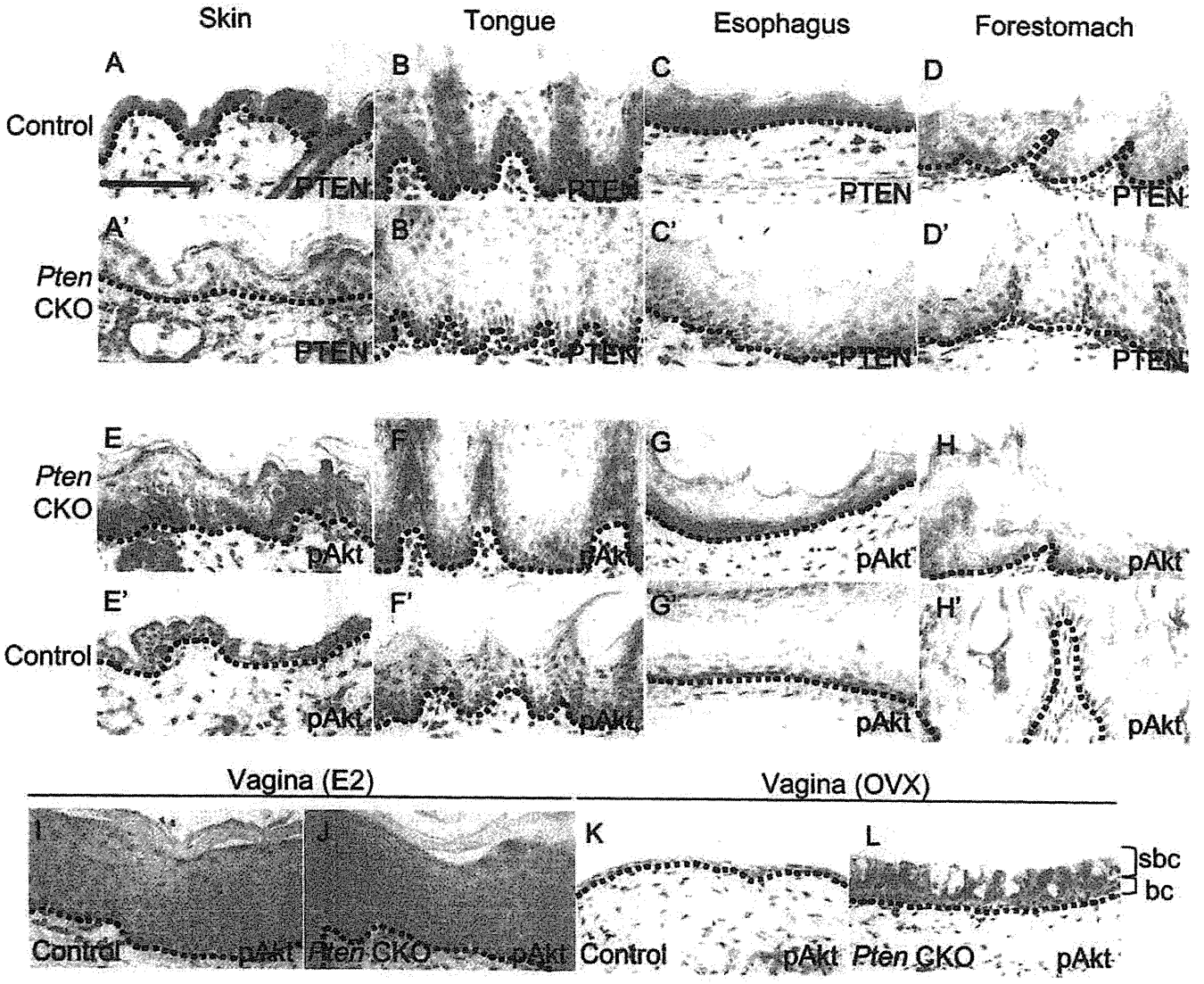
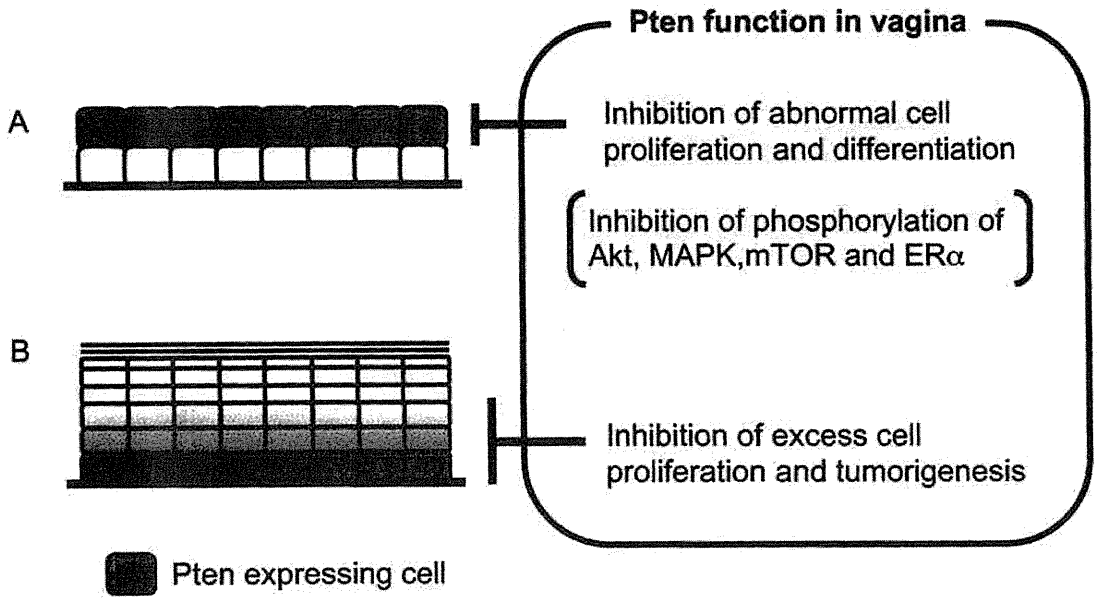

Skin
Tongue
Esophagus
Forestomach
A
B
C
D
Control
PTEN
A'
B'
C'
D'
Pten CKO
E
F
G
H
pAkt
E'
F'
G'
H'
Vagina (E2)
Vagina (OVX)
I
J
K
L
sbc
bc
Control
Pten CKO

Fig. 8


Pten function in vagina
A
Inhibition of abnormal cell proliferation and differentiation
Inhibition of phosphorylation of Akt, MAPK,mTOR and ERα
B
Inhibition of excess cell proliferation and tumorigenesis
Pten expressing cell

in vivo 27: 333-338 (2013)

# Epithelial–stromal Interactions in the Mouse Vagina Exposed Neonatally to Diethylstilbestrol

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**Abstract.** *Background: In neonate mice exposed to diethylstilbestrol (neoDES), vaginal epithelium shows persistent proliferation and stratification even after ovariectomy. Tissue recombination studies suggest that neonatally-estrogenized vaginal stroma can induce vaginal epithelial hyperplasia depending on the stromal age. This study examined the proliferative effect of the vaginal stroma from 8-day-old mice treated with DES on the vaginal epithelia of 8-day-old and adult mice. Materials and Methods: Vaginal epithelium and stroma from 8-day-old and adult mice was recombined, and grafted to ovariectomized host mice. Results: The 5-bromo-2'-deoxyuridine (BrdU)-labeled cells in the epithelium and the number of epithelial cell layers were not significantly different between epithelia from 8-day-old and adult mice when combined with stroma from 8-day-old control mice. BrdU-labeled cells in the vaginal epithelia from both age groups combined with the stroma from 8-day-old neoDES mice exhibited higher values. The epithelium from neoDES adult mice had a lower percentage of BrdU-labeled cells. Conclusion: The stroma from 8-day-old neoDES mice induces epithelial cell proliferation, but lower stromal cell proliferation.*

The mouse vagina consists of stroma and stratified squamous epithelium, with 2 to 12 cell layers. During estrous cycles, vaginal epithelial cells proliferate and stratify (up to 12 layers) and the superficial cells undergo keratinization in response to estrogen. Overiectomy induces a decrease in the number of epithelial cell layers, epithelial thickness and mitotic index, accompanied by an increase of apoptotic cells (1, 2).

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*Key Words:* Epithelial–stromal interactions, vagina, mouse, diethylstilbestrol.

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Diethylstilbestrol (DES), a synthetic estrogen, was routinely prescribed to several millions of pregnant women from 1946 to 1971 for the prevention of abortion (3). It is now well-known that *in utero* exposure to DES induces vaginal clear-cell adenocarcinoma in young women (4). In mice, neonatal exposure to estrogens, including DES, induces ovary-independent persistent proliferation and cornification in vaginal epithelium (5). Vaginal epithelial cells from ovariectomized adult mice exposed neonatally to DES (neoDES) exhibit a decrease in the initial rate of cell proliferation and an altered sensitivity to epidermal growth factor and insulin *in vitro*, suggesting that epithelial cells isolated from DES-exposed vaginae in mice are different in sensitivity on hormones and growth factors, from those of control mice (6, 7). Thus, neonatal DES exposure alters the characteristics of epithelial cells and sensitivity to hormones and growth factors in the mouse vagina.

Cunha *et al.* (8) examined the epithelial-stromal interactions of ovary-independent vaginal hyperplasia using a tissue recombination technique. While recombinants of the epithelium and the stroma from adult control mice exhibited an atrophied epithelium in ovariectomized hosts, control epithelium associated with the stroma from adult mice treated with estrogen neonatally-exhibited hyperplasia, suggesting that the stroma from neonatally-estrogenized mice induced cell proliferation in the control epithelium. On the other hand, control epithelium from 8-day-old mice exhibited atrophied epithelium in ovariectomized hosts when it was recombined with 8-day-old mouse stroma treated with estrogen neonatally. These results indicate that vaginal stroma from 8-day-old mice has less inductive activity compared to adult vaginal stroma, or the epithelium from 8-day-old mice is not able to accept the proliferation signals from the estrogenized stroma.

The underlying stroma can induce and specify the regional characteristics of the epithelium (9). Uterine epithelium combined with vaginal stroma differentiates into a vaginal stratified squamous epithelium. Similarly, vaginal epithelium in association with uterine stroma exhibits uterine epithelial

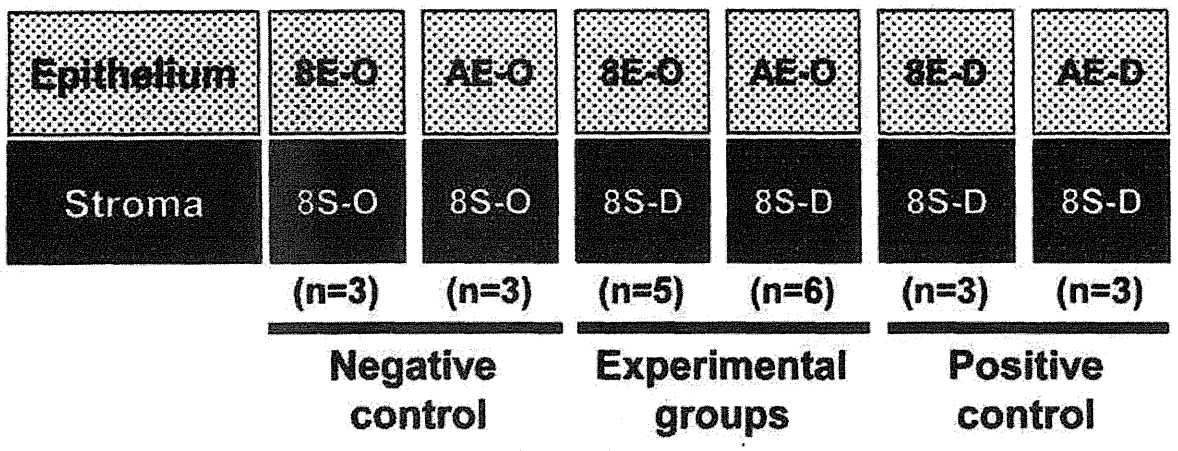


Figure 1. *Diagram indicating the tissue recombinant groups: 8S-O, stroma from vagina from 8-day-old mice; 8E-O, the epithelium from vagina from 8-day-old mice; AE-O, epithelium from adult mouse vagina; 8S-D, stroma from vagina from 8-day-old mice exposed neonatally to diethylstilbestrol (DES); 8E-D epithelium from 8-day-old mouse vagina exposed neonatally to DES; AE-D, epithelium from vagina from adult mice exposed neonatally to DES.*

differentiation. The developmental response of epithelia is age-dependent and neonatal epithelium has a higher sensitivity to induction signals from the stroma than the adult epithelium (9). Thus, the balance of epithelial sensitivity and stromal induction capacity may differ with age.

The aim of this study was to examine whether the vaginal stroma from 8-day-old neoDES mice induced continuous cell proliferation of untreated epithelium. Vaginal epithelium and stroma from 8-day-old neonatally DES- or oil-treated mice were separated, recombined with the epithelium from 8-day-old or adult mice, and transplanted into ovariectomized host mice, the epithelial morphology and proliferative activities of cells in the grafts were then examined.

## Materials and Methods

*Animals.* C57BL/6J mice purchased from CLEA Japan Inc. (Tokyo, Japan) were given commercial diet (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*. All animals were housed under controlled lighting (12 h light/12 h dark) and temperature (23-25°C) conditions and maintained in accordance with the NIH guide for the Care and Use of Laboratory Animals (10) and all experiments were approved by the Institutional Animal Care Committee of the Yokohama City University (#20402).

*Treatments, tissue separation and recombination.* Neonatal C57BL/6J mice were daily injected subcutaneously with 3 μg DES (Sigma Chemical Co., St. Louis, MO, USA) dissolved in 0.02 ml sesame oil or the oil vehicle-alone for five days from the day of birth (=day 0). Vaginae were dissected from oil- or DES-treated 8-day-old mice and 90- to 120-day-old mice ovariectomized 10 days before termination, then incubated with 1% trypsin (Gibco BRL, Gaithersburg, MD, USA) in Hank's balanced salt solution (HBSS) (Sigma Chemical Co.) for 90 min at 4°C. After trypsinization, tissues were placed into 20% fetal bovine serum (Sigma Chemical Co.) in HBSS for 5 min, and vaginal epithelium and stroma were separated by gentle mechanical manipulation using fine forceps. The procedure of tissue recombination was performed according to previous reports (8, 9). Six types of recombinants were prepared (Figure 1): Stroma from vaginae from 8-day-old mice (8S-O) plus epithelium from vaginae from 8-day-old mice (8E-O); 8S-O plus epithelium from vaginae from adult mice (AE-O); stroma from vaginae from 8-day-old mice exposed neonatally to DES (8S-D) plus 8E-O, 8S-D plus AE-O; 8S-D plus epithelium from vaginae from 8-day-old mice exposed neonatally to DES (8E-D); and 8S-D plus epithelium vaginae from adult mice exposed neonatally to DES (AE-D). Tissue recombinants were incubated on agar plates overnight at 37°C in humidified, 5% $CO_2$ atmosphere in air.

*Grafting, harvesting and histological analysis.* Tissue recombinants (n=2-3 of each group) were transplanted under the renal capsules of ovariectomized host mice (n=3-6) for two weeks. Hosts were injected intraperitoneally with 5-bromo-2'-deoxyuridine (BrdU) (Sigma Chemical Co.) (10 mg/100 g body weight) 2 h before sacrifice to identify proliferative cells. Host mouse uterus and vaginae were weighed to confirm the lack of estrogenic stimulation at dissection. Grafts were fixed in 10% formalin neutral buffer solution (Wako Pure Chemical Industries, Osaka, Japan), embedded in paraffin and sectioned at 6 μm of thickness. Sections were stained with hematoxylin and eosin for histological analysis.

*BrdU immunostaining.* To determine the number of proliferative cells on the grafts, BrdU immunostaining was performed. Sections were mounted on glass slides coated with 2% 3-aminopropyltriethoxysilane (Sigma Chemical Co.), de-paraffinized, hydrated with graded ethanol and rinsed in phosphate-buffered saline (PBS) (pH 7.4). Endogenous peroxidase was blocked by 0.3% $H_2O_2$ in methanol for 10 min and immersed in 2 N HCl for 20 min at room temperature to denature the genomic DNA, then neutralized in 0.1 M sodium tetraborate buffer (pH 8.5). After washing with PBS, sections were incubated with 1% trypsin (Sigma Chemical Co.) for 30 min at 37°C for antigen retrieval.

Sections were incubated with 1% bovine serum albumin (BSA) in PBS (BSA/PBS) for 20 min at room temperature and incubated

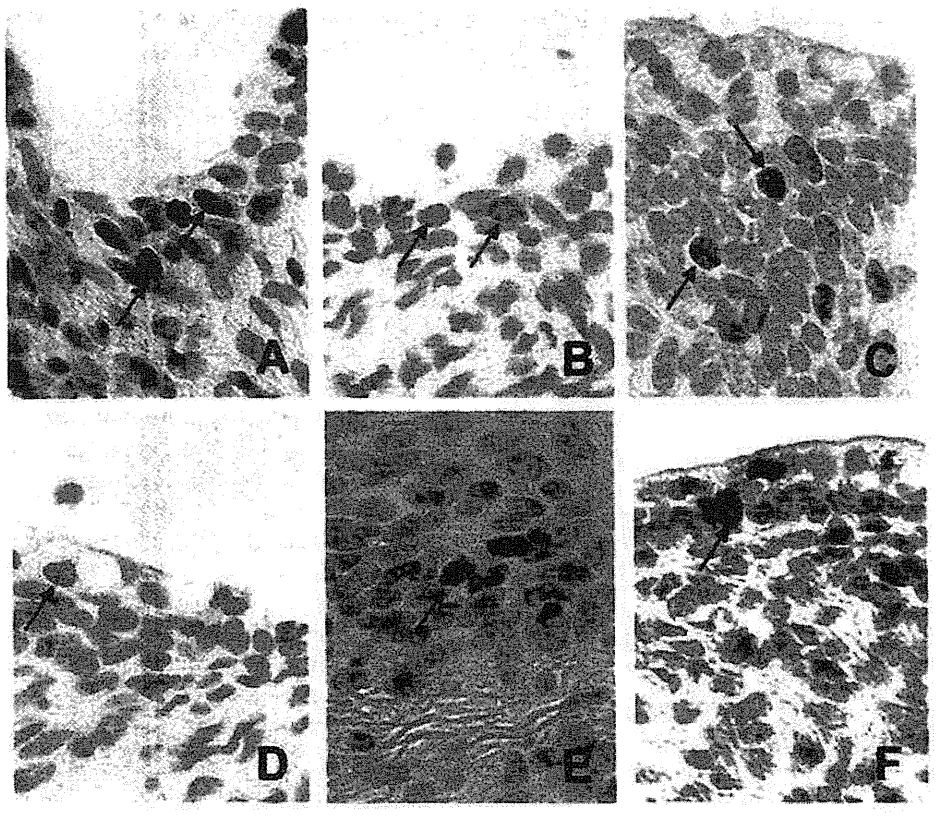


Figure 2. *Representative histological sections of 5-bromo-2'-deoxyuridine (BrdU) immunostaining of the tissue recombinants, 8S-O plus 8E-O (A), 8S-O plus AE-O (B), 8S-D plus 8E-O (C), 8S-D plus AE-O (D), 8S-D plus 8E-D (E) and 8S-D plus AE-D (F), respectively. (See lagend to Figure 1 for groups). BrdU-labeled cells are shown by arrows. Bar=20 μm. The cells indicated by arrows do not appear to stain as densely in all photos.*

with anti-BrdU-peroxidase, Fab fragments (1:15 dilution; Roche Diagnostics GmbH, Mannheim, Germany) in 1% BSA/PBS at 4°C overnight. Peroxidase visualization was performed using 2 mg/ml diaminobenzidine (DAB) (Sigma Chemical Co.) dissolved in 0.05 M Tris/HCl buffer (pH 7.4), containing 1 M imidazole (Sigma Chemical Co.) and 0.1% hydrogen peroxide for 1 min. Sections were counterstained with hematoxylin. The labeling index (%) was estimated by counting the number of BrdU-labeled cells per 200 cells in the grafted epithelium and stroma, separately, in three randomly-selected sections (n=3-6).

*Statistical analysis*. Data are expressed as the mean±standard error. Two-tailed Student's *t*-test after application of F-test was used for the comparison of the two mean values. A statistically significant difference was defined as $p<0.05$.

## Results

Tissue recombinant groups performed in this study are shown in Figure 1. All-tissue recombinants exhibited a squamous epithelial morphology, with 2-3 cell layers typical of normal vaginal epithelium (Figure 2). In the 8S-D plus AE-O group, mucous cells were observed on the superficial cell layers. BrdU-labeled cells were found in both epithelium and stroma in the all-tissue recombinants (Figure 2, arrows).

The percentage of BrdU-labeled cells in the epithelium of 8S-D plus 8E-O was significantly higher than that of 8S-O plus 8E-O (Figure 3A). In the epithelium of 8S-D plus AE-O, the percentage of BrdU-labeled cells was significantly higher than that of 8S-D plus AE-D (Figure 3A). No significant difference was found in the percentage of BrdU-labeled cells in the epithelium between 8S-D plus 8E-O and 8S-D plus AE-O (Figure 3A), however, the percentage of BrdU-labeled cells in the epithelium of 8S-D plus AE-D was significantly lower compared with that of 8S-D plus 8E-D and 8S-D plus AE-O (Figure 3A).

In contrast, BrdU-labeled cells in the stroma of 8S-D plus AE-O was significantly reduced compared to those of 8S-D plus 8E-O, 8S-O plus AE-O and 8S-D plus AE-D (Figure 3B). The percentage of BrdU-labeled cells in the stroma of 8S-D plus 8E-O was significantly lower than that of 8S-O plus 8E-O (Figure 3B). The number of epithelial cell layers in 8S-D plus AE-O was significantly higher compared to that of 8S-O plus AE-O (Figures 2 and 3C).

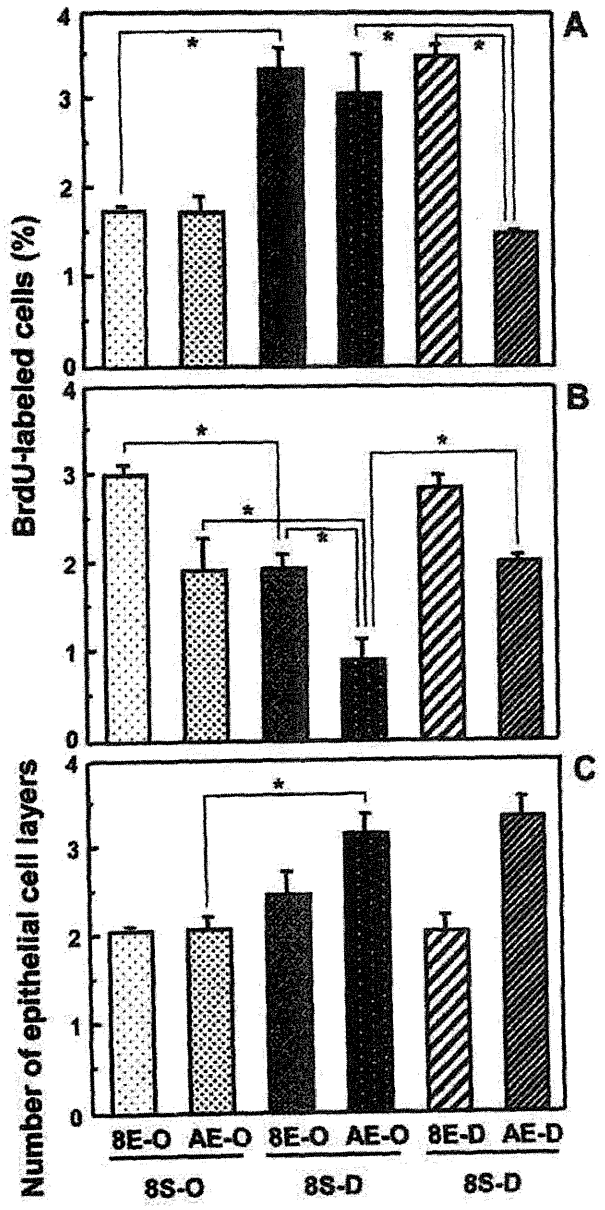


Figure 3. *Percentage of 5-bromo-2'-deoxyuridine (BrdU)-labeled cells in the vaginal epithelium (A) and stroma (B) of tissue recombinants and the number of cell layers in the epithelium of tissue recombinants (C). *Significant difference at $p<0.05$. (See legend to Figure 1 for groups).*

## Discussion

Epithelial–stromal interactions are important in many organs. In male and female genital tracts, the underlying stroma induces and specifies the regional characteristics of the epithelium (11). In tissue recombination experiments, heterotypic recombinants composed of vaginal stroma and uterine epithelium usually exhibit vaginal morphogenesis. Therefore, stroma has an inductive effect on epithelial cells. The developmental responses of these heterotypic recombinants are age-dependent (9). Recently, we reported that fibroblast growth factor (FGF)7- and FGF10-FGFR2IIIb from the vaginal stroma stimulate differentiation in vaginal epithelial cells *via* the mitogen activated protein kinase (MAPK)1/3 pathway (12). Cunha *et al.* (8) also examined the epithelial-stromal interactions of ovary-independent vaginal hyperplasia using a tissue recombination technique and reported that the vaginal stroma from estrogenized mice induces stratification in the untreated vaginal epithelium.

In the present study, all-tissue recombinants exhibited a squamous epithelium that consisted of two to three cell layers. The percentage of BrdU-labeled cells in the epithelium of 8S-D plus 8E-O recombinants was significantly higher than that of 8S-O plus 8E-O, suggesting that the stroma from 8-day-old mice exposed neonatally to DES induced epithelial cell proliferation. In the 8S-D plus AE-O recombinants, mucification of vaginal epithelium was observed in the superficial cell layers, indicating that AE-O recombined with 8S-D could be stimulated by the DES-exposed mouse stroma. In addition, epithelial age did not correlate with epithelial cell proliferation induced by the stroma because no significant difference was found in the epithelium between 8S-D plus 8E-O and 8S-D plus AE-O groups. Our previous report showed that activated Hedgehog signaling stimulates epithelial cell proliferation in neonatal uterus and vagina but inhibits stromal cell proliferation in neonatal uterus (13), therefore, neonatal exposure to DES could alter these signaling pathways from the stroma. The number of vaginal epithelial cell layers was not different among groups except 8S-D plus AE-O, suggesting that 8S-D also has the ability to induce stratification and cornification only in the epithelium from adult mice. Interestingly, AE-D exhibited low cell proliferation even if it was recombined with 8S-D, therefore, the epithelium from adult mice exposed neonatally to DES did not respond to the stimulative signals from the stroma.

The percentage of BrdU-labeled cells in the stroma of 8S-D plus 8E-O recombinants was significantly decreased compared with that in 8S-O plus 8E-O, suggesting that the stroma from 8-day-old mice exposed neonatally to DES exhibited less cell proliferation. In the case of adult epithelium recombinants, neonatally DES-exposed stroma also exhibited decreased cell proliferation. In addition, the percentage of BrdU-labeled cells in the stroma of 8S-D plus AE-O recombinants was significantly decreased compared with that in 8S-D plus 8E-O. This fact suggests that stromal cell proliferation is affected by the age of the recombined epithelium. Therefore, adult vaginal epithelium inhibited stromal cell proliferation, but vaginal epithelium from 8-day-old mice did not. The epithelium from both 8-day-old and adult mice exposed neonatally to DES did not inhibit the stromal cell proliferation. Thus, the epithelium from DES-exposed mice may lose its inhibitory effects on the stroma.

Immature uterine stroma but not adult stroma exhibits 17β-estradiol ($E_2$)-induced cell proliferation (14). Uterine and vaginal stromal cells from immature mice are mitogenically stimulated by $E_2$ *in vitro* (15) and by DES *in vivo* (16). Normal uterine growth is independent of the ovaries and adrenals prior to postnatal day 10 (17). These results indicate that immature stromal cells can proliferate with or without $E_2$ compared to adult stroma. This allows us to speculate that paracrine regulatory signals from adult epithelial cells prevent proliferation of the stroma. In rat uterine stromal cells, $p27^{Kip1}$ protein, a cell cycle-dependent kinase inhibitor (CKI) and exerting a negative control on cell-cycle progression, is detected by immunohistochemistry and $E_2$ induces a heterogeneous and a 'gradient-like' expression pattern of $p27^{Kip1}$ (18). The expression of $p27^{Kip1}$ protein could be affected by the epithelial tissue, resulting in regulation of uterine and vaginal stromal cell proliferation. $p21^{Cip1}$, another CKI, acts as a positive cell cycle regulator in mouse uterine epithelium (19). These results suggest that $p27^{Kip1}$ and $p21^{Cip1}$ might be positive and/or negative regulators of the cell cycle in the stroma of female reproductive organs affected by the epithelium. Further studies are needed to investigate the role of $p27^{Kip1}$ in both vaginal epithelium and stroma.

In conclusion, vaginal stroma from 8-day-old neoDES-mice induces vaginal epithelial cell proliferation, however, the epithelium of adult mice exposed neonatally to DES did not respond to the stroma. The vaginal epithelium of adult mice inhibits cell proliferation of stroma from 8-day-old mice neonatally-exposed to DES, but not of the epithelium. Therefore, the characteristics of the epithelium and the stroma are permanently changed by neonatal exposure to DES.

## Acknowledgements

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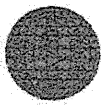

ENVIRONMENTAL HEALTH

COMMENTARY Open Access

# Science and policy on endocrine disrupters must not be mixed: a reply to a "common sense" intervention by toxicology journal editors

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See related Editorial: http://www.ehjournal.net/content/12/1/70/abstract

**Abstract**

The "common sense" intervention by toxicology journal editors regarding proposed European Union endocrine disrupter regulations ignores scientific evidence and well-established principles of chemical risk assessment. In this commentary, endocrine disrupter experts express their concerns about a recently published, and is in our considered opinion inaccurate and factually incorrect, editorial that has appeared in several journals in toxicology. Some of the shortcomings of the editorial are discussed in detail. We call for a better founded scientific debate which may help to overcome a polarisation of views detrimental to reaching a consensus about scientific foundations for endocrine disrupter regulation in the EU.

**Keywords:** Endocrine disrupting chemicals, Environment, Health, Precautionary principle, Regulatory toxicology

## Commentary

*"Common sense is the collection of prejudices acquired by age eighteen"*

- Albert Einstein

As experts and practitioners of endocrine disrupter research, several of whom were invited to prepare some recent international status reports of the topic [1-4], we, the authors, would like to comment on the recent editorial "Scientifically unfounded precaution drives European Commission's recommendations on EDC regulation, while defying common sense, well-established science and risk assessment principles" by Dietrich et al. [5].

We are concerned that the Dietrich editorial appears to be intended as an intervention designed to impact imminent decisions by the European Commission concerning endocrine disrupting chemicals (EDCs), countering the views recently expressed by the 129 signatories of the Berlaymont Declaration on endocrine disrupters [6] and by the Collegium Ramazzini [7]. Given the prominent nature of the authors as members of several EU scientific committees and the importance of these decisions, we would have expected a more accurate analysis of the situation. In contrast, the editorial confuses and conflates several aspects of the current debate that are important to clarify. In general, their fears appear to be founded on a 'common sense' that largely ignores the continued efforts of many scientific expert groups at European and international level as well as the expertise and competence of European decision makers.

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First, in describing endocrine systems as "... play[ing] a fundamental role in the physiological response to changes in the environment with the aim of keeping an organism's response within the homeostatic space" Dietrich et al. seek to define the endocrine system in overly simplistic terms to reduce the task of identifying endocrine disruption to making distinctions "between those effects that are within this adaptive range and effects that go beyond the boundaries of this space and thus can be called adverse" [5]. It is perplexing that editors of international toxicology journals seem to be unaware of the fact that endocrine systems also have a programming role during development, and that disruption of these programming events leads to irreversible effects that go far beyond disturbances of homeostasis [1]. Such phenomena (for example disruption of androgen action in fetal life and the malformations that arise from this) have been described for decades in the scientific literature and provide some of the cause for concerns about endocrine disrupting chemicals. These and other clearly demonstrated cases necessitate the identification of specific windows of vulnerability and this poses considerable challenges to established toxicity testing paradigms, all of which Dietrich et al. [5] ignore.

## Thresholds and no thresholds

Dietrich et al. [5] claim that the "currently drafted EU framework" is based on an *a priori* default assumption of no thresholds for regulating endocrine disrupters, but no document is referenced to substantiate this claim. The latest publicly available document from the European Commission is the Report of the Endocrine Disrupters Expert Advisory Group (ED EAG) published by Directorate General Joint Research Centre (JRC) [8] which is intended to provide the underpinnings of the future EU regulatory framework for endocrine disrupters. The Report was prepared by an expert group comprised of 43 members from competent authorities representing 19 member countries of the European Union as well as other stakeholders including environment and health, NGOs and the industry-funded scientific association, ECETOC. The circumstances that led up to this Report are at odds with the claim by Dietrich et al. [5] that the proposed regulatory framework "is based on virtually complete ignorance of all well-established and taught principles of toxicology and pharmacology, of opinions raised by the European Commission's own competent expert authority (...), and of critical statements made by EU member states...". In the JRC document [8], no reference is made to a presumed *a priori* assumption of no thresholds for endocrine disrupters.

From a scientific standpoint, the issue of the existence of a threshold for endocrine disrupters and other non-genotoxic toxicants remains under debate. As Dietrich et al. [5] rightly point out, absence of effect cannot be statistically demonstrated in an experimental setting. It derives from this that regardless of the mode-of-action and the existence or non-existence of a mechanistic threshold, such a threshold cannot be demonstrated experimentally. If science prides itself in the robustness of its experimental approach to evidence, it should be stressed that the current argument can be modelled or theorised upon, but cannot currently be definitively experimentally tested. Regarding the claim that "...the weight of evidence (...) clearly demonstrates the presence of threshold for non-genotoxic compounds including EDCs...", Dietrich et al. [5] ignore that this evidence is far from established. In international toxicology journals, not under the editorship of Dietrich et al. [5], widely accepted biometrical and mathematical principles about the impossibility of establishing thresholds at the level of populations, independent of the status of the chemicals in terms of genotoxicity or non-genotoxicity have been elaborated [9,10].

## Adversity of effects

It is also unclear where the claim by Dietrich et al. [5] that "the currently drafted EU framework for EDCs foresees a priori regulation of agents that may show presumably endocrine-mediated effects in some experimental system (*in vitro, in silico, in vivo...*)" derives from. The JRC report clearly states that for a substance to be identified as an endocrine disrupter, evidence not only of an endocrine mode-of-action but also of an adverse effect is required, as well as some plausible link between mode-of-action and adversity. This is consistent with the widely accepted IPCS definition [11] of endocrine disrupters which the JRC report accepted.

Concerning assays or endpoints that would be considered adequate for assessments of evidence of adverse effects, the JRC report makes detailed reference to level 4 or level 5 of the assays included in the OECD Conceptual Framework for the assessment of endocrine disrupters. This framework is the result of expert efforts over many years [12]. Although many endpoints relevant to endocrine disruption are not included in the OECD study guidelines, the tests that form part of the current framework are validated, robust, reproducible methods that have been tested in many laboratories before approval to ensure consistent, valid results that are also recognised worldwide under the OECD Mutual Acceptance of Data. These can hardly be qualified as "irrelevant tests" as Dietrich et al. [5] have done.

## *A priori* assumption of human relevance

Referring to a statement by the European Commission (again not referenced) that "relevance of the data to

humans should be assumed in the absence of appropriate data demonstrating non-relevance", Dietrich et al. [5] declare: "The mere statement demonstrates the lack of attention paid by the European Commission to the weight of scientific evidence that clearly demonstrates the presence of a threshold for non-genotoxic compounds including EDC". Here, the authors conflate the statistical impossibility of demonstrating the absence of effects (and thresholds) with the issue of demonstrating human relevance of toxicity data derived from testing on animals. In doing so they reveal ignorance of important risk assessment principles elaborated in an IPCS Framework document [11] for assessing the human relevance of non-cancer endpoints [13]. The default assumption under that framework is of human relevance, unless there is evidence of toxicodynamic or toxicokinetic differences between the animal test species and humans that shows that the effect seen in animals is not expected to occur in humans. The applicability of that default assumption was tested through a number of case studies [13]. The alternative *a priori* assumption (that effects seen in animals are not relevant for humans) would be unworkable and would undermine the sense of conducting toxicological testing in animals at all.

## "Scientifically unfounded precaution", and the distinction between hazard assessment and risk management

The most worrying aspect of the editorial by Dietrich et al. [5] is the blurring of the border between what constitutes science and what belongs to the realm of political, societal and democratic choices.

The Precautionary Principle is enshrined in European Law in the EC Treaty as well as in International Law [14]. This principle was elaborated at the 1992 Rio Conference on the Environment and Development, during which the Rio Declaration was adopted. Principle 15 states that: "in order to protect the environment, the precautionary approach shall be widely applied by States according to their capability. Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation" [14]. Defined in this way, the precautionary principle is a legal concept for addressing scientific uncertainty, and not a scientific concept. Its interpretation and application is a matter for politicians and lawyers. The state of the science on endocrine disruption has been reviewed and summarised in several recent reports published by the UNEP/WHO or commissioned by the European Commission [1,2,8,15]. Already over 10 years ago, it was concluded that the state of the science justified regulatory action [13]. Decisions as to what kind of action may be justified by the level of available evidence and proportionate to the potential risks is a matter for politicians and risk managers, and not the exclusive domain of scientists. Yet Dietrich et al. [5] express strong reservations regarding the application of EU law but do not engage with the scientific basis for concern, or with widely published scientific evidence.

In contrast, the JRC report [8] made a clear distinction between hazard identification and characterisation on the one hand, which they considered within the remit of their expertise, and risk management on the other.

Scientific truths about endocrine disruption as a phenomenon resulting from disturbances of the programming effects of the endocrine system during development seem to have been ignored by Dietrich et al. [5]. It is to be hoped that this editorship of international toxicological journals will be able to engage in a better founded scientific debate which may help to overcome a polarisation of views detrimental to reaching a consensus about scientific foundations for endocrine disrupter regulation in the EU.

#### Abbreviations

EC: European communities; ECETOC: European centre for ecotoxicology and toxicology of chemicals; ED EAG: Endocrine disrupter expert advisory group; EDC: Endocrine disrupting chemical; EU: European union; IPCS: International programme for chemical safety; NGO: Non-governmental organisation; OECD: Organisation for economic cooperation and development.

#### Competing interests

All authors declare that they have no competing interests. Several of the authors were invited by the European Commission and UNEP/WHO, as scientific experts, to prepare some recently published international reports on state of the science of endocrine disrupters.

#### Authors' contributions

A core group of the authors first drafted the manuscript and circulated it for comments. All authors contributed actively to the revision of the draft. All authors approved the final version.

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# The Impact of Endocrine Disruption: A Consensus Statement on the State of the Science

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In 2002, the joint International Programme on Chemical Safety (IPCS) of the World Health Organization (WHO), the United Nations Environment Programme (UNEP), and the International Labour Organisation (ILO) published a report titled *Global Assessment of the State-of-the-Science of Endocrine Disruptors* (http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/). Since 2002, intense scientific work has improved our understanding of the impacts of endocrine-disrupting chemicals (EDCs) on human and wildlife health, such that in 2012, the UNEP and WHO, in collaboration with international experts, have produced an updated document on EDCs, *State of the Science of Endocrine Disrupting Chemicals - 2012* (http://www.who.int/ceh/publications/endocrine/en/index.html) that includes scientific information on human and wildlife impacts and lists key concerns for decision makers and others concerned about the future of human and wildlife health.

The basis for these key concerns is described in the *State of the Science of Endocrine Disrupting Chemicals - 2012* (http://www.who.int/ceh/publications/endocrine/en/index.html) and includes extensive references to the science behind the concerns. A shorter summary, primarily for decision makers, elaborates on the key concerns listed below and and also on suggested considerations related to EDCs (*State of the Science of Endocrine Disrupting Chemicals - 2012: Summary for Decision-Makers;* http://www.who.int/ceh/publications/endocrine/en/index.html).

The key concerns noted in the *State of the Science of Endocrine Disrupting Chemicals - 2012* (http://www.who.int/ceh/publications/endocrine/en/index.html) are as follows:

- Human and wildlife health depends on the ability to reproduce and develop normally. This is not possible without a healthy endocrine system.
- Three strands of evidence fuel concerns over endocrine disruptors:
  - The high incidence and the increasing trends of many endocrine-related disorders in humans;
  - Observations of endocrine-related effects in wildlife populations;
  - The identification of chemicals with endocrine disrupting properties linked to disease outcomes in laboratory studies.
- Many endocrine-related diseases and disorders are on the rise.
  - Large proportions (up to 40%) of young men in some countries have low semen quality, which reduces their ability to father children.
  - The incidence of genital malformations, such as non-descending testes (cryptorchidisms) and penile malformations (hypospadias), in baby boys has increased over time or levelled off at unfavourably high rates.
  - The incidence of adverse pregnancy outcomes, such as preterm birth and low birth weight, has increased in many countries.
  - Neurobehavioural disorders associated with thyroid disruption affect a high proportion of children in some countries and have increased over past decades.
  - Global rates of endocrine-related cancers (breast, endometrial, ovarian, prostate, testicular and thyroid) have been increasing over the past 40–50 years.
  - There is a trend towards earlier onset of breast development in young girls in all countries where this has been studied. This is a risk factor for breast cancer.
  - The prevalence of obesity and type 2 diabetes has dramatically increased worldwide over the last 40 years. WHO estimates that 1.5 billion adults worldwide are overweight or obese and that the number with type 2 diabetes increased from 153 million to 347 million between 1980 and 2008.
- Close to 800 chemicals are known or suspected to be capable of interfering with hormone receptors, hormone synthesis or hormone conversion. However, only a small fraction of these chemicals have been investigated in tests capable of identifying overt endocrine effects in intact organisms.
  - The vast majority of chemicals in current commercial use have not been tested at all.
  - This lack of data introduces significant uncertainties about the true extent of risks from chemicals that potentially could disrupt the endocrine system.
- Human and wildlife populations all over the world are exposed to EDCs.
  - There is global transport of many known and potential EDCs through natural processes as well as through commerce, leading to worldwide exposure.
  - Unlike 10 years ago, we now know that humans and wildlife are exposed to far more EDCs than just those that are POPs [persistent organic pollutants].
  - Levels of some newer POPs in humans and wildlife are still increasing, and there is also exposure to less persistent and less bioaccumulative, but ubiquitous, chemicals.

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- New sources of human exposure to EDCs and potential EDCs, in addition to food and drinking-water, have been identified.
- Children can have higher exposures to chemicals compared with adults—for example, through their hand-to-mouth activity and higher metabolic rate.

- The speed with which the increases in disease incidence have occurred in recent decades rules out genetic factors as the sole plausible explanation. Environmental and other non-genetic factors, including nutrition, age of mother, viral diseases and chemical exposures, are also at play, but are difficult to identify. Despite these difficulties, some associations have become apparent:
  - Non-descended testes in young boys are linked with exposure to diethylstilbestrol (DES) and polybrominated diphenyl ethers (PBDEs) and with occupational pesticide exposure during pregnancy. Recent evidence also shows links with the painkiller paracetamol. However, there is little to suggest that polychlorinated biphenyls (PCBs) or dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyltrichloroethane (DDT) are associated with cryptorchidism.
  - High exposures to polychlorinated dioxins and certain PCBs (in women who lack some detoxifying enzymes) are risk factors in breast cancer. Although exposure to natural and synthetic estrogens is associated with breast cancer, similar evidence linking estrogenic environmental chemicals with the disease is not available.
  - Prostate cancer risks are related to occupational exposures to pesticides (of an unidentified nature), to some PCBs and to arsenic. Cadmium exposure has been linked with prostate cancer in some, but not all, epidemiological studies, although the associations are weak.
  - Developmental neurotoxicity with negative impacts on brain development is linked with PCBs. Attention deficit/hyperactivity disorder (ADHD) is overrepresented in populations with elevated exposure to organophosphate pesticides. Other chemicals have not been investigated.
  - An excess risk of thyroid cancer was observed among pesticide applicators and their wives, although the nature of the pesticides involved was not defined.
- Significant knowledge gaps exist as to associations between exposures to EDCs and other endocrine diseases, as follows:
  - There is very little epidemiological evidence to link EDC exposure with adverse pregnancy outcomes, early onset of breast development, obesity or diabetes.
  - There is almost no information about associations between EDC exposure and endometrial or ovarian cancer.
  - High accidental exposures to PCBs during fetal development or to dioxins in childhood increase the risk of reduced semen quality in adulthood. With the exception of these studies, there are no data sets that include information about fetal EDC exposures and adult measures of semen quality.
  - No studies exist that explore the potential link between fetal exposure to EDCs and the risk of testicular cancer occurring 20–40 years later.
- Numerous laboratory studies support the idea that chemical exposures contribute to endocrine disorders in humans and wildlife. The most sensitive window of exposure to EDCs is during critical periods of development, such as during fetal development and puberty.
  - Developmental exposures can cause changes that, while not evident as birth defects, can induce permanent changes that lead to increased incidence of diseases throughout life.
  - These insights from endocrine disruptor research in animals have an impact on current practice in toxicological testing and screening. Instead of solely studying effects of exposures in adulthood, the effects of exposures during sensitive windows in fetal development, perinatal life, childhood and puberty require careful scrutiny.
- Worldwide, there has been a failure to adequately address the underlying environmental causes of trends in endocrine diseases and disorders.
  - Health-care systems do not have mechanisms in place to address the contribution of environmental risk factors to endocrine disorders. The benefits that can be reaped by adopting primary preventive measures for dealing with these diseases and disorders have remained largely unrealized.
- Wildlife populations have been affected by endocrine disruption, with negative impacts on growth and reproduction. These effects are widespread and have been due primarily to POPs. Bans of these chemicals have reduced exposure and led to recovery of some populations.
  - It is therefore plausible that additional EDCs, which have been increasing in the environment and are of recent concern, are contributing to current population declines in wildlife species. Wildlife populations that are also challenged by other environmental stressors are particularly vulnerable to EDC exposures.
- Internationally agreed and validated test methods for the identification of endocrine disruptors capture only a limited range of the known spectrum of endocrine disrupting effects. This increases the likelihood that harmful effects in humans and wildlife are being overlooked.
  - For many endocrine disrupting effects, agreed and validated test methods do not exist, although scientific tools and laboratory methods are available.
  - For a large range of human health effects, such as female reproductive disorders and hormonal cancers, there are no viable laboratory models. This seriously hampers progress in understanding the full scale of risks.
- Disease risk due to EDCs may be significantly underestimated.
  - A focus on linking one EDC to one disease severely underestimates the disease risk from mixtures of EDCs. We know that humans and wildlife are simultaneously exposed to many EDCs; thus, the measurement of the linkage between exposure to mixtures of EDCs and disease or dysfunction is more physiologically relevant. In addition, it is likely that exposure to a single EDC may cause disease syndromes or multiple diseases, an area that has not been adequately studied.
- An important focus should be on reducing exposures by a variety of mechanisms. Government actions to reduce exposures, while limited, have proven to be effective in specific cases (e.g. bans and restrictions on lead, chlorpyrifos, tributyltin, PCBs and some other POPs). This has contributed to decreases in the frequency of disorders in humans and wildlife.
- Despite substantial advances in our understanding of EDCs, uncertainties and knowledge gaps still exist that are too important to ignore. These knowledge gaps hamper progress towards better protection of the public and wildlife. An integrated, coordinated international effort is needed to define the role of EDCs in current declines in human and wildlife health and in wildlife populations.

With the present state of the science of EDCs, we are now poised to have an important impact on disease prevention. The increase in noncommunicable diseases in humans and wildlife over the past 40 years indicates an important role of the environment in disease etiology. EDCs are an important component of the environmental influences on disease, along with nutrition and other factors. Thus, reducing exposures to EDCs could have an important impact on actual disease prevention. Prevention of disease is always better than intervening after the disease occurs, both in terms of cost and human suffering: The benefits of early action outweigh the costs.

To take advantage of our current knowledge to improve human and wildlife health by preventing environmentally induced diseases, we propose the following ideas for consideration (*State of the Science of Endocrine Disrupting Chemicals - 2012: Summary for Decision-Makers*; http://www.who.int/ceh/publications/endocrine/en/index.html):

> ***Strengthening knowledge of EDCs:*** It is critical to move beyond the piecemeal, one chemical at a time, one disease at a time, one dose approach currently used by scientists studying animal models, humans or wildlife. Understanding the effects of the mixtures of chemicals to which humans and wildlife are exposed is increasingly important. Assessment of EDC action by scientists needs to take into account the characteristics of the endocrine system that are being disrupted, including tissue specificity and sensitive windows of exposure across the lifespan. While there are different perspectives on the importance of low-dose effects and non-monotonic dose–response curves for EDCs, this issue is important in determining whether current testing protocols are sufficient to identify EDCs. Interdisciplinary efforts that

combine knowledge from wildlife, experimental animal and human studies are needed to provide a more holistic approach for identifying the chemicals that are responsible for the increased incidence of endocrine-related disease and dysfunction. The known EDCs may not be representative of the full range of relevant molecular structures and properties due to a far too narrow focus on halogenated chemicals for many exposure assessments and testing for endocrine disrupting effects. Thus, research is needed to identify other possible EDCs. Endocrine disruption is no longer limited to estrogenic, androgenic and thyroid pathways. Chemicals also interfere with metabolism, fat storage, bone development and the immune system, and this suggests that all endocrine systems can and will be affected by EDCs. Together, these new insights stress a critical need to acquire a better understanding of the endocrine system to determine how EDCs affect normal endocrine function, how windows of exposure may affect disease incidence (particularly for childhood respiratory diseases) and how these effects may be passed on to generations to come.

Furthermore, new approaches are needed to examine the effects of mixtures of endocrine disruptors on disease susceptibility and etiology, as examination of one endocrine disruptor at a time is likely to underestimate the combined risk from simultaneous exposure to multiple endocrine disruptors. Assessment of human health effects due to EDCs needs to include the effects of exposure to chemical mixtures on a single disease as well as the effects of exposure to a single chemical on multiple diseases. Since human studies, while important, cannot show cause and effect, it is critical to develop cause and effect data in animals to support the studies on humans.

***Improved testing for EDCs:*** Validated screening and testing systems have been developed by a number of governments, and it requires considerable time and effort to ensure that these systems function properly. These systems include both *in vitro* and *in vivo* endpoints and various species, including fish, amphibians and mammals. New approaches are also being explored whereby large batteries of high-throughput *in vitro* tests are being investigated for their ability to predict toxicity, the results of which may be used in hazard identification and potentially risk assessment. These new approaches are important as one considers the number of chemicals for which there is no information, and these high-throughput assays may provide important, albeit incomplete, information. An additional challenge to moving forward is that EDC research over the past decade has revealed the complex interactions of some chemicals with endocrine systems, which may escape detection in current validated test systems. Finally, it will be important to develop weight-of-evidence approaches that allow effective consideration of research from all levels—from *in vitro* mechanistic data to human epidemiological data.

***Reducing exposures and thereby vulnerability to disease:*** It is imperative that we know the nature of EDCs to which humans and wildlife are exposed, together with information about their concentrations in blood, placenta, amniotic fluid and other tissues, across lifespans, sexes, ethnicities (or species of wildlife) and regions. Many information gaps currently exist with regard to what is found in human and wildlife tissues, more so for developing countries and countries with economies in transition and for chemicals that are less bioaccumulative in the body. Long-term records to help us understand changes in exposures exist only for POPs and only for a few countries.

In addition, there is a need to continue expanding the list of chemicals currently examined to include those contained in materials and goods as well as chemical by-products; it is impossible to assess exposure without knowing the chemicals to target. The comprehensive measurement of all exposure events during a lifetime is needed, as opposed to biomonitoring at specific time points, and this requires longitudinal sampling, particularly during critical life stages, such as fetal development, early childhood and the reproductive years.

Wildlife and humans are exposed to a wide variety of EDCs that differ greatly in their physical and chemical properties. Further, these compounds are generally present at trace concentrations and in complex matrices requiring highly selective and sensitive analytical methods for their measurement. The wide range of different compound classes requires a variety of analytical approaches and techniques, making it challenging to understand all of the different chemicals in the environment and in human and wildlife tissues. There is a growing need to develop new analytical techniques and approaches to prioritize the assessment of EDCs. There is global transport of EDCs through natural processes (ocean and air currents) as well as commerce, leading to worldwide exposures. New sources of exposure to EDCs, in addition to food, have been identified and include indoor environments and electronics recycling and dumpsites (of particular concern in developing countries and countries with economies in transition). The sources and routes of exposure to EDCs need to be further investigated.

***Identifying endocrine active chemicals:*** Identifying chemicals with endocrine disrupting potential among all of the chemicals used and released worldwide is a major challenge, and it is likely that we are currently assessing only the "tip of the iceberg". It is possible to trace high production volume chemicals, but that is not the case for the numerous additives and process chemicals. Adding greatly to the complexity, and to the number of chemicals in our environment, are the unknown or unintended by-products that are formed during chemical manufacturing, during combustion processes and via environmental transformations. While the active ingredients in pharmaceuticals and pesticides have to be documented on the final product, this is not the case for chemicals in articles, materials and goods. Personal hygiene products and cosmetics require declarations of the ingredients, and the number of chemicals applied in this sphere of uses counts in the thousands. Many sources of EDCs are not known because of a lack of chemical constituent declarations in products, materials and goods. We need to know where the exposures are coming from.

***Creating supportive environments for scientific advances, innovation and disease prevention:*** Exposure to EDCs and their effects on human and wildlife health are a global problem that will require global solutions. More programs are needed that foster collaboration and data sharing among scientists and between governmental agencies and countries. To protect human health from the combined effects of exposures to EDCs, poor nutrition and poor living conditions, there is a need to develop programs and collaborations among developed and developing countries and those in economic transition. There is also a need to stimulate new adaptive approaches that break down institutional and traditional scientific barriers and stimulate interdisciplinary and multidisciplinary team science.

***Methods for evaluating evidence:*** There is currently no widely agreed system for evaluating the strength of evidence of associations between exposures to chemicals (including EDCs) and adverse health outcomes. A transparent methodology is also missing. The need for developing better approaches for evaluating the strength of evidence, together with improved methods of risk assessment, is widely recognized. Methods for synthesizing the science into evidence-based decisions have been developed and validated in clinical arenas. However, due to differences between environmental and clinical health sciences, the evidence base and decision context of these methods are not applicable to exposures to environmental contaminants, including EDCs. To meet this challenge, it will be necessary to exploit new methodological approaches. It is essential to evaluate associations between EDC exposures and health outcomes by further developing methods for which proof of concept is currently under development.

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*Letter*

# Neonatal exposure to 2,3,7,8-tetrachlorodibenze-p-dioxin increases the mRNA expression of prostatic proteins in C57BL mice

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**ABSTRACT** — The effects of neonatal exposure to low doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on prostatic secretory protein expression were investigated. Male C57BL mice were treated with TCDD at 10, 100, or 1,000 ng/kg body weight at postnatal day (PND) 6. At PND42, the ventral, dorsolateral, and anterior prostatic lobes were dissected and the mRNA expression of prostatic proteins including spermine-binding protein, serine protease inhibitor Kazal type 3, prostate secretory protein 94 (PSP94), immunoglobulin binding protein-like protein (IgGBPLP), experimental autoimmune prostatitis antigen proteins, and peroxiredoxin-6 (Prdx6) was measured by quantitative PCR. There was no significant difference in the weight of the prostatic lobes between the control and TCDD-treated groups. The expression of PSP94 and Prdx6 in the ventral prostate and IgGBPLP in the dorsolateral prostate at PND42 was significantly increased by neonatal TCDD treatment in a dose-dependent manner, while no changes were noted in other prostatic secretions. These data suggest that neonatal exposure to TCDD may have effects on the neonatal differentiation of the prostate and results in the hyper-expression of some prostatic proteins later in life.

**Key words:** 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), Prostatic secretion, Mouse prostate, Neonatal effects

## INTRODUCTION

The developing male reproductive system of laboratory rodents is highly sensitive to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Mably *et al.*, 1992; Roman and Peterson, 1998; Theobald *et al.*, 2000). Its toxic effects include a decrease in the weight of the testis and accessory sex organs, degeneration of germ cells, and decreased spermatogenesis. The adverse effects of maternal exposure to TCDD on the development of the prostate gland have been studied extensively in rats and mice. In Holtzman rats, a single maternal dose of 64 ng/kg body weight (bw) of TCDD caused a significant decrease in ventral prostate (VP) weight (Mably *et al.*, 1992). More recently, it was reported that androgen receptor (AR) mRNA expression was reduced in the VP of Holtzman rats following maternal treatment with as low as 12.5 ng/kg bw TCDD (Ohsako *et al.*, 2001). In the mouse, the C57BL/6J strain appears to be sensitive to TCDD, in which the maternal administration of 5 μg/kg bw TCDD suppressed the development of the VP in the offspring, while the weight of the dorsolateral prostate (DLP) and anterior prostate (AP) decreased by approximately 50% (Lin *et al.*, 2002a). Exposure to TCDD during only the lactational period also resulted in offspring with lower prostate weights, but with less severe changes (Lin *et al.*, 2002b).

Although the previous studies have been clearly demonstrated that TCDD affect the development of the prostate morphologically, it is important to examine the effect on the prostatic function, production of prostatic proteins. We recently reported the identification of the major proteins secreted from the mouse prostate (Fujimoto *et al.*, 2006). The secreted proteins included spermine-binding protein (SBP), serine protease inhibitor Kazal type 3

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(SPI-KT3), prostate secretory protein 94 (PSP94), glucose-regulated protein, 78kDa (GRP78), peroxiredoxin-6 (Prdx6), probasin, experimental autoimmune prostatitis antigen protein (EAPA2), and immunoglobulin binding protein-like protein (IgGBPLP). The expression profile of these proteins would be useful for studying prostatic function and may also provide markers for evaluating the effects of environmental chemicals on the prostate. In the present study, we investigated the effects of neonatal exposure to low doses of TCDD on the mRNA expression of prostatic proteins as well as AR in the prostate.

## MATERIALS AND METHODS

### Animal experiments

The animal experiments were conducted under the approval of the Animal Experiment Committee of the National Institute of Health Sciences (NIHS). All experiments involving TCDD-treated animals were carried out following the rules for the use of TCDD set by NIHS. Five-day-old male C57BL mice were purchased from Charles River Japan Co. and maintained with free access to a basal diet and tap water. At postnatal day (PND) 6, the animals were divided into 4 groups (n = 6, each group): control and 3 TCDD-treated groups. TCDD (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) in corn oil (50 μl) was injected intraperitoneally (ip) at doses of 0, 10, 100, or 1,000 ng/kg bw). At PND42, the animals were killed under ether anesthesia, since our previous study indicated that the mRNA expression of prostatic proteins is matured at PND42 (Fujimoto *et al.*, 2006). The prostatic lobes were dissected under a microscope, then immediately fixed in RNAlater Solution (Life technologies, Grand Island, NY, USA).

### Quantification of mRNA by real-time RT-PCR

Total RNA was prepared from prostatic tissues using an RNA isolation kit (NucleoSpin RNA II; Machery-Nagel GmbH & Co. KG, Düren, Germany). An ABI Prism 7500 (Applied Biosystems/Life Technologies Co., Carlsbad, CA, USA) was employed for the RT-PCR based quantification of prostatic protein mRNAs as described previously (Fujimoto *et al.*, 2006). All mRNA levels were normalized with reference to β-actin mRNA.

### Statistical analysis

Statistical comparisons were made by Dunnett's multiple comparison test.

## RESULTS

### Body and prostate lobe weights

There was no significant difference in body weight between the control and 3 TCDD-treated groups at PND42 (Table 1). There was no significant change in the weight of either the VP, DLP, or AP.

### Expression of prostatic protein and AR mRNAs

SBP and SPI-KT3 were preferentially expressed in the VP, while probasin, EAPA2, and IgGBPLP expression was localized in the DLP and AP (Table 2). PSP94 was expressed in both the VP and DLP, while GRP78 and Prdx6 were expressed in all prostatic lobes. The effects of neonatal treatment of TCDD on mRNA expression were evident for PSP94, Prdx6, and IgGBPLP. The effects were lobe specific; that is, neonatal TCDD increased the expression of PSP94 and Prdx6 mRNA in the VP as well as IgGBPLP mRNA in the DLP in a dose-dependent manner. Neonatal TCDD exposure did not change the expression of AR mRNA in the VP or DLP, but decreased its expression in the AP.

## DISCUSSION

Maternal exposure to TCDD reportedly causes irreversible changes to the reproductive systems of offspring, including reduced sperm count and reduced size of the reproductive organs. The development of the male reproductive organs in rodents, in particular the prostate gland, has been recognized as a sensitive target to

**Table 1.** Weight of body and prostatic lobes at PND42

| Treatment | body weight (g) | VP (mg/g bw) | DLP (mg/g bw) | AP (mg/g bw) |
|---|---|---|---|---|
| control | 16.8 ± 0.28 | 0.20 ± 0.03 | 0.21 ± 0.02 | 0.30 ± 0.02 |
| TCDD 10 | 16.9 ± 0.37 | 0.19 ± 0.02 | 0.23 ± 0.01 | 0.22 ± 0.06 |
| TCDD 100 | 17.7 ± 0.25 | 0.30 ± 0.08 | 0.20 ± 0.02 | 0.34 ± 0.03 |
| TCDD 1000 | 18.9 ± 0.38 | 0.24 ± 0.08 | 0.24 ± 0.01 | 0.31 ± 0.03 |

Mean ± S.E.M. (n = 6). Male C57BL mice were treated with TCDD (10, 100, or 1,000 ng/kg bw) at postnatal day (PND) 6 and sacrificed at PND42.

**Table 2.** mRNA expression of prostatic proteins and AR in prostatic lobes

| Treatment | SBP | SPI-KT3 | PSP94 | GRP78 | Prdx6 | Probasin | EAPA2 | IgGBPLP | AR |
|---|---|---|---|---|---|---|---|---|---|
| VP | | | | | | | | | |
| control | 315 ± 64.4 | 280 ± 85.7 | 12.4 ± 4.2 | 5.7 ± 0.36 | 1.5 ± 0.21 | | | | 7.3 ± 1.24 |
| TCDD 10 | 293 ± 131.2 | 495 ± 95.7 | 40.0 ± 11.9 | 7.6 ± 0.92 | 2.4 ± 0.38 | | | | 8.6 ± 1.08 |
| TCDD 100 | 322 ± 63.6 | 278 ± 44.1 | 65.7 ± 21.1* | 8.3 ± 1.89 | 4.5 ± 0.79* | | | | 4.8 ± 0.27 |
| TCDD 1000 | 450 ± 84.7 | 308 ± 46.5 | 110 ± 16.6** | 7.1 ± 1.68 | 5.6 ± 1.0* | | | | 7.5 ± 0.71 |
| DLP | | | | | | | | | |
| control | | | 92.3 ± 20.2 | 11.2 ± 1.23 | 27.2 ± 4.7 | 7.8 ± 0.65 | 2.5 ± 0.44 | 0.80 ± 0.09 | 4.5 ± 0.96 |
| TCDD 10 | | | 59.0 ± 24.3 | 9.5 ± 1.21 | 31.7 ± 1.93 | 9.1 ± 1.36 | 2.8 ± 0.44 | 2.2 ± 0.59 | 3.9 ± 0.45 |
| TCDD 100 | | | 82.3 ± 20.7 | 8.9 ± 1.07 | 31.6 ± 3.02 | 8.5 ± 0.57 | 2.4 ± 0.29 | 2.7 ± 0.75* | 3.5 ± 0.22 |
| TCDD 1000 | | | 56.9 ± 15.9 | 7.3 ± 0.71 | 31.3 ± 2.46 | 7.9 ± 0.07 | 2.5 ± 0.28 | 3.3 ± 0.47* | 3.2 ± 0.50 |
| AP | | | | | | | | | |
| control | | | | 13.7 ± 3.23 | 38.1 ± 5.4 | 4.5 ± 0.66 | 3.4 ± 0.29 | 19.5 ± 1.95 | 3.6 ± 0.35 |
| TCDD 10 | | | | 18.0 ± 1.59 | 67.1 ± 4.1 | 5.8 ± 0.21 | 3.0 ± 0.33 | 27.8 ± 5.63 | 3.0 ± 0.31 |
| TCDD 100 | | | | 9.8 ± 0.91 | 40.9 ± 6.13 | 3.9 ± 0.52 | 3.0 ± 0.57 | 11.2 ± 1.90 | 1.9 ± 0.22 |
| TCDD 1000 | | | | 14.6 ± 1.90 | 73.6 ± 15.4 | 5.9 ± 0.75 | 3.3 ± 0.37 | 28.3 ± 6.19 | 2.1 ± 0.32 |

Mean ± S.E.M. (n = 5 or 6). Values are mRNA levels divided by beta actin mRNA levels (*$p < 0.05$ and **$p < 0.01$ vs. control).
Male C57BL mice were treated with TCDD (10, 100, or 1,000 ng/kg body weight) at postnatal day (PND) 6 and sacrificed at PND42.