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.

the targets of newer insecticides, Perry *et al.* have used mutagenesis of *Drosophila* to create strains resistant to spinosad or neonicotinoids; they pinpointed the acetylcholine receptor subunits that are most sensitive to these toxins (*17*).

The goal of reducing the use of chemical insecticides has spurred the search for biologically based alternatives, a strategy encouraged by Carson [chapter 17 in (*1*)]. Insecticidal protein toxins from the bacterium *Bacillus thuringiensis* (Bt) are now expressed in more than 58 million hectares of transgenic cotton and maize worldwide to deter lepidopteran pests (*18*). When Bt cotton was first introduced in the United States and Australia, government-mandated, industry-implemented resistance management plans were in place. These "high dose/refuge" strategies aimed to slow the process of natural selection, first by ensuring that transgenic plants expressed enough toxin to kill all but the most resistant insects, and second by providing non-Bt crops as "susceptibility refuges" on which Bt-susceptible pests could develop to adulthood and mate with the relatively few survivors from the Bt crop. These strategies to delay resistance are working so far in most cases (*19*).

What if they fail? Estimation of the frequency of rare Bt resistance alleles before they become common enough to cause unsustainable crop damage can provide advance warning of developing resistance. Using methods based on the inbreeding of large field samples, Downes and Mahon have detected alleles for resistance to the Cry2Ab toxin at frequencies of 0.5 to 0.9% in two species of bollworms in Australia (*20*). When the resistance gene is known, DNA sequencing can also be used; Zhang *et al.* have correlated mutations in a 12-cadherin-domain protein with bollworm resistance to the Cry1Ac toxin in China (*21*). Modified Bt toxins have been engineered to circumvent this type of resistance and show promise on other Bt resistance mechanisms as well (*22*).

Coexpression of an additional toxin, Vip3A, with a different mode of action has been commercialized to delay pest resistance to transgenic crops; however, the Vip3A-resistant allele frequency is already 2.7% in one pest, which is very high given that there has been no prior exposure to this toxin (*23*).

Forewarned by the long history of insecticide resistance, the deployment of transgenic crops for insect control has incorporated resistance management plans from the beginning. Unfortunately, this has not been the case for transgenic crops engineered for herbicide tolerance. Greatly increased spraying to control weeds in these new crops has led to a rapid rise of herbicide resistance in several weed species (*24*), and agronomists must now follow entomologists in learning the hard lessons of the past 50 years.

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ECOLOGY

Life in a Contaminated World

Exposure to pesticides and other chemicals can have complex long-term health effects.

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Until the early 1960s, pesticide use was perceived as a benefit to agriculture and public health, with few detrimental consequences. This perception changed dramatically with the publication 50 years ago of Rachel Carson's *Silent Spring* (*1*). The book was the start of a debate that continues to this day on the relative benefits and risks of not just pesticides but all synthetic chemicals.

Pesticides are unquestionably beneficial for food production (*2*), but there is a growing awareness of the risks to human and ecological health associated with their use. Over the past decade, a growing literature (*3*–*6*) has examined how early-life exposure to an array of chemical agents, found not only in pesticides but also in personal care products and plastics, can affect human health. The effects on endocrine signaling (and thus endocrine disruption) have been observed in the exposed generation and also in succeeding generations, but the conclusions are not without controversy.

"It is ironic to think that man might determine his own future by something so seemingly trivial as the choice of an insect spray" wrote Carson in 1962 [p. 8 in (*1*)]. Although she had no mechanism to explain her observations, it is now well documented that exposure early in embryonic development to commonly used chemicals alters gene expression patterns that can lead to altered health later in life (*7*).

But what dose is required to cause an effect? A large literature in the fields of endocrinology and general physiology demonstrates not only that different effects can be induced at different doses but also that the mechanisms driving those effects can differ as well (*7*). A report from the Endocrine Society states that different effects should be expected when comparing high- and low-dose regimens of endocrine disruptors (*3*). Studies using acute high-dose exposures may thus be of limited value for predicting what might occur following the chronic low-dose exposures that almost every population on Earth is subjected to today, often at low but detectable concentrations.

Early-life exposure to chemicals with endocrine disruption potential has been shown to alter gene expression profiles that

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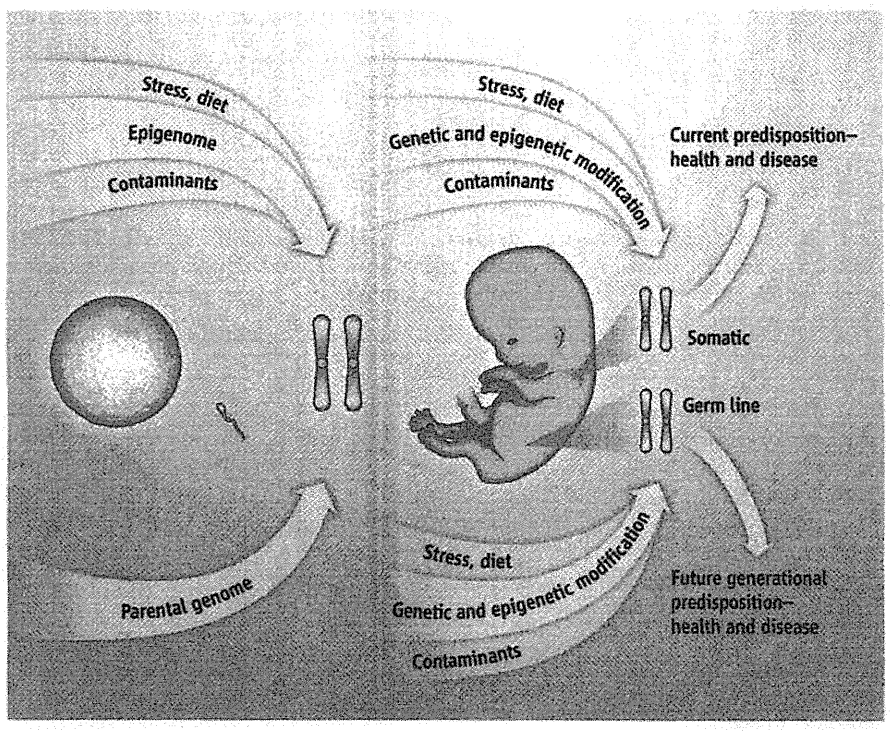
are linked to altered morphology and physiology, such as compromised fertility and reproductive tract development, altered metabolism, obesity, and altered behavior (*8–11*). The multigenerational relationship between chemical exposure and health has been observed in laboratory models and wildlife (*4*). Given the heritable nature of some epigenetic modifications (termed germ line–dependent epigenetic modification) (*12*), the results indicate that the classic "gene by environment" paradigm used to understand environmental impacts on health is incomplete: The parental genome is based on both the genome inherited from the parents (which includes accumulated gene mutations) and the epigenetic modifications that occurred to that genome before fertilization of the new offspring (see the figure).

Much has been made of the inability of some research groups to replicate the endocrine disruptive effects of some chemicals reported by other laboratories (*13–15*). For example, Hayes *et al.* (*16*) reported effects on the development of frogs after exposure to environmentally relevant concentrations of atrazine, but other groups were unable to replicate these findings (*15*, *17*). However, differences in the design of these experiments did exist, including the source of the animals used and the density at which they were housed (*18*). Disparate outcomes have also been reported in studies of bisphenol A in rodents that used different designs or methodologies (*19*) and in studies of human semen quality or genital development (*20*).

Blount *et al.* have found that in men, perchlorate (a contaminant that affects thyroid function) showed no relationship with the concentrations of thyroid biomarkers. In contrast, in women, raised urinary iodine levels were associated with an influence of perchlorate on thyroid biomarker concentrations (*21*). Thus, dietary iodine, a key factor influencing urinary iodine output, influenced whether an effect was observed or not. How many studies examining potential effects of contaminants on thyroid function record the iodine concentration in the diet of their research animals?

Other studies also illustrate the complexity of the response to environmental endocrine disruptors. For example, Spearow and colleagues have shown in a series of studies that differing strains of mice respond dramatically differently when exposed to the same estrogenic drugs or doses (*22*). Further, a physical factor such as hypoxia can down-regulate the mixed-function oxygenase enzyme (CYP1A) that metabolizes polycyclic aromatic hydrocarbons and polychlorinated biphenyls, thereby affecting the biotransformation of environmental contaminants, thus altering persistence or even the metabolites present (*23*). These complex genetic and environmental effects must be taken into account in studies assessing the health effects of environmental pollutants.

Rachel Carson was right: Chemical contaminants play an important role in our health and the health of the environment. We must continue her legacy and focus on how exposure to environmental contaminants, stress, and diet interacts with the human germline genome and epigenome to establish predispositions for disease that are influenced by secondary exposures later in life (*5*). Understanding this complexity is essential to our understanding of the multiple roles of the environment in promoting those factors that lead to health.



The role of the environment. Environmental factors, including numerous contaminants, have been shown to modify the parental genome, so that the genetic makeup of any offspring is a combination of a parental inherited genome (itself likely influenced by epigenetic mechanisms of the germ line) and environmental influences on that germ line during maturation. Environmental factors such as diet, stress, and contaminants can also modify the genome of the developing embryo by classic selection and mutation or by epigenetic mechanisms at both the somatic and germline levels. These modifications can produce predispositions for health and disease in the current lifetime of the individual. Future transgenerational effects could also be established through modifications in the germline genome or epigenome after exposures during the lifetime of that individual.

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Wnt family genes and their modulation in the ovary-independent and persistent vaginal epithelial cell proliferation and keratinization induced by neonatal diethylstilbestrol exposure in mice

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ABSTRACT

Proliferation and differentiation of cells in female reproductive organs, the oviduct, uterus and vagina, are regulated by endogenous estrogen. In utero exposure to a synthetic estrogen, diethylstilbestrol (DES), induces vaginal clear-cell adenocarcinoma in humans. In mice, perinatal exposure to DES results in abnormalities such as polyovular follicles, uterine circular muscle disorganization and persistent vaginal epithelial cell proliferation. We reported the persistent gene expression change such as interleukin-1 (IL-1) related genes, insulin-like growth factor-I (IGF-I) and its downstream signaling in the mouse vagina exposed neonatally to DES. In this study, we found persistent up-regulation of *Wnt4* and persistent down-regulation of *Wnt11* in the vagina of mice exposed neonatally to DES and estrogen receptor α specific ligand. Also *Wnt4* expression in vagina is correlated to the stratification of epithelial cells with the superficial keratinization of vagina, but not epithelial cell stratification only.

1. Introduction

Estrogen-induced cell proliferation and differentiation in female reproductive organs such as oviduct, uterus and vagina are long being studied by several group of researchers (Takasugi et al., 1962; Dunn and Green, 1963; Takasugi and Bern, 1964; Forsberg, 1969; Herbst et al., 1971; McLachlan et al., 1980; Newbold and McLachlan, 1982; Newbold et al., 1985; Iguchi et al., 1986; Iguchi, 1992). Diverse biological effects of estrogens are primarily mediated via the activation of nuclear estrogen receptors, ERα and ERβ, which are ligand-inducible transcription factors (Tsai and O'Malley, 1994; Beato et al., 1995). Increase in specific gene expressions via ERα or ERβ after estrogen exposure in mice has been silenced by an ER antagonist, ICI 182,780 (Miyagawa et al., 2004a,b).

Vaginal epithelium is an intriguing model for analyzing the estrogen action in mice. It undergoes characteristic changes from a non-keratinized to a fully keratinized epithelium depending on the levels of the endogenous estrogen, estradiol (E_2), during the estrous cycle (Miller et al., 1998).

Estrogen exposure, during a critical period in the early development in mice, induces persistent, ovary-independent proliferation and keratinization in the vaginal epithelium at adulthood (Takasugi et al., 1962; Takasugi and Bern, 1964). In humans, trans-placental exposure to a synthetic estrogen, diethylstilbestrol (DES), which was routinely prescribed to pregnant women for prevention of miscarriages from the 1940s to 1970s in the USA and European countries, resulted in vaginal clear-cell adenocarcinoma in young women (Herbst et al., 1971). Although perinatal estrogenic chemical exposure induces various abnormalities, i.e., polyovular follicles, oviductal tumors, uterine epithelial metaplasia, persistent vaginal stratification and keratinization, vaginal adenosis, and cervico-vaginal carcinomas (Takasugi et al., 1962; Dunn and Green, 1963; Takasugi and Bern, 1964; Forsberg, 1969; Newbold and McLachlan, 1982; Newbold et al., 1985; Iguchi et al., 1986; Iguchi, 1992; Suzuki et al., 2002), the critical period of estrogen action during mouse development varies from organ to organ (Iguchi et al., 2002). DES exposure during critical developmental period results in alterations of the response to estrogens in mouse vagina, leading to a set of

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Table 1
Sequences of gene primer sets for real-time quantitative RT-PCR.

Gene	Primer (5′–3′)[a]	Product size (bp)	Gene accession no.
Wnt4	F: CATCGAGGAGTGCCAATACCA R: GACAGGGAGGGAGTCCAGTGT	70	NM.009523
Wnt11	F: ATGTGCCGGACAACCTCAGCTA R: CGCATCAGTTTATTGGCTTGG	100	NM.009519

[a] F, forward; R, reverse.

subsequent abnormalities. Among them, vaginal epithelial proliferation persists even after ovariectomy in mice exposed to sufficient doses of DES during the early neonatal period (Takasugi et al., 1962; Takasugi and Bern, 1964).

Wnt genes are the vertebrate homologs of *wingless*, the Drosophila segment polarity gene is comprised of 16 members. They are a large group of highly conserved secreted glycoproteins, and play crucial roles in embryonic developmental processes (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998; Smalley and Dale, 1999), tumorigenesis (Tsukamoto et al., 1988; Smalley and Dale, 1999; Lustig and Behrens, 2003) and reproduction (Parr and McMahon, 1998; Vainio et al., 1999) mostly via Frizzled (Fz) receptor (Dale, 1998). Fzs constitute a large family of seven transmembrane G protein-coupled receptors and possess an extracellular cysteine-rich domain (CRD) for Wnt/binding (Wang et al., 1996; Liu et al., 1999). Among several Wnt-mediated intracellular signaling pathways (Willert and Nusse, 1998; Huelsken and Birchmeier, 2001; van Noort and Clevers, 2002), the canonical Wnt β-catenin pathway has been well studied.

The Wnt singling is highly responsive to variable hormone concentration and location (Weber-Hall et al., 1994). It is well known that Wnt singling plays roles in epithelial–mesenchymal interactions and cellular organization during embryonic and postembryonic development, involving in cell proliferation and differentiation, cell fate specification and cell-to-cell communication (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998; Smalley and Dale, 1999). Wnt signaling also plays a key role in murine female reproductive tract development (Miller et al., 1998; Daikoku et al., 2004), and has been suggested as a target for potential endocrine disruptors (Sassoon, 1999). Miller et al. (1998) reported that three Wnt family genes, *Wnt4*, *Wnt5a* and *Wnt7a*, were expressed in the uterus and cervix in specific epithelial–mesenchymal interactions during postnatal development and in the adult. However, the expression of Wnt genes in vagina has not yet been elucidated.

Previously, we examined the global expression of mRNA, focusing on factors involved in cell signaling in the vagina of mice exposed neonatally to DES showing persistent hyperplasia and the superficial keratinization (Miyagawa et al., 2004b). In the present study, we report that neonatal exposure of DES and ERα specific ligand induced persistent up-regulation of *Wnt4* and persistent down-regulation of *Wnt11* in mouse vagina. In addition, to clarify the role of Wnt4 in vaginal histological modulation by estrogen, we used Wnt4 hetero ($Wnt4^{+/-}$) mice, since $Wnt4^{-/-}$ mice exhibit fetal lethality (Stark et al., 1994; Vainio et al., 1999). *Wnt4* expression was correlated to epithelial keratinization, in mouse vagina exposed neonatally to DES.

2. Materials and methods

2.1. Reagents

Diethylstilbestrol (DES) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Estrogen receptor α (ERα) specific ligand, 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (propyl pyrazole triol, PPT), ERβ specific ligand, 2,3-bis(4-hydroxyphenyl)-propionitrile (diarylpropionitrile, DPN) and estrogen receptor antagonist, ICI 182,780, were obtained from Tocris Bioscience (Ellisville, MO, USA). Sesame oil and dimethyl sulfoxide (DMSO) were obtained from Kanto Chemical (Tokyo, Japan).

2.2. Animals and treatments

C57BL/6J mice and 129^{+Ter}/Sv mice were purchased from CLEA Japan (Tokyo, Japan). Wnt4 mutant mice (129^{+Ter}/Sv strain) were from Jackson Laboratory (Bar Harber, ME, USA) through Prof. K.-I. Morohashi. They were maintained under 12 h light/12 h dark at 23–25 °C and fed laboratory chow (CE-2, CLEA) and tap water ad libitum. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at the National Institute for Basic Biology, National Institutes of Natural Sciences.

C57BL female newborn mice were given 5 daily subcutaneous (s.c.) injections of 0.025 ($n=6$), 0.25 ($n=6$) or 2.5 μg ($n=6$) DES/g body weight (bw) dissolved in sesame oil or the oil vehicle alone ($n=6$) beginning from day 0 (the day of birth). Ovariectomy was performed in all mice exposed neonatally to DES, since the aim of the present study was to understand the underlying molecular mechanisms of ovary (estrogen)-independent persistent vaginal changes. These mice ovariectomized at 8 weeks and sacrificed at 10 weeks of age were used for DNA microarray analysis, reverse transcriptase polymerase chain reaction (RT-PCR), histology and immunohistochemistry. In addition, mice exposed to 2.5 μg DES/g bw neonatally and ovariectomized as adults ($n=8$) were given 5 daily intraperitoneal injections of 5 μg ICI 182,780/g bw or oil vehicle alone beginning from day 65 and killed 24 h after the last injection. Tissues were used for real-time quantitative RT-PCR and histological examination for counting number of vaginal epithelial cell layers.

Newborn female C57BL mice were given 5 daily s.c. injections of 2.5 μg DES/g bw ($n=4$), 25 μg/g bw PPT ($n=4$) or DPN ($n=4$) dissolved in 5.6% DMSO or the vehicle alone ($n=4$) beginning from day 0. These mice ovariectomized at 13 weeks were sacrificed at 15 weeks of age, and used for real-time quantitative RT-PCR and histology.

$Wnt4^{+/+}$ and $Wnt4^{+/-}$ newborn mice were given 5 daily s.c. injections of 2.5 μg DES/g bw dissolved in oil ($n=10$ or 4, respectively) or the oil vehicle alone ($n=5$ each). These mice ovariectomized at 8 weeks were sacrificed at 10 weeks of age, and analyzed *Wnt4* mRNA expression and histology.

2.3. DNA microarray analysis

Total RNA from vaginae exposed neonatally to 0.025, 0.25 or 2.5 μg DES/g bw or oil vehicle alone were extracted using TRIZOL (Invitrogen, Carlsbad, CA, USA) and purified using an RNeasy mini kit (QIAGEN, Chatsworth, CA, USA). Quality and quantity of total RNA were confirmed by the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). cRNA probes were prepared from the purified RNA using an Affymetrix cRNA probe kit (Affymetrix, Santa Clara, CA USA) according to the manufacturer's protocol. All preparations met the recommended criteria of Affymetrix for use on their expression array. The amplified cRNA was hybridized to high-density oligonucleotide arrays (Mouse U74A; Affymetrix) containing approximately 12,500 genes, and the scanned data were analyzed with GeneChip software (Affymetrix) and processed as described previously (Watanabe et al., 2004). To confirm the estrogen-related changes in gene expression revealed by DNA microarray analysis, we independently repeated the same experiment twice. The expression data were analyzed with GeneSpring software (Agilent) as described previously (Watanabe et al., 2004).

For the clustering analysis, genes expressed more than 2-fold or less than a half by neonatal DES treatment to controls were selected, and similarities between experiments and expression levels were measured by standard correlation using the GeneSpring program as described previously (Watanabe et al., 2002, 2003, 2004).

2.4. RT-PCR and real-time quantitative RT-PCR

Total RNA, isolated with RNeasy kit (QIAGEN, Chatsworth, CA, USA) from each group of vaginae, was used in RT-PCR or real-time quantitative RT-PCR reactions carried out with SuperScript III reverse transcriptase (Invitrogen). RT-PCR was carried out using AmpliTaq Gold (TAKARA, Ohtsu, Japan). Sequences of gene primer sets are given in Table 1. PCR conditions were as follows: 94 °C for 10 min, and 32 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and 72 °C for 10 min in 25 μl volumes.

Changes in gene expression were confirmed and quantified using ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and 36 cycles of 95 °C for 15 s and 60 °C for 1 min in 15 μl volumes. Relative RNA equivalents

Table 2
Microarray date of Wnt genes in vaginas of adult mice (10-week old) exposed neonatally to DES.

Gene accession no.	Name	Fold change			Prove set ID
		0.025	0.25	2.5	
NM_021279	Wingless-related MMTV integration site 1	NC	NC	NC	1425377_at
NM_023653	Wingless-related MMTV integration site 2	NC	NC	NC	1449425_at
NM_009520	Wingless-related MMTV integration site 2b	NC	NC	NC	1421465_at
NM_009521	Wingless-related MMTV integration site 3	NC	NC	NC	1450763_x_at
NM_009522	Wingless-related MMTV integration site 3A	NC	NC	NC	1422093_at
NM_009523	Wingless-related MMTV integration site 4	**4.15**	**5.95**	**3.23**	1450782_at
NM_009524	Wingless-related MMTV integration site 5A	0.82	0.91	1.33	1436791_at
NM_009524	Wingless-related MMTV integration site 5A	1.29	1.13	1.03	1448818_at
NM_009525	Wingless-related MMTV integration site 5B	NC	NC	NC	1422602_a_at
NM_009525	Wingless-related MMTV integration site 5B	NC	NC	0.80	1439373_x_at
NM_009526	Wingless-related MMTV integration site 6	NC	NC	NC	1419708_at
NM_009527	Wingless-related MMTV integration site 7A	NC	NC	NC	1423367_at
NM_001163634	Wingless-related MMTV integration site 7B	NC	**2.42**	1.97	1420891_at
NM_001163634	Wingless-related MMTV integration site 7B	NC	NC	NC	1420892_at
NM_009290	Wingless-related MMTV integration site 8A	NC	NC	NC	1422228_at
NM_011720	Wingless-related MMTV integration site 8b	NC	NC	NC	1421439_at
NM_011720	Wingless-related MMTV integration site 8b	NC	NC	NC	1421440_at
NM_139298	Wingless-type MMTV integration site 9A	NC	NC	NC	1425889_at
NM_011719	Wingless-type MMTV integration site 9B	NC	NC	NC	1451711_at
NM_009518	Wingless-related MMTV integration site 10a	NC	NC	NC	1460657_at
NM_011718	Wingless-related MMTV integration site 10b	NC	NC	NC	1426091_a_at
NM_009519	Wingless-related MMTV integration site 11	**0.21**	**0.21**	**0.29**	1450772_at
NM_053116	Wingless-related MMTV integration site 16	NC	NC	NC	1422941_at

The values shown as bold indicate statistically significant change compared to controls.

for each sample were obtained by standardization of ribosomal protein L8 levels. Sequences of gene primer sets are given in Table 1. More than three pools of samples per group were run in 3–7 groups to determine sample reproducibility, and the average relative RNA equivalents per sample were used for further analysis. Error bars represent the standard error, with all values represented as fold change compared to the control group normalized to an average of 1.0.

2.5. HE staining and immunohistochemistry

Tissues were fixed in neutral buffered 10% formalin, embedded in paraffin and sectioned at 8 μm. Some sections were stained with standard hematoxylin and eosin. Other sections deparafinized were incubated with 0.3% H_2O_2 in methanol for 15 min to eliminate endogenous peroxidase. After washing with PBS, the sections were incubated anti-Wnt4 antibody (R&D Systems, Inc., Minneapolis, MN, USA) at 1:200 dilution in PBS containing 1% BSA (Sigma) overnight at 4 °C. The sections were visualized with LSAB™ 2 kit, Universal (Dako, Carpinteria, CA, USA) according to the manufacturer-supplied protocol. For negative controls, normal goat immunoglobulin fraction (Dako) was used at the same dilution.

2.6. Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA), Student's *t*-test or Welch's *t*-test followed by *F*-test as appropriate. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. DNA microarray analysis

Microarray analyses were performed to get an idea about the expression profiles of different Wnt genes, especially, *Wnt4*, *Wnt5a*, *Wnt5b*, *Wnt7b* and *Wnt11* mRNA in the mouse vagina (Table 2). Surprisingly, only *Wnt4* and *Wnt7b* showed higher (3 to 6-fold) to moderate (1.97 to 2.42-fold) spikes in the neonatally DES-exposed mouse vagina than controls. On the other hand, *Wnt11* showed a decrease (0.21 to 0.29-fold) after DES treatment. However, other Wnt genes remained unaffected in vaginal epithelia after neonatal DES treatment. To verify the results of microarray analysis, we examined the expression of *Wnt4* and *Wnt11* mRNA using RT-PCR. Similar to microarray analysis, *Wnt4* or *Wnt11* expression was up- or down-regulated, respectively, in the vaginal epithelium of DES-exposed mice than controls (data not shown). Interestingly, mRNAs of all *Frizzled* family (*Fz 1–10*) were detected in the mouse vagina regardless of the neonatal DES exposure (data not shown). Henceforth, further studies were conducted with *Wnt4* and *Wnt11* only.

3.2. Estrogen responsive changes of Wnt genes in mouse vagina

Neonatal DES exposure induced vaginal epithelial stratification with superficial keratinization which was not abolished by ovariectomy (Fig. 1A and D). By contrast, neonatally oil-treated control mice had atrophied vaginal epithelium after ovariectomy (Fig. 1C and D). Expressions of *Wnt4* mRNA was high and *Wnt11* mRNA was low in the vagina of ovariectomized mice exposed neonatally to DES, however, the expression patterns in these genes in the vagina of ovariectomized mice exposed neonatally to oil vehicle alone were reversed (Fig. 1E and F). To investigate the transcriptional regulation of *Wnt4* and *Wnt11* mRNA by exogenous estrogen, we administrated ER antagonist, ICI 182,780, to neonatally 2.5 μg DES-exposed, ovariectomized mice, showing vaginal epithelial stratification and superficial keratinization. *Wnt4* expression in the neonatally DES-exposed mouse vagina, which treated with ICI 182,780, was significantly decreased, but *Wnt11* expression was not changed by anti-estrogen exposure. Surprisingly, the number of vaginal epithelial cell layers in ICI 182,780-treated mice exposed neonatally to DES, were significantly decreased (Fig. 1B, E, and F). This suggested that the DES-responsive changes in Wnt expressions and estrogen responsive epithelial cell proliferation are actually correlated.

To ascertain the role of Wnt genes in vaginal epithelial cell proliferation, we performed immunohistochemistry (IHC) of Wnt4. Ten-week-old ovariectomized mice exposed neonatally to 2.5 μg DES or oil vehicle alone, were used for IHC with anti-Wnt4 antibody (Fig. 2). Wnt4 staining was observed in the basal and middle layers of epithelial cells in vagina of mice exposed neonatally to DES (Fig. 2A), but no Wnt4 staining was observed in oil-treated control mouse vagina (Fig. 2C). This suggests that Wnt4 might be associated with epithelial cell proliferation and further keratinization. We also found that *Wnt4* was expressed in the vagina showing epithelial cell proliferation, while *Wnt11* was restricted to the atrophic vagina having 2–3 epithelial cell layers.

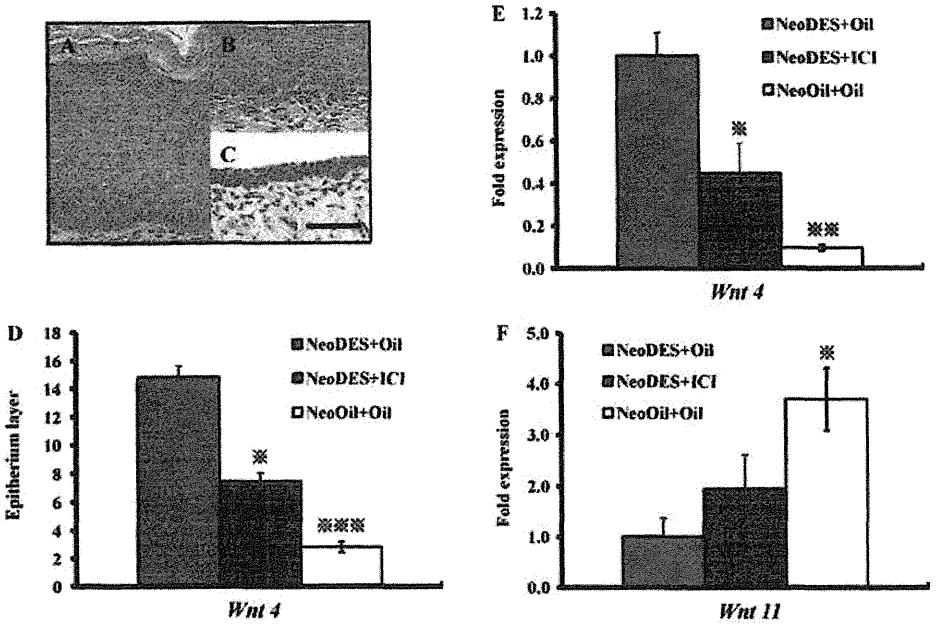

Fig. 1. Administration of anti-estrogen, ICI 182,780, reduced proliferation of vaginal epithelial cells in 10-week-old, ovariectomized mice exposed neonatally to 2.5 μg DES. Vaginal histology of ovariectomized mice exposed neonatally to DES (NeoDES) treated with oil vehicle [NeoDES + oil (A)] or 5 μg ICI 182,780/g bw [NeoDES + 5 μg/g bw ICI 182,780 (B)] and ovariectomized mice exposed neonatally to oil vehicle alone and oil before sacrifice [NeoOil + oil (C)]. Sections were stained with hematoxylin and eosin. Number of vaginal epithelial cell layers was significantly decreased in mice treated with ICI 182,780 (D). Expression profiles of Wnt gene mRNA in vagina of NeoDES mice treated with oil or ICI 182,780. *Wnt4* mRNA expression was significantly decreased in mice treated with ICI 182,780 (E), but *Wnt11* mRNA was not changed (F). Bar: 50 μm. $^{*}P<0.05$ vs. NeoDES + oil, $^{**}P<0.01$ vs. NeoDES + oil, $^{***}P<0.001$ vs. NeoDES + oil (Student's *t*-test or Welch's *t*-test followed by *F*-test).

To pinpoint the role of specific estrogen receptor on such transcriptional modulation of Wnt genes and related cell proliferation, we analyzed both *Wnt4* and *Wnt11* mRNA expression and epithelial cell proliferation and keratinization in vagina of 15-week-old ovariectomized mice treated neonatally with 25 μg DPN, 25 μg PPT or 2.5 μg DES. The vaginal epithelium of these ovariectomized mice exhibited epithelial cell proliferation, stratification and superficial keratinization (Fig. 3A–D). *Wnt4* expression was found to increase after neonatal DES or PPT treatment (Fig. 3E). A simultaneous decrease in *Wnt11* expression was also observed in DES- or PPT-treated vagina (Fig. 3F). However, DPN treatment neither changed the *Wnt4* and *Wnt11* expression nor epithelial cell proliferation. Vaginal epithelia of ovariectomized mice treated neonatally with oil (Fig. 3A) or 25 μg DPN (Fig. 3D) were composed of 2–3 layers of cuboidal cells only. This highlights only *Wnt4*, but not *Wnt11*, is responsible for the persistent vaginal epithelial cell proliferation and persistent activation of ERα (Miyagawa et al., 2004a).

To clarify the role of Wnt4 in vaginal histological modulation by estrogen, we used Wnt4 hetero ($Wnt4^{+/-}$) mice, since

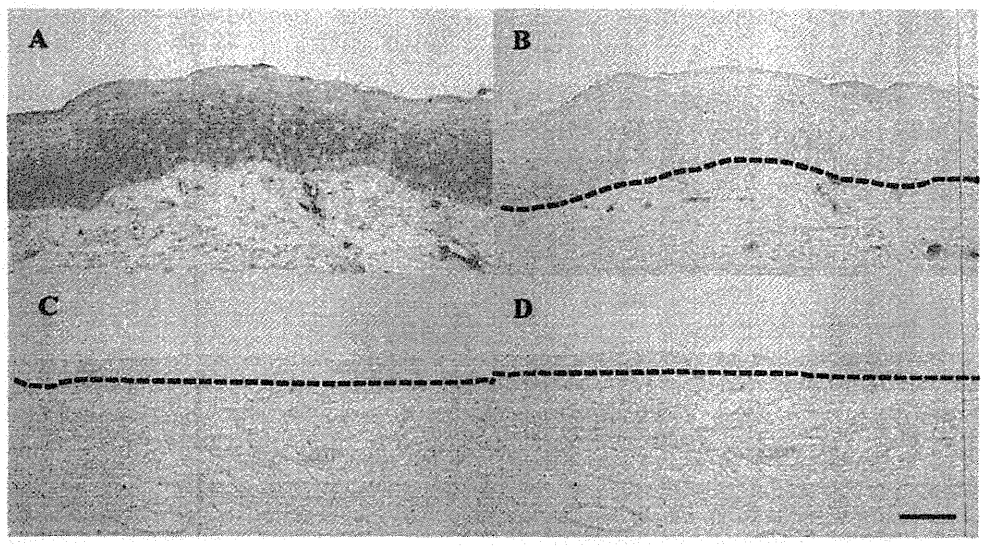

Fig. 2. Immunohistochemistry by Wnt4 antibody. Vaginae of 10-week-old, ovariectomized mice exposed neonatally to 2.5 μg DES (A, B) or oil vehicle alone (C, D). Wnt4 localized in epithelium cells of DES-exposed vagina, especially, in basal layer and middle layers (A). Control mouse vagina was not expressed Wnt4 protein (C). No immunostaining was noted when sections were incubated with preimmune serum instead of primary antibody (B, D). Bar: 50 μm. The boundary between epithelium and stroma is indicated by a dotted line.

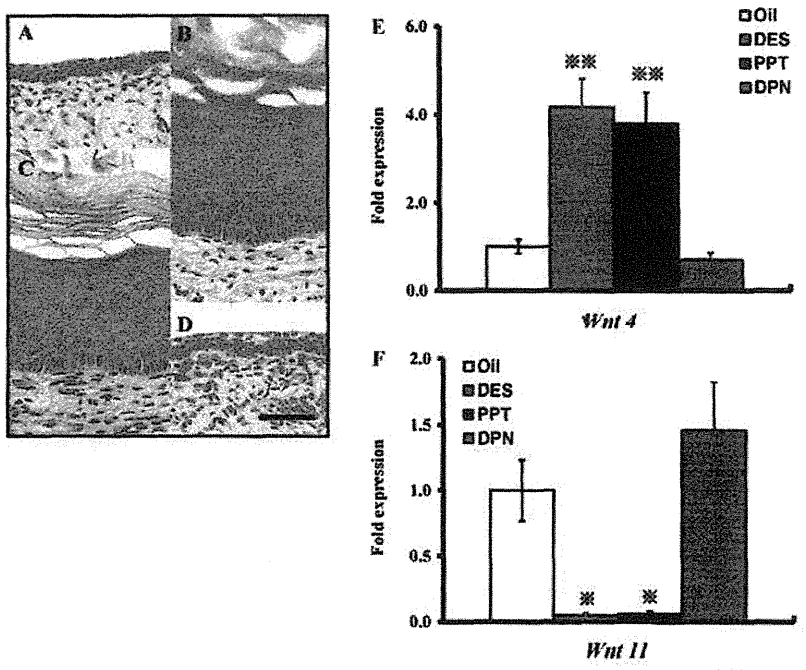

Fig. 3. Histology of vaginae of 15-week-old, ovariectomized mice exposed neonatally to oil (A), 2.5 μg DES (B), 25 μg PPT (C) and 25 μg DPN (D) for the first 5 days. Note ovary-independent persistent proliferation of vaginal epithelium in DES- and PPT-treated mice. Neonatal 2.5 μg DES or 25 μg PPT treatment for the first 5 days induced persistent up-regulation of *Wnt4* mRNA (E), and persistent down-regulation of *Wnt11* mRNA (F) in mouse vagina. The expression of each mRNA in vagina of the oil-treated controls was regarded as the basal level (1.0). Bar: 50 μm. $^{*}P<0.05$ vs. controls, $^{**}P<0.01$ vs. controls (one-way ANOVA).

$Wnt4^{-/-}$ mice exhibit fetal lethality (Stark et al., 1994; Vainio et al., 1999). We thought that Wnt4 expression levels in the vagina of wild type ($Wnt4^{+/+}$) mice were higher than $Wnt4^{+/-}$ mice. All $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice treated neonatally with oil, vaginal epithelia were composed of 2–3 layers of cuboidal cells (Table 3). While, all neonatally DES-exposed $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice exhibited vaginal epithelial stratification or stratification with superficial keratinization (Table 3). *Wnt4* expression levels and histology in vaginae between $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice were not different. *Wnt4* was highly expressed in neonatally DES-exposed mice both in $Wnt4^{+/+}$ and in $Wnt4^{+/-}$ mice (Fig. 4A), showing epithelial stratification with superficial keratinization (Fig. 4B and C). The vagina of $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice exposed neonatally to DES having only epithelial stratification show no up-regulation of Wnt4 expression (Fig. 4B and C) suggesting that Wnt4 plays a role in epithelial keratinization in the vagina.

4. Discussion

In the present study, we intended to clarify the mechanism of ovary-independent proliferation of vaginal epithelial cells. First, we analyzed global gene expression patterns in the DES-exposed mouse vagina. Both microarray analysis and RT-PCR showed differential interplay of Wnt family genes after DES-exposure. Especially, neonatal DES and ERα specific ligand exposure induced persistent up-regulation of *Wnt4* or persistent down-regulation of *Wnt11* in mouse vagina. In addition, we found that DES induces ER-mediated epithelial stratification and keratinization regulated by *Wnt4*.

During embryonic development, members of the Wnt gene family express in a diverse fashion. Pavlova et al. (1994) have previously noted that murine Wnt gene family, *Wnt5a*, were abundant in the adult female reproductive tract, but become relatively scarce during gestation. In addition to *Wnt4*, *Wnt5a* and *Wnt7a* are also detected at high levels in the murine female reproductive tract and had a specific mesenchymal–epithelial expression pattern (Miller et al., 1998). However, these expressions fluctuate along with estrus cycle progression (Miller et al., 1998). In present study, we confirmed the expressions of several Wnt family genes, i.e., *Wnt4*, *Wnt5a*, *Wnt5b*, *Wnt7b* and *Wnt11* mRNA in the neonatally DES-exposed or oil control mouse vagina using DNA microarray analysis. Although we recorded an elevated expression for *Wnt4* and *Wnt7b*, and reduced expression of *Wnt11*, but *Wnt5a* and *Wnt5b* remain unchanged. Therefore, we decided to focus on *Wnt4* and *Wnt11* genes in the vagina exposed neonatally to DES.

Wnt4 is known to be involved in multiple development processes, such as the formation of kidney, adrenal gland, female reproductive tracts and various cancers (Connoly and Schnitt, 1993; Stark et al., 1994; Kispert et al., 1998; Brisken et al., 2000; Smalley and Dale, 2001; Jeays-Ward et al., 2004; Yu et al., 2006). *Wnt11* is a non-canonical Wnt family, regulates ureteric branching (Majumdar

Table 3
Effect of neonatal treatment of DES on vaginae of $Wnt4^{+/-}$ and $Wnt4^{+/+}$ mice ovariectomized 2 weeks before sacrifice.

Treatments	Genotypes	No. of mice used	No. of mice showing vaginal epithelial		
			Atrophy	Stratification	Stratification with keratinization
Oil	$Wnt4^{+/+}$	5	5	0	0
	$Wnt4^{+/-}$	5	5	0	0
2.5 μg/g bw DES	$Wnt4^{+/+}$	10	0	3	7
	$Wnt4^{+/-}$	4	0	1	3