**Table 2.** Uterine Gland Genesis before Puberty in Control and PNDs 1–5 or PNDs 1–15 OP-treated Rats*

| | No. of uterine gland / section (Mean ± SD) | | |
|---|---|---|---|
| | Control | PNDs 1–5 | PNDs 1–15 |
| PND 10 | 0 | 0 | 0 |
| PND 14 | 3.94 ± 0.5 | 4.05 ± 1.5 | 0.1 ± 0.13** |
| PND 21 | 4.58 ± 0.6 | 5.57 ± 1.7 | 2.55 ± 1.5** |
| PND 28 | 6.42 ± 1.5 | 7.83 ± 1.3 | 3.14 ± 2.2** |

*: Yoshida *et al.*, Carcinogenesis 2002; 23: 1745–1750.
**: Significantly different from the control value (P<0.05).

**Table 3.** Sequential Changes in Incidences of Persistent Estrus in Control and PNDs 1–5 or PNDs 1–15 OP-treated Rats*

| | Incidence of persistent estrus (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Group | 1.5 | 2 | 3 | 4 | 5 | 6 | 8 | 10 | 11 | (Months of age) |
| Control | 0 | 0 | 0 | 2.6 | 17.9 | 30.8 | 64.1 | 85.7 | 100 | |
| OP-treated (PNDs 1–5) | 4.9 | 12.2 | 53.7** | 70.1** | 87.8** | 100** | 100** | 100 | 100 | |
| OP-treated (PNDs 1–15) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | |

*: Yoshida *et al.*, Carcinogenesis 2002; 23: 1745–1750.
**: Significantly different from the control value (P<0.05).

to EDCs including estrogens or androgens, but information on such delayed effects is limited. In our recent study, exposure after birth to 100 mg/kg t-OP for the first 5 days (PNDs 1–5) caused a "delayed" influence which was characterized by accelerated appearance of atrophic ovary, manifested by early-occurring and long-term continuing persistent estrus, whereas no abnormalities could be found with regard to growth and development of the reproductive organs and the hypothalamo-pituitary-gonadal control system up to maturation[37], thus differing from the case of exposure for PNDs 1–15 to the same dose of t-OP[34] (Tables 2 and 3). Previously, we confirmed neonatal OP-treatment of 50 mg/kg/day every other day for PNDs 1–15 did not affect estrous cyclicity[34], the total administration-dose (400 mg/kg) being higher than that (300 mg/kg) in the PNDs 1–5 study. This result indicates that the differences were due to the treatment period, rather than the total dosing volume.

## Postnatal Exposure

Chronic administration of OP to adult male rats causes alteration in hormonal secretions[38], and also induces atrophies of the testis and other genital organs[39]. We therefore tested estrogenic effects of t-OP using adult OVX Donryu rats given daily subcutaneous injections of 6.25, 12.5, 25, 50 or 100 mg/kg for 2 or 14 days. t-OP was detected in serum at doses of 25 mg/kg and above for 2 days and of 12.5 mg/kg and above for 14 days, and uterine weights and luminal epithelial heights were increased dose-dependently. OP-treatment for 2 days caused a dose-related increase in proliferation of uterine luminal, glandular and stromal cells and vaginal epithelial cells, and the effects were fundamentally related to the serum OP levels[40].

Effects of t-OP on the female reproductive tract of normal cycling rats were also investigated. F344 and Donryu rats were used, and t-OP was subcutaneously injected for 28 days at similar concentrations to those applied to OVX rats. The most notable changes were disappearance of normal cyclicity in 50 mg/kg or more OP-treated rats and appearance of persistent estrus in the 100 mg/kg group. In rats showing abnormal cyclicity, the uterine morphology deviated from the normal at each estrous stage of cycling rats, and cell proliferation in the endometrium was slightly increased. However, the data for uterine weights, luminal epithelial cell heights and/or numbers of epithelial cells in the endometrium demonstrated only equivocal alteration. In treated rats, the serum E2 levels were decreased with 50 mg/kg of OP or more. Donryu and F344 rats showed similar sensitivity to estrogenic effects of OP, no strain difference being evident. The results indicate that vaginal cytology may be the most sensitive endpoint for the detection of estrogenic activity of potential EDCs in studies using adult female rats[41]. It was also demonstrated that vaginal cytology or its morphological features might be very useful in animal toxicity studies for assessment of the individual hormonal milieu including dysfunction of the hypothalamo-pituitary-gonadal control system[42].

The suitability of the 28-day repeated oral-dosing study for risk assessment of EDCs or strain differences was investigated in adult SD, F344 and Donryu female rats given 60 or 250 (150) mg/kg/day of NP, or 5 or 50 mg/kg/day of atrazine by stomach tube for 28 days. No morphological changes were noted in any reproductive organs of the treated animals, although abnormal estrous cycles were detected in high-dose groups of all strains, without any strain differences[43]. The results also indicate that vaginal smear is the most sensitive parameter for detection of effects of estrogenic or anti-estrogenic chemicals, when normal

cycling animals are used. Although atrazine is an agrochemical having weak estrogen-antagonistic activity, an anti-estrogenic property was not clear in the study. However, effects were detected in the immature rat uterotropic assay, in which atrazine alone was not associated with any changes in uterine weight, but co-treatment with atrazine and E2 reduced E2-induced increase of uterine weight[44].

## Effects on Uterine Carcinogenesis

While the etiology of uterine adenocarcinomas in women is still inconclusive, hormones such as estrogens are considered to be of essential importance[2,3]. The carcinogenic effects on the female genital tract in mammals, including humans, are considered to be one of the most important adverse consequences of EDCs with estrogenic activity. However, there have been only a few reports of unequivocal induction of carcinomas in experimental animals by EDCs, except with diethylstilbestrol (DES), as reviewed previously[1]. In humans, the causation of vaginal and uterine cancers by prenatal exposure to DES is a striking example of environmental carcinogenesis[45]. In experimental animals also, the effects of prenatal DES exposure have been studied in rats and mice, as reviewed by Marselos and Tomatis[46]. Vaginal and uterine adenocarcinomas were induced in mice exposed prenatally to DES[47,48]. In rats following in utero DES exposure, however, mammary and vaginal tumors, rather than uterine tumors, were observed[46]. Thereafter, uterine carcinomas were also induced in Donryu rats by transplacental administration of DES[49]. In the study, interestingly, data for persistent estrus incidence indicate a "delayed" influence in offspring exposed prenatally, similar to our recent report[37].

Tamoxifen (TAM) is a non-steroidal anti-estrogen which competes with estrogen for binding to ER. However, its pharmacology is very complex, and both estrogen agonistic and antagonistic properties have been found, depending on the species, age, exposure duration, dose, route and organs in experimental studies[50]. It has been pointed out that the risk of endometrial cancer may be increased in postmenopausal women exposed to TAM for mammary cancer therapy, the agent acting on the uterus as a weak estrogen agonist[51,52]. In experimental studies using adult rats and mice, however, it has been impossible to cause endometrial cancers by TAM treatment, although endometrial carcinomas were induced in mice treated neonatally[53]. Also the incidences of uterine and cervical/vaginal cancers increased in rats, in the absence of any estrogen agonistic effect, when tamoxifen was administered orally on days 2–5 after birth[54]. Previously we reported that TAM showed potent anti-estrogenic effects on the adult rat uterus and inhibited the development of endometrial adenocarcinomas in our two-stage uterine carcinogenesis model[55]. In that study, however, the dose levels used might have been high. Quite recently, we also reported that TAM showed promotion, but not progression, effects on mouse uterine carcinogenesis, so that the influence in the progression stage appears to be different from the estrogen agonism reported for human beings, although TAM did show estrogen agonistic effects in the promotion stage[56].

In one study, atrazine slightly increased the incidence of endometrial adenocarcinomas in female F344 rats, when given in the diet[57]. Quite recently, however, it was reported that atrazine administered in diet has no modifying effects on uterine carcinogenesis in ICR mice initiated with N-ethyl-N-nitrosourea[58]. Vinclozoline, a pesticide also showing an anti-estrogenic effect, induced uterine adenocarcinomas in female Wistar rats, as well as ovarian sex cord-stromal tumors, when given orally[59]. The carcinogenic mechanisms of these chemicals with anti-estrogenic activity are not clear and further studies are needed to elucidate them.

Dioxin (2,3,7,8-TCDD) is known to exert its modulatory actions through the Ah receptor, and there is experimental evidence suggesting that it can also act in both estrogenic and anti-estrogenic manners, depending on the dose, species, and organ system involved. In rodents, TCDD induces mainly hepatocellular tumors. In addition, in an initiation-promotion study, morphological changes were also noted in both the uterus and the ovary. Although there is no evidence that TCDD can induce tumors in the female genital tract of rodents, it was reported to cause endometriosis in monkeys[60].

Another interesting example is ethylenethiourea (ETU), a metabolic product of ethylenebisthiocarbamate fungicides such as maneb and zineb, which are also listed as EDCs. ETU itself is a well established carcinogen, inducing thyroid tumors in rats and hepatic and lymphoid tumors in mice. In addition, it reacts with nitrite under acidic conditions in vitro and in vivo to form a mutagenic and carcinogenic compound, N-nitroso ETU[61]. Concurrent oral administration of ETU and sodium nitrite is reported to induce uterine endometrial adenocarcinomas in mice[62]. In our two-stage uterine carcinogenesis model using Donryu rats, concurrent oral administration of ETU (80 mg/kg) and sodium nitrite (56 mg/kg) resulted in uterine endometrial carcinomas without initiation by intrauterine administration of ENNG, and also promoted development of the tumors in animals initiated by ENNG, presumably by influencing the hormonal balance[63]. Both ETU and nitrite are known environmental chemicals which are included in foods. Our confirmation that endometrial adenocarcinomas can be induced in this way in rats as well as mice, thus points to an importance of the oral route of exposure to these chemicals, although the doses used in the study were much higher than those in the diet.

The effects of high-dose t-OP on uterine carcinogenesis were investigated using adult Donryu rats initiated with a single intrauterine treatment of ENNG at 11 weeks of age and exposed thereafter to 100 mg/kg/day t-OP by s.c. injections until 15 months of age. Adult OVX rats were also treated in the same way. t-OP had no effect on the occurrence of persistent estrus in non-OVX rats, although uterotropic effects were obvious in the OVX case. At the

**Table 4.** Uterine Adenocarcinomas in ENNG-initiated Rats with Exposure to High-dose OP*

| Group | No. of rats examined | Incidence of endometrial lesions | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Hyperplasia | | | Adenocarcinoma | | | |
| | | + | ++ | +++ | total | differentiation** | | |
| | | | | | | G1 | G2 | G3 |
| 1. Control | 23 | 2 | 8 | 7 | 4 | 4 | 0 | 0 |
| 2. OP-treated (Adulthood) | 26 | 1 | 8 | 5 | 12*** | 9 (G1 and/or G2) | | 3 |
| 3. Control | 23 | 3 | 7 | 5 | 6 | 6 | 0 | 0 |
| 4. OP-treated (PNDs 1–5) | 28 | 1 | 3 | 5 | 18*** | 17 | 1 | 0 |
| 5. OP-treated (PNDs 1–15) | 22 | 2 | 2 | 1 | 8 | 1*** | 3 | 4*** |

*: Groups 1–2: Katsuda *et al.*, Jpn J Cancer Res 2002; 93: 117–124.
Groups 3–5: Yoshida *et al.*, Carcinogenesis 2002; 23: 1745–1750.
**: Histological grades of uterine adenocarcinomas by tumor differentiation.
G1: well differentiated; G2: moderately differentiated; G3: poorly differentiated.
***: Significantly different from the control value ($p<0.05$).

end of the experiment, however, development of uterine adenocarcinomas was significantly increased in animals exposed to t-OP during adulthood, but no tumors developed in OVX rats. This finding suggests that high-dose t-OP has tumor-promoting effects on the ENNG-treated endometrium of rats, possibly due to direct action on the uterus, as indicated by the uterotropic effect of OP[64] (Table 4).

Uterine carcinogenesis in Donryu rats treated neonatally with a high-dose of t-OP has also been investigated. Female pups were subcutaneously administered 100 mg/kg/day t-OP every other day for the first 5 days after birth (PNDs 1–5), or the first 2 weeks (PNDs 1–15). Thereafter, they received a single intrauterine injection of 20 mg/kg ENNG at 11 weeks of age and were observed until 15 months of age. PNDs 1–5 OP-treated animals showed normal development of the female reproductive system, including uterine gland genesis before weaning and normal estrous cycling immediately after vaginal opening. However, the treatment accelerated the occurrence of persistent estrus after 6 weeks of age, and increased the number of well-differentiated uterine adenocarcinomas at the end of the experiment (15 months of age), as compared with controls. This indicates that PNDs 1–5 OP-treatment resulted in delayed modulation of the hypothalamus-pituitary-ovarian hormonal control system, and thus increased the serum E2:P ratio, leading to promotion of uterine carcinoma development. On the other hand, PNDs 1–15 OP-treatment demonstrated immediate and irreversible influences on the control system, called "androgenization", and induced suppression of uterine gland genesis as well as abnormal uterine development manifested by prolonged persistent estrus immediately after vaginal opening, similar to our previous report[34]. In addition, at the end of the experiment, uterine tumor malignancy as assessed by morphological and biological properties was clearly increased, although there was no significant alteration in the total incidence of adenocarcinomas. The total incidence of hyperplasias was significantly lowered, probably related to suppression of uterine gland genesis (Table 4). That study provided evidence that neonatal exposure during PNDs 1–5 or 1–15 to high-dose t-OP enhances uterine carcinogenesis in ENNG-initiated rats, and that the type of uterine tumor is changed by the period of neonatal treatment[37].

Concerning the histogenesis of endometrial adenocarcinomas in Donryu rats, the tumors are considered to arise from hyperplasias of the luminal or glandular epithelium, especially the latter[28]. In humans, it has been pointed out that the presence or absence of hyperplasia as the background is important for the biological behavior of endometrial adenocarcinomas. High-dose OP treatment at PNDs 1–15 induced luminal epithelial hyperplasia in the uteri of rats at 8 weeks of age[34], and finally increased development of undifferentiated adenocarcinomas, although the incidence of hyperplasias was decreased[37]. Carthew *et al.* also reported that tamoxifen induced uterine adenocarcinomas, including biologically malignant examples, in rats in the absence of endometrial hyperplasia, when given on days 2–5 after birth[54]. These results are very interesting in consideration of the histogenesis of uterine adenocarcinomas.

As mentioned above, estrogen and related compounds are reported to increase the risk of endometrial adenocarcinoma development in women. Estrogens occur naturally within the normal body, and are mainly metabolized in the liver by two separate pathways, producing either catechol estrogens (2- or 4-hydroxylated products) or 16$\alpha$- or 16$\beta$-hydroxylated products. 2-Hydroxylation of estradiol or estrone to a catechol is a major metabolic pathway, and the catechol estrogens 2-OHE2 and 2-OHE1 have much weaker hormonal potency than their parent hormones, and lack carcinogenic potency when given to adult animals. On the other hand, 4-hydroxyestradiol (4-OHE2) and the two 16$\alpha$-hydroxylated forms, 16$\alpha$-OHE1 and 16$\alpha$-OHE2, retain potent hormonal activity by acting on classical estrogen receptor and also are tumorigenic[65]. In fact, induction of preneoplastic and neoplastic lesions by estrogen and its steroid metabolites (16 steroids) were studied with our two-stage mouse uterine carcinogenesis

model, and 2-OHE1 or 2-OHE2 exerted promoting, but not progressing, effects, while 16$\alpha$- and 16$\beta$-OHE1 caused both promotion and progression[66].

It is known that indole-3-carbinol binds to the Ah receptor, similar to TCDD, and induces cytochrome p450 metabolic enzymes mainly in the liver. It has been reported that this chemical shows a chemopreventive effect on spontaneous endometrial adenocarcinoma development in Donryu rats when given orally, the effect being speculated to be due to enhanced 2-hydroxylation[67]. We also assessed the effect of indole-3-carbinol on uterine carcinogenesis using our two-stage rat uterine carcinogenesis model. Contrary to expectation, however, the incidences of endometrial carcinomas were increased. In rats given indole-3-carbinol, elevated liver weights and centrilobular enlargement of hepatocytes were also observed, the results indicating an effect on estrogen metabolism in the liver, and further studies are now under way, to clarify the discrepancy (Yoshida *et al.* unpublished data).

## Effects of Low-doses of EDCs

The concentrations of EDCs including OP in the environment are very low, and the main exposure route is oral, rather than cutaneous, in humans. In general, the toxicokinetics of chemicals including EDCs in animals is known to be influenced by the method of administration. It has been reported that low doses of estrogens and EDCs such as OP might be removed from the blood during the first passage through the liver, when given orally[68,69]. For risk assessment of EDCs, it is very important to investigate oral dose effects at human exposure levels and thus we have also focused on relatively low doses of OP (t-OP or n-OP) by oral administration. Female Donryu rats initiated by intrauterine administration of ENNG were given diets containing 100 or 1000 ppm t-OP (about 5 or 50 mg/kg/day) or 100 ppm n-OP (about 5 mg/kg/day) from 11 weeks of age to 15 months of age. Although the concentrations are higher than those in the environment, no significant increase in the incidences of uterine adenocarcinomas was observed in any treated group at the end of the experiment, and also there was no difference in tumor malignancy among the groups (Yoshida *et al.* unpublished data).

As detailed above, exposure to high doses of estrogens or EDCs in the fetal or new born period exerts irreversible androgenization of the female reproductive organs, because of heightened sensitivity. In addition, "delayed" influences on these organs may occur after puberty or sexual maturation. Therefore, relatively long-term comprehensive studies on the endocrinological and morphological aspects may be necessary for determination of prenatal and/or neonatal effects of low doses of EDCs regarding toxicity/carcinogenicity in the female genital organs. Low doses of EDCs such as NP or bisphenol A (BPA) were given orally to pregnant rats, and offspring were observed until 15 months of age, to investigate the prenatal and neonatal effects on growth and development of the female reproductive system and uterine carcinogenesis. In the reproductive toxicity studies reported by others, high doses of NP caused estrogenic effects on pubertal development in male and female rats[70,71]. However, maternal or neonatal exposure to relatively low doses demonstrated no adverse influence on the reproductive tract[72]. In our study with NP, dams were administered 0.1, 10 and 100 mg/kg daily by gavage from gestation day 2 up to the day before weaning of their offspring. Then, all female pups at 11 weeks of age were administered a single dose of 20 mg/kg ENNG into a uterine horn, and observed until 15 months of age. The low level, 0.1 mg/kg, was selected as a dose relevant to human daily intake (1 mg/kg) of isoflavones, uterotropic activity of NP being reported to be 10 times stronger that that of daidzein, one of the major isoflavones, and the middle-dose, 10 mg/kg, was selected as near the no observed effect level in a multi-generation reproductive study using rats[71]. None of the treated groups demonstrated any alteration in reproductive ability. In their offspring also, uterine growth and development, vaginal opening and hormonal secretion until puberty were not changed and there were no effects on estrous cyclicity and morphology of the reproductive organs after maturation, or on uterine carcinogenesis in animals initiated with ENNG[73].

BPA, a volume chemical used in the manufacture of polycarbonate plastics and found in canned foods, lacquered containers and composite dental sealant, is one of the most representative EDCs with weak estrogenic activity, and uterotropic potential has been demonstrated in the immature rat assay[74]. A study conducted by the National Toxicity Program (NTP) in the USA demonstrated that maternal exposure to high doses of BPA at 0.5 or 1.0% in the diet (approximately daily intakes of 875 and 1750 mg/kg/day) reduced the number of live pups per litter and litters per pair in first generation mice[75], although pre- and/or postnatal high-dose BPA exposure did not have any apparent adverse effects on pubertal development in female rats or reproductive functions in rats and mice[76–78]. Recently, however, perinatal treatment with BPA at much lower doses has been described to influence male reproductive organ parameters such as weight of the testis, prostate, preputial gland and epididymis, and the efficiency of sperm production in rodents[79–81], and neonatal treatment advanced puberty in mice[82], although there are also some reports of no treatment-related effects at low dose levels when given to pregnant mice and rats[83–85], and to rats in a three-generation reproductive toxicity study[86]. To further assess the risk, we also investigated effects of maternal exposure to low-doses of BPA, including a human exposure-level, on growth and development of the female reproductive system, and also uterine carcinogenesis in Donryu rats. Dams were administered BPA (0, 0.006 and 6 mg/kg/day) daily by gavage from gestation day 2 up to the day before weaning (PND 21). The concentration of 0.006 mg/kg was selected as consistent with the 63 ppb defined as the average daily intake from canned food in human beings, and 6 mg/kg was selected as appropriate to simulate the maximum dose level

**Table 5.** Proliferative Uterine Endometrial Lesions in Rats Given Low-doses of BPA*

| Dose | Incidence of lesions (%) | | | |
|---|---|---|---|---|
| | Hyperplasia | | | Adenocarcinoma** |
| | + | ++ | +++ | |
| 0 mg/kg/day | 21 | 21 | 13 | 33 |
| 0.006 mg/kg/day | 20 | 20 | 17 | 33 |
| 6 mg/kg/day | 13 | 47 | 17 | 20 |

*: Yoshida *et al.*, J Reprod Dev 2004; 50: 349–360.
**: All adenocarcinomas in the three groups were well differentiated and limited to the uterus.

(80 ppm) detected in plastic plates[87]. The treatment did not exert any influences on the reproductive system of female offspring in either treated group, in terms of prepubertal uterine growth and gland-genesis, vaginal opening and gonadotropin secretion. After maturation also, no effects were evident with regard to estrous cyclicity, age-matched sequential changes of the reproductive organs, and uterine carcinogenesis until 15 months of age (Table 5). The results demonstrated that maternal exposure to BPA at human exposure-levels did not have any adverse effects on the female reproductive organs of offspring in rats[88].

For determination of effects of EDCs on offspring by maternal treatment, biotransfer of the chemicals from dam to offspring is crucial, because the impact is fundamentally related to the serum EDC level[40]. However, data for transfer of the test chemical via the placenta or milk to offspring, or toxicokinetics of low-dose EDCs are very limited[89]. In one of our studies, NP at 10 and 100 mg/kg doses was transferred from dams to their offspring via the milk, but the compound could not be detected in their serum or liver[73]. Furthermore, BPA levels in the milk of dams, and those in the serum and liver in offspring were comparable between control and treated groups, although the serum level of BPA in dams receiving 6 mg/kg was significantly elevated[88].

Quite recently, it was reported that cadmium has potent estrogen-like activity in vivo[90]. Thus, exposure to low-dose cadmium (a single ip injection at a dose of 5 μg/kg) increased uterine net weight accompanied by proliferation of the endometrium, promoted growth and development of the mammary glands, and induced hormone-regulated genes in ovariectomized rats. In utero exposure to the metal (i.p. injections of 0.5 or 5 μg/kg on days 12 and 17 gestation) mimicked also the effects of estrogens, and female offspring experienced an earlier onset of puberty and an increase in the epithelial area and the number of terminal end buds in the mammary gland. The amounts of cadmium used in the study were environmentally relevant, because the WHO-recommended Provisional Tolerable Weekly Intake Level is 7 μg/kg/week. Although the administration route was intraperitoneal, not oral, the ability of environmentally relevant amounts of cadmium to mimic the effects of estradiol is very important and the metal may represent a new class of EDC.

## Species Differences in Toxicologic/carcinogenic Effects of EDCs, Effects of EDCs based on the Molecular Biology and Extrapolation of the Effects to Humans

It is well known that the toxicokinetics of chemicals in animals are influenced by many factors, including species, strain, sex, age, dosage and/or administration method, as mentioned above. In particular, species differences are very important for the risk assessment in humans. Species differences in occurrence of toxicologic/carcinogenic effects of EDCs may be an indication of variation in endogenous hormonal factors, in addition to susceptibility to exogenous agents. Mice are generally more sensitive to estrogens than rats, and uterine adenocarcinomas can be induced in mice by estrogen alone, but not in rats. In mice, perinatal exposure to estrogens was found to induce ovary-independent proliferation of the vaginal epithelium, which could not be abolished by ovariectomy[91]. On the other hand, the vagina in rats neonatally exposed to high-dose t-OP became atrophic immediately after ovariectomy[34]. Adenocarcinoma development in the ENNG-initiated endometrium of Donryu rats exposed to high-dose t-OP was also ovary-dependent[64]. In mice, however, E2 promoted uterine adenocarcinoma development ovary-independently[56]. Differences in the reaction to estrogens or EDCs with estrogenic activity may help to explain species differences in toxicity/carcinogenicity, though further studies on this point, focusing on metabolism of estrogens or EDCs and localization of ER expression, are needed.

The prenatal and/or neonatal periods are more sensitive to estrogens, and also EDCs, than the adult period. As mentioned above, it has been pointed out that tamoxifen increases the risk of endometrial cancer in women. In rodents, TAM can induce uterine carcinomas when given to newborn animals, but not adults. Similarly, 2-OHE2 has weak estrogenic, but not carcinogenic effects, although 4-OHE2 is a potent estrogenic catechol causing uterine tumors in adult mice. However, both catechols induced tumors when given on days 1–5 of neonatal life, although carcinogenic activity of 4-OHE2 is stronger than that of 2-OHE2[92]. Further studies on age-dependent differences in the mechanism of EDCs' effects on the female genital organs are also needed.

Various methods such as DNA micro-array techniques based on gene expression levels have recently been used for the evaluation of hazardous effects of various chemicals, because they should reveal very early changes. Up till now, however, there have only been a few reports concerning EDCs. The fact that changes in ontogenic expression of ER alpha and not of ER beta occur in the fetal female rat reproductive tract, provides fundamental information critical for clarifying species-specific physiological roles of ER subtypes during fetal development and for investigating the tissue-specific mechanisms underlying prenatal responses to estrogen and E2 agonists[93]. Genome-wide analysis of early gene expression has furthermore suggested a basis for the

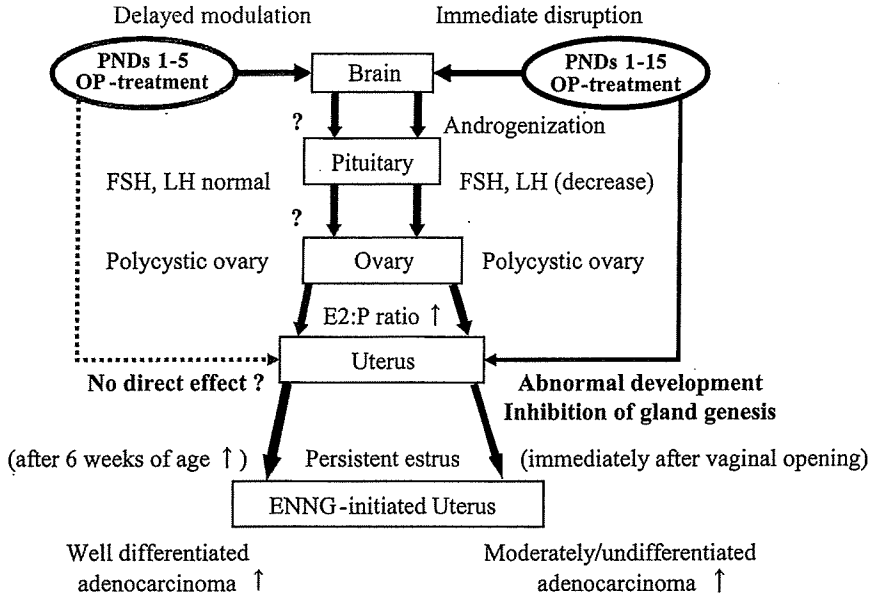


**Fig. 3.** Hypothesis of the scheme for "androgenization effects" or "delayed modulation effects" on the hypothalamo-pituitary-gonadal system in rats exposed neonatally to high-dose OP.

drastic uterotropic effects following estrogen administration[94]. Although DNA micro-array techniques presently demonstrate problems with reliability and reproducibility, future precise analysis should facilitate understanding of the mechanisms underlying effects of estrogenic EDCs[95].

Almost all EDCs exist at only very low concentrations in the environment, but humans may be exposed for long periods. In many animal studies, various toxicologic effects of EDCs on the female genital organs were demonstrated when very high doses were given, but no obvious effects were detected with low-doses. In humans also, low doses may show no adverse effects, because of homeostasis, although there are notable exceptions in animal and human studies. In fish, the ova-testis is known to be a good indicator of estrogenic effects of EDCs in females, but in female rodents, we are still lacking a consensus regarding equivalent reliable endpoint markers. Thus, more comprehensive studies of the endocrinological, morphological and also biomolecular aspects are necessary in animal studies using rodents for extrapolation of EDCs' effects to humans.

## Conclusion

It is well known that the prenatal and/or neonatal period is particularly sensitive to various chemicals, including EDCs, in humans and rodents. Inappropriate exposure may exert irreversible influence, resulting in androgenization of the female genital system. In addition, it has also been reported that a delayed influence may be exerted. Neonatal exposure to a high dose of t-OP (100 mg/kg s.c. injection every other day from PND 1 to PND 15) induced various long-term persistent irreversible effects on the female reproductive system of Donryu rats, such as lower gonadotropin levels at prepuberty, inhibition of uterine gland genesis, persistent estrus shown by vaginal cytology and polycystic ovaries. Neonatal treatment of high-dose EDCs having estrogenic activity can thus affect gonadotropin secretion during the developmental period of sexual maturation with direct masculinization of the hypothalamic function. Abnormal differentiation in the developing rat uteri may be induced via abnormal ER expression and subsequent alteration of cell proliferating activity. However, exposure limited to the first 5 days after birth to 100 mg/kg t-OP caused "delayed" influence which was characterized by accerelated appearance of atrophic ovary, manifested by an early-occurring and long-term continuing persistent estrus status after puberty, whereas no abnormalities could be found with regard to growth and development of the reproductive organs and the hypothalamo-pituitary-gonadal control system up to maturation. The hypothetic scheme for "androgenization effects" or "delayed modulation effects" on the hypothalamo-pituitary-gonadal system in rats exposed neonatally to high-dose OP is shown in Fig. 3.

On the other hand, the most notable effect on the female reproductive system when normal cycling rats were exposed to a high-dose of t-OP for a short time (28 days), was disappearance of the estrous cycle, and no clear changes were detected in other parameters such as uterine weight and morphology. These results indicate that the vaginal smear is the most sensitive parameter for the detection of effects of EDCs in normal cycling rats.

Well or moderately differentiated adenocarcinomas

were increased in Donryu rats initiated by ENNG, when high dose t-OP was given subcutaneously during adulthood. Neonatal exposure to a high dose of t-OP also showed promoting effects on uterine adenocarcinoma development in a two-stage rat uterine carcinogenesis model using Donryu rats, with slight to higher malignancy with more prolonged treatment.

For the risk assessment of EDCs to human health, it is very important to investigate the effects of low doses at actual human exposure levels, because the concentrations of agents, including alkylphenols and BPA, in the environment are very low. In addition, the main exposure route to EDCs is oral, not subcutaneous, in humans. Thus, we have focused on effects of maternal exposure to low doses of EDCs, such as NP and BPA, by the oral route, which have shown no effects on growth and development of the female reproductive system or uterine carcinogenesis. Transfer of low doses of BPA from dams to offspring via the placenta and/or milk was not unequivocal, although NP was transferred when relatively high doses were given.

These results indicate that dietary exposure to low doses of EDCs might not induce any adverse effects on the female genital system in mammals, including humans, because of the effects of homeostasis and clearance from the blood stream on first passage through the liver.

## References


1. Maekawa A, Takahashi M, Ando J, and Yoshida M. Uterine carcinogenesis by chemicals/hormones in rodents. J Toxicol Pathol 1999; **12**: 1–11.
2. Zeil HK. Estrogen's role in endometrial cancer. Obstet Gynecol 1982; **60**: 509–515.
3. Fox H. Endometrial carcinogenesis and its relation to estrogens. Pathol Res Pract 1984; **179**: 13–19.
4. Nagaoka T, Takegawa K, Takeuchi M, and Maekawa A. Effects of reproduction on spontaneous development of endometrial adenocarcinomas and mammary tumors in Donryu rats. Jpn J Cancer Res 2000; **91**: 375–382.
5. Ando-Lu J, Takahashi M, Imai S, Ishihara R, Kitamura T, Iijima T, Takano S, Nishiyama K, Suzuki K, and Maekawa A. High-yield induction of uterine endometrial adenocarcinomas in Donryu rats by a single intra-uterine administration of N-ethyl-N'-nitro-N-nitrosoguanidine via the vagina. Jpn J Cancer Res 1994; **85**: 789–793.
6. Vollmer G. Endometrial cancer: experimental models useful for studies on molecular aspects of endometrial cancer and carcinogenesis. Endocrine-Related Cancer 2003; **10**: 23–42.
7. Hoare RM. Comparative developmental aspects of selected organ system. II. Gastrointestinal and urogenital systems. Environ Health Perspect 1976; **18**: 61–66.
8. Branham WS, Sheehan DM, Zehr DR, Ridlon E, and Nelson CJ. The postnatal ontogeny of rat uterine glands and age-related effects of 17$\beta$-estradiol. Endocrinology 1985; **117**: 2229–2237.
9. Fishman RB, Branham WS, Streck RD, and Sheehan DM. Ontogeny of estrogen receptor messenger ribonucleic acid expression in the postnatal rat uterus. Biol Reprod 1996; **55**: 1221–1230.
10. Cheng HC and Johnson DC. Serum estrogens and gonadotropins in developing androgenized and normal female rats. Neuroendocrinology 1974; **13**: 357–365.
11. Dussault JH, Walker P, Dubois JD, and Labrie F. The development of the hypothalamo-pituitary axis in the neonatal rat: Sexual maturation in male and female rats assessed by hypothalamic LHRH and pituitary and serum LH and FSH concentrations. Can J Physiol Pharmacol 1977; **55**: 1091–1097.
12. Sell S, Nichols M, Becker FF, and Leffert HL. Hepatocyte proliferation and $\alpha$1-fetoprotein in pregnant, neonatal, and partially hepatectomized rats. Cancer Res 1974; **34**: 865–871.
13. Meijs-Roelofs HM and Kramer P. Maturation of the inhibitory feedback action of oestrogen on follicle-stimulating hormone secretion in the immature female rat: A role for alpha-fetoprotein. J Endocrinol 1979; **81**: 199–208.
14. Gorski RA, Gordon JH, Shryne JE, and Southam AM. Evidence for a morphological sex difference within the medial preoptic area of rat brain. Brain Res 1978; **148**: 333–346.
15. Jacobson CD, Csernus VJ, Shryne JE, and Gorski RA. The influence of gonadectomy, androgen exposure or a gonadal graft in the neonatal rat on the volume of the sexually dimorphic nucleus of the preoptic area. J Neurosci 1981; **1**: 1142–1147.
16. Doehler KD. The pre- and postnatal influence of hormones and neurotransmitters on sexual differentiation of the mammalian hypothalamus. Int Rev Cytology 1991; **131**: 1–57.
17. Houtsmuller EJ, Brand T, de Jonge FH, Joosten RN, Van de Poll NE, and Slob AK. SDN-POA volume, sexual behavior and preference of male rats affected by perinatal treatment with ATD. Physiol Behav 1994; **56**: 535–541.
18. Davis EC, Popper P, and Gorski RA. The role of apoptosis in sexual differentiation of the rat sexually dimorphic nucleus of the preoptic area. Brain Res 1996; **734**: 10–18.
19. Furguson SA, Scallet AC, Flynn KM, Meredith JM, and Schwetz BA. Developmental neurotoxicity of endocrine disruptors: focus on estrogen. Neurotoxicol 2000; **21**: 947–956.
20. Doehler KD and Jarzab B. The influence of hormone antagonists on sexual differentiation of the brain. J Clean Technol Environ Toxicol & Occup Med 1998; **7**: 195–220.
21. Pinos H, Collado P, Rodrigues-Zafra M, Rodriguez C, Segovia S, and Guillamon A. The development of sex difference in the locus coeruleus of the rats. Brain Res Bull 2001; **56**: 73–78.
22. Orikasa C, Kondo Y, Hayashi S, McEwen BS, and Sakuma Y. Sexually dimorphic expression of estrogen receptor $\beta$ in the anteroventral periventricular nucleus of the rat preoptic area: Implication in luteinizing hormone surge. Proc Natl Acad Sci USA 2002; **99**: 3306–3311.
23. Henry WW Jr, Medlock KL, Sheehan DM, and Scallet AC. Detection of estrogen receptor (ER) in the hypothalamus using rat anti-ER monoclonal IgG with the unlabeled antibody method. Histochem 1991; **96**: 157–162.
24. Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, and Gustafsson JA. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors $\alpha$ and $\beta$. Endocrinol 1997; **138**: 863–870.

25. Del Vecchio FR. Normal development, growth, and aging of the female genital tract. In: Pathology of the Aging Rat (Vol. 1), Mohr U, Dungworth DL and Capen CC (eds), Washington DC: ILSI Press, 331–336, 1992.
26. Katsuda S, Yoshida M, Watanabe T, Kuroda H, Ando-Lu J, Takahashi M, Hayashi H, and Maekawa A. Estrogen receptor mRNA in uteri of normal estrous cycling and ovariectomized rats by in situ hybridization. Proc Soc Exp Biol Med 1999; **221**: 207–214.
27. Ando-Lu J, Sasahara K, Nishiyama K, Takano S, Takahashi M, Yoshida M, and Maekawa A. Strain-differences in proliferative activity of uterine endometrial cells in Donryu and Fischer 344 rats. Exp Toxic Pathol 1998; **50**: 185–190.
28. Nagaoka T, Takeuchi M, Onodera H, Matsushima Y, Ando-Lu J, and Maekawa A. Sequential observation of spontaneous endometrial adenocarcinoma development in Donryu rats. Toxicol Pathol 1994; **22**: 261–269.
29. OECD. OECD guideline and protocol for the prevalidation of the rat uterotropic screening assay. ENV/JM/TG/EDTA(99)2/ANN1. Joint Meeting of the Chemical Committee and the Working Party on Chemicals, Paris, 1999.
30. Dodge JA, Glasebrook AL, Magee DE, Phillips DL, Sato M, Short LL, and Bryant HU. Environmental estrogens: effects on cholesterol lowering and bone in the ovariectomized rat. J Steroid Biochem Mol Biol 1996; **59**: 155–161.
31. Iguchi T and Takasugi N. Occurrence of permanent changes in vaginal and uterine epithelia in mice treated neonatally with progestin, estrogen, and aromatizable or non-aromatizable androgens. Endocrinol Jpn 1976; **23**: 327–332.
32. Colborn T and Clement C. Chemically-induced alterations in sexual and functional development: the wildlife/human connection. In: Advances in Modern Environmental Toxicology. Colborn T and Clement C (eds), Princeton, NJ: Princeton Scientific Publishing, 1–403, 1992.
33. Blake CA and Ashiru OA. Disruption of rat estrous cyclicity by the environmental estrogen 4-tert-octylphenol. Proc Soc Exp Biol Med 1997; **216**: 446–451.
34. Katsuda S, Yoshida M, Watanabe G, Taya K, and Maekawa A. Irreversible effects of neonatal exposure to p-tert-octylphenol on the reproductive tract in female rats. Toxicol Appl Pharmacol 2000; **165**: 217–226.
35. Yoshida M, Takenaka A, Katsuda S, Kurokawa Y, and Maekawa A. Neonatal exposure to p-tert-octylphenol causes abnormal expression of estrogen receptor $\alpha$ and subsequent alteration of cell proliferating activity in the developing Donryu rat uterus. Toxicol Pathol 2002; **30**: 357–364.
36. MacLusky NJ and Naftolin F. Sexual differentiation of the central nervous system. Science 1981; **211**: 1294–1303.
37. Yoshida M, Katsuda S, Tanimoto T, Asai S, Nakae D, Kurokawa Y, Taya K, and Maekawa A. Induction of different types of uterine adenocarcinomas in Donryu rats due to neonatal exposure to high-dose p-t-octylphenol for different periods. Carcinogenesis 2002; **23**: 1745–1750.
38. Blake CA and Boockfor FR. Chronic administration of the environmental pollutant 4-tert-octylphenol to adult male rats interferes with the secretion of luteinizing hormone, follicle-stimulating hormone, prolactin, and testosterone. Biol Reprod 1997; **57**: 255–266.
39. Boockfor FR and Blake CA. Chronic administration of 4-tert-octylphenol to adult male rats causes shrinkage of the testes and male accessory sex organs, disrupts spermatogenesis, and increases the incidence of sperm deformities. Biol Reprod 1997; **57**: 267–277.
40. Katsuda S, Yoshida M, Isagawa S, Asagawa Y, Kuroda H, Watanabe T, Ando J, Takahashi M, and Maekawa A. Dose- and treatment duration-related effects of p-tert-octylphenol on female rats. Reprod Toxicol 2000; **14**: 119–126.
41. Yoshida M, Katsuda S, Ando J, Kuroda H, Takahashi M, and Maekawa A. Subcutaneous treatment of p-tert-octylphenol exerts estrogenic activity on the female reproductive tract in normal cycling rats of two different strains. Toxicol Lett 2000; **116**: 89–101.
42. Nagaoka T, Takegawa K, and Maekawa A. The relationship between endocrine imbalance and alteration of rat vaginal epithelium. J Toxicol Pathol 2002; **15**: 103–109.
43. Aso S, Anai M, Noda S, Imatanaka N, Yamasaki K, and Maekawa A. Twenty-eight-day repeated-dose toxicity studies for detection of weak endocrine disrupting effects of nonylphenol and atrazine in female rats. J Toxicol Pathol 2000; **13**: 13–20.
44. Yamasaki K, Sawaki M, Noda S, Muroi T, and Maekawa A. Immature rat uterotropic assay of diethylstilbestrol, ethynyl estradiol and atrazine. J Toxicol Pathol 2000; **13**: 145–149.
45. Herbst AL, Ulfelder H, and Poskanzer DC. Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. N Engl J Med 1971; **184**: 878–881.
46. Marselos M and Tomatis L. Diethylstilbestrol. II. Pharmacology, toxicology and carcinogenicity in experimental animals. Eur J Cancer 1993; **29**: 149–155.
47. Newbold RR and McLachlan JA. Vaginal adenosis and adenocarcinoma in mice exposed prenatally or neonatally to diethylstilbestrol. Cancer Res 1982; **42**: 2003–2011.
48. Walker BE. Uterine tumors in old female mice exposed prenatally to diethylstilbestrol. J Natl Cancer Inst 1983; **70**: 477–484.
49. Kitamura T, Nishimura S, Sasahara K, Yoshida M, Ando J, Takahashi M, Shirai T, and Maekawa A. Transplacental administration of diethylstilbestrol (DES) causes lesions in female reproductive organs of Donryu rats, including endometrial neoplasia. Cancer Lett 1999; **141**: 219–228.
50. Pasqualini JR, Sumida C, and Giambiagi N. Pharmacodynamic and biological effects of anti-estrogens in different models. J Steroid Biochem 1988; **31**: 613–643.
51. Killackey MA, Hakes TB, and Pierce VK. Endometrial adenocarcinoma in breast cancer patients receiving antiestrogens. Cancer Treat Rep 1985; **69**: 237–238.
52. IARC. Tamoxifen. In: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans (Vol. 66), Lyon: IARC, 253–365, 1996.
53. Newbold RR, Jefferson WN, Padilla-Burgos E, and Bullock BC. Uterine carcinoma in mice treated neonatally with tamoxifen. Carcinogenesis 1997; **18**: 2293–2298.
54. Carthew P, Edwards RE, Nolan BM, Martin EA, Heydon RT, White INH, and Tucker MJ. Tamoxifen induces endometrial and vaginal cancer in rats in the absence of endometrial hyperplasia. Carcinogenesis 2000; **21**: 793–797.
55. Yoshida M, Kudoh K, Katsuda S, Takahashi M, Ando J, and Maekawa A. Inhibitory effects of uterine endometrial carcinogenesis in Donryu rats by tamoxifen. Cancer Lett 1998; **134**: 43–51.
56. Takahashi M, Shimomoto M, Miyajima K, Iizuka S, Watanabe T, Yoshida M, Kurokawa Y, and Maekawa A.

Promotion, but not progression, effects of tamoxifen on uterine carcinogenesis in mice initiated with N-ethyl-N'-nitro-N-nitrosoguanidine. Carcinogenesis 2002; **23**: 1549–1555.

57. Pinter A, Torok G, Borzsonyi M, Surjan A, Csik M, Kelecsenyi Z, and Kocsis Z. Long-term carcinogenicity bioassay of the herbicide atrazine in F344 rats. Neoplasia 1990; **37**: 533–544.
58. Watanabe T, Kashida Y, Ueda M, Onodera H, Hirose M, and Mitsumori K. Promoting effects of ethinylestradiol but not atrazine on N-ethyl-N-nitrosourea-induced uterine carcinogenesis in ICR mice. J Toxicol Pathol 2003; **16**: 139–145.
59. IPCS (International Programme on Chemical Safety). Vinclozolin. In: Pesticide Residues in Food-1995 (Joint FAO/WHO Meeting of Pesticide Residues), Geneva: WHO, 375–404, 1996.
60. Rier SE, Martin DC, Bowman RE, Dmowski WP, and Becker JL. Endometriosis in rhesus monkeys (Macaca mulatta) following chronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Fund Appl Toxicol 1993; **21**: 433–441.
61. Moriya M, Mitsumori K, Kato K, Miyazawa T, and Shirasu Y. Carcinogenicity of N-nitroso-ethylenethiourea in female mice. Cancer Lett 1979; **7**: 339–342.
62. Yoshida A, Harada T, Hayashi S, Mori I, Miyajima H, and Maita K. Endometrial carcinogenesis induced by concurrent oral administration of ethylenethiourea and sodium nitrite in mice. Carcinogenesis 1994; **15**: 2311–2318.
63. Nishiyama K, Ando-Lu J, Nishimura S, Takahashi M, Yoshida M, Sasahara K, Miyajima K, and Maekawa A. Initiating and promoting effects of concurrent oral administration of ethylenethiourea and sodium nitrite on uterine endometrial adenocarcinoma development in Donryu rats. In Vivo 1998; **12**: 363–368.
64. Katsuda S, Yoshida M, Kuroda H, Ando J, Takahashi M, Kurokawa Y, Watanabe G, Taya K, and Maekawa A. Uterine adenocarcinoma in N-ethyl-N'-nitro-N-nitrosoguanidine-treated rats with high-dose exposure to p-tert-octylphenol during adulthood. Jpn J Cancer Res 2002; **93**: 117–124.
65. Zhu BT and Conney AH. Functional role of estrogen metabolism in target cells: review and perspectives. Carcinogenesis 1998; **19**: 1–27.
66. Takahashi M, Shimomoto T, Miyajima K, Yoshida M, Katashima S, Uematsu F, Maekawa A, and Nakae D. Effects of estrogens and metabolites on endometrial carcinogenesis in young adult mice initiated with N-ethyl-N'-nitro-N-nitrosoguanidine. Cancer Lett 2004; in press.
67. Kojima T, Tanaka T, and Mori H. Chemoprevention of spontaneous endometrial cancer in female Donryu rats by dietary indole-3-carbinol. Cancer Res 1994; **54**: 1446–1449.
68. Certa H, Fedtke N, Wiegand HJ, Muller AMF, and Bolt HM. Toxicokinetics of p-tert-octylphenol in male Wistar rats. Arch Toxicol 1996; **71**: 112–122.
69. Upmeier A, Degan GH, Schuhmacher US, Certa H, and Bolt HM. Toxicokinetics of p-tert-octylphenol in female DA/Han rats after single i.v. and oral application. Arch Toxicol 1999; **73**: 217–222.
70. NTP. NTP final report on the reproductive toxicity of nonylphenol (CAS# 84852-15-3) administered by gavage to Sprague-Dawley rats. NTP Report # RACB 94021, 1997.
71. Chapin RE, Delaney J, Wang Y, Lanning L, Davis B, Collins B, Minz N, and Wolfe G. The effects of 4-nonylphenol in rats: a multi-generation reproduction study. Toxicol Sci 1999; **52**: 80–91.
72. Nagano T, Wada K, Marumo H, Yoshimura S, and Ono H. Reproductive effects of nonylphenol in rats after gavage administration: a two-generation study. Reprod Toxicol 2001; **15**: 293–315.
73. Yoshida M, Shimomoto T, Katashima S, Shirai T, Nakae D, Watanabe G, Taya K, and Maekawa A. Effects of maternal exposure to nonylphenol on growth and development of the female reproductive system and uterine carcinogenesis in rats. J Toxicol Pathol 2003; **16**: 259–266.
74. Ashby J and Tinwell H. Uterotropic activity of bisphenol A in the immature rat. Environ Health Perspect 1998; **106**: 719–720.
75. Morrissey RE, Lamb JC. IV, Morris RW, Chapin RE, Gulati DK, and Heindel JJ. Results and evaluations of 48 continuous breeding reproduction studies conducted in mice. Fund Appl Toxicol 1989; **13**: 747–777.
76. Morrissey RE, George JJ, Price CJ, Tyl RW, Marr MC, and Kimmel CA. The developmental toxicity of bisphenol A in rats and mice. Fund Appl Toxicol 1987; **8**: 1294–1302.
77. Nagao R, Saito Y, Usumi K, Kuwagata M, and Imai K. Reproductive function in rats exposed neonatally to bisphenol A and estradiol benzoate. Reprod Toxicol 1999; **13**: 303–311.
78. Kwon S, Stedman DB, Ilswick RC, Cattley RC, and Welsch F. Pubertal development and reproductive functions of Crl:CD BR Sprague-Dawley rats exposed to bisphenol A during prenatal and postnatal development. Toxicol Sci 2000; **55**: 399–406.
79. Sharpe RM, Fisher J, Milar MR, Jobling S, and Sumpter JS. Gestational and/or neonatal exposure of rats to environmental estrogenic chemicals results in reduced testis size and daily sperm production in adulthood. Environ Health Perspect 1995; **103**: 1136–1143.
80. vom Saal FS, Cooke PS, Buchanan DL, Palanza P, Thayer KA, Nagel SC, Parmigiani S, and Welshons WV. A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm-production, and behavior. Toxicol Ind Health 1998; **14**: 239–260.
81. Fisher JS, Turner KJ, Brown D, and Sharpe RM. Effects of neonatal exposure to estrogenic compounds on development of the excurrent ducts of the rat testis through puberty to adulthood. Environ Health Perspect 1999; **107**: 397–405.
82. Howdeshell KL, Hotchkiss AK, Thayer KA, Vanderbergh JG, and vom Saal FS. Exposure to bisphenol A advances puberty. Nature 1999; **401**: 763–764.
83. Ashby J, Tinwell H, and Haseman J. Lack of effects for low dose levels of bisphenol A and diethylstilbestrol on the prostate gland of CF1 mice exposed in utero. Regul Toxicol Pharmacol 1999; **30**: 156–166.
84. Cagen SZ, Waechter JM Jr, Dimond SS, Breslin WJ, Butala JH, Jekat FW, Joiner RL, Shiotsuka RN, Veenstra GE, and Harris LR. Normal reproductive organ development in CF-1 mice following prenatal exposure to bisphenol A. Toxicol Sci 1999; **50**: 36–44.
85. Cagen SZ, Waechter JM Jr, Dimond SS, Breslin WJ, Butala JH, Jekat FW, Joiner RL, Shiotsuka RN, Veenstra GE, and Harris LR. Normal reproductive organ development in

Wistar rats exposed to bisphenol A in the drinking water. Regul Toxicol Pharmacol 1999; **30**: 130–139.
86. Tyl RW, Myers CB, Marr MC, Thomas BF, Keimowitz AR, Brine DR, Veselica MM, Fail PA, Chang TY, Seely JC, Joiner RL, Butala JH, Dimond SS, Cagen SZ, Shiotsuka RN, Stropp GD, and Waechter JM. Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. Toxicol Sci 2002; **68**: 121–146.
87. Kawamura Y, Inoue K, Nakazawa H, Yamada T, and Maitani T. Cause of bisphenol A migration from cans for drinks and assessment of inproved cans. Syokuhin Eiseigaku Zassi 2001; **42**: 13–17.
88. Yoshida M, Shimomoto T, Katashima S, Watanabe G, Taya K, and Maekawa A. Maternal exposure to low doses of bisphenol A has no effects on development of female reproductive tract and uterine carcinogenesis in Donryu rats. J Reprod Dev 2004; **50**: 349–360.
89. Takahashi O and Oishi S. Disposition of orally administered 2,2-bis(4-hydroxyphenol)propane (bisphenol A) in pregnant rats and the placental transfer to fetuses. Environ Health Perspect 2000; **108**: 931–935.
90. Johnson MD, Kenney N, Stoica A, Hilakivi-Clarke L, Singh B, Chepko G, Clarke R, Sholler P, Lirio AA, Foss C, Reiter R, Trock B, Paik S, and Martin MB. Cadmium mimics the in vivo effects of estrogen in the uterus and mammary gland. Nature Med 2003; **9**: 1081–1084.
91. Takasugi N. Cytological basis for permanent vaginal changes in mice treated neonatally with steroid hormones. Int Rev Cytol 1976; **44**: 193–224.
92. Newbold RR and Liehr JG. Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens. Cancer Res 2000; **60**: 235–237.
93. Okada A, Ohta Y, Buchanan DL, Sato T, Inoue S, Hiroi H, Muramatsu M, and Iguchi T. Changes in ontogenic expression of estrogen receptor alpha and not of estrogen receptor beta in the female rat reproductive tract. J Molecular Endocrinol 2002; **28**: 87–97.
94. Watanabe H, Suzuki A, Mizutani T, Khono S, Lubahn DB, Handa H, and Iguchi T. Genome-wide analysis of changes in early gene expression induced by estrogen. Genes to Cells 2002; **7**: 497–507.
95. Watanabe H and Iguchi T. Evaluation of endocrine disruptors based on gene espression using a DNA microarray. Environ Sci 2003; **10**: 061–067.

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# Catechin derivatives: Specific inhibitor for caspases-3, 7 and 2, and the prevention of apoptosis at the cell and animal levels

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**Abstract Tea-catechin derivatives are shown to inhibit activities of caspases-3, 2 and 7 in vitro, and prevented experimental apoptosis at the cell and animal levels. Epigallo-catechin-gallate showed the strongest inhibition at $1 \times 10^{-7}$ M to these caspases, but cysteine cathepsins and caspase-8 were not inhibited. Caspase-3 inhibition showed a 2nd-order allosteric-type, but the inhibition of caspases-2 and 7 showed a non-competitive-type. The apoptosis-test using cultured HeLa cells was inhibited by these catechins. In rat hepatocytes, apoptosis was induced by D-galactosamine in vivo. In this case, caspase-3 activity in the cytoplasm, the serum aminotransferases and dUTP nick formation detected by TUNNEL-staining were effects, and these elevations were suppressed by administration of catechin.**


*Keywords:* Catechin; Caspase-3; Apoptosis; D-Galactosamine; Serum aminotransferase

## 1. Introduction

Various pharmacological functions of tea-catechin derivatives have been extensively studied in recent years. Their anti-oxidant effects are well established; in addition, the possibility for prevention of oncogenesis by tea-catechins from the aspect of epidemiological statistics has been advocated. However, no reasonable explanation exists for the prevention of oncogenesis at the molecular level (see Section 4). The direct effect of tea-catechins on specific caspases with respect to apoptosis has not yet been reported. The synthetic inhibitors of substrate analogues for caspases have been reported; however, natural inhibitors have not been identified. Allosteric inhibition of caspase-3 by synthetic inhibitors was reported by Hardy et al., therefore the tertiary structures of caspases are flexible (see Section 4) [11]. We have previously shown that some tea-catechin derivatives strongly inhibited caspases-3, 2 and 7, in vitro and in vivo [1,2,5–9].

The inhibition of cultured HeLa cell apoptosis test, which is reported by Wells et al., was studied [4]. Liver injury induced by D-galactosamine with lipopolysaccharide (LPS) in vivo is well characterized to induce hepatocyte apoptosis within the pathological field, assessed by TUNNEL-staining and DNA fragmentation [1–4]. The activity of caspase-3 in the liver cytoplasm was significantly elevated, and aspartate (AST) and alanine (ALT) aminotransferases in the serum were also significantly elevated in the D-galactosamine induced apoptotic liver. These increases were suppressed by epigallo-catechin-gallate (EGCG) in vivo. EGCG is the main component of green tea. The specific inhibition of activities of caspases-3, 2 and 7 by tea-catechin derivatives in vitro and the prevention of liver cell apoptosis in vivo are reported in this paper.

## 2. Materials and methods

### 2.1. Materials

Recombinant human caspases-3, 7, 8 and 2 were purchased from Bio-Vision Co. Catechin derivatives were purchased from Wako Co. Cathepsin B and L were purchased from Sigma.

### 2.2. Methods

*2.2.1. Inhibition assays of caspases-3, 7, 2 and 8 activities by catechin derivatives.* An established method for the assay of activities of caspase-3 and caspase-7 was used [9], using the recombinant pure caspases and DEVD-AFC as the substrate. Ac-IETD-MCA was used for caspase-8 and AC-VDVAD-MCA was used for caspase-2. Enzyme activity was expressed as the released AFC (or MCA) formed nM/h/mg protein.

*2.2.2. Cell-free apoptosis test using cultured HeLa cell S-100.* The apoptosis assay system reported by Wells et al. is composed of cultured HeLa cell cytoplasm S-100 (4 mg protein/ml), cytochrome *c* (80 μM) and Ac-DEVD-MCA (40 μM) as the substrate for formed caspase-3 [12]. Preparation of S-100 from cultured HeLa cells was followed using the method described by Wells and Nguyen [12]. Following incubation at 37 °C for 40 min, the released fluorescent MCA in the S-100 fraction was assayed as formed caspase-3 from procaspase-3 in the S-100. Caspase-3 activity without addition of cytochrome *c* was used as the negative control.

*2.2.3. Administration method of D-galactosamine and tea-catechin derivatives in rats.* Liver apoptosis was induced according to Muntane's method, by intraperitoneal injection of D-galactosamine [3,4]. A single dose of D-galactosamine was administered intraperitoneally

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*Abbreviations:* AST, aspartate aminotransferase; ALT, alanine aminotransferase; EGCG, epigallo-catechin gallate; ECG, epi-catechin gallate; CG, catechin gallate; EC, epi-catechin; EGC, epigallo-catechin; C, catechin; GC, gallo-catechin; G, gallate; LPS, lipopolysaccharide; TdT, terminal transferase; MCA, methyl coumaryl amide

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(0.5 g/kg), and rats were sacrificed 12 h after the injection. Two doses of EGCG with 50 μg/kg of LPS were administered intraperitoneally at 1 h before and after the D-galactosamine administration. EGCG was further administered twice at 3-h intervals.

*2.2.4. Preparation of liver cytoplasm for assay of caspase-3 activity.* Liver cytoplasm fraction for caspase-3 activity assay was prepared by sequential centrifugation method for cell organelle separation according to a method described by Fleisher and Kervina [16].

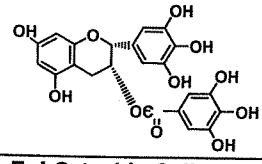
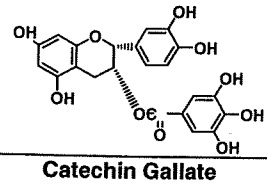
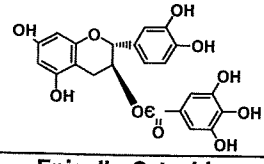
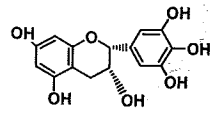
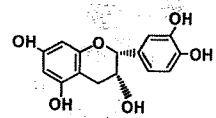
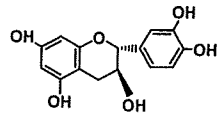
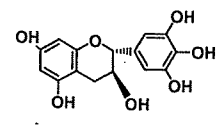
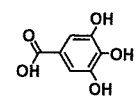
*2.2.5. TdT-mediated dUTP nick end labeling (TUNNEL) assay.* Apoptotic cells were detected in sections using the in situ Apoptosis Kit (Takara Kyoto, Japan). Frozen sections of liver tissues were fixed in 3% paraformaldehyde, incubated with protease K (20 μg/ml) for 10 min, and then presoaked in terminal transferase (TdT) buffer (0.5 μM/L cacodylate, 1 μM/L CoCl, 0.5 μM/L dithiothreitol, 0.05% bovine serum albumin, and 0.15 M/L NaCl) for 10 min. Sections were incubated for 1 h at 37 °C in 25 ml of TdT solution, containing 1× terminal transferase buffer, 0.5 nM of biotin-dUTP, and 10 U of TdT. After the TdT reaction, sections were soaked in TdT blocking buffer (300 nM/L NaCl and 30 mM/L tri-sodium citrate-2-hydrate), incubated with HRP-conjugated streptavidin for 30 min at room temperature, and developed for 10 min in phosphate-buffered citrate (pH 5.8) containing 0.6 mg/ml DAB. Nuclei were counterstained with hematoxylin.

## 3. Results

### 3.1. Inhibition of caspase-3 activity by various catechin derivatives in vitro

Caspase-3 plays a central role as an executive enzyme of apoptosis in the final step of various apoptotic cascades [5–9]. Caspase-3 activity was completely inhibited by EGCG at $1 \times 10^{-7}$ M and was inhibited to 50% at $1 \times 10^{-8}$ M in vitro.

Table 1
Comparison of inhibition of caspase-3 activity in vitro and the apoptosis test using cultured HeLa cells by tea-catechin derivatives

| Catechin Derivatives | *in vitro* 50% Inhibition of caspase-3 | HeLa cell apoptosis test 50% Inhibition of apoptosis test |
|---|---|---|
| Epigallo-Catechin Gallate  | $1\times10^{-8}$ M | $1\times10^{-6}$ M |
| Epi-Catechin Gallate  | $1\times10^{-7}$ M | $1\times10^{-4}$ M |
| Catechin Gallate  | $1\times10^{-6}$ M | $5\times10^{-4}$ M |
| Epigallo-Catechin  | $1\times10^{-6}$ M | $5\times10^{-4}$ M |
| Epi-Catechin  | $1\times10^{-6}$ M | $5\times10^{-4}$ M |
| Catechin Gallate  Gallo-Catechin  Gallic Acid  | | No Inhibitions by $1\times10^{-4}$ M |

The left-hand column shows the concentrations of catechin derivatives. inducing 50% inhibition of caspase-3 activity in vitro. The right-hand column shows the 50% inhibition of the apoptosis test units of cultured HeLa cells. The assay methods are described in the text [10,11]. The 50% inhibition concentrations of various catechin derivatives are illustrated ($n = 3$, the mean ± S.E.M. with $^{*}P < 0.01$).

Epi-catechin gallate (ECG) showed 50% inhibition at $1 \times 10^{-7}$M, and catechin gallate (CG), epi-catechin (EC) and epigallo-catechin (EGC) had induced inhibition at $1 \times 10^{-6}$ M. Catechin (C), gallo-catechin (GC) and gallate (G) showed no inhibition as Table 1 shows. The stereo-binding form of –OH to the catechin-ring should be an epi-structure to display inhibitory activity. The presence of either component, catechin gallate (CG) and/or epi-form catechin (EC), is essential.

Relationship of velocity and substrate concentration of caspase-3 in the presence of EGCG showed a typical sigmoidal curve and the Lineweaver–Burk relationship did not give a straight line, but showed a logarithmic curve. When the abscissa was taken as $1/[S]^2$, the logarithmic curve changed to a straight line (Fig. 1A). The inhibition kinetics of these catechin derivatives appear to be a 2nd-order sigmoidal allosteric inhibition as follows:

$$1/v = Km/V(1/[S]^2) + 1/V.$$

The other four effective catechin derivatives, such as ECG, CG, EC and EGC, also showed the same type of allosteric inhibition to caspase-3 as that by EGCG (figures are abbreviated).

The binding site of the catechins appeared to be different from the substrate-binding site. The allosteric nature of caspase-3 using synthetic inhibitors was reported by Hardy et al. [11] (see Section 4). The molecular weight of caspase-3 did not appear to change in the presence of EGCG and/or substrate using Superdex G-75. Therefore, polymerization or depolymerization was not observed using these allosteric inhibitors (data not shown).

### *3.2. Inhibitions of activities of caspases-7 and 2 activities by EGCG in vitro*

Caspases-7 and 2 are also known to participate in various apoptosis cascades. The activities of caspases-7 and 2 were also strongly inhibited by EGCG, and the 50% activities were inhibited at $1 \times 10^{-6}$ M. However, the mode of inhibitions of caspases-7 and 2 were different from that of caspase-3. The $V_{max}$ decreased in the presence of EGCG and the Lineweaver–Burk relationship showed a non-competitive type inhibition (Fig. 1B and C). The binding site to EGCG is the same as the substrate-binding site or located near the active site. Caspase-8, cathepsins B and L, which are the same cysteine proteases, were not inhibited at $1 \times 10^{-5}$ M of EGCG. Therefore, the inhibitions of caspases are not due to an attack to the active site –SH of these enzymes by the scavenger effect of catechins.

### *3.3. Inhibition of caspase-3 in HeLa cell apoptosis test induced by cytochrome c by catechin derivatives*

Wells et al. developed a cell-free apoptosis test using cultured HeLa cells [12]. The S-100 prepared from cultured HeLa cell cytoplasm contains sufficient amounts of procaspase-3 and the activating enzyme system except cytochrome *c*. Caspase-3 activity in the S-100 increased following the addition of cytochrome *c*, as shown in Fig. 2. The 70% of the apoptosis unit was inhibited by EGCG at a concentration of $1 \times 10^{-5}$ M. The strengths of suppression by the various catechin derivatives were in the same order as the inhibitions of caspase-3 activity in vitro, as shown in Table 1, right column.

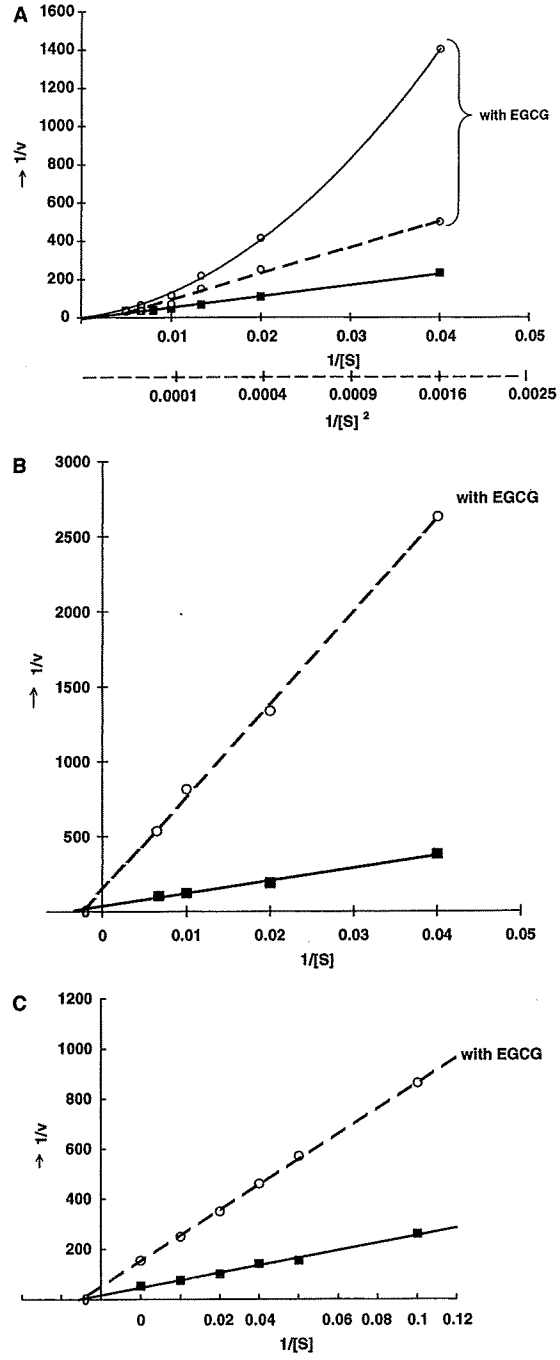



Fig. 1. Mode of inhibitions of caspase-3, 2 and 7 by EGCG in Lineweaver–Burk relationship. (A) Caspase-3 inhibition by $1 \times 10^{-7}$ M of EGCG. The $1/v$ values to the $1/[S]$ in the presence of EGCG are expressed as open circles with a thin solid line (O – O). The $1/v$ values to the $1/[S]$ in the absence of EGCG are illustrated as closed squares with a solid line. The $1/v$ values to the $1/[S]^2$ illustration in the presence of EGCG are expressed as open circles with a broken line (O – O). (B) and (C) Caspase-2 or 7 inhibition by $1 \times 10^{-6}$ M of EGCG. The activities in the absence of EGCG are illustrated as solid line with a solid line and the activities in the presence of EGCG are illustrated as open circles with a broken line. (B) shows caspase-2 inhibition by EGCG.; the Lineweaver–Burk relationship. (C) shows caspase-7 inhibition by EGCG. All symbols and lines are the same as those in (B).

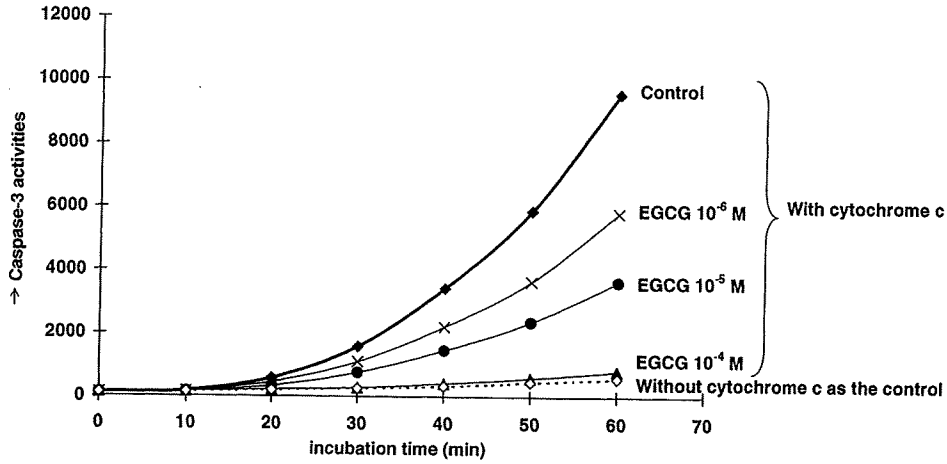



Fig. 2. Inhibition of caspase-3 activities in apoptotic test using cultured HeLa cells induced by cytochrome *c* by catechin derivatives. Caspase-3 inhibition in S-100 of cultured HeLa cells by various catechin derivatives was assayed using Nguyen and Wells' apoptosis test as shown in Fig. 3 [12]. The inhibitory activities by various catechin derivatives are compared as 50% inhibition concentrations as shown in Fig. 1 in right panel. The catechin derivatives added were expressed as the final concentrations in the S-100 fraction. All data are the means ± S.E.M. ($n = 5$) with $^{*}P < 0.01$.

### *3.4. Liver apoptosis induced by D-galactosamine plus LPS, and its prevention by EGCG in vivo*

Sufficient amounts of procaspase-3 are present and active caspase-3 is not present in the normal hepatocyte cytoplasm. However, procaspase-3 in the cytoplasm is activated to form active caspase-3 by the effective apoptotic signal. It is well known within the pathological field that hepatocyte injury induced by D-galactosamine results in hepatocyte apoptosis, as assessed by the TUNNEL-staining and the DNA ladder formation [3,4,10].

(1) Elevations of liver caspase-3 activity and serum aminotransferases in D-galactosamine induced hepatocyte apoptosis, but were prevented by cotreatment with EGCG, as shown in Table 2. The both elevations were prevented by cotreatment with EGCG in a dose-dependent manner, and treatments with 50 mg/head EGCG suppressed the activity to the normal level. Furthermore, the macroscopic liver profile was protected and resembled to normal level.

However, the mechanism of procaspase-3 activation cascade induced by D-galactosamine remains unknown (see Section 4).

(2) TUNNEL-staining method, which is the most established DNA nick formation in the nucleus, was examined in these livers. As shown in Fig. 3, the significant nick staining of nuclear DNA was observed in the livers treated with D-galactosamine, while nick formations was significantly suppressed by cotreatment with EGCG. These data show that D-galactosamine induced liver injury resulted in caspase-3 mediated apoptosis and the apoptosis was significantly suppressed by EGCG administration.

(3) Increased activities of AST and ALT in the serum by D-galactosamine administration, which are the established marker for hepatocyte injury, were also completely suppressed by cotreatment with EGCG dose-dependently as shown in Table 2. EGCG showed an effective protecting effect for the liver injury mediated by caspase-3.

## 4. Discussion

There are several papers on cancer prevention by tea-catechin derivatives, which appear to contradict our own data.

Table 2
Elevation of caspase-3 activities in rat liver cytoplasm in vivo and the activities of AST and ALT in the serum following D-galactosamine administration, and the preventions by EGCG treatment in vivo

| | Caspase-3 activities in liver (AFC nM/mg/h) | Aminotransferases in serum (IU/l) | |
|---|---|---|---|
| | | AST | ALT |
| Control LPS | <100 | <37.8 | <5.8 |
| D-GalN | 1000.0 | 450.0 | 300.0 |
| D-GalN + LPS | 5500.0 | 5229.3 | 1438.3 |
| D-GalN + LPS + EGCG 10 mg | 3500.0 | – | – |
| D-GalN + LPS + EGCG 30 mg | 300.0 | 320.5 | 114.0 |
| D-GalN + LPS + EGCG 50 mg | 100.0 | <100 | <100 |

0.5 g/kg D-galactosamine with 50 μg/kg LPS was administered once intraperitoneally. The D-galactosamine administration method and the preparations of the liver cytoplasm for the caspase-3 assay are described in Section 2.2. The injection doses of EGCG are mg/head. Elevation of caspase-3 activities in the liver cytoplasm in D-galactosamine-induced apoptosis and the preventions by EGCG. The dose-dependent suppressions of caspase-3 activities by EGCG administration are represented in the left columns. All data represent the means ± S.E.M. ($n = 5$) with $^{*}P < 0.01$.

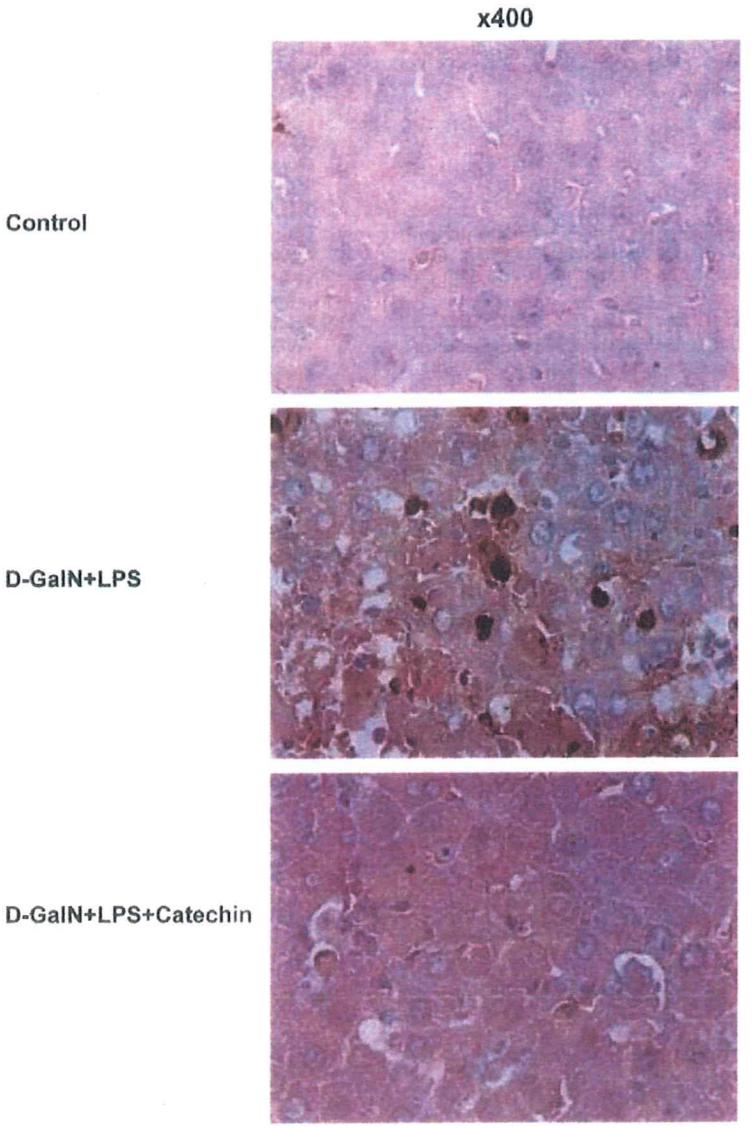



Fig. 3. Hepatocyte apoptosis images using TUNNEL-staining induced by D-galactosamine plus LPS and its prevention by EGCG cotreatment. The staining method is described in Section 2.2. These images are 400× magnification. Top image shows the control liver, the middle image shows liver administrated D-galactosamine plus LPS and the suppression profile by EGCG is shown in the bottom image.

However, this is completely different phenomenon from the following reasons; the reported effective concentration of catechin for cancer prevention is very high $10^{-3}$–$10^{-4}$ M [13], these concentrations are not physiological and appear to be toxic concentration. On the other hand, inhibition of caspase-3 by catechins was $10^{-6}$–$10^{-7}$ M in vitro and in vivo. Furthermore, these papers do not mention on the relationship between cancer cell death and apoptosis mediated by caspases [13–15]. Some papers reported that catechin stimulates release of TNF-α and enhances effect of anticancer drugs in vivo. While there is data demonstrating the prevention of oncogenesis in vivo, there is no research at the molecular level [14,15].

There are two possible mechanisms by which catechin suppresses hepatocyte apoptosis induced by D-galactosamine administration. One is due to direct inhibition of caspase-3 activity and the other is due to elimination of $O_2^-$, which is produced by D-galactosamine-protein binding through Maillard reaction. Both mechanisms are likely.

Caspase-3 is constructed from a heterotetramer, which is composed of two pairs of heterodimers. Each unit is composed of a long chain and a short chain. The substrate-binding site is located in the long chains. The interaction between the long chain and short chain and also the unit-to-unit interactions are susceptible to allosteric effectors. For example, it has been reported by Hardy et al. [11] using synthetic allosteric inhibitors that the inhibitor-binding site of the caspase-3 molecule is different from the substrate binding site. They also reported that the –SH of these inhibitors can form a disulfide bond with the cysteine-SH at amino acid 290th of the enzyme, which is different from the active site cysteine in the long chain. The practical conformational change by EGCG will be made clear using X-ray co-crystallography.

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## References

[1] Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. and Nagata, S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391, 43–50.
[2] Liu, X., Zou, H., Slaughter, C. and Wang, X. (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 89, 175–184.
[3] Muntane, J., Montero, J.L., Marchal, T., Perez-Seoane, C., Lozano, J.M., Fraga, E., Pintado, C.O., de la Mata and Mino, G. (1998) Effect of $PGE_1$ on TNF-α status and hepatic D-galactosamine-induced apoptosis in rats. J. Gastroenterol. Hepatol. 13, 197–207.
[4] Galanos, C., Freudenberg, M.A. and Reutter, W. (1979) Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc. Natl. Acad. Sci. USA 76, 5939–5943.
[5] Korsmeyer, S.J. (1995) Regulators of cell death. Trends Genet. 11, 101–105.
[6] Williams, G.T. and Smith, C.A. (1993) Molecular regulation of apoptosis: genetic controls on cell death. Cell 74, 777–779.
[7] Villa, P., Kaufmann, S.H. and Earnshaw, W.C. (1997) Caspase and caspase inhibitors. Trends Biochem. Sci. 22, 388–393.
[8] Stennicke, H.R., Jurgensmeier, J.M., Shin, H., Deveraux, Q., Wolf, B.B., Yang, X., Zhou, Q., Ellerby, H.M., Ellerby, L.M., Bredesen, D., Green, D.R., Reed, J.C., Froelich, C.J. and Salvesen, G.S. (1998) Pro-caspase-3 is a major physiologic target of caspase-8. J. Biol. Chem. 273, 27084–27090.
[9] Salvesen, G.S. and Dixit, V.M. (1997) Intracellular signaling by proteolysis. Cell 91, 443–446.
[10] Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., Miller, D.K, Molineaux, S.M., Weidner, J.R, Aunins, J., Elliston, K.O., Ayala, J.M., Casano, F.J., Chin, J., Ding, G.J.-F., Egger, L.A., Gaffney, E.P., Limjuco, G., Palyha, O.C., Raju, S.M., Ralond, M., Salley, J.P., Yamin, T.T., Lee, T.D., Shively, J.E., MacCross, M., Mumford, R.A., Schumidt, J.A. and Tocci, M.J. (1992) A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature 356, 768–774.
[11] Hardy, J.A., Lam, J., Nguyen, J.T., O'Brien, T. and Wells, J.A. (2004) Discovery of an allosteric site in the caspases. Proc. Natl. Acad. Sci. USA 101, 12461–12466.
[12] Nguyen, J.T. and Wells, J.A. (2003) Direct activation of the apoptosis machinery as a mechanism to target cancer cells. Proc. Natl. Acad. Sci. USA 100, 7533–7538.
[13] Okabe, S., Ochiai, Y., Aida, M., Park, K., Kim, S.J., Nomura, T., Suganuma, M. and Fujiki, H. (1999) Mechanistic aspects of green tea as a cancer preventive: effect of components on human stomach cancer cell lines. Jpn. J. Cancer Res. 90, 733–739.
[14] Suganuma, M., Okabe, S., Kai, Y., Sueoka, N., Sueoka, E. and Fujiki, H. (1999) Synergistic effects of (−)-epigallocatechin gallate with (−)-epicatechin, sulindac, or tamoxifen on cancer-preventive activity in the human lung cancer cell line PC-9. Cancer Res. 59, 44–47.
[15] Suganuma, M., Sueoka, E., Sueoka, N., Okabe, S. and Fujiki, H. (2000) Mechanisms of cancer prevention by tea polyphenols based on inhibition of TNF-alpha expression. Biofactors 13, 67–72.
[16] Fleischer, S. and Kervina, M. (1974) Subcellular fractionation of rat liver. Methods Enzymol. 31, 6–41.

nature genetics

# Mutations in the gene encoding fibroblast growth factor 10 are associated with aplasia of lacrimal and salivary glands

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**Autosomal dominant aplasia of lacrimal and salivary glands (ALSG; OMIM 180920 and OMIM 103420) is a rare condition characterized by irritable eyes and dryness of the mouth. We mapped ALSG to 5p13.2–5q13.1, which coincides with the gene fibroblast growth factor 10 (*FGF10*). In two extended pedigrees, we identified heterozygous mutations in *FGF10* in all individuals with ALSG. *Fgf10*$^{+/-}$ mice have a phenotype similar to ALSG, providing a model for this disorder. We suggest that haploinsufficiency for FGF10 during a crucial stage of development results in ALSG.**

ALSG has variable expressivity, and affected individuals may have aplasia or hypoplasia of the lacrimal, parotid, submandibular and sublingual glands and absence of the lacrimal puncta[1]. The disorder is characterized by irritable eyes, recurrent eye infections, epiphora (constant tearing) and xerostomia (dryness of the mouth), which increases the risk of dental erosion, dental caries, periodontal disease and oral infections[2]. Individuals affected with ALSG are sometimes misdiagnosed with the more prevalent disorder Sjögren syndrome, an autoimmune disorder characterized by keratoconjunctivitis sicca and xerostomia[3]. Both sporadic and familial cases of ALSG have been described[2,4,5]. We recently identified two extended families of Swedish origin with ALSG (**Fig. 1a**). The phenotypes of the affected individuals are summarized in **Supplementary Table 1** online. In total, 16 individuals from both families were diagnosed with ALSG (**Supplementary Methods** online). We investigated the lacrimal and major salivary glands by magnetic resonance imaging (**Supplementary Fig. 1** online), which showed aplasia or hypoplasia of several major salivary glands in all affected individuals and absent or hypoplastic lacrimal glands in 13 of 14 affected individuals. We observed absence of one or several lacrimal puncta in 13 of 14 affected individuals. We observed no other abnormalities, and the affected individuals had normal lifespans.

Inheritance of ALSG in both families is autosomal dominant, and the segregation pattern suggested full penetrance. A genome-wide screen with 400 polymorphic microsatellite markers showed linkage of ALSG to 5p13.2–5q13.1 flanked by microsatellite markers *D5S395* and *D5S2046* (**Fig. 1a**). We obtained a maximum cumulative lod score of 5.72 ($\theta = 0$) at the marker locus *D5S398* for both families (**Supplementary Table 2** online). The gene fibroblast growth factor 10 (*FGF10*) is located in the linked region[6]. Mouse FGF10, which is 93% identical to human FGF10, is crucial for the development of several organs, including lacrimal and salivary glands[7–9]. *Fgf10*$^{-/-}$ mice die shortly after birth[9,10]. No abnormalities have been described in *Fgf10*$^{+/-}$ mice.

We considered *FGF10* as a candidate gene for ALSG. Sequence analysis of the three exons of *FGF10* in samples from family 1 showed no alterations compared with sequences in the National Center for Biotechnology Information database. To identify deletions, we genotyped the family members for SNPs and microsatellite markers in *FGF10*. The affected members of family 1 were hemizygous with respect to two dinucleotide repeats (TA53 and CA17) and three SNPs (rs10060548, rs6881797 and rs2290070; **Fig. 1b,c**). After genotyping, we characterized the deletion breakpoint by long-range PCR and sequencing across the breakpoint (**Supplementary Fig. 2** online). We determined the size of the deletion to be 53 kb, including exons 2 and 3, without the involvement of any flanking genes (**Fig. 1b**). In family 2, DNA sequence analysis of *FGF10* identified a heterozygous stop mutation in exon 3 (R193X; 577C→T) resulting in a predicted truncated protein in the four affected members (**Fig. 1d**).

We then reexamined *Fgf10*$^{+/-}$ mice, which were previously described as apparently normal[8,10]. We dissected adult mice and carried out a macroscopical and histological examination of the lacrimal and salivary gland apparatuses. *Fgf10*$^{+/-}$ mice had aplasia of lacrimal glands and hypoplasia of salivary glands (**Fig. 2**). These findings are consistent with the phenotype of individuals with ALSG. Other internal organs, including lung, liver, spleen, heart, stomach, thyroid, pancreas, intestines and ovaries, were macroscopically normal in *Fgf10*$^{+/-}$ mice.

To clarify whether *FGF10* mutations cause dry eyes and dry mouth in sporadic cases with symptoms identical to those of individuals with ALSG, we screened DNA samples from 74 individuals for mutations in *FGF10*. These individuals had been evaluated and diagnosed with dry eyes and/or dry mouth, without fulfilling the criteria for Sjögren syndrome[11,12]. We found no sequence alterations in the coding region of *FGF10* in samples from these individuals,

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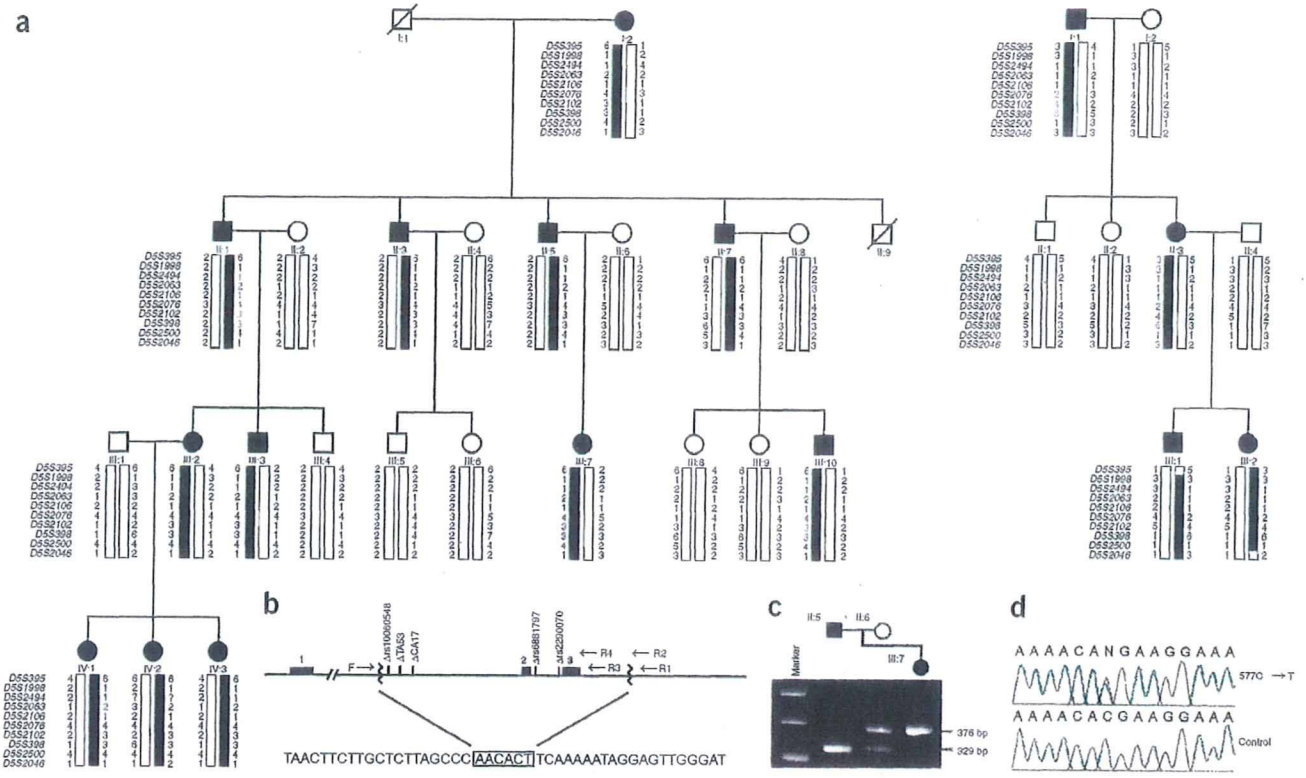


**Figure 1** ALSG mapping. **(a)** Two pedigrees segregating for ALSG. Marker haplotypes on chromosome 5p13.2–5q13.1 that are linked to ALSG are indicated by black bars. **(b)** Schematic overview of *FGF10* and the 53-kb deletion inherited with ALSG in family 1 (figure not drawn to scale). Black boxes denote exons 1–3, and wavy vertical lines indicate deletion breakpoints. Genomic sequence spanning the breakpoint is shown. **(c)** Genotyping of the SNP rs6881797, located in the deletion found in family 1 and 37 bp 3′ of exon 2, by digestion with *Bsr*I. Undigested PCR product (376 bp) corresponds to the T allele and digested PCR product (329 bp) corresponds to the A allele. The absence of a paternal A allele in individual III:7 in family 1 indicates hemizygosity with respect to rs6881797. **(d)** The upper sequence chromatogram illustrates the heterozygous (R193X; 577C→T) mutation found in the affected members of family 2. The lower sequence chromatogram illustrates the corresponding normal sequence.

suggesting that mutations in *FGF10* are uncommon in individuals with unspecific sicca syndromes.

In family 1, the affected individuals are hemizygous with respect to exons 2 and 3 of *FGF10*. In family 2, the premature stop codon (R193X; 577C→T) in exon 3 predicts a truncated protein with a loss of 16 amino acids. The truncation abolishes one predicted cAMP- and cGMP-dependent protein kinase phosphorylation site (residues 194–197) and one predicted *N*-linked glycosylation site (residues 196–198)[13]. Furthermore, the truncation eliminates one of the sites for the interaction between FGF10 and fibroblast growth factor receptor 2b (FGFR2b) at residues 202 and 204 (ref. 14). If produced, the truncated FGF10 is probably unstable or nonfunctional. Both mutations in *FGF10* that we identified are consistent with the idea that haploinsufficiency with respect to FGF10 underlies ALSG.

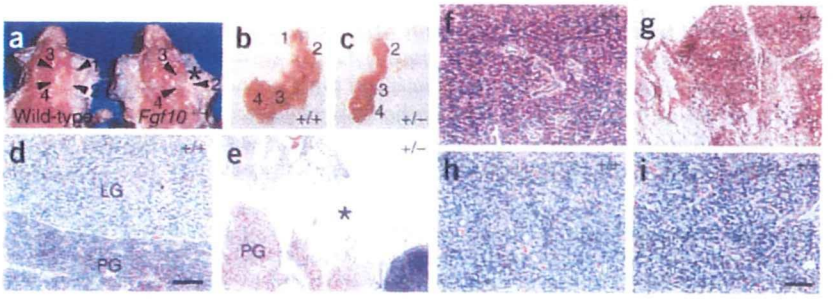


**Figure 2** Salivary and lacrimal gland apparatuses of wild-type and *Fgf10*$^{+/-}$ mice. **(a)** Macroscopic examination of wild-type (left) and *Fgf10*$^{+/-}$ (right) adult mice. Ventral view of the mandibular region. 1, lacrimal; 2, parotid; 3, sublingual; 4, submandibular glands. The asterisk indicates the expected site for the lacrimal gland, which was absent in the heterozygote. Other glands were hypoplastic in the heterozygote. **(b,c)** Dissected salivary and lacrimal glands from the wild-type **(b)** and *Fgf10*$^{+/-}$ **(c)** mice. The salivary glands from the *Fgf10*$^{+/-}$ mouse were hypoplastic and the lacrimal gland was absent. **(d–i)** Histology of wild-type **(d,f,h)** and *Fgf10*$^{+/-}$ **(e,g,i)** glands. **(d)** Wild-type lacrimal (LG) and parotid (PG) glands. **(e)** The lacrimal gland was replaced by adipose tissue (asterisk) in the *Fgf10*$^{+/-}$ mouse. **(f)** Wild-type parotid gland. **(g)** Parotid gland from *Fgf10*$^{+/-}$ mouse, which appears atrophic. **(h,i)** Submandibular glands have similar histology in the wild type and heterozygote, as did sublingual glands (not shown). Scale bars: **d** (for panels **d** and **e**), 0.5 mm; **i** (for panels **f–i**), 0.1 mm.

The clinical examinations and medical histories of the affected family members illustrate that one intact copy of *FGF10* is sufficient for development of essential organs in humans. The restricted phenotype associated with heterozygosity with respect to *FGF10* in both humans and mice suggests that the response to FGF10 is dosage-sensitive. This is probably related to a specific embryonic stage and occurs at the site of lacrimal and salivary gland formation. A possible explanation for the absence of generalized effects in *FGF10* hemizygotes is a functional overlap with other FGFR2b ligands, such as FGF1, FGF3 and FGF7 (ref. 15).

The identification of mutations in *FGF10* as causing ALSG will hopefully result in increased diagnostic accuracy. In a larger context, this report clarifies the phenotypic effects of mutations in *FGF10* and may lead to a better understanding of the mechanisms involved in lacrimal and salivary gland formation.

We obtained informed consent from all participants in the study under a protocol approved by the Ethical Committee at Uppsala University or by the collaborating Universities.

**URLs.** Primer 3 is available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/. The National Center for Biotechnology Information Entrez Genome Map Viewer is available at http://www.ncbi.nlm.nih.gov/mapview. The Ensembl Human Genome Server database is available at http://www.ensembl.org/. The Genome Database is available at http://www.gdb.org/.

**Accession numbers.** GenBank: human *FGF10*, NM_004465; human chromosome 5 clones containing *FGF10*, AC093537.2 and AC093289.2. GenBank Protein: human FGF10, NP_004456.1.

*Note: Supplementary information is available on the Nature Genetics website.*

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Wiedemann, H.R. *Am. J. Med. Genet.* **68**, 222–224 (1997).
2. Ferreira, A.P. *et al. Am. J. Med. Genet.* **94**, 32–34 (2000).
3. Bolstad, A.I. & Jonsson, R. *Arthritis Res.* **4**, 353–359 (2002).
4. Young, W. *et al. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **92**, 38–48 (2001).
5. Milunsky, J.M., Lee, V.W., Siegel, B.S. & Milunsky, A. *Am. J. Med. Genet.* **37**, 371–374 (1990).
6. Emoto, H. *et al. J. Biol. Chem.* **272**, 23191–23194 (1997).
7. Makarenkova, H.P. *et al. Development* **127**, 2563–2572 (2000).
8. Ohuchi, H. *et al. Biochem. Biophys. Res. Commun.* **277**, 643–649 (2000).
9. Min, H. *et al. Genes Dev.* **12**, 3156–3161 (1998).
10. Sekine, K. *et al. Nat. Genet.* **21**, 138–141 (1999).
11. Vitali, C. *et al. Arthritis Rheum.* **36**, 340–347 (1993).
12. Vitali, C. *et al. Ann. Rheum. Dis.* **61**, 554–558 (2002).
13. Bagai, S. *et al. J. Biol. Chem.* **277**, 23828–23837 (2002).
14. Yeh, B.K. *et al. Proc. Natl. Acad. Sci. USA* **100**, 2266–2271 (2003).
15. De Moerlooze, L. *et al. Development* **127**, 483–492 (2000).

# Development of Autoimmunity against Transcriptionally Unrepressed Target Antigen in the Thymus of Aire-Deficient Mice[1]

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**Autoimmune regulator (AIRE) gene mutation is responsible for the development of organ-specific autoimmune disease with monogenic autosomal recessive inheritance. Although Aire has been considered to regulate the elimination of autoreactive T cells through transcriptional control of tissue-specific Ags in thymic epithelial cells, other mechanisms of AIRE-dependent tolerance remain to be investigated. We have established Aire-deficient mice and examined the mechanisms underlying the breakdown of self-tolerance. The production and/or function of immunoregulatory T cells were retained in the Aire-deficient mice. The mice developed Sjögren's syndrome-like pathologic changes in the exocrine organs, and this was associated with autoimmunity against a ubiquitous protein, α-fodrin. Remarkably, transcriptional expression of α-fodrin was retained in the Aire-deficient thymus. These results suggest that Aire regulates the survival of autoreactive T cells beyond transcriptional control of self-protein expression in the thymus, at least against this ubiquitous protein. Rather, Aire may regulate the processing and/or presentation of self-proteins so that the maturing T cells can recognize the self-Ags in a form capable of efficiently triggering autoreactive T cells. With the use of inbred Aire-deficient mouse strains, we also demonstrate the presence of some additional factor(s) that determine the target-organ specificity of the autoimmune disease caused by Aire deficiency.** ***The Journal of Immunology,*** **2005, 174: 1862–1870.**

Autoimmune diseases are mediated by sustained adaptive immune responses specific for self-Ags through unknown mechanisms. Although breakdown of self-tolerance is considered to be the key event in the disease process, the mechanisms that allow the production of auto-Abs and/or autoreactive lymphocytes are largely enigmatic (1). The situation seems to have become more complicated due to the existence of multiple factors that influence the disease process, such as environmental factors, immune dysregulation, and genetic predisposition. In this regard, although only a small number of genes genetically relevant to the pathogenetic processes for the development of autoimmune diseases have been found so far (2), genetic engineering of such genes in mice should enable us to establish disease models and facilitate an understanding of the disease mechanisms to a large extent. One of these genes is the autoimmune regulator (*AIRE*)[3] mutation, which is responsible for the development of autoimmune-polyendocrinopathy-candidiasis ectodermal dystrophy (APECED; Online Mendelian Inheritance in Man 240300) with autosomal recessive inheritance (3–6).

The *AIRE* gene encodes a predicted 58-kDa protein carrying a conserved nuclear localization signal, two plant homeodomain (PHD)-type zinc fingers, four LXXLL motifs or nuclear receptor interaction domains, and the recently described homogeneously staining region (HSR) and SAND domains (3, 4); the HSR and SAND domains have been suggested to function in homodimerization and DNA binding, respectively (7, 8). Based on the fact that PHD resembles the RING finger, which can function as an E3 ubiquitin ligase, in both sequence and structure (9), we have recently found that AIRE acts as an E3 ubiquitin ligase through the N-terminal PHD domain (PHD1) (10). Because the ubiquitin-proteasome pathway plays an essential role in diverse cell functions such as cell cycle progression, signal transduction, cell differentiation, DNA repair and apoptosis (11, 12), we speculate that AIRE should play a fundamental role by facilitating polyubiquitinylation of the substrate(s) in yet undetermined processes. The significance

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[3] Abbreviations used in this paper: AIRE, autoimmune regulator; APECED, autoimmune-polyendocrinopathy-candidiasis ectodermal dystrophy; TEC, thymic epithelial cell; mTEC, medullary TEC; PHD, plant homeodomain; HEL, hen egg lysozyme; 3d-Tx mice, mice thymectomized 3 days after birth; SS, Sjögren's syndrome; Treg, immunoregulatory T cell; BM, bone marrow.



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