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Effects of neonatal diethylstilbestrol exposure on c-*fos* and c-*jun* protooncogene expression in the mouse uterus

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Summary. Quantitative and cell-type-specific expression of c-*fos* and c-*jun* genes after 17ß-estradiol (E2) stimulation, was investigated in the uteri of neonatally diethylstilbestrol (DES)-exposed and ovariectomized adult mice (neoDES-mice), employing Northern blot analysis, immunohistochemistry and *in situ* hybridization. The c-*fos* mRNA level before E2 injection (at baseline) was about 2.2-fold higher in neoDES-mice than in vehicle-treated control mice. In controls, E2 treatment transiently increased c-*fos* mRNA levels, showing a peak value (15.8-fold relative to the baseline) after 2 hours. In neoDES-mice, c-*fos* mRNA level reached a peak showing a 2.1-fold increase compared with its baseline value 1 hour after E2 injection. Immunohistochemistry and *in situ* hybridization revealed that c-*fos* protein (Fos) and mRNA are induced in the epithelium and vascular endothelium in both groups. Most uterine epithelia of neoDES-mice revealed low sensitivity to the c-*fos* expression after E2 administration compared with those of vehicle-treated controls, whereas few epithelia showed high c-*fos* mRNA expression even at baseline. The c-*jun* mRNA concentration in the neoDES-mice uteri at baseline was 70% of that in vehicle-treated controls. At 1 hour after E2 injection, c-*jun* mRNA levels increased 1.8-fold in controls and 1.3-fold in the neoDES-mice relative to each baseline value. There were no significant differences in the distribution pattern of c-*jun* protein (Jun) and mRNA in the uteri of either groups; E2 stimulated c-*jun* mRNA expression in the stromal and myometrial cells but suppressed it in the epithelial cells, whereas intensity of c-*jun* immunostaining increased in the three cell types. The permanent changes in the expression of estrogen-regulated protooncogenes, c-*fos* and c-*jun* genes, by neonatal DES exposure may be responsible for the wide range of abnormalities in the genital tract of mature animals.

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Introduction

It is well known that estrogen administration to prenatal or neonatal animals elicits a wide range of irreversible abnormalities including carcinogenesis in the reproductive organs (Forsberg, 1969; Bern et al., 1975; McLachlan et al., 1975). Takasugi (1963) demonstrated that ovary-independent proliferation and cornification of vaginal epithelium developed in adult mice exposed perinatally to 17ß-estradiol (E2). Newbold et al. (1990) reported that uterine adenocarcinoma in mice exposed neonatally to artificial estrogen, diethylstilbestrol (DES), for 5 days occurred in a time- and dose-related manner, and that these DES-induced uterine tumors depended on postpubertally secreted estrogen.

Estrogen stimulates cell proliferation and differentiation in the female reproductive organs. Hormone-occupied estrogen receptors (ERs) bind to the estrogen responsive elements of target genes and modulate the transcription of these genes (Klein-Hitpass et al., 1986; Kumar and Chambon, 1988; Lannigan and Notidis, 1989). ERs also mediate gene transcription from an AP-1 enhancer element that requires ligand and AP-1 transcription factors, dimer of Fos and Jun family proteins, for the transcriptional activation (Gaub et al., 1990; Umayahara et al., 1994; Paech et al., 1997). These two regulatory pathways are thought to be interactive through a signal transducing pathway which is mediated by cell membrane with growth factors such as epidermal growth factor (EGF) and insulin-like growth factor (van der Burg et al., 1990; Katzenellenbogen, 1996).

The expression pattern of Fos and Jun, major components of AP-1 complexes, might play an important role in epithelial proliferation, not only in the uteri of normal mice but also in neonatally estrogen-exposed mice, and might participate in the development of various abnormalities in the uteri of neonatally DES-

exposed mice (Persico et al., 1990; Kirkland et al., 1992; Webb et al., 1993; Kamiya et al., 1996; Yamashita et al., 1996). Recent studies have demonstrated that both c-*fos* and c-*jun* protooncogene expression was under direct control of estrogen (Loose-Mitchell et al., 1988; Weisz and Bresciani, 1988). In the uteri of ovariectomized rodents, estrogen transiently activated c-*fos* expression in the epithelial cells, and stimulated c-*jun* expression in stromal and myometrial cells but suppressed c-*jun* expression in epithelial cells (Bigsby and Li, 1994; Nephew et al., 1994; Yamashita et al., 1996).

However, a few studies have been carried out to demonstrate the expression of c-*fos* and c-*jun* protooncogenes in the reproductive organs of perinatally estrogenized animals. Kamiya et al. (1996), using Northern blot analysis, reported that expression of c-*fos* and c-*jun* mRNAs was greater (6- and 3-fold) in the uteri of neonatally DES-exposed 50-day-old ovariectomized mice. They also showed that both c-*fos* and c-*jun* mRNA expression were stimulated transiently in the uteri of DES-unexposed control mice after single estrogen injection, whereas c-*fos* and c-*jun* expression were not changed in the uteri of neonatally DES-exposed mice. By employing immunohistochemistry, Falck and Forsberg (1996) showed that Fos was more highly expressed in the uterine epithelium and stromal cells of 8-week-old mice which were treated with DES neonatally and ovariectomized at the age of 6 weeks, than in those cells of controls.

In this study, we tried to verify the quantitative and cell-type-specific expression of c-*fos* and c-*jun* protooncogenes, and to analyze the temporal changes of expression of these genes in the uteri of neonatally DES-treated or untreated 12-week-old mice which were ovariectomized at the age of 10 weeks by employing Northern blot analysis, immunohistochemistry and *in situ* hybridization.

Materials and methods

Animals and tissue collection

Pregnant CD-1 mice were obtained from Clea Japan Inc. (Tokyo, Japan). Mice were housed at 21 to 22 °C with a 12-hour alternating light-dark cycle at Keio University Animal Facility (Tokyo, Japan). Neonatal mice were given subcutaneous injections of 4 μg of DES (Sigma Chemical Co., St. Louis, MO) dissolved in 0.02 ml sesame oil (neoDES-mice) or vehicle alone for 5 days, starting within 24 hours after birth. The female mice were ovariectomized at the age of 10 weeks. At 2 weeks after ovariectomy, the animals received an intraperitoneal injection of 0.1 ml of 17ß-estradiol (E2, 20 μg/kg bw; Sigma) and were killed at 0 (without treatment, baseline), 1, 2, and 3 hours after the injection. For immunohistochemical analysis, small pieces of uterine horn were mounted in OCT compound and frozen in dry-ice-cooled acetone. The tissue blocks for the *in situ* hybridization were frozen rapidly in isopentane quenched with liquid nitrogen and subsequently freeze-substitution fixed in acetone containing formalin and then embedded in paraffin as previously described (Yamashita and Yasuda, 1992; Yamashita et al., 1996). Uteri collected from 6-7 animals were combined for each time point and processed for immunohistochemistry and *in situ* hybridization histochemistry. For Northern blot analysis, uteri from 6-8 mice were pooled, cut into small pieces and directly frozen in the liquid nitrogen and stored until isolation of RNA.

Immunohistochemistry

Immunohistochemistry of ERs, and Fos and Jun has been previously reported (respectively: Yamashita and Korach, 1989; Yamashita et al., 1996). Frozen sections, 6-μm thick, were fixed with Zamboni's fixatives for 30 minutes at room temperature. After incubation with 0.2% glycine in phosphate-buffered saline (PBS), and successively with 10% normal goat serum and 1% bovine serum albumin in PBS, the sections were incubated with anti-Fos rabbit antibody (Oncogene Sciences Inc., MA) or anti-Jun rabbit antibody (Oncogene Sciences), each a 50-fold dilution with the blocking solution, or anti-ER monoclonal rat antibody (Abbott Laboratories, Abbott Park, IL) overnight at 4 °C. They were then treated with horseradish peroxidase-linked $F(ab')_2$ fragments (Amersham Japan, Tokyo, Japan), 100-fold dilution, for 2 hours at room temperature. The peroxidase activity was detected with 3,3'-diaminobenzidine solution containing nickel and cobalt ions (Adams, 1981). For controls, normal rabbit serum or antibodies absorbed with the respective peptides antigens were used as the primary antibodies; S-14-C or T-15-D (Oncogene Sciences) were incubated with anti-Fos or anti-Jun antibodies (Yamashita et al., 1996). Normal rat IgG was used as the primary antibody for the control immunostaining of ER.

In situ hybridization

Cloning of murine cDNA fragments of c-*fos* and c-*jun* has been previously described (Yamashita et al., 1996). The cDNA fragments of c-*fos* (195 bp) and c-*jun* (198 bp) were inserted at the Hinc II site of pSPT18 (Boehringer Mannheim, Mannheim, Germany). Digoxigenin (Dig)-labeled RNA antisense and sense probes were prepared employing a Dig-RNA mixture (Boehringer Mannheim) and an AmpliScribe Transcription Kit (Epicebtre Technol., Badison, WI). Antisense probes of c-*fos* and c-*jun* were synthesized by T7 RNA polymerase using Hind III-digested plasmids as templates, and sense probes were transcribed by SP6 RNA polymerase using Xba I-digested plasmids.

We have previously reported the procedures of the *in situ* hybridization in detail (Yamashita et al., 1996). After deparaffinization, the sections were treated with 0.2 N HCl, and with protease K (Sigma, P-2308), and

finally fixed with a 4% paraformaldehyde solution. The sections were incubated with Dig-labeled antisense or sense RNA probes at 45 °C overnight. After RNase A (Sigma, R-5250) treatment and washing, the sections were incubated with alkaline phosphatase-labeled anti-Dig Fab fragment (Boehringer Mannheim). The alkaline phosphatase enzyme activity was visualized with 5-bromo-4-chloro-3-indoxyl phosphate and nitroblue tetrazolium.

Total RNA isolation

Total RNA was isolated from the uterine horn. The frozen uteri were homogenized in 2.5 ml of ice-cold RNAzol B (Tel-Test, Inc., Friendswood, TX) using a glass-teflon homogenizer. Total RNA was precipitated by addition of isopropanol, washed with 75% ethanol and finally dissolved in 1 mM EDTA. The concentration of total RNA was determined by measuring the optical density at 260 and 280 nm.

Northern blot analysis

Each sample, 5 μg of total RNA in the buffer containing 45% formamide, 6% formaldehyde and 0.25 mg/ml ethydium bromide, was separated on a formaldehyde -1% agarose gel and then transferred to nylon membrane by capillary blotting in 10 x SSC buffer. The blotted RNA was cross-linked under UV irradiation. The membrane was incubated with prehybridization solution mixture and single strand DNA for 4 hours at 42 °C, then hybridized with ^{32}P-labeled DNA probes overnight at 42 °C. After hybridization, the membranes were washed with 1% SDS in 2 x SSC twice for 30 minutes each at 65 °C and with 0.1% SDS in 0.1 x SSC twice for 30 minutes each at 65 °C, and they were then exposed to Bas 2000 imaging plates and scanned using a Bas 2000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).

The c-*fos* and c-*jun* inserts were PCR-amplified and ^{32}P-labeled by random priming using a Random Primer Extension Labeling System (NEN Life Science Products Inc., Boston, MA) and [^{32}P]-CTP (Amersham Japan).

Results

Morphology

In control 12-week-old mice that were ovariectomized at 10 weeks of age, the uterine luminal epithelia were lined with simple low-columnar epithelium, and glandular epithelia with the narrow uterine lumens were lined with cuboidal cells. In 12-week-old neonatally DES-exposed mice which were ovariectomized at 10 weeks of age (neoDES-mice), endometrial epithelium was composed of simple columnar epithelium but pseudostratified columnar epithelium was also present in some regions. No squamous metaplasia was noted and the lumens of uterine glands were enlarged compared with those of vehicle-treated control mice (Fig. 4C-E). Apoptotic epithelial cells and clusters of epithelial cells being sloughed into the uterine lumens were observed more frequently in the neoDES-mice than in controls. Also, the myometrium was thin in the neonatally estrogenized mice. In particular, the inner circular layers were disordered showing sparse cell density (Fig. 5D), and uterine glands sometimes penetrated into the inner muscular layers.

Localization of ERs

ERs were localized in the nuclei of all major uterine cell-types, i.e., epithelial cells, stromal cells and muscular cells in the vehicle-treated control and neoDES-mice (Fig. 1). In the control mice uteri, glandular epithelium showed stronger ER-immunostaining than luminal epithelium. In neonatally

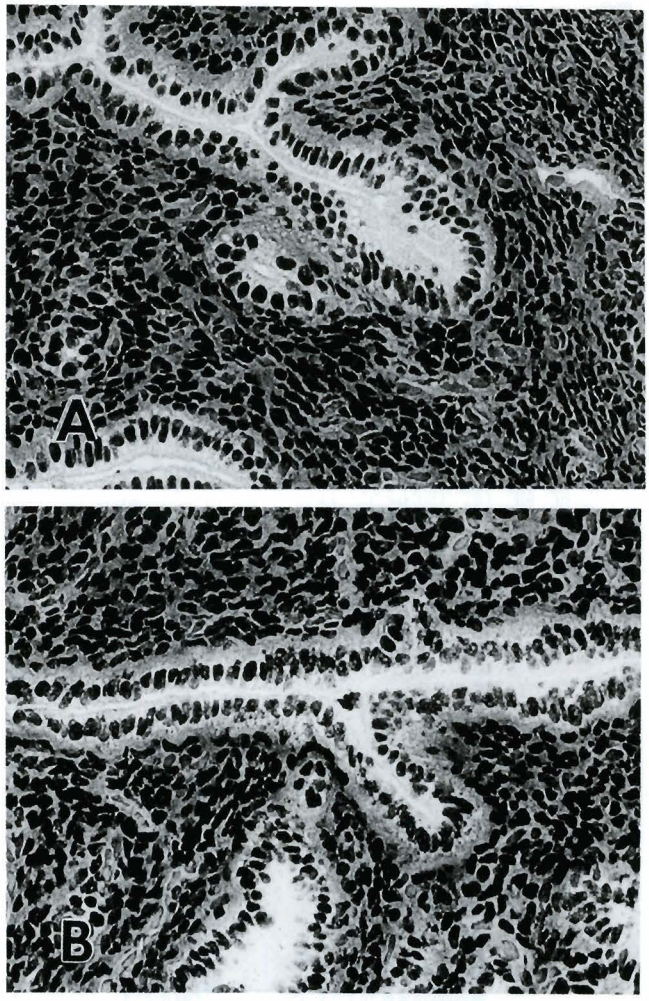
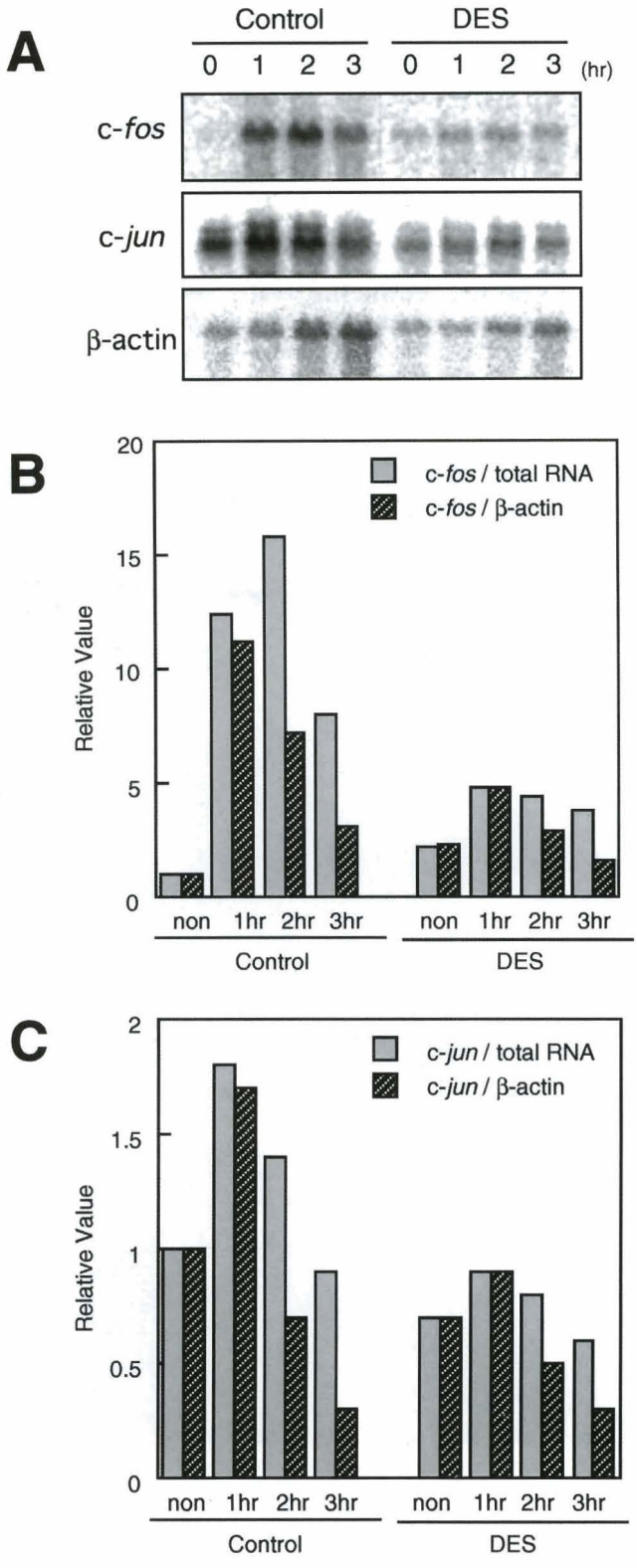


Fig. 1. Immunohistochemistry of estrogen receptors (ERs) in the uteri of mice. ERs are localized in the uteri of 12-week-old control mouse **(A)** and neonatally diethylstilbestrol (DES)-exposed mouse (neoDES-mouse) **(B)**, which were ovariectomized at 10 weeks of age. x 300

estrogenized mice, intensity of ER-immunostaining in the stromal and smooth muscle cells was almost the same as that in the controls, although uterine epithelial cells seemed to show the same or slightly weaker reaction than those of control mice.



Northern blot analysis of protooncogenes

a) c-*fos* mRNA

Total RNA was extracted from the uteri of control and neoDES-mice which were sacrificed either before (0 hour, baseline) or at 1, 2, and 3 hours after E2 administration. All sample preparations showed single 2.2 kb transcript on Northern blot analysis (Fig. 2A). Very low levels of c-*fos* mRNA were present in the uterine tissues of vehicle-treated control mice at baseline. A peak expression of c-*fos* mRNA, 15.8-fold relative to the baseline, was seen at 2 hours after E2 injection, when the signals were analyzed on the basis of total RNA concentration (Fig. 2B). When the amount of c-*fos* transcripts was normalized on the basis of the ß-actin mRNA level in the same blot, the peak of c-*fos* mRNA expression was at 1 hour after E2 injection; 11.2-fold more than the E2-unstimulated animals. In the uteri of neoDES-mice, the c-*fos* mRNA level was 2.2-fold higher than that of vehicle-treated control mice at baseline (Fig. 2B). E2 treatment also stimulated c-*fos* mRNA expression in neonatally estrogenized mice uteri, however, the inductive effect was low compared with controls. At 1 hour after E2 injection, the c-*fos* mRNA levels reached a peak showing a 2.1-fold increase compared with the value at baseline, even though total RNA or ß-actin mRNA concentrations were used for the normalization (Fig. 2B). The c-*fos* mRNA concentration was about 3-fold higher in the vehicle-treated control animals than in the neoDES-mice at 2 and 3 hours after E2 administration.

b) c-*jun* mRNA

All specimens displayed 2.7 kb major bands and 3.2 kb minor bands in Northern blotting (Fig. 2A); presence of these two forms of c-*jun* transcripts in rodents has been reported by many investigators (Persico et al., 1990; Webb et al., 1993; Bigsbi and Li, 1994). Since it appeared that the ratio of the two bands was almost constant, we analyzed 2.7 kb major bands. The c-*jun* transcript levels in the uteri of vehicle-treated controls, rapidly increased showing a peak at 1 hour after E2 treatment and returned to the baseline level at 3 hours; c-

Fig. 2. Time course of c-*fos*, c-*jun* and ß-actin mRNA expression in the uteri of neonatally DES-exposed and unexposed control mice. The 12-week-old mice ovariectomized at the age of 10 weeks, are killed before (baseline) and 1, 2 and 3 hours after E2 injection and c-*fos*, c-*jun* and ß-actin mRNA levels are analyzed by Northern blot analysis of total RNA using ^{32}P-labeled cDNAs, respectively. **A.** The mRNA signals in control mice (lanes 1-4) and neoDES-mice (lanes 5-8). Lanes 1 and 5, at baseline; lanes 2 and 6, 1 hour after E2 injection; lanes 3 and 7, 2 hours after E2 injection; lanes 4 and 8, 3 hours after E2 injection. Quantitative analysis of c-*fos* **(B)** and c-*jun* **(C)** mRNA levels after E2 injection. The mRNA concentrations are normalized on the basis of total RNA levels and ß-actin mRNA levels, respectively.

jun mRNA peak level was 1.8-fold higher compared to the baseline value (Fig. 2C). When c-*jun* mRNA concentration was normalized with ß-actin transcript levels, it was about 65% and 30% of the baseline value at 2 and 3 hours after E2 injection, respectively. The uterine c-*jun* mRNA level in the neoDES-mice was 70% of that in vehicle-treated control mice before E2 injection. E2 injection also elicited an increase in c-*jun* mRNA levels, although the increase was smaller than that of controls. At the 1 hour peak after E2 injection, c-*jun* mRNA concentration was 1.3-fold higher than compared with that at baseline, when it was normalized on the basis of total RNA or ß-actin transcript levels. The concentration then decreased rapidly at 3 hours.

Expression of c-fos protooncogene

a) Immunohistochemistry

In the control and neoDES-mice, single E2 injection induced *c-fos* protein in endometrial epithelium and vascular endothelium, but not in stromal and myometrial cells; however, the induction was weak in the neoDES-mice compared to controls. Before E2 injection, very faint immunoreaction was detectable in the nuclei of uterine epithelial cells in vehicle-trated controls (Fig. 3A), and some epithelial cells showed a weak Fos-immunostaining in the neoDES-mice (Fig. 3C). The nuclear Fos-immunostaining gradually increased and peaked at 2 to 3 hours after E2 administration; Fos-immunoreaction was strong in the luminal epithelium and weak to moderate in the glandular epithelium (Fig. 3B). In the uteri of neoDES mice, Fos-immunostaining in the luminal epithelium was slightly stronger than that in the glandular epithelium after 2 or 3 hours. The Fos-immuoreaction was heterogeneous among the epithelial cells (Fig. 3D), whereas it was almost homogeneous in the endometrial epithelium of control animals. No apparent differences in the Fos-immunostaining were observed between pseudostratified epithelium and simple columnar epithelium in the neoDES-mice uteri. In both neonatally vehicle treated-control and estrogenized-mice, vascular endothelium expressed Fos-immunoreaction in the nucleus at 2 hours after E2

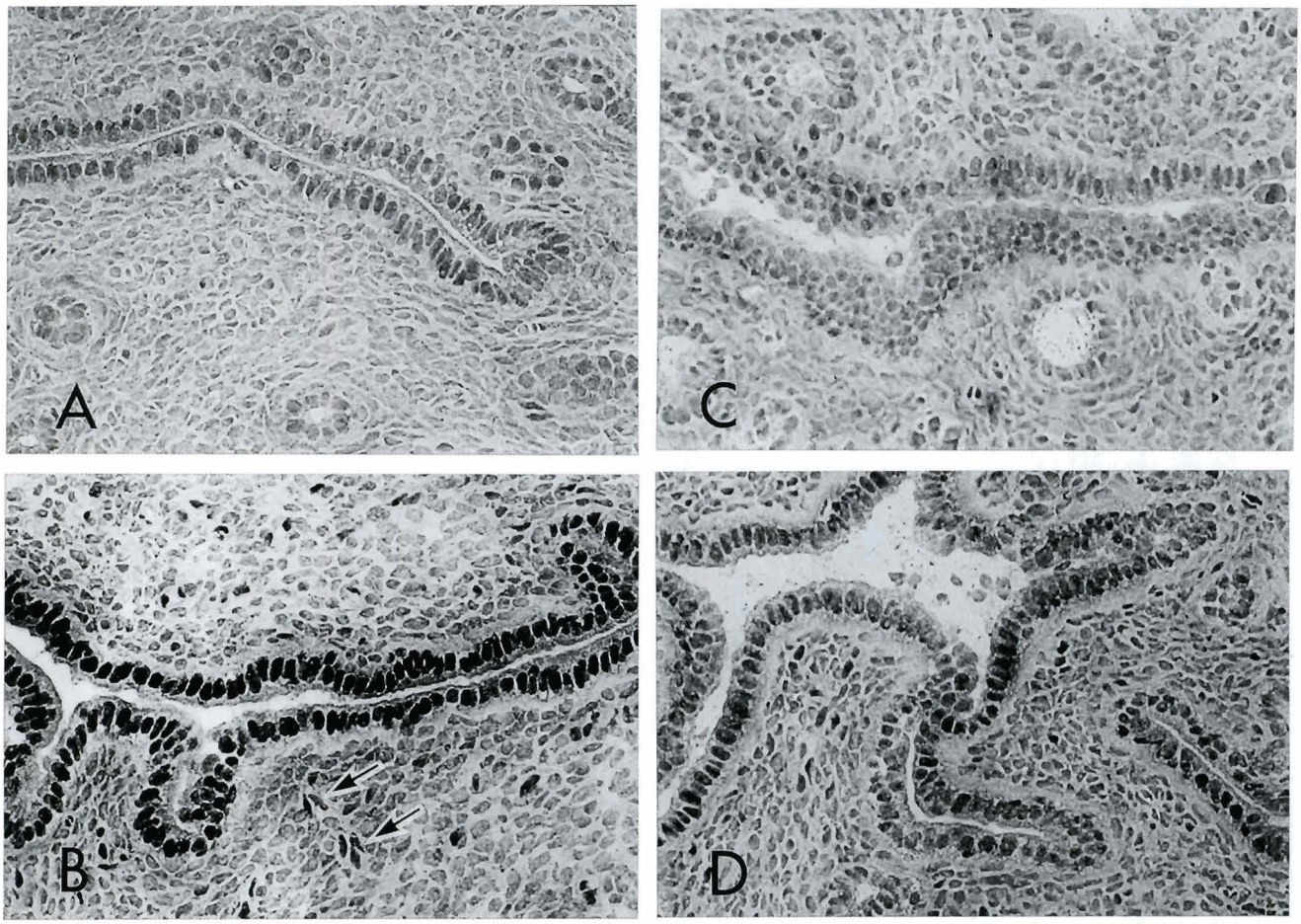


Fig. 3. Immunohistochemistry of Fos in the uterus of mouse. Fos is localized in the uteri of control **(A and B)** and neoDES-mice **(C and D)**. Animals sacrificed before E2 injection **(A and C)** and 2 hours after injection **(B and D)**. Arrows indicate Fos-immunoreaction in vascular endothelia. x 300

injection (Fig. 3B).

b) *In situ* hybridization

E2 treatment induced c-*fos* mRNA rapidly and transiently in the uterine epithelia and vascular endothelia of vehicle-treated control and neoDES-mice (Fig. 3). Expression of c-*fos* transcript was not evident in any of the uterine cell types of control mice before E2 stimulation (Fig. 4A) but it reached a maximum in the epithelial cells at 2 hours (Fig. 4B) and in the endothelial cells at 1 hour after E2 injection, respectively. In the neoDES-mice at baseline, most uterine epithelia showed negative or very faint c-*fos* mRNA signals (Fig. 4C), while some epithelia displayed strong c-*fos* mRNA expression (Fig. 4D). Endometrial epithelium of neoDES-mice showed either the same or slightly stronger c-*fos* mRNA signals at 1 hour after E2 administration and weaker signals at 2 hours than those of vehicle-treated control animals. Glandular epithelium showed a weaker reaction than luminal epithelium in the uteri of both groups. Faint c-*fos* hybridization signals were present in the epithelium of both control and neoDES-mice 3 hours after E2 injection.

Expression of c-jun protooncogene

a) Immunohistochemistry

E2 elicited differential c-*jun* expression responses in all uterine cell types: Jun-immunoreaction was localized in the nuclei of uterine cells (Fig. 5). There were no significant differences in the expression pattern of Jun between control and neoDES-mice. Epithelial cells displayed weak to moderate Jun-immunoreaction at baseline (Fig. 5A,C). The intensity of Jun-immunostaining increased in the epithelial, stromal and myometrial cells from 2 to 3 hours after E2 treatment. Weak Jun-immunostaining was present in the endothelial cells of blood vessels at 2 hours.

b) *In situ* hybridization

Uteri of control and neoDES-mice provided almost the same c-*jun* mRNA expression pattern after E2 administration. Strong c-*jun* mRNA signals were present

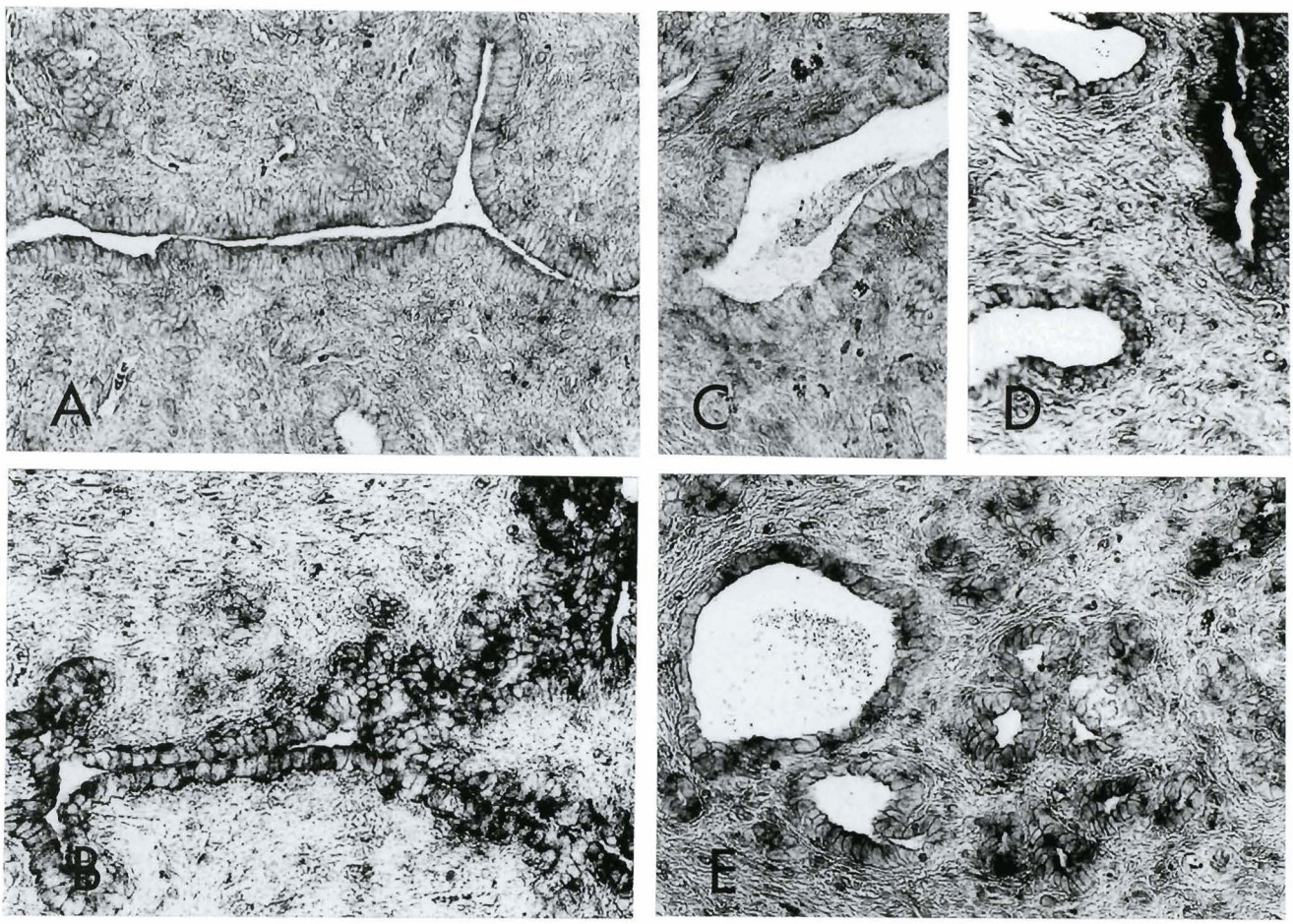


Fig. 4. *In situ* localization of c-*fos* mRNAs in the mice uteri. Uteri of control **(A and B)** and neoDES- mice **(C-E)**. The c-*fos* mRNAs are detected in the uterine sections before E2 injection **(A, C and D)** and at 2 hours after E2 injection (**B and E**), by employing *in situ* hybridization histochemistry with digoxigenin (Dig)-labeled RNA probes. x 250

in the luminal epithelium and a weak reaction was seen in the glandular epithelium in the uteri of vehicle-treated control and neoDES-mice before E2 injection (Fig. 6A,B). The amount of c-*jun* transcripts in the epithelial cells decreased at 1, 2 and 3 hours after E2 treatment. No detectable c-*jun* mRNA was seen in the stromal or muscular cells at baseline and the mRNA was transiently induced and peaked at 1 to 2 hours after E2 injection (Figs. 6C,D). Vascular endothelium displayed *c-jun* mRNA signals at 1 hour.

Discussion

We investigated temporal and quantitative expression of c-*fos* and c-*jun* protooncogenes by E2 stimulation in the uteri of neoDES-mice. In Northern blot analysis, the c-*fos* mRNA concentration reached a peak at 2 hours after E2 injection in the uteri of vehicle-treated control mice, when the mRNA levels were compared with the total RNA concentration of each sample preparation (Fig. 2). However, c-*fos* mRNA expression peaked at 1 hour in neoDES-mice, when normalized on the basis of the ß-actin mRNA levels; a similar result was shown when compared with rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels (data not shown). Results of *in situ* hybridization revealed that the strongest c-*fos* mRNA signals were in the uterine epithelia at 2 hours after E2 administration. This finding agreed with our previous data obtained in adult mice, which were fed without neonatal DES-exposure and were ovariectomized at 12 week of age (Yamashita et al., 1996). Postpubertal estrogen treatment also stimulated the expression of ß-actin and GAPDH mRNAs in the mouse uterus. Therefore, the use of total mRNA levels seems to be reliable for the normalization of not only c-*fos* but also c-*jun* mRNA concentrations in the uteri of E2-stimulated mice by the Northern blot analysis.

The apoptotic epithelial cells and clusters of swollen epithelial cells being sloughed into the uterine lumens were observed more frequently in the uteri of neoDES-mice than in controls. Mori (1975) and Iguchi et al. (1985) reported that steady-state mitotic rates are higher in the uterine epithelia of ovariectomized adult mice that were exposed to DES neonatally than in those of vehicle-treated controls. These results indicate that the uterine epithelia of neonatally-estrogenized mice turn

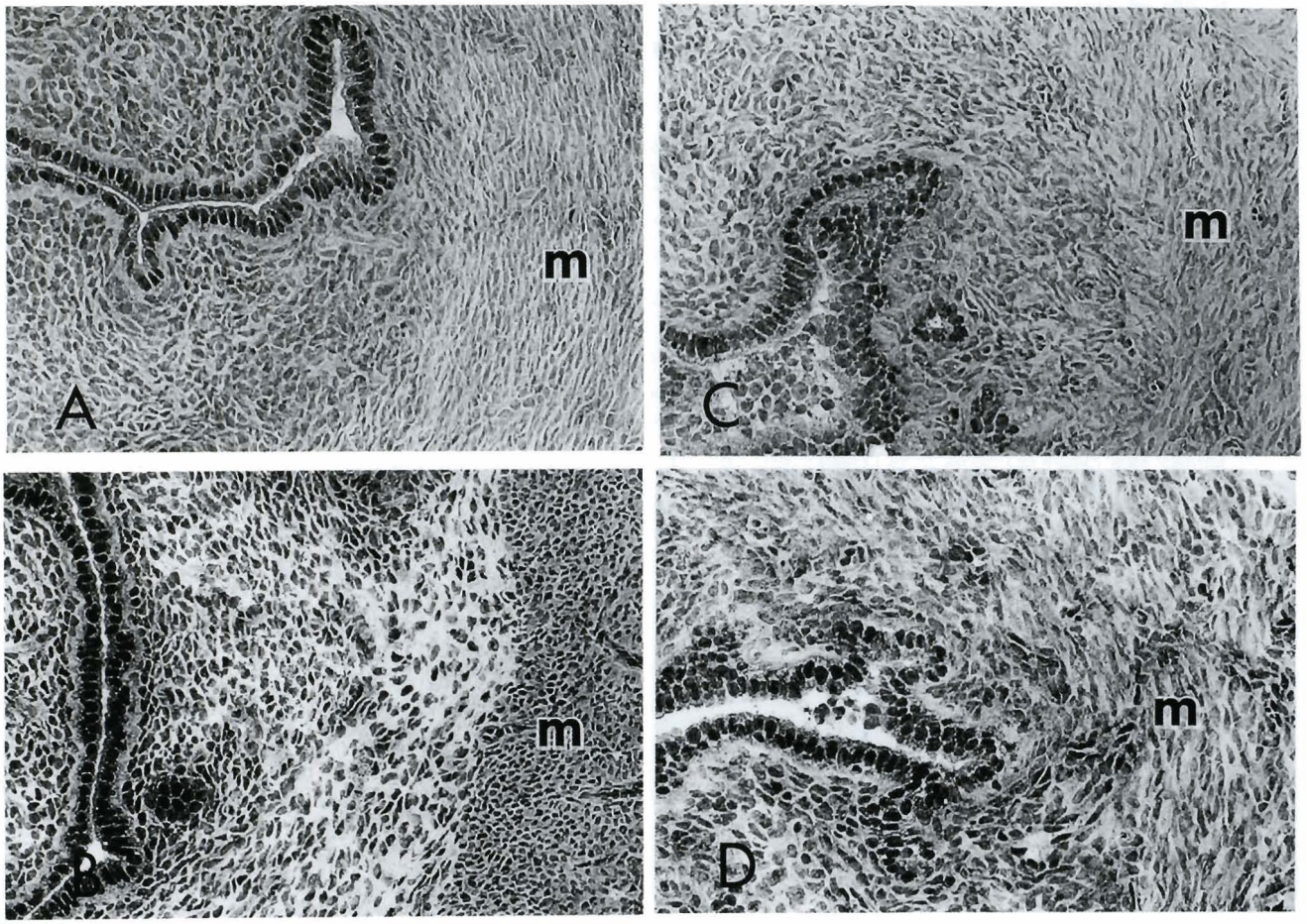


Fig. 5. Immunohistochemistry of Jun in control **(A and B)** and neoDES-mice **(C and D)**. Localization of Jun is demonstrated in the uterine sections before **(A and C)** and 2 hours after E2 injection **(B and D)**. m: myometrium. x 300.

over more rapidly compared with control animals even in the absence of estrogen stimulation. Hendry III et al. (1997) revealed that hyperplastic epithelium is also an active site of cell death by apoptosis in the uteri of neonatally DES-exposed and postpubertally estrogen-stimulated hamsters. The present data from Northern blot analysis revealed that the baseline level of c-*fos* mRNA was higher and c-*jun* mRNA was lower in the uteri of neoDES-mice than in those of vehicle-treated controls. Therefore, the persistent high c-*fos* and low c-*jun* mRNA levels might be responsible for the high-mitotic rates in the uterine epithelia of neonatally-estrogenized mice.

Kamiya et al. (1996), employing Northern blot analysis, showed that the steady-state concentrations of both c-*fos* and c-*jun* mRNA were higher in the neonatally DES-exposed mice than in the vehicle-treated control animals. The reason for the inconsistency concerning the c-*jun* mRNA expression is unclear, and may result from the differences in the strain and age of mice. In the present study, nuclear Jun-immunostaining increased in the uterine epithelium of both control and neoDES-mice after E2 injection but c-*jun* mRNA levels decreased in these cells. The reason for the difference in the results by immunohistochemistry and *in situ* hybridization may be due to that accessibility of antibody to Jun in the nuclei became easy because of a change of nuclear structure after E2 injection, or that translation of c-*jun* mRNA may be activated in the E2-stimulated epithelial cells.

The c-*fos* gene expression appeared heterogeneous in the uterine epithelium of neoDES-mice. The c-*fos* mRNA and protein were synthesized more rapidly in some populations of the uterine epithelia in the neoDES-mice than in the vehicle-treated controls. Some epithelium showed strong and others exhibited faint immunostaining and *in situ* hybridization signals at 2 hours after E2 injection, while the epithelium of control animals showed almost homogeneous c-*fos* expression (Figs. 3, 4). In addition, few epithelial cells displayed high c-*fos* mRNA expression in the uteri of neonatally-estrogenized mice without E2 injection (Fig. 4D). These results suggest that the uterine epithelia of neonatally DES-treated mice are composed of a heterogeneous

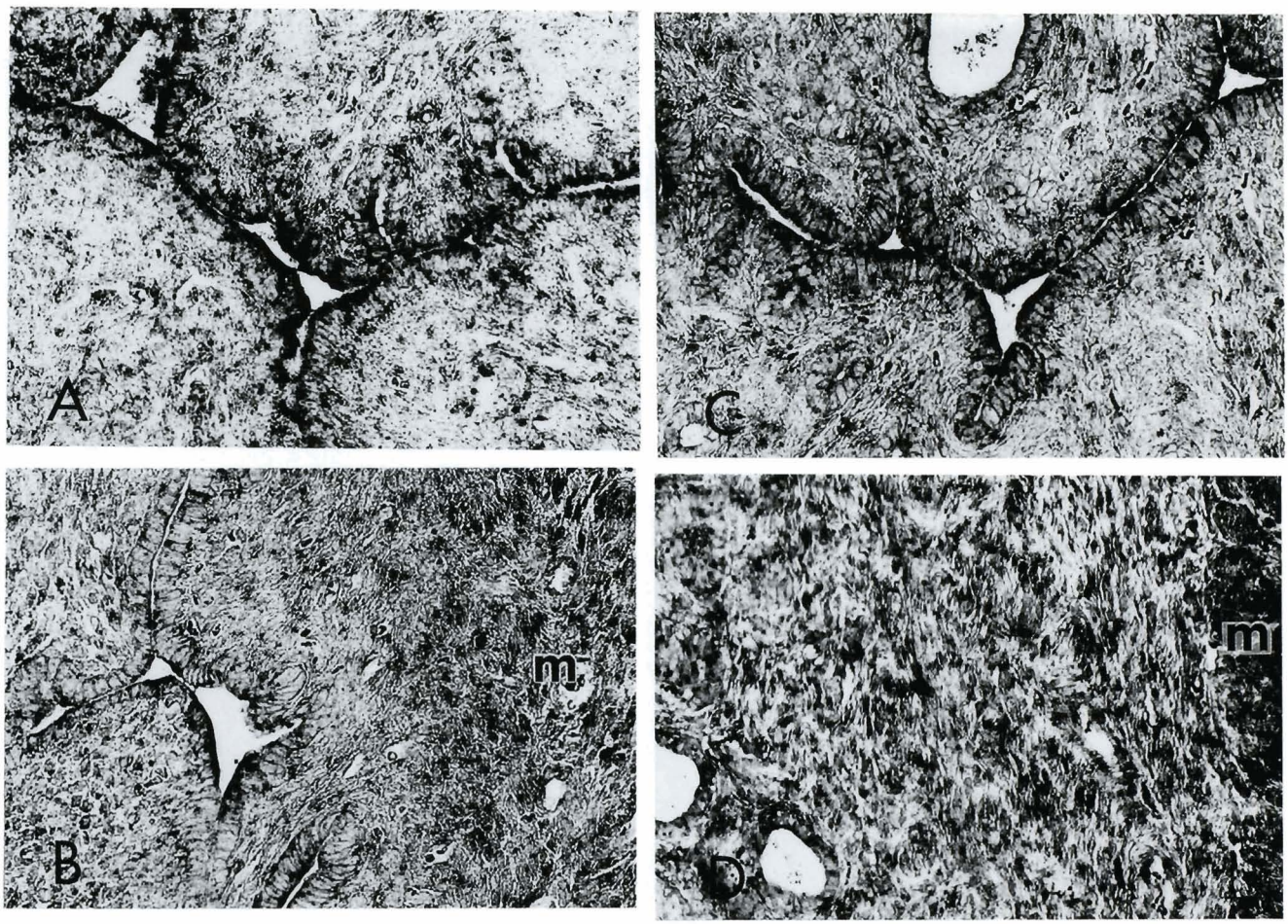


Fig. 6. *In situ* localization of c-*jun* mRNAs in the mice uteri in control **(A and B)** and neoDES-mice **(C and D)**. Expression of c-*jun* mRNAs is seen in the uteri before **(A and C)** and 2 hours after E2 injection **(B and D)**, by employing *in situ* hybridization with Dig-labeled RNA probes. m: myometrium. x 250

population in relation to their responses to postpubertal estrogen stimulation. Mori (1975) pointed out that uterine epithelia of ovariectomized neonatally DES-exposed mice consisted of a mixed population of cells; total mitotic rate of uterine epithelia in the neonatally DES-exposed mice was about 30% of that for control mice after a single injection of E2, but the epithelia rapidly entered the mitotic stage.

Nelson et al. (1994) demonstrated that neonatal DES exposure results in the persistent ovary-independent activation of estrogen-regulated genes, such as lactoferrin and EGF genes in the mouse uterus and vagina. Iguchi et al. (1993) showed that EGF receptor levels in neonatally DES-treated mice were almost the same in the uterus and oviduct but significantly lower in the vagina compared with those of control animals. The present study also revealed that the baseline levels of estrogen-regulated protooncogenes alter in the uteri of neoDES-mice. One of the mechanisms of alteration of estrogen-regulated gene expression may be based on the demethylation or methylation of DNA in the genital tract of neonatally-estrogenized mice. Li et al. (1997) reported that neonatal DES-treatment imprinted an abnormal, site-specific demethylation of immediately upstream from the estrogen responsive element of lactoferrin promoter, and that the demethylation was maintained in uterine tumors developed from neonatally DES-treated mice. In addition, persistent EGF expression in the uterus of neonatally DES-exposed mice may lead to the ovary-independent expression of c-*fos* transcripts, because the c-*fos* mRNA expression is shown to be stimulated through the EGF signaling pathway system independently of ER systems in the mouse uterus (Curtis et al., 1996).

The low sensitivity of c-*fos* expression to E2 administration in the uterine epithelia of neoDES-mice may not be simply proportional to the concentration of ERs, because there were no significant differences in ER expression pattern between neoDES-mice and controls (Fig. 1). Sato et al. (1992) showed almost the same ER-immunostaining pattern in the uteri of neonatally vehicle-treated control and DES-exposed mice.

Neonatal DES exposure elicited alterations in expression patterns of estrogen-regulated nuclear protooncogens, c-*fos* and c-*jun*, in the mice uteri; the c-*fos* mRNA expression at baseline was higher and that of *c-jun* was lower in the uteri of neoDES-mice compared with vehicle-treated control animals. Almost the same results were observed in the uteri of 3-week-old neoDES-mice (Yamashita S, Takayanagi A, Shimizu N, unpublished data). In the male reproductive tissues, Salo et al. (1997) demonstrated that neonatal DES treatment results in a increase of c-*fos* mRNA expression in the epithelial cells of prostatic urethra and all prostatic lobes compared with non-treated control mice at of 3-5 months of age. *In situ* hybridization and immunohistochemistry displayed that c-*fos* is expressed primarily in the epithelium of posterior periurethral prostatic collecting ducts, which are sites that show the most pronounced morphological changes in neoDES-mice. Long-term expression of Fos was shown to cause loss of epithelial polarity irreversibly and trigger epithelial-fibroblastoid cell conversion (Reichmann et al., 1992). A function of Jun in the epithelium appears to modulate epithelial polarity and tissue organization; expression of Jun elicits a loss of mammary epithelial polarity involving a destabilization of adherens junctions (Fialka et al., 1996). The persistent changes in the expression of estrogen-regulated genes such as c-*fos*, c-*jun* and EGF which regulate the cell differentiation and proliferation, should be responsible for the wide ranges of abnormalities in the genital tract of perinatally-estrogenized animals.

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References

Adams J.C. (1981). Heavy metal intensification of DAB-based HRP reaction product. J. Histochem. Cytochem. 29, 775.

Bern H.A., Jones L.A., Mori T. and Young P.N. (1975). Exposure of neonatal mice to steroids: Longterm effects on the mammary gland and other reproductive structures. J. Steroid Biochem. 6, 673-676.

Bigsby R.M. and Li A. (1994). Differentially regulated immediate early genes in the rat uterus. Endocrinology 134, 1820-1826.

Curtis S.W., Washburn T., Sewall C., DiAugustine R., Lindzey J., Couse J.F. and Korach K.S. (1996). Physiological coupling of growth factor and steroid receptor signaling pathways: estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor. Proc. Natl. Acad. Sci. USA 93, 12626-12630.

Falck L. and Forsberg J.G. (1996). Immunohistochemical studies on the expression and estrogen dependency of EGF and its receptor and *c-fos* proto-oncogene in the uterus and vagina of normal and neonatally estrogen-treated mice. Anat. Rec. 245, 459-471.

Fialka I., Schwarz H., Reichmann E., Oft M., Busslinger M. and Beug H. (1996). The estrogen-dependent c-Jun ER protein causes a reversible loss of mammary epithelial cell polarity involving a destabilization of adherens junctions. J. Cell Biol. 132, 1115-1132.

Forsberg J.G. (1969). The development of atypical epithelium in the mouse uterine cervix and vaginal fornix after neonatal oestradiol treatment. Br. J. Exp. Pathol. 50, 187-195.

Gaub M.P., Bellard M., Scheuer I., Chambon P. and Sassone-Gorsi P. (1990). Activation of ovalbumin gene by estrogen receptor involves the Fos-Jun complex. Cell 63, 1267-1276.

Hendry III W.J., Zheng X., Leavitt W.W., Branham W.S. and Sheehan D.M. (1997). Endometrial hyperplasia and apoptosis following neonatal diethylstilbestrol exposure and subsequent estrogen stimulation in both host and transplanted hamster uteri. Cancer Res. 57, 1903-1908.

Iguchi T., Ostrander P.L., Mills K.T. and Bern H.A. (1985). Induction of abnormal epithelial changes by estrogen in neonatal mouse vaginal transplants. Cancer Res. 45, 5688-5693.

Iguchi T., Edery M., Tsai P.-S., Ozawa S., Sato T. and Bern H.A. (1993). Epidermal growth factor receptor levels in reproductive organs of

female mice exposed neonatally to diethylstilbestrol. Proc. Soc. Exp. Biol. Med. 204, 110-116.

Kamiya K., Sato T., Nishimura N., Goto Y., Kano K. and Iguchi T. (1996). Expression of estrogen receptor and proto-oncogene messenger ribonucleic acids in reproductive tracts of neonatally diethylstilbestrol-exposed female mice with or without postpuberal estrogen administration. Exp. Clin. Endocrinol. Diabetes 104, 112-122.

Katzenellenbogen B.S. (1996). Estrogen receptors: bioactivities and interactions with cell signalling pathways. Biol. Reprod. 54, 287-293.

Kirkland J.L., Murthy L. and Stancel G.M. (1992). Progesterone inhibits the estrogen-induced expression of c-*fos* messenger ribonucleic acid in the uterus. Endocrinology 130, 3223-3230.

Klein-Hitpass L., Schorpp M., Wagner V. and Ryffel G.U. (1986). An estrogen-response element derived from the 5' flanking region of the Xenopus vitellogenin A2 gene functions in transfected human cells. Cell 46, 1053-1061.

Kumar V. and Chambon P. (1988). The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell 55, 145-156.

Lannigan D.A. and Notides S. (1989). Estrogen selectively binds the "coding strand" of an estrogen responsive element. Proc. Natl. Acad. Sci. USA 86, 863-867.

Li S., Washburn K.A., Moore R., Uno T., Teng C., Newbold R.R., McLachlan J.A. and Negishi M. (1997). Developmental exposure to diethylstilbestrol elicits demethylation of estrogen-responsive lactoferrin gene in mouse uterus. Cancer Res. 57, 4356-4359.

Loose-Mitchell D.S., Chiappetta C. and Stancel G.M. (1988). Estrogen regulation of *c-fos* messenger ribonucleic acid. Mol. Endocrinol. 2, 946-951.

McLachlan J.A., Newbold R.R. and Bullock B. (1975). Reproductive tract lesions in male mice exposed prenatally to diethylstilbestrol. Science 190, 991-992.

Mori T. (1975). Effects of postpuber oestrogen injections on mitotic activity of vaginal and uterine epithelial cells in mice treated neonatally with oestrogen. J. Endocrinol. 64, 133-140.

Nelson K.G., Sakai Y., Eitzman B., Steed T. and McLachlan J.A. (1994). Exposure to diethylstilbestrol during a critical developmental period of the mouse reproductive tract leads to persistent induction of two estrogen- regulated genes. Cell Growth Differ. 5, 595-606.

Nephew K.P., Tang M. and Khan S.A. (1994). Estrogen differentially affects c-*jun* expression in uterine tissue components. Endocrinology 134, 1827-1834.

Newbold R.R., Bullock B.C. and McLachlan J.A. (1990). Uterine adenocarcinoma in mice following developmental treatment with estrogens: a model for hormonal carcinogenesis. Cancer Res. 50, 7677-7681.

Paech K., Webb P., Kuiper G.G., Nilsson S., Gustafsson J., Kushner P.J. and Scanlan T.S. (1997). Differential ligand activation of estrogen receptors ERα and ERß at AP1 sites. Science 277, 1508-1510.

Persico E., Scalona M., Cicatiello L., Sica V., Bresciani F. and Weisz A. (1990). Activation of 'immediate-early genes' by estrogen is not sufficient to achieve stimulation of DNA synthesis in rat uterus. Biochem. Biophys. Res. Commun. 171, 287-292.

Reichmann E., Schwarz H., Deiner E.M., Leitner I., Eilers M., Berger J., Bausslinger M. and Beug H. (1992). Activation of an inducible c-Fos ER fusion protein causes loss of epithelial polarity and triggers epithelial-fibroblastoid cell conversion. Cell 71, 1103-1116.

Salo L.K., Makela S.I., Stancel G.M. and Santti R.S. (1997) Neonatal exposure to diethylstilbestrol permanently alters the basal and 17 ß-estradiol induced expression of c-*fos* proto-oncogene in mouse urethroprostatic complex. Mol. Cell. Endocrinol. 126, 133-141.

Sato T., Okamura H., Ohta Y., Hayashi S., Takamatsu Y., Takasugi N. and Iguchi T. (1992). Estrogen receptor expression in the genital tract of female mice treated neonatally with diethylstilbestrol. In Vivo 6, 151-156.

Takasugi N. (1963). Vaginal cornification in persistent-estrous mice. Endocrinology 72, 607-619.

Umayahara Y., Kawamori R., Watada H., Imano E., Iwama N., Morishima T., Yamasaki Y., Kajimoto Y. and Kamada T. (1994). Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. J. Biol. Chem. 269, 16433-16442

van der Burg B., de Groot R. P., Isbrucker L., Kruijer W. and de Laat S.W. (1990). Stimulation of TPA-responsive element activity by a cooperative action of insulin and estrogen in human breast cancer cells. Mol. Endocrinol. 4, 1720-1726.

Webb D.K., Moulton B.C. and Khan S.A. (1993). Estrogen induces expression of c-*jun* and *jun*-B protooncogenes in specific rat uterine cells. Endocrinology 133, 20-28.

Weisz A. and Bresciani F. (1988). Estrogen induces expression of c-*fos* and c-*myc* protooncogenes in rat uterus. Mol. Endocrinol. 2, 816-824.

Yamashita S. and Korach K.S. (1989). A modified immunohistochemical procedure for the detection of estrogen receptor in mouse tissues. Histochemistry 90, 325-330.

Yamashita S. and Yasuda K. (1992). Freeze-substitution fixation for immunohistochemistry at the light microscopic level: effect of solvent and chemical fixatives. Acta Histochem. Cytochem. 25, 641-650.

Yamashita S., Takayanagi A. and Shimizu N. (1996). Temporal and cell-type specific expression of c-*fos* and c-*jun* protooncogenes in the mouse uterus after estrogen stimulation. Endocrinology 137, 5468-5475.

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