Table 3
c-*myc* target genes induced by high-dose DES treatment in EMTOKA cells

GB#	Symbol	Function	Regulation	ER	Organism	Reference
J03075	PKCSH	Signal transduction	–	–	H	www.[a]
X04106	CAPNS1	Apoptosis	up	–	H	Xynos et al. [24]
X17620	NME1	Transcription Factor	up	–	H, R, C	www.[a]
L16785	NME2	Metabolism	up	–	R	www.[a]
AF040105	C6orf108	Cell growth	up	–	H, R	Lewis et al. [22]
M26708	PTMA	DNA repair	up	P	H, M, R	www.[a]
X54942	CKS2	Cell cycle	up	P	H, R	www.[a]
U07418	MLH1	DNA repair	*	–	Y	Partlin et al. [25]
U51004	HINT	Signal transduction	–	–	H	www.[a]
D15057	DAD1	Anti-apoptosis	up	–	H	Xynos et al. [24]
M81757	RPS19	Protein synthesis	up	–	H, R	www.[a]
M97935	STAT1	Transcription factor	up	–	H, M	Ramana et al. [23]
X16707	FOSL1	Transcription factor	up	–	H	www.[a]

All the data, c-*myc* target genes in Table 3 were picked up from Table 2. GB#, GeneBank accession number; –, unknown; *, interaction with c-*myc*; P, chimeric myc-estrogen receptor system was employed; H, human; R, rat; M, mouse; C, chicken; Y, yeast.
[a] www., Myc Cancer Gene (http://www.myccancergene.org/index.asp).

female reproductive system including uterus, cervix, and vaginal development [27,28], but there have been no reports regarding the roles of estrogen including ERs expression in EMTOKA cells. ERβ was expressed in EMTOKA cells and the cell proliferation in response to both E2 and DES, but ER inhibitor, ICI, could not reverse or prevent these reactions. HD-DES could stimulate cell proliferation of EMTOKA cells, but LD-DES and HD-E2 could not necessarily increase the cell numbers. In human breast cancer, MCF-7 cell proliferation assay (E-screen assay) [29], DES exhibited a broad peak ranging from 10^{-9} to 10^{-6} M (low-dose to high-dose), and E2 exhibited a peak in 3×10^{-11} M. The status of cell proliferation was also evaluated at the treatment of the same concentrations described above for both E2 and DES except for LD-DES in EMTOKA cells. These E2 and DES induced cell proliferations could not be reversed or prevented by ICI, which also indicated that this cell proliferation may not be exerted through ERs or may be though ER independent pathway. In vivo study using fetal female rat, DES increased Müllerian duct cell proliferation in the proximal epithelium and mesenchyme, but decreased cell proliferation in caudal epithelium, and DES was postulated to exert these effects through both ERβ1 and ERβ2 [30]. Altered expression of ERα, but not of ERβ was also reported in adult male and female reproductive tracts after neonatal DES exposure in reproductive tracts of ERα and/or β knockout mouse, although developments were normal during the prenatal and neonatal stages [30]. Couse et al. [31] further reported that the absence of DES-induced abnormalities in reproductive tract of ERα knockout mouse was consistent with a predominant role of ERα in mediating the detrimental effects of DES in female reproductive tract development. Expression of ERα and β in normal human embryo and/or fetal tissues were largely unknown, but Takeyama et al. [32] reported that ERβ was predominantly expressed in the genital tract of human fetal female tissues. In the human embryo/fetus, ERβ is considered to play some roles in the maintenance of development, but HD-DES may stimulate EMTOKA cell proliferation through ERβ independent pathway. It awaits further investigations for clarification.

Estrogen induced cell proliferation in quiescent cells is generally associated with increased expression of immediate early estrogen response genes such as proto-oncogenes such as c-*jun*, c-*fos*, and c-*myc* [26]. Zheng and Hendry [26] demonstrated that immunoreactivities of c-*jun*, c-*fos*, and c-*myc* were all increased in DES-treated ovariectomised hamster uterus. In our microarray analysis, both c-*jun* and c-*fos* were not up regulated by both E2 and LD-DES treatments. However, in HD-DES-treated EMTOKA cells, expression of c-*myc* and its response genes was markedly increased, while low levels of c-*jun* and

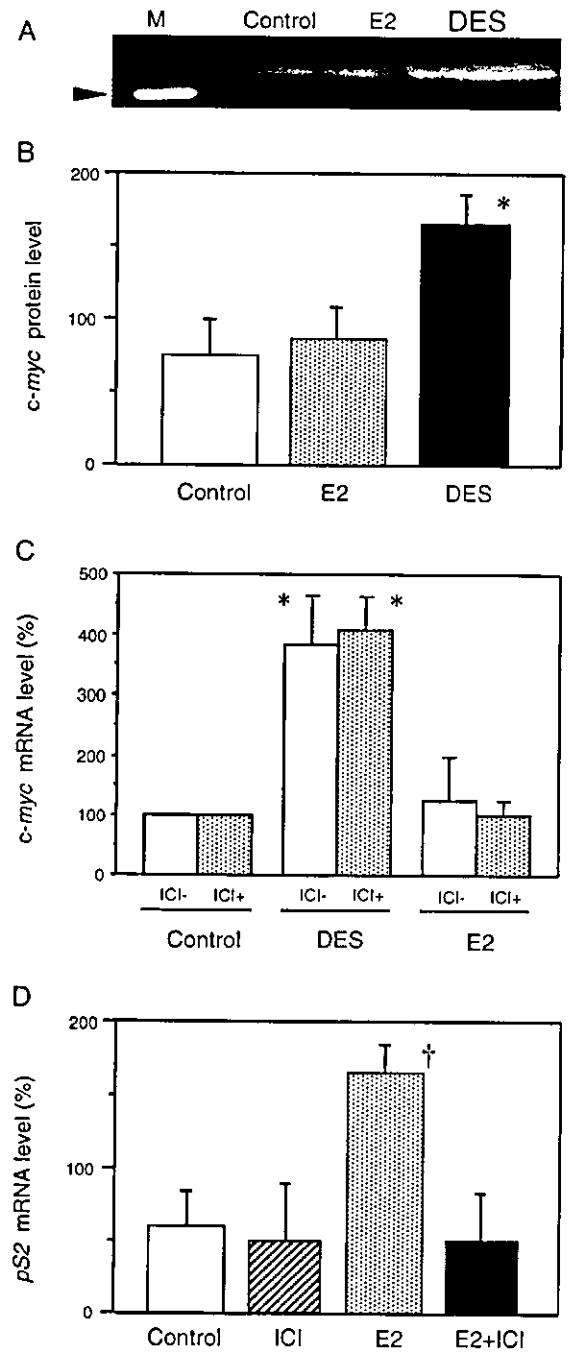

Fig. 5. c-*myc* protein and mRNA expressions in 1 μM DES or 10 pM E2 exposed EMTOKA cells for 48 h. (A) Weak immunoreactivity of c-*myc* was detected in the vehicle (0.05% ethanol) control and E2 exposed cells. Marked immunoreactivity of c-*myc* was detected in DES exposed cells (arrow head, 60 kDa). (B) c-*myc* protein expression was low in the intact and E2 exposed cells, whereas the significantly high levels of c-*myc* protein were detected in DES exposed cells. c-*myc* protein level was demonstrated as relative OD (arbitrary unit). (C) The effects of estrogen receptor antagonist ICI on c-*myc* transcripts in DES or E2 treated EMTOKA cells. DES and E2 were treated with (ICI+) or without (ICI−) ICI. There were no changes of ICI pre-treatment in both vehicle (0.05% ethanol) control (Control) and E2. ICI could not diminish the effects of DES on induction of c-*myc* mRNA transcript. (D) ICI decreased c-*myc* mRNA transcript in MCF-7 induced by E2 (10 pM) treatments. *, $P<0.05$ vs. vehicle (0.05% ethanol) control (Control) or E2. †, $P<0.05$ vs. Control, ICI, and E2+ICI.

c-*fos* expressions were detected in these systems. c-*myc* has ER response element, and has been demonstrated to be induced by E2 and DES treatment via ER [26] (Table 4). In this study, c-*myc* mRNA transcript and protein were demonstrated to be increased by HD-DES, but not by LD-E2 and LD-DES. Furthermore, ICI compound failed to decrease the effects of HD-DES on induction of both c-*myc* mRNA transcript and protein. DES was strongly correlated with the abnormalities and/or carcinogenesis in embryo to fetal reproductive development by induction of c-*myc* oncogene through ER independent pathway. Overexpression of the c-*myc* gene in cervical squamous cell carcinoma has been demonstrated to be associated with significant increment of early disease relapse following surgery [33]. c-*myc* is therefore postulated to play important roles in carcinogenesis induced by DES in human reproductive system. Expression of the c-*myc* gene is in general closely related to the G1-S transition by activating cyclin–cyclin dependent kinases (CDK) complexes [34]. Results of our present study also demonstrated up-regulation of CDKs including CDK 10 and 5 consistent with those report above. c-*myc* interacts with the MutL homologue MLH1 that is related to DNA repair [25]. Furthermore, markedly up-regulated genes also included *rcl* in our study, a c-*myc* responsive growth related gene, cell cycle regulators such as G1/S specific cyclin D1, and apoptosis regulators including calpain and defender against cell death (DAD1) [24]. The expression of c-*myc* is regulated by the c-*myc* transcriptional factor PuF [24]. These findings all suggest that various genes up- or down regulated by c-*myc* may lead to cell turnover and differentiation in human primitive genital tract. At one level, both c-*myc* and N-*myc* are considered essential for vertebrate development [35]. In normal

Table 4
Genes induced by E2 and DES treatment in EMTOKA cells

No.	GB #	Gene	Symbol	Ratio		
				L-E	L-D	H-D
1	V00568	v-Myc myelocytomatosis viral oncogene homolog (avian)	MYC	−1.4	–	2.2
2	J04111	v-Jun sarcoma virus 17 oncogene homolog (avian)	JUN	–	−1.4	1.4
3	K00650	v-Fos FBJ murine osteosarcoma viral oncogene homolog	FOS	–	–	1.5
4	M26679	Homeo box A5	HOXA5	–	–	−2.7
5	M92299	Homeo box B5	HOXB5	–	–	−1.5

GB#, GeneBank accession number; L-E, low-dose (10 pM) estradiol; L-D, low-dose (10 pM) diethylstilbestrol; H-D, high-dose (10 μM) diethylstilbestrol; Ratio, compared with control gene expressions.

Müllerian duct cells, the expression of c-*myc* result in inhibition of programmed cell death in normal development and/or apoptosis in the cells associated with DNA damages. In addition, accumulation of these effects may result in permanental genomic imprinting. Uncontrolled expression of imprinting genes can be associated with hereditary and sporadic cancer development [36]. These findings all suggest that disorganized imprinting may affect embryogenesis and may result in several disease symptoms including DES-adenosis. In addition to DES-daughter, mother treated with DES result in increased risks for breast cancer [37]. Li et al. [38] demonstrated that neonatal, but not mature mice treated with DES were associated with demethylation of estrogen-responsive gene (lactoferrin), which is consistent with permanental genomic imprinting. However, it is also true that c-*myc* is associated with a wide variety of functions [34], and it awaits further investigations for clarifying about DES induced inherited abnormality and/or directly carcinogenesis of reproductive system.

The importance of homeobox (HOX) genes in the regulation of mammalian development has been well established, and differential expression of HOX genes occurs in several cancers [39]. In human uterine or cervical cells, HOX genes including HOXA9 and HOXB5 genes were expressed in normal cervical tissues [40]. In addition, DES induced HOXA9 and HOXA10 genes expression to levels approximately 2-fold compared to estradiol [41]. In our present study, DES and E2 treatment did not affect levels of HOXA9 and HOXA10 genes expression, but did down regulate HOXA5 and HOXB5 genes. HOXA5 gene has been demonstrated to activate transcription of the key tumor suppressor gene p53, and down regulation of HOXA5 gene leads may result in loss of p53 gene expression [42]. Reduction of HOXA5 as a result of HD-DES treatment may be also subsequently result in decreased p53. Further investigations were required for clarifications.

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Estrogen-independent activation of erbBs signaling and estrogen receptor α in the mouse vagina exposed neonatally to diethylstilbestrol

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Growth factors and estrogen receptor (ER) signaling cooperate to play essential roles in cell proliferation, differentiation and tumor progression in mouse reproductive organs. Treatment of neonatal mice with diethylstilbestrol (DES) induces an estrogen-independent persistent proliferation and cornification of the vaginal epithelium, which results in cancerous lesions later in life. However, the mechanisms of the estrogen-dependent and -independent pathways essentially remain unknown. We characterized the expression of epidermal growth factor (EGF)-like growth factors (EGF, transforming growth factor α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), amphiregulin (APR), epiregulin (EPR) and neuregulin (NRG) 1) and erbB receptors (EGF receptor (EGFR), erbB2/neu, erbB3 and erbB4) in the vaginae of mice treated either neonatally (0–4 day) or as adults (55–59 day) with estrogens. EGFR and erbB2 were activated in the vaginal epithelium of mice by estrogen treatment. This activation was also encountered in vaginae from neonatally DES-exposed mice, along with the expression of EGF, TGF-α, HB-EGF, BTC, APR, EPR and NRG1. Immunohistochemical analysis indicated that erbB2 was primarily expressed in vaginal epithelium. Finally, we found that serine 118 and 167 located in the AF-1 domain of ERα were phosphorylated in these vaginae. AG825, AG1478 or ICI 182,780 administration blocked proliferation of vaginal epithelium induced by neonatal DES exposure. Thus, signal transduction via EGFR and erbB2 could be related to the estrogen-induced vaginal changes and persistent erbBs phosphorylation and sustained expression of EGF-like growth factors, leading to ERα activation that may result in cancerous lesions in vaginae from neonatally DES-exposed mice later in life.
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Keywords: mouse; diethylstilbestrol; vagina; erbBs signaling; estrogen receptor

Introduction

The proliferation and differentiation of mouse vaginal epithelial cells are strongly regulated by ovarian estrogens, such as 17β-estradiol (E_2). The vaginae of ovariectomized (OVX) mice show an atrophied epithelium of 2–3 cell layers, but estrogen administration rapidly induces epithelial cell proliferation, stratification and cornification. In rodents, crosstalk between growth factor signaling and estrogen receptor α (ERα) contribute to estrogen action in uterus and vagina. Epidermal growth factor (EGF) has mitogenic effects on the mouse uterus similar to estrogens (Ignar-Trowbridge *et al.*, 1992) in that administration of EGF or transforming growth factor α (TGF-α) to OVX adult mice induces cell proliferation and differentiation in the female reproductive tracts (Nelson *et al.*, 1991, 1992). However, in ERα-deficient mice, both estrogen and EGF stimulation of uterine growth was disrupted (Curtis *et al.*, 1996). EGF-like growth factors are composed of EGF, TGF-α, heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), amphiregulin (APR), epiregulin (EPR) and neuregulins (NRGs) and interact with the erbB receptor tyrosine kinases: EGF receptor (EGFR)/erbB1, erbB2/neu, erbB3 and erbB4. The members of the erbB receptor family interact as homo- or heterodimers upon ligand binding, which leads to the crossactivation of the receptors. ErbBs have the potential to recruit and activate interacting proteins, thereby initiating signaling cascades that culminate in distinct cellular responses, such as cell proliferation, differentiation and morphogenesis. Overexpression of erbBs is associated with carcinogenesis in the reproductive organs (Dickson and Lippman, 1995) and erbB2 is frequently amplified and overexpressed in cancer cells (Hynes and Stern, 1994).

Epidemiological and laboratory studies have shown that estrogens are important for the normal proliferation that occurs in reproductive organs. Moreover, long-term estrogenic stimulation is a well-known risk factor for carcinogenesis in human reproductive organs (Marselos and Tomatis, 1992a). Beginning in the 1940s, a synthetic estrogen, diethylstilbestrol (DES) was routinely prescribed to pregnant women for the prevention of miscarriages. To date, it is well known that *in utero* exposure to DES induces vaginal clear-cell adenocarci-

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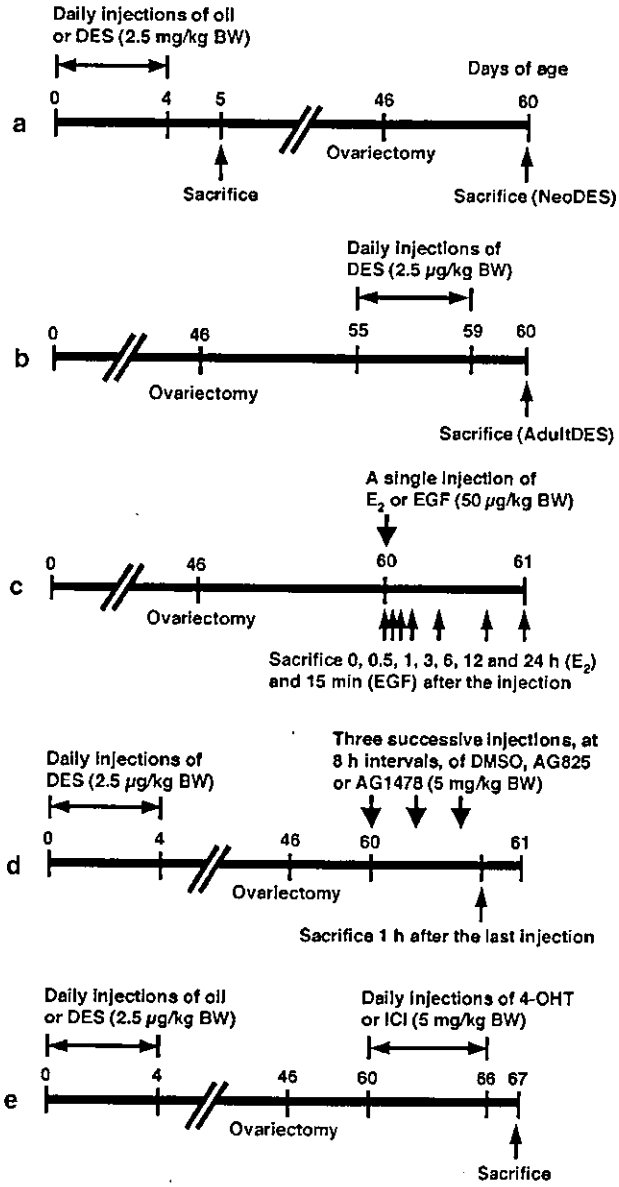

Figure 1 Treatment timelines. (a) Female newborn mice were given five daily injections of 2.5 mg DES/kg BW or the vehicle alone from day 0 (the day of birth). They were OVX on day 46 and killed on day 60. (b) To examine the effects of DES on adult mouse vagina, intact mice were OVX on day 46 and given five daily injections of 2.5 μg DES/kg BW from day 55, then killed 24 h after the last injection. (c) Effects of E_2 or EGF on phosphorylation of erbB2, EGFR and ERα were examined in OVX adult mice 0.5, 1, 3, 6, 12 and 24 h (E_2) or 15 min (EGF) after a single injection of 50 μg E_2 or 50 μg EGF/kg BW. (d) Three injections of AG825 or AG1478 (5 mg/kg BW) were administered every 8 h. BrdU was injected at the last injections of AG825 or AG1478, and killed 1 h later. (e) 4-OHT and ICI (5 mg/kg BW/day) were administered to mice that had been OVX on day 46 for 7 days from days 60 to 66, and then killed 24 h after the last injection

noma in young women (Herbst *et al*., 1971) and various malformation in the reproductive tracts (Marselos and Tomatis, 1992a; Herbst, 2000). In males, *in utero* DES exposure is associated with an increased risk of testicular cancer and epididymal cysts (Marselos and Tomatis, 1992a; Herbst, 2000). Like humans, perinatal female mice exposed to natural or synthetic estrogens develop estrogen-independent persistent cell proliferation, stratification and cornification of the vaginal epithelium, resulting in hyperplastic lesions and vaginal cancer later in life (Takasugi *et al*., 1962; Dunn and Green, 1963; Forsberg, 1979; McLachlan *et al*., 1980; Iguchi, 1992). This rodent model, which simulates the effects of developmental DES exposure in humans, has been characterized, yet the underlying mechanisms remain poorly understood. This irreversible proliferation and differentiation of the vaginal epithelium may be attributable to estrogen-independent, persistent activation of downstream growth factor expression. Indeed, high levels of EGF and TGF-α are expressed in the vaginae of DES treated mice, even after ovariectomy (Nelson *et al*., 1994; Sato *et al*., 1996a). However, the role of crosstalk between erbB signaling and ER in estrogen-independent effects induced by neonatal DES treatment has not yet been elucidated.

Cell proliferation in reproductive organs is tightly regulated by hormones, particularly estrogens. Tumors of reproductive organs can exhibit 'hormone-independent cell proliferation'. Thus, the vaginae of mice exposed neonatally to estrogens can be used as a model system to understand the mechanisms leading to the change from normal to aberrant cell proliferation and differentiation, and tumor formation *in vivo*. The importance of growth factor induction of ER transcriptional activity in normal physiology and in tumor progression is essentially unknown. In this report, we characterized estrogen-independent vaginal epithelial cell proliferation and differentiation by focusing on the crosstalk between erbBs signaling and ERα. We found that neonatal DES treatment led to increased phosphorylation of EGFR, erbB2 and ERα persistently. These phosphorylations are each capable of activating the receptors in a ligand-independent manner, suggesting a possible mechanism whereby neonatal DES treatment leads to persistent, estrogen-independent proliferation of the vaginal epithelium.

Results

Expression of EGF-like growth factors

We quantified the time-course mRNA expression of growth factors (EGF, TGF-α, HB-EGF, BTC, APR, EPR, insulin-like growth factor (IGF)-I, IGF-II and keratinocyte growth factor (KGF)) in 60-day-old OVX mice after a single injection of E_2 by quantitative real-time RT–PCR (Q-PCR) (see Figure 1 for experimental design). Although it has been reported that estrogen administration induces EGF and TGF-α mRNA expression in mouse uterus (DiAugustine *et al*., 1988; Nelson *et al*., 1992), a single injection of E_2 did not induce significant changes in the expression of growth factor genes in vagina. APR mRNA was the highest

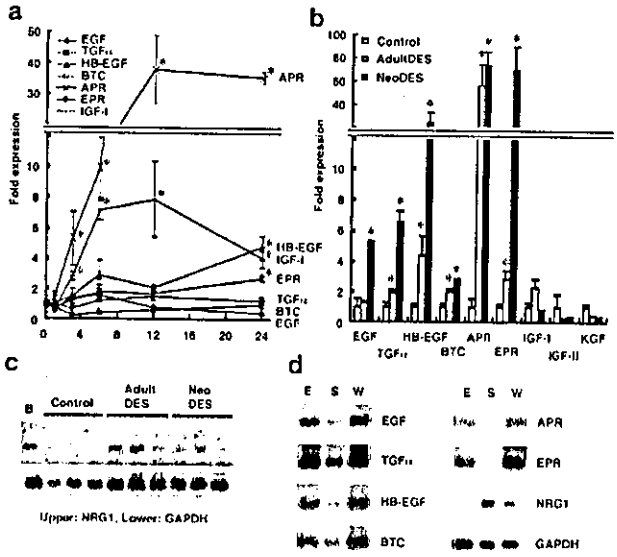

Figure 2 Expression of growth factor mRNA in mouse vagina. (a) Time-course analysis of growth factor mRNA expression. The samples were from vaginae of mice 0 (control), 1, 3, 6, 12 and 24 h after a single injection of 50 μg E_2/kg BW. (b) Expression profiles of growth factor mRNA in vaginae of 60-day-old OVX mice treated with oil (control), adultDES and neoDES. (c) Expression of NRG1 was examined by RT-PCR following electrophoresis. B; brain cDNA used as positive control. (d) Tissue distribution of growth factor mRNA expression in neoDES vaginae. E; epithelium, S; stroma, W; whole tissue

among E_2-responsive growth factors in this study (Figure 2a).

We also quantified the expression level of mRNAs encoding ligands for erbB receptors in vaginae of oil-treated OVX control, 60-day-old OVX mice after five daily injections of DES (adultDES) and 60-day-old OVX mice treated neonatally with DES for 5 days (neoDES mice). In neoDES vaginae, all ligands for erbB receptors were highly expressed (Figure 2b). Successive DES treatments induced the expression of erbBs in adultDES vaginae that closely resembled that seen in neoDES vaginae, but the magnitude of the induction was lower (Figure 2b). The NRGs primarily bind to erbB3 and erbB4 and have been shown to be a complex gene family with many alternatively spliced forms. Therefore, we examined NRG1 transcript expression by RT–PCR and gel electrophoresis to allow detection of splicing variants. NRG1 transcripts were expressed in both adultDES and neoDES vaginae, with adultDES tissue showing higher expression levels (Figure 2c).

IGF-I signaling is involved in E_2-induced responses, such as epithelial cell proliferation in rodent uterus (Klotz *et al*., 2000); therefore, we also examined IGF-I mRNA expression. In mouse vagina, a single injection of E_2 induced IGF-I mRNA expression in a time-dependent manner (Figure 2a). In adultDES and neoDES vaginae, IGF-I mRNA levels were not altered when compared to controls. Similarly, other growth factors, IGF-II and KGF did not show changes in mRNA expression in vaginae from adultDES nor neoDES mice (Figure 2b) and OVX mice given a single injection of E_2 (data not shown). Although we do not exclude a possible role of IGF-I in vaginal responses, persistent vaginal epithelial cell proliferation and differentiation is mainly contributed by ligands of erbB receptors in neoDES vagina.

To study the tissue distribution of growth factors, epithelium and stroma were separated by incubating in a trypsin solution, and followed by RT–PCR analysis. Excluding NRG1, which was expressed in the stroma only, the other ligands were primarily expressed in the epithelium. In particular, APR and EPR were only detected in the epithelium (Figure 2d).

ErbB mRNA and protein expression in vagina

We confirmed the expression of mRNAs encoding all four erbB types in both adult and neonatal mouse vagina by RT–PCR (data not shown) and the erbB proteins by immunoblot analysis (Figure 3a). Q-PCR was employed to quantitate the response of the erbB mRNAs to DES treatment. In vaginae of adultDES, there was no significant decrease in the expression of erbB mRNAs (Figure 3b). In contrast, EGFR and erbB2 mRNA expression was significantly decreased in neoDES vaginae (Figure 3b). Reduction of EGFR expression following neonatal DES exposure has been reported previously (Iguchi *et al*., 1993). However, we have now observed that erbB2 expression was also significantly decreased in neoDES vagina. ErbB3 and erbB4 mRNA levels also appeared to decrease but these changes were not statistically significant. We also assessed the expression of erbB mRNAs in vaginae

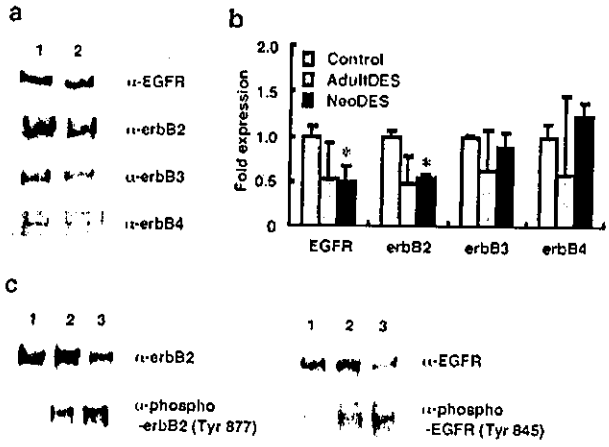

Figure 3 ErbB mRNA and protein expression pattern in vaginae of OVX control, adultDES and neoDES mice. (a) Immunoblots of erbBs in vaginae of oil control (lane 1) and neoDES (lane 2). Each contained the same amount of protein. (b) Quantification of gene expression in vaginae of 60-day-old mice of oil (control), adultDES and neoDES using Q-PCR. The expression of each receptor mRNA in vaginae of the oil-treated control mice was regarded as the basal level (1.0). (c) Phosphorylation of erbB2 and EGFR was detected by antiphospho-erbB2 and EGFR antibodies. The samples are from vaginae of OVX mice treated with oil (lane 1), adultDES (lane 2) and neoDES (lane 3)

from 5-day-old mice and found that successive DES treatment from the day of birth suppressed the expression of erbB2 mRNA (data not shown). The expression of EGFR and erbB4 mRNAs was slightly, but not significantly decreased (Figure 3b). ErbB2 protein expression was slightly reduced in neoDES vaginae (Figure 3a). Thus, erbB2 downregulation appears to be correlated with vaginal tissue morphology.

Phosphorylation of erbB2 and EGFR in vagina

The phosphorylation status of erbB2 in vaginae from adultDES and neoDES mice was examined using antiphospho-erbB2 antibody, which recognizes phosphotyrosine 877 from human protein sequences. Phospho-erbB2 was detected in vaginae from both adultDES and neoDES mice but not in vaginae from controls, although erbB2 protein was expressed in controls (Figure 3c). Tyrosine 845 of EGFR was also phosphorylated in vaginae of neoDES and adultDES mice (Figure 3c). Taken together, these data suggest that DES treatment induces phosphorylation of erbB2 and EGFR, which would be expected to upregulate the downstream signaling pathways under their control.

Considering the correlation between DES treatment and erbB2 expression, we next investigated the distribution of erbB2 mRNA in mouse vagina using immunohistochemistry. Intense membrane staining for erbB2 was observed in epithelial cells of vaginae from 60-day-old OVX mice. Apical cells were more strongly stained than basal cells whereas stromal cells beneath the basement membrane were weakly stained (Figure 4a).

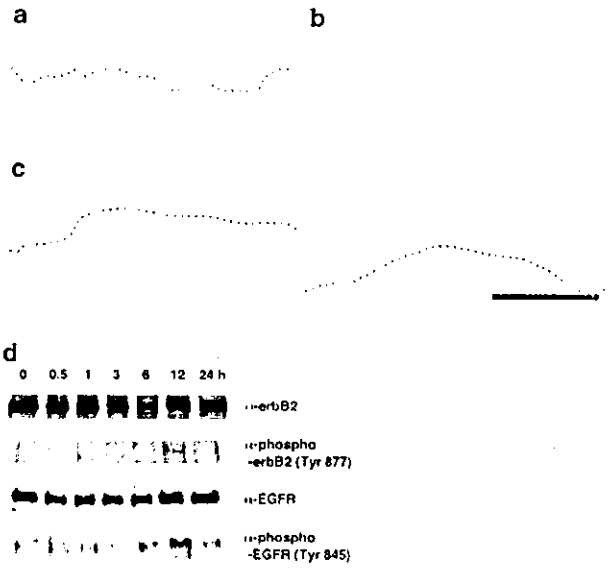

Figure 4 ErbB2 activation status and distribution in the mouse vagina. (a–c) Immunohistochemical detection of erbB2 in vaginae of 60-day-old mice from control (a) and neoDES (b). No immunostaining was observed with normal rabbit serum (c). The boundary between epithelium and stroma is indicated by a dotted line. Scale bar = 50 μm. (d) Time-course analysis of erbB2 and EGFR phosphorylation. The samples are from vaginae of mice 0 (control), 0.5, 1, 3, 6, 12 and 24 h after a single injection of 50 μg E_2/kg BW

In neoDES vaginae, erbB2 immunostaining was found in basal epithelial cells, whereas the differentiating cells that migrated toward the surface of the epithelium exhibited decreased erbB2 expression (Figure 4b). Stromal cells were not stained (Figure 4b). Immunoreactivities were abolished by incubation with control rabbit IgG demonstrating the specificity of the erbB2 staining (Figure 4c).

The time course of erbB2 phosphorylation was examined in vaginae from 60-day-old OVX mice after a single injection of E_2. Phospho-erbB2 was transiently detected in vaginae 12 h after E_2 injection (Figure 4d). The phosphorylation pattern of EGFR was similar to that observed for erbB2 (Figure 4d). These data suggested the existence of a crosslink between erbB2 phosphorylation and estrogen action in the mouse vagina.

Phosphorylation of ERα in vagina

To investigate the downstream of erbB2 and EGFR phosphorylation further, we tested whether increased erbB signaling would affect downstream target genes. ErbBs activation derived from administration of EGF, the most well-known ligands for erbBs, leads to vaginal epithelial cell proliferation and differentiation in OVX mice (Nelson *et al*., 1991). It is known that erbBs can stimulate the transcriptional activity of ERα by phosphorylating serine 118 and 167 of this receptor (located within the AF-1 region) through mitogen-activated protein kinase (MAPK) and/or Akt cascades (Lannigan, 2003). Actually, EGF administration led to phosphorylation of ERα with erbB2 and EGFR activation in vagina (Figure 5a).

ERK1/2 exhibited a high basal level of phosphorylation even in OVX mice, and phosphorylation of ERK1/2 above basal levels did not occur in adultDES and neoDES vaginae as previously reported using uterine tissue from mice (Klotz *et al*., 2002). However, p90 ribosomal S6 kinase (RSK), which is one of the substrates for ERK1/2 and can phosphorylate ERα (Joel *et al*., 1998), was activated in neoDES and adultDES vaginae (Figure 5b). Furthermore, both serine residues of ERα were phosphorylated in neoDES and adultDES vaginae (Figure 5c), confirming that erbBs phosphorylation could lead to the activation of ERα independently of the continuing presence of estrogens in vaginal epithelium. Detectable levels of ERα are present in both stromal and epithelial compartments of the vaginae obtained from neonatally DES-treated mice (Sato *et al*., 1996b). After tissue separation by trypsin, we weakly detected total and phosphorylated ERα protein in vaginal epithelial tissue, but in stromal tissue, we could not detect even total ERα protein due to the long trypsin incubation (data not shown).

We examined AF-1 function of ERα in mouse vagina using 4-hydroxytamoxifen (4-OHT), which acts as an agonist for AF-1, but as an antagonist for AF-2 (Berry *et al*., 1990; McDonnell *et al*., 1995; Metzger *et al*., 1995). Administration of 4-OHT to intact OVX mice induced vaginal epithelial stratification and cornification

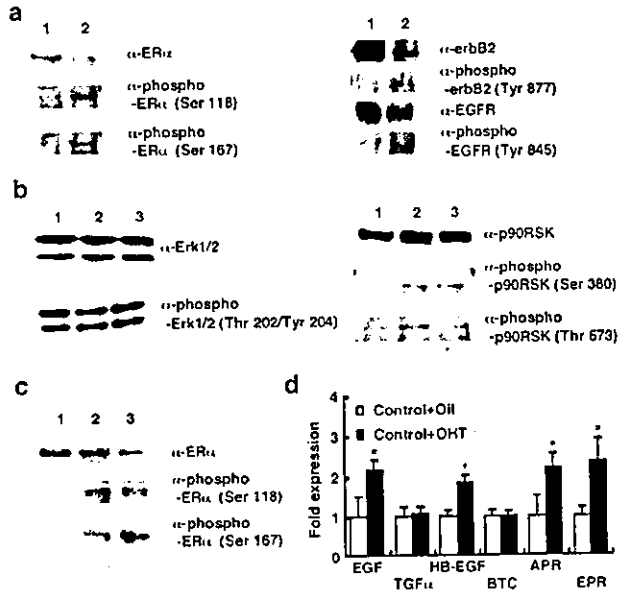

Figure 5 Activation status of erbB downstream signaling in the mouse vagina. (a) ERα, EGFR and erbB2 phosphorylation status in vaginae of mice treated with saline (lane 1) or EGF (lane 2). (b) Erk1/2 and p90RSK activation status was detected by antiphospho-Erk1/2 and p90 RSK antibodies. The samples were from vaginae of OVX mice treated with oil (lane 1), adultDES (lane 2) and neoDES (lane 3) (a and b). (c) ERα activation status was detected by antiphospho-ERα antibodies. (d) Expression profiles of growth factor mRNA in vaginae of intact OVX mice treated with oil control or 4-OHT

(data not shown), accompanied with sustained mRNA expression of EGF-like growth factors, such as EGF, HB-EGF, APR and EPR (Figure 5d). These data indicated that only AF-1 activation of ERα induced ligands for erbB receptors. Further, ERα and erbB signaling formed on activation loop without E_2, which initially must regulate this signaling pathway.

This hypothesis was supported by the experiment using inhibitors of EGFR (AG1478) and erbB2 (AG825). The number of BrdU-incorporated cells was slightly and significantly reduced in neoDES vagina treated with AG825 and AG1478, respectively (Figure 6a–c). The level of phosphorylation of ERα, in particular Ser 118, was also reduced in these vaginae treated with the inhibitors (Figure 6d).

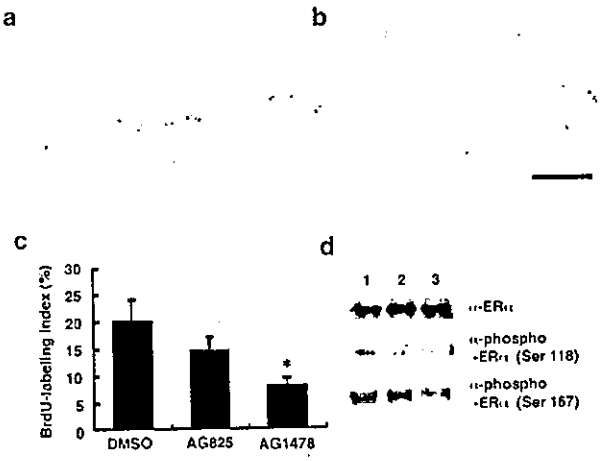

Figure 6 Administration of inhibitors of erbBs blocked BrdU incorporation and ERα phosphorylation. (a and b) BrdU-labeling cells in neoDES vagina treated with DMSO (a) and AG1478 (b). Sections were counterstained with methyl green. Scale bar = 100 μm. (c) BrdU-labeling index. Index is presented using percent values based on the number of BrdU-incorporated cells per 100 cells in the epithelial basal layer. The bars indicate standard error ($n = 5$–6). *, $P < 0.05$ *vs* control (DMSO). (d) ERα phosphorylation status after injections of erbB inhibitors. The samples are from neoDES vagina treated with DMSO (lane 1), AG825 (lane 2) and AG1478 (lane 3)

Effects of ICI 182,780 in NeoDES vagina

We next tested whether ICI 182,780 (ICI), an antagonist for both AF-1 and AF-2 of the ERα, could block the irreversible effects in neoDES vaginae. As shown in Table 1 and Figure 7, ICI administration partially blocked epithelial differentiation, accompanied by low expression of growth factors, erbB receptors and their phosphorylation levels in neoDES vagina killed 24 h after the last ICI injection. These data confirm that deregulation of AF-1 leads to estrogen-independent ERα action. Administration of ICI to intact OVX mice did not induce vaginal epithelial stratification (data not shown).

Discussion

Integrated hormonal signaling networks modulate the reproductive systems in animals. E_2 is responsible for

Table 1 Effects of ICI on neoDES mice

Treatment (No. of mice)	*No. of mice showing vaginal epithelial*		*Thickness of vaginal epithelium (μm)*[a]
	Cornification	*Stratification*	
neoDES + oil (9)	9	9	133 ± 6.0
neoDES + ICI (11)	3[b,c]	11	55 ± 9.4[d]

[a]Mean thickness of vaginal epithelium was estimated by measuring the epithelial cell layers in vaginae of three random regions (mean ± s.e.). [b]These mice showed cornified epithelium in partial region. [c]Statistical difference between neoDES + Oil and neoDES + ICI group by Fisher's exact probability test ($P < 0.05$). [d]Statistical difference between neoDES + Oil and neoDES + ICI group by Student's *t*-test or Welch's *t*-test followed by F-test ($P < 0.05$)

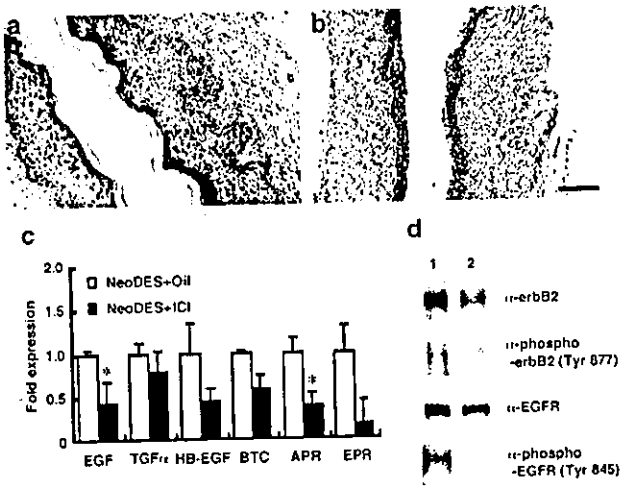

Figure 7 Administration of ICI blocked proliferation of vaginal epithelial cells in neoDES mice. (a and b) Histology of vaginae from 60-day-old neoDES (a) and neoDES + ICI (b) mice. Sections were stained with hematoxylin and eosin. Scale bar = 100 μm. (c) Expression profiles of growth factor mRNA in vaginae of neoDES mice treated with oil control or ICI. (d) ErbB2 and EGFR phosphorylation status in neoDES vaginae of mice treated with oil (lane 1) or ICI (lane 2)

promoting estrus, influences the development and maintenance of female sex characteristics, including the induction of behavioral and physiological processes in a variety of organ systems. Like most hormones, E_2 exhibits acute and transient actions in its target organs. For example, E_2 administration increases organ weight and promotes cell proliferation and differentiation in the adult female reproductive tracts (Iguchi *et al*., 1986). E_2 withdrawal induces rapid involution of uteri and vaginae, resulting in atrophy (Sato *et al*., 2003). This reversible and specific effect of E_2 is important in maintaining homeostasis and is required for normal health and reproduction. In contrast, long-term exposure to estrogens induces an imbalance in cell proliferation and increases the risk of cancer of the reproductive organs in rodents and humans (Marselos and Tomatis, 1992a, b). It was reported that *in utero* exposure to DES caused vaginal clear-cell adenocarcinoma in a subset of exposed females (Herbst *et al*., 1971). To help understand this phenomenon, a laboratory rodent model has been characterized; mice treated neonatally with DES develop estrogen-independent proliferation and cornification in the vaginal epithelium and tumors later in life (Takasugi, 1976; Forsberg, 1979; Iguchi, 1992). Since the differentiative response of the vaginal epithelium is complex and involves the generation and differentiation of multiple suprabasal layers, an appropriate *in vitro* system for studying vaginal epithelium has not been established. For example, isolated vaginal epithelium *in vitro* fails to stratify or cornify in response to E_2 (Iguchi *et al*., 1983; Tsai *et al*., 1991). Therefore, to elucidate mechanisms of estrogen-dependent and -independent effects, which involve the induction of tumors, we used neonatal DES-treated mice as a model system. The present study was focused on the signaling of ER and erbBs and their ligands, the EGF-like growth factors in mouse vagina, since many cancers of estrogen-target organs can be related to these factors (Kurokawa *et al*., 2000; Atanaskova *et al*., 2002).

Although many growth factors have been reported to be related to reproductive organ growth, EGF-like growth factors were hypothesized as major factors involved in estrogen-dependent and -independent growth. It is notable that HB-EGF, APR and EPR were expressed at higher levels than EGF or TGF-α, which are considered to be powerful mitogens in the reproductive tracts of adult mouse (Nelson *et al*., 1991). Interestingly, it was reported that APR was induced by progesterone but not by estrogens in rodent uterus (Das *et al*., 1995). On the contrary, APR contributed to the autocrine growth of keratinocytes and tumor progression (Normanno *et al*., 1994; Piepkorn *et al*., 1994). This is the first report that APR is an estrogen-inducible growth factor and may be involved in vaginal epithelial cell growth, such as stratification and cornification. The sustained expression of other growth factors may be induced by APR, as EGF-like growth factors are capable of inducing other EGF-like growth factors, the so called crossinduction (Hashimoto *et al*., 1994).

In addition to increased EGF-like growth factors, neonatal DES treatment led to increases in phosphorylation of EGFR and erbB2 even after ovariectomy. The erbB family has been implicated in numerous physiological processes and is generally regarded as a major contributor to cell proliferation and tumor progression. Amplification or overexpression of erbB2 has been reported in various cancer cells (Hynes and Stern, 1994), thus it is expected that erbB mRNAs would be increased in vaginae from neoDES mice. Instead, we observed a reduction of EGFR and erbB2 mRNA and protein expression in vaginae from both neoDES and adultDES mice, accompanied by an increase in phosphorylations of these receptor proteins. Phosphorylation of erbB2 is probably due to ligand binding and ultimately results in downregulation of the receptor, a property commonly known to receptor tyrosine kinases (Pastan and Willingham, 1983). ErbB expression in neoDES vaginae was quite similar to that in adultDES vaginae, even in the absence of stimulation by estrogens. It should be noted that although erbB expression differs between neoDES vaginae and vaginal tumors, persistent phosphorylation and activation of erbB2 have been suggested to be an early stage in tumor formation. Although all erbBs were expressed in mouse vagina and have the potential as mediators of estrogen action, EGFR likely interacts with erbB2 since we found that both EGFR and erbB2 were phosphorylated in neoDES vaginae, erbB2 is expressed in the vaginal epithelium and it was previously reported that EGFR was expressed in mouse vaginal epithelium (Falck and Forsberg, 1996).

Finally, we found that ERα is phosphorylated at serine 118 and 167 within the AF-1 domain in neoDES vaginae. Phosphorylation of these residues regulates estrogen-independent transactivation by the receptor. The activity of the ERα AF-1 domain is ligand

independent and constitutive, whereas the function of AF-2 in the ligand-binding domain is dependent on ligand binding. When E_2 dissociates from the receptor, ERα becomes transcriptionally inactive because the potential function of the AF-1 is suppressed by the unliganded ligand-binding domain (Kato *et al.*, 2000). Phosphorylation of the AF-1 domain in mouse ERα also affects the transcriptional function of AF-2 (Lahooti *et al.*, 1995). It is well established that growth factors can activate ERα in the absence of its ligand through phosphorylation in AF-1 (Kato *et al.*, 1998; Yee and Lee, 2000). Although participation of other growth factor signaling pathways cannot be ignored, erbBs can activate AF-1 of ERα by MAPK and Akt cascades (Lannigan, 2003). In our study, activated erbB signal induced ERα phosphorylation via at least p90RSK, which has the potential to phosphorylate serine 167 of ERα (Joel *et al.*, 1998), while phosphorylated ERα induces EGF-like growth factors, which activates erbB receptors. Thus, estrogen-independent actions in the vagina were characterized by the formation of an activation loop between ERα and erbB signaling.

Normal E_2-induced epithelial proliferation in the vagina is mediated through stromal ERα (Buchanan *et al.*, 1998), and the alterations in stromal–epithelial interactions may lead to the onset and/or progression of carcinogenesis. In our study, epithelial cells in neoDES vagina seem to exhibit direct mitogenic and differentiation responses in epithelial cells, and they lose their normal stromal association. Actually, tissue recombinant experiments showed that the reciprocal recombination of neonatally estrogenized vaginal epithelium and untreated vaginal stroma exhibited ovary-independent hyperplasia (Cunha *et al.*, 1977). This is the first report that ligand-independent ER action induces a precancerous status in estrogen-target organ *in vivo*.

It has not been determined whether a major contributor to estrogen-independent effects in neoDES vaginae are the erbBs or the ER. Our study demonstrated that, at least, ERα might be critical for estrogen-independent vaginal changes in neoDES mice, even in the absence of estrogen. AF-1 activation following the administration of 4-OHT induced expression of ligands for erbB receptors in vaginae. Furthermore, the administration of ICI to neoDES mice blocked phosphorylation of EGFR and erbB2, resulting in a reduction in the number of vaginal epithelial cell layers. As to erbBs, the administration of EGF rapidly induced the phosphorylation of ERα in the present study. The administration of EGF leads to vaginal cell proliferation and differentiation (Nelson *et al.*, 1991). Thus, erbBs play roles in the phosphorylation of ERα and mediation of ER action in vaginal cells. We also demonstrated that inhibitors of erbBs blocked or slightly reduced BrdU incorporation and ERα phosphorylation of serine 118 in neoDES mice. These data suggests that ERα and erbBs make a crosstalk and form activation loop, then it leads to estogen-independent activation in neoDES vagina.

The mechanisms driving the constitutive activation of ERα that occurs only in neoDES mice remain unknown. DES activates both ERα and erbBs in the vagina of normal adult mice in the similar manner to that observed in the neoDES mice. However, the activation loop has not been established in the normal adult mice and the effect of estrogen is always reversible. Additional factors, such as dysregulation of phosphatase or signals through other receptor tyrosine kinases may be involved in the persistent proliferation and differentiation of vaginal epithelial cells.

Estrogen signals acting through ERα cause both proliferation and differentiation of the vaginal epithelial cells. While hyperproliferation leads to cancer, the superactivation of differentiation signal may prevent cancer development. The balance of these two signaling cascades seems to be an important factor in estrogen-induced cancers. The constitutive activation of the ERα-erbB signaling loop seems to induce both proliferation and differentiation. Hence, an alternation in this activation loop may shift the tissue response toward more proliferation than differentiation during carcinogenesis. Therefore, further analyses are needed in order to clarify the activation status of ERα and erbB family members in the precancerous and cancer stages.

Despite decades of research, the mechanisms by which developmental exposure to estrogens results in persistent alteration of growth and differentiation of reproductive tracts in humans and rodents remain unknown. As generation of women exposed to DES becomes older, with the possibility of a second rise in DES-associated vaginal clear-cell adenocarcinoma (Herbst, 2000). Various studies to date have shown that DES-like effects can be induced following exposure to xenoestrogens in laboratory animals and wildlife (McLachlan, 2001). Therefore, further characterization of the DES model is needed to advance our knowledge of the potential risk of the carcinogenetic effects of estrogens, including developmental effects of xenoestrogens. In conclusion, we demonstrated that estrogen-independent pathway required for ERα, and activation of crosstalk between erbBs signaling and ER leads to ligand-independent activation of ERα which, in turn, leads to persistent, estrogen-independent vaginal changes and may lead to cancer in the mouse vagina later in life.

Materials and methods

Animals and treatments

Female C57BL/6J mice were maintained under 12 h light/12 h dark at 23–25°C and fed laboratory chow (CA-1, CLEA, Tokyo, Japan) and tap water *ad libitum*. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at the National Institute for Basic Biology, Okazaki National Research Institutes.

Treatment time lines are shown in Figure 1. Female newborn mice were given a daily subcutaneous (s.c.) injection of 2.5 mg DES (Sigma, St Louis, MO, USA)/kg body weight (BW)/day dissolved in sesame oil or the vehicle alone beginning from days 0 (the day of birth) to 4. Some mice were killed 24 h after the last injection, and others were OVX at day 46 and killed at day 60 (referred to as neoDES mouse) (Figure 1a). Subsets of these mice were administered three injections of AG825 or AG1478 (Tocris, Ellisville, MO, USA)

(5 mg/kg BW) dissolved in dimethyl sulfoxide (DMSO), at 8 h intervals. BrdU (50 mg/kg BW) (Sigma) was injected at the last injection of the inhibitors and killed 1 h later (Figure 1d). The other subset of mice were administered with daily 4-OHT (Sigma) or ICI 182,780 (ICI, Tocris) (5 mg/kg BW/day) dissolved in sesame oil for a week and killed 24 h after the last injection (Figure 1e). To examine the acute and reversible effects of estrogens on the vagina, untreated mice were OVX at day 46 and treated with a daily injection of 2.5 μg DES/kg BW/day for 5 days, then killed 24 h after the last injection (referred to adultDES mouse) (Figure 1b). This treatment was sufficient to induce stratified and keratinized vaginal epithelium in OVX mice. For examining direct effects of estrogen and EGF, a single injection of 50 μg E_2 (Sigma) or 50 μg EGF (Biomedical, Stoughton, MA, USA)/kg BW was given to 60-day-old mice, which were OVX on day 46, and killed 0.5, 1, 3, 6, 12 and 24 h (E_2) or 15 min (EGF) after the injection (Figure 1c).

For tissue separation, neoDES vagina were cut into small pieces, placed into 1% trypsin (Difco, Kansas, MO, USA) in Hanks' balanced salt solution (Invitrogen, Carlsbad, CA, USA) and digested at 4°C for 90 min. The vaginal epithelium and stroma were then separated mechanically using fine surgical forceps.

Immunohistochemistry and BrdU immunostaining

Tissues were fixed in neutral buffered 10% formalin, embedded in paraffin and sectioned at 6 μm. Deparaffinized sections were incubated in 0.3% H_2O_2 in methanol for 15 min to eliminate endogenous peroxidases. After washing with PBS, the sections were stained with Histofine (Nichirei, Tokyo, Japan) according to the manufacturer-supplied protocol. Anti-erbB2 antibody was obtained from Novocastra (Newcastle, UK). The sections were incubated at a 1 : 500 dilution in PBS containing 1% BSA (Sigma) for 60 min at room temperature. For negative controls, normal rabbit immunoglobulin fraction (Dako, Carpinteria, CA, USA) at the same dilution of each antibody was used.

For BrdU-immunostaining, deparaffinized, sections were incubated in 3% H_2O_2 in methanol for 30 min and immersed in 2 N HCl for 20 min in order to denature the genomic DNA. After washing with PBS, the sections were incubated with anti-BrdU antibody (Boehringer Mannheim, Mannheim, Germany) diluted 1 : 10 in PBS containing 1% BSA for 60 min at room temperature. The sections were subsequently incubated with 3,3-diaminobenzidine tetrahydrochloride containing hydrogen peroxide. BrdU-labeling index was estimated by counting the number of BrdU-incorporated cells in the vaginal epithelial basal layer. Statistical analysis was performed using Student's *t*-test or Welch's *t*-test followed by F-test.

RT–PCR

Changes in gene expression were confirmed and quantified using Q-PCR with the ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Total RNA (2.5 μg), isolated with an RNeasy kit (QIAGEN, Chatsworth, CA, USA) from each groups, was used in RT–PCR reactions carried out with SuperScript II reverse transcriptase (Invitrogen) and SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min in 15 μl volumes. Sequences of gene primer sets are given in Table 2. Relative RNA equivalents for each sample were obtained by standardization of ribosomal protein L8 (L8) levels. More than three pools of samples per group were run in triplicate to determine sample reproducibility, and the average relative RNA equivalents per sample was used for further analysis. Error bars represent the standard error, with all values represented as fold change compared to the control treatment group normalized to an average of 1.0. Statistical analysis was performed using Student's *t*-test or Welch's *t*-test followed by F-test; differences with $P<0.05$ were considered significant.

The expression of NRG1 was examined using RT–PCR following electrophoresis. The annealing temperature was 60°C and 31 cycles for NRG1 and 24 cycles for glyceraldehyde-3-phosphate dehydrogenase.

Protein preparation and immunoblotting

Vaginae removed from mice were immediately homogenized in buffer A (20 mM HEPES, 2 mM EDTA, 2 mM EGTA, 250 mM sucrose, 100 mM β-glycerophosphate, 2 mM Na_3VO_4 and protease inhibitor cocktail (Complete Mini; Boehringer Mannheim, Germany), pH 7.5). The homogenates were centrifuged at 900 *g* for 15 min at 4°C. The pellets were suspended in buffer B (20 mM HEPES, 2 mM EDTA, 2 mM EGTA, 250 mM NaCl, 1% Triton X-100, 100 mM

Table 2 Sequences of gene primer sets for RT–PCR

	Primer sequences (5′-3′)	
Gene	*Forward*	*Reverse*
EGFR	ATTCATGCGAAGACGTCACATT	GTTCCACGAGCTCTCTCTCTTGA
erbB2	GCTGCCCGAAACGTGCTA	CCGTGCCAGCCCGAA
erbB3	AGGCTTGTCTGGATTCTGTGGTT	GGGATCGGGTGCAGAGAGA
erbB4	GGAGGCTGCTCAGGACCAA	ACGCACGCTCCACTGTCAT
EGF	TTCACAGAGCACACCTCAAAGGT	GAATGTAAGCGTGGCTTCC
TGF-α	CCAGATTCCCACACTCAGT	GGAGGTCTGCATGCTCACA
HB-EGF	CAAGGTTCCCAGACAGGATCTC	GGAGGACAGCGAGGTTCCA
BTC	AGATGCCGCTTCGTGGTG	CGAGCCCCAAAGTAGCCTT
APR	CCGGTGGAACCAATGAGAACT	CCTAAGACCAGCAGCAACAGC
EPR	GCTGCACCGAGAAAGAAGGA	GGGAACCTAGACAAAGCAGCG
IGF-I	TTCAGTTCGTGTGTGGACCGAG	TCCACAATGCCTGTCTGAGGTG
IGF-II	TTCGCCTTGTGCTGCATC	TCAACAAGCTCCCCTCCG
KGF	GAAAGGGACCCAGGAGATGAA	TGATTGCCACAATTCCAACTG
L8	ACAGAGCCGTTGTTGGTGTTG	CAGCAGTTCCTCTTTGCCTTGT
GAPDH	AACGACCCCTTCATTGACCTC	CCTTGACTGTGCCGTTGAATT
NRG-1	TGAAAGACCTTTCAAACCCCTC	CTCTTCTGGTACAGCTCCTCCG

β-glycerophosphate, 2 mM Na_3VO_4 and protease inhibitor, pH 7.5) and used as nuclear samples. Supernatant fluids were recentrifuged at 105 000 *g* for 60 min and the resulting pellets were suspended in buffer B. Protein contents were determined using the Bradford Assay (Protein Assay Reagent, BioRad, Hercules, CA, USA).

The samples mixed with Laemmli sample buffer were boiled then electrophoresed on SDS–polyacrylamide gels; proteins were transferred onto a nitrocellulose membrane. The membranes were preincubated with 3% BSA in TBS contained 0.1% Tween-20 over night at 4°C. Incubations with each antibody were performed at a dilution of 1 : 1000 at room temperature for 2 h in TBS contained 0.1% Tween-20 with or without 1% BSA. Anti-erbB2, anti-erbB3, anti-ERα and anti-p90RSK-1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-erbB4 was obtained from NeoMarkers, anti-EGFR, anti-Erk1/2 and antiphospho-EGFR (Tyr 845 of human sequence corresponding to Tyr 847 of mouse sequence), -erbB2 (Tyr 877; mouse sequences corresponding to human Tyr 877 is not known because N-terminal sequence is not determined), -Erk1/2 (Thr 202/Tyr 204 to Thr 203/Tyr 205 of mouse sequence), -p90RSK (Thr 574 to Thr 611 of mouse sequence) and -ERα (Ser 118 and Ser 167 to Ser 122 and Ser 171 of mouse sequences) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). The numbering of amino-acid residues in this paper is according to the human protein sequences as the manufacturer's product name and specificity of these phosphospecific antibodies against mouse proteins is described in the manufacturer's instruction. Signals were detected with the ECL kit (Amersham, Arlington Heights, IL, USA).

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Persistent gene expression in mouse vagina exposed neonatally to diethylstilbestrol

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Abstract

Developmental exposure to a synthetic estrogen, diethylstilbestrol (DES), induces carcinogenesis in human and laboratory animals. In mice, neonatal DES treatment induces persistent proliferation and keratinization of the vaginal epithelium, even in the absence of the ovaries, resulting in cancerous lesions later in life. To understand the mechanisms underlying this persistent cell proliferation and differentiation, we characterized the gene expression patterns in the neonatally DES-exposed mouse vagina using DNA microarray and real-time quantitative RT-PCR. We found that genes related to cellular signaling, which are candidates for mediating the persistent proliferation and differentiation, were altered, and genes related to the immune system were decreased in the neonatally DES-exposed mouse vagina. We also noted high expression of interleukin-1 (IL-1)-related genes accompanied by phosphorylation of JNK1. In addition, expression IGF-I and its binding proteins was modulated and led to phosphorylation of IGF-I receptor and Akt, which is one of the downstream factors of IGF-I signaling. This led us to characterize the expression as well as the phosphorylation status of IL-1 and IGF-I signaling pathway components which may activate the phosphorylation cascade in the vagina of mice exposed neonatally to DES. These findings give insight into persistent activation in the vagina of mice exposed neonatally to DES.

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Introduction

Estrogens tightly regulate cell proliferation and differentiation in target organs, particularly the reproductive tracts (oviduct, uterus and vagina) and mammary gland. The vaginal epithelium is a particularly intriguing model for estrogen-regulated gene expression. It undergoes characteristic changes from non-keratinized to a fully keratinized epithelium depending on the levels of the endogenous estrogen, estradiol (E_2), during the estrous cycle. Under this process, E_2-induced cell proliferation is restricted to the basal layer. The suprabasal cells are no longer mitogenic but differentiate while moving up through the epithelium; finally, the apical cells exhibit keratinization, losing their nuclei and cytoplasm and substituted by the keratin proteins. The fully stratified and keratinized epithelium in the vagina resembles epidermis in the skin, however, again, differentiation of vaginal epithelium is regulated by estrogen.

Long-term estrogenic stimulation is a known risk factor for carcinogenesis in laboratory animals and humans (Marselos & Tomatis 1992*a*,*b*). In humans, transplacental exposure to a synthetic estrogen, diethylstilbestrol (DES), which was routinely prescribed to pregnant women for preventing miscarriages in 1940s to the 1970s, induces vaginal clear-cell adenocarcinoma in young women (Herbst *et al.* 1971). As generations of women exposed to DES approach menopause, concern has

arisen about their health risk, because it has been hypothesized that *in utero* DES exposure influences the incidences of breast cancer, squamous neoplasia of the cervix and vagina, and vaginal clear-cell adenocarcinoma later in life (Herbst 2000, Hatch *et al.* 2001, Palmer *et al.* 2002). Rodent models of DES exposure have been developed to understand the mechanistic basis of DES effects in humans. In mice, developmental exposure to estrogens within a critical developmental period elicits various permanent alternations in female reproductive tracts. For example, in the vagina, neonatal estrogen administration induces persistent cell proliferation and cornification of vaginal epithelium even after ovariectomy (OVX), resulting in hyperplastic lesions later in life (Takasugi *et al.* 1962, Forsberg 1979, McLachlan *et al.* 1980). We previously reported that persistent erbB receptor phosphorylation and sustained expression of epidermal growth factor-like growth factors contributed to persistent action in the neonatally DES-exposed vagina (Miyagawa *et al.* 2004). However, the underlying mechanisms remain unknown, therefore, additional factors must be present. In order to understand the underlying mechanisms of persistent action, it is important to understand cellular events, such as mRNA/protein expression and the phosphorylation status of signaling molecules.

In this report, we characterized the mRNAs expression patterns using DNA microarray and real-time quantitative RT-PCR (Q-PCR). In the vagina of mice exposed neonatally to DES, expressions of various genes were modulated, and interleukin-1 (IL-1) and insulin-like growth factor-I (IGF-I) signaling were activated without estrogen stimulation. In particular, IGF-I receptor (IGF-IR) and its downstream factor, Akt, were phosphorylated, which may lead to persistent cell proliferation and differentiation in the vagina. In addition, genes related to the immune system were suppressed in the neonatally DES-exposed mouse vagina. These findings give insight into persistent phenomena in vaginas exposed neonatally to DES in mice, and also in humans.

Materials and methods

Animals and treatments

Female C57BL/6J mice were maintained under 12 h light:12 h darkness at 23–25 °C and fed laboratory chow (CE-2; CLEA, Tokyo, Japan) and tap water freely. All procedures were approved by the Institutional Animal Care and Use Committee at the National Institute for Basic Biology, Okazaki National Research Institutes.

Female newborn mice were given a daily s.c. injection of 2·5 μg DES (Sigma)/g body weight (BW) per day dissolved in sesame oil (Kanto Chemical, Tokyo, Japan) or the vehicle alone beginning from day 0 (the day of birth) to day 4. These mice underwent OVX at day 46 and were killed at day 60 (referred to as neoDES mouse) (see Fig. 1A). For examining effects of estrogen and IGF-I, a single injection of 50 μg E_2 (Sigma) or 500 μg long R3 IGF-I (JRH Biosciences, Lenexa, TS, USA)/g BW was given to 60-day-old mice, which underwent OVX at day 46, and were killed 0·5, 1, 3, 6, 12, 24, 36 and 48 h (E_2) or 15 min (IGF-I) after the injection. Some mice underwent OVX at day 46 and were treated with a daily injection of 2·5 ng DES/g BW per day for 5 days, then killed 24 h after the last injection. This treatment was sufficient to induce stratified and keratinized vaginal epithelium in adult OVX mice. For tissue separation, neoDES vaginas were cut into small pieces, placed into 1% trypsin in Hanks' balanced salt solution and incubated at 4 °C for 90 min. Then, the vaginal epithelium and stroma were separated mechanically using fine surgical forceps. More than three mice were pooled per each experimental group.

Immunohistochemistry

Tissues were fixed in neutral buffered 10% formalin, embedded in paraffin and sectioned at 6 μm. Deparaffinized sections were incubated in 0·3% H_2O_2 in methanol for 15 min to eliminate endogenous peroxidases. After washing with PBS, the sections were stained with Histofine (Nichirei, Tokyo, Japan) according to the manufacturer-supplied protocol. Antibody for Ki-67 antigen was obtained from Novocastra Laboratories Ltd (Newcastle upon Tyne, UK). The sections were incubated at 1:1000 dilutions in PBS containing 1% BSA (Sigma) for 60 min at room temperature.

DNA microarray analysis

Preparation of cRNA and microarray analysis were essentially performed as described previously

(Watanabe *et al.* 2003). Total RNA was isolated with the TRIZOL reagent (Invitrogen) and purified using the RNeasy kit (Qiagen). Total RNA (10 μg) was converted into double-strand cDNA using the SuperScript Choice System (Invitrogen) with the T7-$(dT)_{24}$ primer (Amersham), which was used for first-strand cDNA synthesis. Biotin-labeled cRNA was synthesized using the ENZO BioArray HighYield RNA transcript labeling kit (Amersham). The cRNA was purified using RNeasy kit and partly hydrolyzed in fragmentation buffer (40 mM Tris, 100 mM potassium acetate and 30 mM magnesium acetate, pH 8·1) by 94 °C heat treatment for 35 min. The fragmented cRNA was mixed with hybridization buffer containing 100 mM 2-(N-morpholino)ethanesulfonic acid, 1 M NaCl, 20 mM EDTA and 0·01% Tween-20, and control oligonucleotides. The quality of the cRNA was assessed by analysis with Test 3 array (Affymetrix; Amersham) containing housekeeping genes. All preparations met Affymetrix's criteria for use on their expression arrays. After checking the quality of the cRNAs, 15 μg cRNA were hybridized to the high-density oligonucleotide arrays, Murine U74A GeneChip Expression Arrays (Affymetrix; the references to array design are available on the manufacturer's website, http://www.affymetrix.com) for 16 h at 45 °C. The arrays were then washed, stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR, USA) in an Affymetrix fluidix station and scanned with an argon-ion laser confocal scanner (Affymetrix).

Data were analyzed with GeneChip software Microarray Suite 5·0 (Affymetrix) as described elsewhere (Lockhart *et al.* 1996). Briefly, each gene is represented by 20 perfectly matched (PM) and one-base-mismatched (MM) 25 base oligonucleotides. As the MM probes are used to detect background level and cross-hybridization signals, the relative level of the gene expression is represented by differences between the levels of fluorescent intensity of the PMs and MMs, which are averaged into the so-called average difference. To normalize the data, the average differences were adjusted to produce an average intensity that equaled 2500.

The signals obtained from experimental groups were performed twice, independently, and the differences in intensities were calculated. In order to maintain reproducibility, we only selected the genes whose expression level changes were less than 2-fold between two independent experiments under the same conditions and average values were used as gene expression levels. Genes showing 3-fold alteration in the neonatally DES-treated mice as compared with oil controls were selected and analyzed further.

Real-time Q-PCR

Changes in gene expression were confirmed and quantified using Q-PCR using the ABI Prism 5700 Sequence Detection System with SYBR Green Master Mix according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Total RNA (2·5 μl) was used in the RT reaction carried out with SuperScript II reverse transcriptase (Invitrogen) and random primers (9-mer; Takara, Ohtsu, Japan) for 60 min at 42 °C. PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min in 15 μl volumes. The primers were chosen to amplify short PCR products (less than 100 bp) with the assistance of Primer Express software (Applied Biosystems). The representative primer sequences are as follows: IL-1α (X01450), AGG AGA GCC GGG TGA CAG TAT, TCA CTG AAA CTC AGC CGT CTC TT; IL-1β (X04964), TCA GGC AGG CAG TAT CAC TCA TT, GGA AGG TCC ACG GGA AAG A; IL-1 receptor type I (IL-1RI) (M20658), CCT GTG CGG GAC ACT AAG GA, GCC CAC TTT TGC CAT GCT A; IL-1 RII (X59769), GTT TAT CTC GGC TGC TTA CCC A, CAA AAA TCA GCG ACA CTT CCA C; IL-1 receptor antagonist (IL-1ra) (L32838), CCT TCT CAT CCT TCT GTT TCA TTC A, CTT GCA TCT TGC AGG GTC TTT; IL-1-like protein 1 (IL-1L1) (AJ250429), GGG CCA AGG AAT CAA AGA GC, CGG ATT CGA AGC TGG AGG TA; IGF-I (X04480), TTC AGT TCG TGT GTG GAC CGA G, TCC ACA ATG CCT GTC TGA GGT G; keratinocyte growth factor (KGF) (NM_008008), GAA AGG GAC CCA GGA GAT GAA, TGA TTG CCA CAA TTC CAA CTG; and ribosomal protein L8 (U67771), ACA GAG CCG TTG TTG GTG TTG, CAG CAG TTC CTC TTT GCC TTG T. The other primer sequences are available upon request. Melting curve analysis using 5700 software was performed to confirm the primer efficiency. Relative RNA equivalents for each sample were obtained by

standardization of ribosomal protein L8 levels. The expression of granulocyte–macrophage colony stimulation factor (GM-CSF) was quantified using the ABI Prism 7000 System with FAM-labeled Taqman probe and primers (Assays-on-Demand Gene Expression Products; Applied Biosystems) because an efficient primer set could not be established using the SYBR Green Q-PCR. The relative RNA equivalent for GM-CSF was obtained by standardization of ribosomal protein S5 levels. For quantifying gene expression on DNA microarray data, more than two pools of samples per group were run in triplicate to determine sample reproducibility, and the average relative RNA equivalents per sample were used for further analysis. All values are represented as fold change compared with the control treatment group average as 1·0. For analyses of IGF-I, KGF, GM-CSF and IL-1-related genes, three pools of samples per group were used. Statistical analysis was performed using Student's *t*-test or Welch's *t*-test followed by an F-test. Points of statistical significance vs control are indicated by asterisks (*), with $P<0·05$.

Protein preparation and immunoblotting

Dissected mouse vaginas were immediately homogenized in buffer (20 mM Hepes, 2 mM EDTA, 2 mM EGTA, 250 mM sucrose, 2 mM Na_3VO_4, 2 mM NaF, 100 mM β-glycerophosphate and protease inhibitor cocktail (Complete Mini; Boehringer Mannheim, Mannheim, Germany), pH 7·5). The homogenates were centrifuged at 900 ***g*** for 15 min at 4 °C. Supernatant fluids were recentrifuged at 105 000 ***g*** for 60 min and the resulting supernatant fluids were used for detection for stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and Akt. The pellets were suspended in homogenization buffer without sucrose but containing 250 mM NaCl and 1% Triton X-100, and they were used for detection of IGF-IR. Protein contents were determined using the Bradford Assay (Protein Assay reagent; BioRad, Hercules, CA, USA).

After electrophoresing on SDS-polyacrylamide gels, proteins were subsequently transferred to nitrocellulose membranes. The membranes were preincubated with 3% BSA in Tris-buffered saline contained 0·1% Tween-20 (TBST) overnight at 4 °C. Incubations with each antibody were performed at a dilution of 1:1000 at room temperature for 2 h in TBST (IGF-IR, SAPK/JNK, Akt, phospho-SAPK/JNK and phospho-Akt) or 1:1000 at 4 °C overnight in TBST and 5% BSA (phospho-IGF-IR). Anti-IGF-IR antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-SAPK/JNK, -Akt, -phospho-SAPK/JNK (Thr183/Tyr185 of human sequence corresponding to Thr183/Tyr185 of mouse sequence), -IGF-IR (Tyr 1131 to Tyr 1163 of mouse sequence) and -phospho-Akt (Ser 473 and Thr 308 of mouse sequence) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). For experimental reproducibility, more than two pools of experimental groups were used. Signals were detected with an ECL kit (Amersham).

Results

Histology of the neoDES vagina

Persistent epithelial proliferation and keratinization were induced by five daily injections of DES from the day of birth as shown in Fig. 1A. The vaginal epithelium of neonatally oil-injected 60-day-old OVX mice (control) was composed of two or three layers of cuboidal cells, whereas the epithelium of the age-matched, neonatally DES-exposed, OVX mice (neoDES) exhibited stratification and superficial keratinization (Fig. 1B). In the neoDES vagina, basal epithelial cells showed high proliferative activity, which was confirmed by Ki-67 antigen immunostaining (Fig. 1C). In contrast, the basal epithelial cells of control animals did not.

Microarray analysis of gene expression changes in the neoDES vagina

Neonatal DES treatment causes persistent cell proliferation and differentiation, however, molecular mechanisms underlying estrogen-induced changes have not been elucidated. We compared gene expression of vaginas of OVX mice treated neonatally with either oil (control) or DES (neoDES) using DNA microarray. The oligo-microarray system (Affymetrix GeneChip – Murine U74A) was used for analysis of the gene expression pattern. The scanned data were analyzed and fold changes in expression levels were determined in the 9977 genes. The genes showing changes in expression (increased or decreased more than

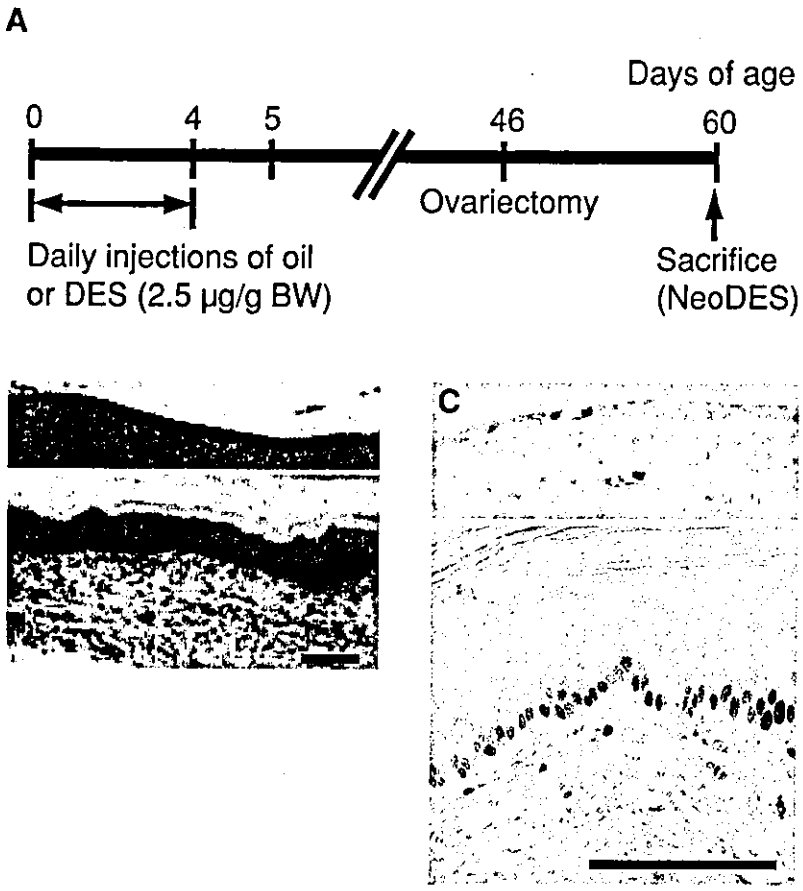

Figure 1 (A) Treatment timeline of neonatal DES treatment and histological analysis. Female newborn mice were given five daily injections of 2·5 μg DES/g BW or the vehicle alone from day 0. They underwent OVX at day 46 and were killed at day 60. (B) Histology of vaginas from a 60-day-old OVX control (upper panel) and neoDES mice (lower) at low magnification. The sections were stained with hematoxylin and eosin. Scale bar=100 μm. (C) Immunohistochemical staining of Ki-67 antigen with methyl green. The samples are from control (upper) and neoDES vaginas (lower). Scale bar=100 μm.

3-fold) between control and neoDES vaginas were selected for further analysis. Thus, 115 increased genes (22 genes were expressed sequence tags (ESTs)) and 160 decreased genes (53 genes were ESTs) were identified by microarrays.

As expected, the expression of cytokeratin (CK) mRNAs and components of the cornified cell envelope were highly increased in the neoDES vagina (Table 1). Expression of marker genes related to keratinization was also increased; e.g. metallothionein IV (93·7-fold compared with controls) (Quaife *et al.* 1994), transglutaminase 3 (17·1-fold) (Kim *et al.* 1993), peptidylarginine deiminase IV (5·1-fold) (Ishigami *et al.* 1998). In contrast, expression of CK8 and CK18, which are both preferentially expressed in simple epithelial cells and basal cells in stratified epithelium, was decreased (Bosch *et al.* 1988). Intriguingly, expression of several genes related to the immune system were decreased in neoDES vaginas, as compared with controls (Table 2).

In order to further characterize the neoDES vagina, we focused on aberrant signaling related to proliferation and differentiation of vaginal epithelium. Hence, genes involved in signal transduction were studied in detail. As to genes related to cell signaling from microarray data, Q-PCR was employed to validate the microarray data and