Effect of diethylstilbestrol on cell proliferation and expression of epidermal growth factor in the developing female rat reproductive tract

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Abstract

To evaluate mechanisms of cell proliferation in the fetal female rat reproductive tract, diethylstilbestrol (DES) effects on cell division and estrogen receptor (ER), epidermal growth factor (EGF) and EGF receptor (EGF-R) expressions were determined from gestational day (GD) 15.5 to 21.5. Reproductive tracts were evaluated within three regions along the Müllerian duct axis; these were proximal, middle and caudal, which differentiate into oviduct, uterus and upper vagina respectively. In fetuses from non-treated dams, epithelial and mesenchymal proliferation, as evaluated by 5-bromo-2'-deoxyuridine incorporation, was decreased with development in all regions of the Müllerian duct. EGF levels were determined by immunohistochemistry. Müllerian epithelial EGF immunoreactivity was intense in the proximal and middle regions on GDs 15.5 and 17.5. EGF staining remained intense only in the proximal epithelia by GD 19.5 and was weak in the caudal epithelium, but substantially reduced throughout epithelia in all regions by GD

21.5. Thus, decreased cell proliferation correlated with decreased EGF expression in the developing Müllerian duct. DES (100 µg/kg body weight) was injected from GD 15 to 19 and female fetuses were collected on GD 19.5. DES increased Müllerian duct cell proliferation in the proximal epithelium and mesenchyme but decreased it in the caudal epithelium compared with oil-treated controls. No proliferative DES effect was observed in any cell type in the middle region. Müllerian duct EGF immunoreactivity was suppressed by DES compared with oil. Competitive RT-PCR indicated DES also decreased mRNAs for EGF, ERβ1 and ERβ2, but not ERα and EGF-R. These results indicate EGF may be an important regulatory factor of Müllerian duct cell proliferation, and that DES may alter cell proliferation by disrupting normal EGF, ERβ1 and ERβ2 expression in the developing female rat reproductive tract.

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Introduction

In mammals, male reproductive organs such as epididymis, vas deferens and seminal vesicles arise from the mesonephric or Wolffian duct, and female reproductive organs such as oviduct, uterus and upper vagina arise from the paramesonephric or Müllerian duct. After gonadal differentiation, androgens produced by Leydig cells induce Wolffian duct development, and Müllerian inhibiting substance (MIS) produced by Sertoli cells induces Müllerian duct degeneration in male fetuses. In female fetuses, Müllerian duct development and Wolffian duct degeneration occur due to a lack of androgens and MIS after gonadal differentiation (Josso & Picard 1986). The pattern of cell proliferation in the female reproductive tract

has been demonstrated in immature and adult rodents (McCormack & Glasser 1980, Quarmby & Korach 1984, Li 1994). However, such patterns in prenatal rodents are not well understood. Similarly, 17β-estradiol effects on reproductive tract cell proliferation have been well reported in immature and adult rodents (McCormack & Glasser 1980, Quarmby & Korach 1984). However, α-fetoprotein limits fetal 17β-estradiol exposure *in utero*. Diethylstilbestrol (DES), a non-steroidal synthetic estrogen, is known to have high affinity for estrogen receptors (ERs), but not α-fetoprotein (Raynaud *et al.* 1971, Raynaud 1973). Therefore, DES is a powerful tool for understanding the mechanisms of estrogen action in fetal rodents. Prenatal exposure to DES causes a variety of abnormalities in reproductive tracts including oviduct

uncoiling, hyperplasia and adenocarcinoma of uterus and vagina in aged mice and rats (reviewed by Herbst & Bern 1981), but the influence of DES exposure on Müllerian duct development during gestation has not been investigated in rats.

Two subtypes of ERs have been identified in rats: the classical receptor, ER α (Koike *et al.* 1987) and a novel receptor, ER β (Kuiper *et al.* 1996). Recently, a new isoform of ER β named ER β 2 was cloned in rats, and the original ER β was renamed ER β 1. ER β 2 has an additional 54 bp sequence in the ligand-binding domain and is expressed in various rat tissues (Maruyama *et al.* 1998, Petersen *et al.* 1998). Although physiological roles of the ER β isoforms are unknown, they are thought to regulate estrogen action since both have different binding affinities for ligands including 17 β -estradiol (Petersen *et al.* 1998). ER α binding affinity for DES is slightly greater than that of ER β 1 and ER β 2, but in all cases affinity for DES is much greater than that for 17 β -estradiol (Kuiper *et al.* 1997, Petersen *et al.* 1998). Since ER α is expressed in the fetal mouse Müllerian duct (Greco *et al.* 1991), prenatally administered DES may act through ER α and cause functional reproductive tract abnormalities in the female. ER β expression has not been reported in the Müllerian duct. Altered c-jun, c-fos, epidermal growth factor (EGF) receptor (EGF-R) and ER α levels were observed in adult mouse reproductive tract exposed neonatally to DES (Iguchi *et al.* 1993, Kamiya *et al.* 1996, Sato *et al.* 1996), suggesting that DES may induce reproductive abnormalities by changing the normal expression levels of growth factors, their receptors and/or proto-oncogenes. To understand the effect of DES on Müllerian duct function and clarify the mechanism of cell proliferation, we subdivided the Müllerian duct into three main regions (proximal, middle and caudal) along the cranial-caudal axis and examined cell proliferation and ontogenetic expression of EGF within the defined regions during development. Prenatal effects of DES on EGF, EGF-R, ER α , ER β 1 and ER β 2 mRNA expression were also determined.

Materials and Methods

Animals and DES treatments

Male and female Sprague–Dawley rats were obtained from Charles River Japan, Inc. (Kanagawa, Japan). Animals were housed individually in stainless-steel cages with controlled temperature ($23 \pm 2^\circ\text{C}$) and relative humidity ($55 \pm 10\%$), and a 13 h light:11 h darkness cycle (0800–2100 h). Pellet food (CRF-1; Oriental Yeast Co., Ltd, Tokyo, Japan) and municipal tap water were freely available. Females were cohabited overnight with males. The day on which sperm was found in a vaginal smear was designated as gestational day (GD) 0. All animals were maintained in accordance with the institutional guidelines for care and use of laboratory animals.

To investigate DES effects on cell proliferation and expression of EGF, EGF-R, ER α , ER β 1 and ER β 2, pregnant rats were given a single s.c. injection of DES (Sigma Chemical Co., St Louis, MO, USA) dissolved in peanut oil, or oil vehicle alone, on each day beginning on GD 15 through to 19 at a dose of 100 $\mu\text{g}/\text{kg}$ body weight. To monitor the extent of cell proliferation, pregnant rats were injected (i.p.) 5 h prior to killing with 100 mg/kg 5-bromo-2'-deoxyuridine (BrdU) (Sigma).

Tissue preparation

Pregnant rats were killed by exsanguination from the abdominal aorta under ether anesthesia on GD 15.5, 17.5, 19.5 and 21.5 for normal development and control studies and on GD 19.5 for DES studies. Fetuses were removed and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4°C , then dehydrated through a graded series of ethanol concentrations and xylene. Tissues were then paraffin embedded and sectioned to 6 μm .

Cell proliferation was studied at three designated regions along the axis of the Müllerian duct as per Visser *et al.* (1998). The proximal region is identified at the level of the fetal ovary and is the region that differentiates into oviduct. The uterus arises from the middle region where the Müllerian and Wolffian ducts cross. The caudal region is near the urogenital sinus and differentiates into the upper part of the vagina.

Immunohistochemistry for BrdU

Tissue sections were deparaffined with xylene and hydrated in decreasing ethanol concentrations, rinsed three times in distilled water (DW) and incubated with 0.1% trypsin (Sigma) in 0.1% CaCl_2 for 30 min at 37°C . After washing in DW, sections were incubated with 0.3% H_2O_2 in methanol for 30 min at room temperature (RT) to block endogenous peroxidases. After rinsing in 10 mM PBS, pH 7.2, sections were incubated with monoclonal anti-BrdU antibody (Cell Proliferation kit; Amersham Pharmacia Biotech, Amersham, Bucks, UK) or PBS for 60 min at RT. Sections were then rinsed in PBS and treated with peroxidase anti-mouse IgG2a (Cell Proliferation kit; Amersham Pharmacia Biotech) for 30 min at RT. Sections were again rinsed in PBS and incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (Cell Proliferation kit; Amersham Pharmacia Biotech) for 10 min at RT. Finally, sections were counterstained with Mayer's hematoxylin. Cell proliferation was calculated as a percentage of the total BrdU-labeled cells counted; all epithelial cells in each section evaluated were counted. For mesenchymal stroma, all cells in the five layers most adjacent to the epithelium in each section were counted.

Immunohistochemistry for EGF and ER α

For ER α immunohistochemical staining, deparaffined and rehydrated sections were autoclaved at 121 °C for 15 min in 10 mM citrate buffer, at pH 6.0. Sections were subsequently rinsed in DW three times and incubated with 0.3% H₂O₂ in methanol for 30 min at RT to block endogenous peroxidases. After rinsing in PBS, 10% normal goat serum blocking solution (Zymed Laboratories, Inc., San Francisco, CA, USA) was applied for 30 min at RT. Sections were incubated overnight at 4 °C with polyclonal anti-rat EGF antibody (IgG Co., Nashville, TN, USA) at a dilution of 1:50 or normal rabbit immunoglobulin fraction (DAKO A/S, Glostrup, Denmark) for EGF staining. For ER α staining, monoclonal anti-human ER α antibody (NCL-ER-6F11; Novocastra Laboratories, Ltd, Newcastle upon Tyne, UK) at a dilution of 1:50 or normal mouse IgG1 (DAKO A/S) in 10% normal goat serum blocking solution was used. Following incubation, sections were rinsed in PBS and treated with biotinylated goat anti-mouse IgG (DAKO LSAB Kit; DAKO Corporation, Carpinteria, CA, USA) for 1 h at RT. Tissue sections were again rinsed in PBS and incubated with streptavidin-conjugated horseradish peroxidase (DAKO LSAB Kit) for 1 h at RT. After a final PBS wash, sections were treated with 0.01% DAB (Dojindo Laboratories, Kumamoto, Japan) in 0.05 M Tris-HCl, at pH 7.6 including 0.068% imidazole (Sigma) and 0.02% H₂O₂ for 10 min at RT. For the evaluation of binding specificity of polyclonal anti-EGF and monoclonal anti-ER α antibodies to rat tissues, submandibular glands isolated from adult male rats, or uterus and ovary isolated from adult female rats were immunostained with anti-EGF or anti-ER α antibodies respectively as positive controls. Tissues were also incubated with normal rabbit immunoglobulin fraction or anti-EGF antibody pre-absorbed with 50 ng EGF peptide (Sigma) overnight at 4 °C, or normal mouse IgG1 as negative controls.

Total RNA preparation and competitive RT-PCR

To collect fetal tissues, three pregnant rats were killed by exsanguination from the abdominal aorta under ether anesthesia on GD 15.5, 17.5, 19.5 and 21.5. The female fetal reproductive tract was collected into ice-chilled diethylpyrocarbonate-treated PBS, and the gonad was dissected away under a dissecting microscope. Reproductive tracts were pooled from approximately 20 female fetuses per gestational day. Tissues were homogenized through a 23 G sterile needle in Trizol (Gibco-BRL, Gland Island, NY, USA), and total RNA was isolated according to the manufacture's instructions. Total RNA concentration was assessed by A₂₆₀ and RNA was stored at -80 °C until needed.

Template total RNA (1 µg) was reverse-transcribed by using SuperScript II RNase H⁻ reverse transcriptase

(Gibco-BRL) with oligo(dT)₁₂₋₁₈ primer for 55 min at 70 °C and then chilled on ice. The generated cDNA was amplified with specific primers, various amounts of competitors constructed using Competitive DNA Construction Kit (Takara Shuzo Co., Ltd, Tokyo, Japan) and Taq DNA polymerase. PCR cycle parameters were 95 °C for 30 s, 62 °C for 30 s and 72 °C for 60 s, repeated for 33 cycles for EGF and EGF-R; and 95 °C for 60 s, 60 °C for 60 s and 72 °C for 120 s, repeated for 25 and 35 cycle for ER α , and ER β 1 and ER β 2 respectively. The RT-PCR product was confirmed by digestion with PstI for EGF and ER α , BglII for EGF-R, and AatII for ER β 1 and ER β 2. The RT-PCR product was electrophoresed on a 2% agarose gel, stained with ethidium bromide (EtBr), and photographed. Gel bands of amplified target and competitor were quantified by SCION Image Version 1.55 (Scion Co., Frederick, MD, USA) software and the ratio of target to competitor was calculated.

Statistics

Statistical analysis was carried out by Duncan's multiple comparison test for cell proliferation in Müllerian epithelium and mesenchyme during fetal development. Student's *t*-test or Welch's *t*-test was performed in cases of equal variance or unequal variance respectively, after ANOVA between GD 15.5 and 17.5 for cell proliferation in the Wolffian epithelium and mesenchyme. To determine the presence of significance for DES effects on cell proliferation and gene expression, data were analyzed by ANOVA followed by Student's *t*-test or Welch's *t*-test in cases of equal variance or unequal variance respectively. Data are reported as means $\pm$ S.E.M. and considered significantly different at *P* < 0.05.

Results

Cell proliferation

Cell proliferation was evaluated by BrdU immunostaining in the developing female rat reproductive tract. BrdU-labeled cells were detected in epithelial and mesenchymal cells in both Müllerian and Wolffian ducts (Fig. 1). The percentage of BrdU-labeled cells in the Müllerian epithelium and mesenchyme are shown in Fig. 2A and B respectively. On GD 15.5, cell proliferation was high at 32–40% in both epithelium and mesenchyme in the proximal and middle regions of the Müllerian duct (Fig. 2A and B). In the caudal region, the Müllerian duct is not yet formed by GD 15.5. However, by GD 17.5, Müllerian epithelial and mesenchymal cell proliferation was high and similar in the caudal and middle Müllerian duct regions (Fig. 2A and B). Both epithelial and mesenchymal cell proliferation was decreased dramatically in the proximal region on GD 17.5 and in the middle and caudal regions by GD 19.5 and 21.5 in the Müllerian duct (Fig. 2A and B).

Figure 1 BrdU immunostaining in the proximal reproductive tract of fetal female rats on GD 15.5 (A), GD 17.5 (B), GD 19.5 (C) and GD 21.5 (D). BrdU-labeled cells were detected in epithelial and mesenchymal cells in both Müllerian and Wolffian ducts (arrows). Staining was abolished by incubating with PBS instead of primary antibody (E). M: Müllerian duct, W: Wolffian duct. Bar: 50 µm.

Cell proliferation levels in the Wolffian epithelium and mesenchyme are shown in Fig. 2C and D respectively. On GD 15.5, cell proliferation in the Wolffian epithelium and mesenchyme was high at 22–36% and 31–38% respectively in all regions (Fig. 2C and D). On GD 17.5, cell proliferation in the Wolffian mesenchyme remained high at 20–33% in all regions, but epithelial proliferation dramatically decreased to 3.0 ± 0.54 , 8.8 ± 1.78 and $21.0 \pm 1.36\%$ in proximal, middle and caudal regions

respectively (Fig. 2C and D). By GD 19.5, the Wolffian duct had degenerated and could not be observed in the specimen.

Ontogenetic immunolocalization of EGF

Staining specificity of polyclonal anti-EGF antibody is illustrated in Fig. 3. Positive staining was detected in the glandular convoluted tubule cells of submandibular glands

Figure 2 Cell proliferation of Müllerian epithelium (A), Müllerian mesenchyme (B), Wolffian epithelium (C) and Wolffian mesenchyme (D) during fetal rat development in the absence of DES. The caudal Müllerian duct region was not yet formed by GD 15.5 (A, B). Cell proliferation in Müllerian epithelium and Müllerian mesenchyme decreased in all regions with development. In the Wolffian duct, epithelial proliferation decreased sharply in all regions on GD 17.5 compared with GD 15.5. The Wolffian duct had fully regressed by GD 19.5 (C, D). Data are represented as means $\pm$ S.E.M. ($n=6$). a and b: $P < 0.05$ and $P < 0.01$ vs GD 15.5 respectively. c: $P < 0.01$ vs GD 17.5. d and e: $P < 0.05$ and $P < 0.01$ vs GD 19.5 respectively. The S.E.M. value for GD 15.5 in (A) is very small and cannot be depicted.

from adult male rats and confirmed EGF immunoreactivity (Poulsen *et al.* 1986, Fig. 3B). No positive immunoreactivity was detected when the specimen was incubated with normal rabbit immunoglobulin or pre-absorbed anti-EGF antibody (Fig. 3A and C), confirming antibody specificity.

Ontogeny of EGF protein expression in the female rat reproductive tract is summarized in Table 1. Cell-type-specific expression of EGF was observed. In the Müllerian

duct, epithelial cells in the proximal and middle regions showed intense EGF staining on GD 15.5 and 17.5, and staining remained intense in the proximal region on GD 19.5 (Fig. 4A, B, D, E and G). In the caudal Müllerian epithelium, EGF staining was weak on GD 17.5, and was also weak in the middle and caudal regions on GD 19.5 and throughout all regions by GD 21.5 (Fig. 4F, H–L). On the other hand, Wolffian epithelial cells showed marked immunoreactivity on GD 15.5 and 17.5 in all regions

Figure 3 Staining specificity of polyclonal anti-rat EGF antibody. Paraffin sections of submandibular glands of adult male rat immunostained with normal rabbit serum (A), anti-EGF antibody (B) or anti-EGF antibody pre-absorbed with EGF peptide (C). Positive immunostaining was detected in the granular convoluted tubule cells (B). No immunostaining was observed with normal rabbit serum (A), or with pre-absorbed antibody (C). Bar: 50 μ m.

except for the caudal region on GD 17·5 (Fig. 4A–F). Mesenchymal cells in both the Müllerian and Wolffian ducts showed weak or no EGF staining in all regions during the examination period (GD 15·5–21·5).

Table 1 Ontogenetic immunolocalization of EGF in the fetal female rat reproductive tract

	GD	Region	Epithelium	Mesenchyme
Müllerian duct	15·5	Proximal	+	±
		Middle	+	±
		Caudal	NF	NF
	17·5	Proximal	+	—
		Middle	+	±
		Caudal	±	±
	19·5	Proximal	+	—
		Middle	±	±
		Caudal	±	±
	21·5	Proximal	±	—
		Middle	±	—
		Caudal	±	—
Wolffian duct	15·5	Proximal	+	±
		Middle	+	±
		Caudal	+	±
	17·5	Proximal	+	±
		Middle	+	±
		Caudal	±	±

+: marked staining; ±: slight staining; –: not detected; NF: not formed.

Effects of prenatal DES exposure

Effects of DES (100 μ g/kg, GD 15–19) on cell proliferation was examined in the Müllerian duct on GD 19·5. Cell proliferation was altered by DES in a region- and cell-type-specific manner. In the proximal region, a 2- to 3-fold increase in cell proliferation was observed in DES-exposed Müllerian epithelium and mesenchyme, compared with oil-exposed tissues (Fig. 5). In contrast, DES decreased cell proliferation in the caudal epithelium (Fig. 5). DES had no effect on cell proliferation in mesenchymal cells in the caudal region or in epithelial and mesenchymal cells in the middle region (Fig. 5).

To understand the effects of DES on EGF and ER α expression in the fetal female rat reproductive tract, immunohistochemistry was performed in control and DES-exposed tissues on GD 19·5. DES inhibited EGF expression in the Müllerian epithelium in both the proximal and middle regions, but no influence was observed in the Müllerian mesenchyme (Fig. 6). Positive nuclear staining of ER α was detected in the Müllerian duct using monoclonal anti-ER α antibody. Binding specificity of the antibody to rat ER α was previously evaluated by Fisher *et al.* (1997). To confirm binding specificity in the present study, adult rat uterus and ovary were immunostained with this antibody. In uterus, intense staining was observed in both luminal and glandular epithelia and in stromal and myometrial tissues (Fig. 7A). In ovary, theca cells, interstitial cells and germinal epithelia also showed intense staining (Fig. 7B). ER α staining was abolished by incubation with normal mouse IgG1 in uterus (Fig. 7C) and ovary (Fig. 7D). These observations were in agreement with previous reports (Hiroi *et al.* 1999, Pelletier

Figure 4 Immunolocalization of EGF in the fetal female rat reproductive tract. The reproductive tract subdivided into proximal (A, D, G and J), middle (B, E, H and K) and caudal (C, F, I and L) regions was immunostained with anti-EGF antibody on GD 15.5 (A–C), GD 17.5 (D–F), GD 19.5 (G–I) and GD 21.5 (J–L). Positive immunostaining was observed in Müllerian epithelium and Wolffian epithelium on GD 15.5, GD 17.5 and GD 19.5 (A–I). EGF was decreased in the proximal epithelium on GD 19.5 and GD 21.5 (G–L). M: Müllerian duct, W: Wolffian duct. Bar: 50 µm.

Figure 5 Effect of DES on cell proliferation in the GD 19.5 Müllerian ducts. DES stimulated cell proliferation in epithelium and mesenchyme of the proximal region, but reduced it in epithelium of the caudal region. Data are represented as means $\pm$ S.E.M. ($n=3-6$). * $P<0.05$, ** $P<0.01$.

et al. 2000). In the oil-exposed GD 19.5 reproductive tract, positive immunoreactivity for ER α was localized mainly in the proximal Müllerian epithelium, and in Müllerian mesenchyme in the middle and caudal regions (Fig. 8A–C). DES did not affect ER α expression (Fig. 8D–F).

Competitive RT-PCR was employed to examine DES influence on expression of mRNAs encoding EGF, EGF-R, ER α , ER β 1 and ER β 2 in the fetal female rat Müllerian duct. The primers used in this study are shown in Table 2. Amplified products of all target genes using these primers were detected in oil-exposed tissues, but they were abolished by omitting the reverse transcriptase, and were digested with specific restriction enzymes to the expected fragment sizes (data not shown). For quantification, the log ratio of target vs competitor was plotted against competitor DNA copy numbers. The plotted curves are shown by linear regression (Fig. 9). DES significantly inhibited expression of EGF, ER β 1 and ER β 2 (60 ± 6.8 , 41 ± 12.8 and $53 \pm 2.9\%$ respectively vs the oil group), but not that of ER α and EGF-R (Fig. 10).

Discussion

In the present study, region-specific ontogenetic changes in cell proliferation and EGF immunolocalization were

examined in the fetal female rat reproductive tract. The findings described provide highly relevant characterization of the pattern and correlation of changes in cell proliferation and EGF expression during normal gestational development in the rat. Inhibitory effects of DES on EGF, ER β 1 and ER β 2 expression were shown by immunohistochemistry and/or competitive RT-PCR. These results will serve to better understand the mechanism of DES effects on female reproductive tracts in mammals.

In female fetuses, the Wolffian duct has regulatory influences on early development of the Müllerian duct, which develops in a cranial to caudal direction during gonad formation (Dohr & Tarmann 1984, Byskov & Høyer 1994). In this study, the Müllerian duct was not yet formed at the caudal region, while the proximal and middle regions had high proliferative activity on GD 15.5. By GD 17.5, the caudal region of the Müllerian duct had formed and showed high proliferation. Morphogenesis of the proximal Müllerian duct may have been completed on GD 17.5, since a dramatic decrease in cell proliferation was observed. On GD 19.5 and 21.5, decreases in cell proliferation were also observed in the middle and caudal Müllerian duct, indicating a termination of primary morphogenesis.

In mice, cell proliferation indicated by [3 H]thymidine incorporation and immunohistochemical staining of proliferating cell nuclear antigen occurred in more than 95%

Figure 6 Effect of DES on EGF expression in the GD 19.5 Müllerian ducts. Positive epithelial EGF immunostaining was observed in the proximal, middle and caudal regions in the oil group (A–C). DES reduced epithelial EGF expression (D–F). Me: Müllerian epithelium, Mm: Müllerian mesenchyme. Bar: 50 μ m.

of uterine cells and more than 80% of oviduct cells on postnatal day 1 (Li 1994). Therefore, it was hypothesized that cell proliferation in the rat Müllerian duct would be high and then decrease during morphogenesis from mid to late gestation. Subsequently, the developing reproductive tract would show high proliferative activity again during morphological and functional differentiation after birth. Although the Wolffian duct regresses in females, cell proliferation in the Wolffian epithelium and mesenchymal stroma showed high proliferation, similar to the Müllerian duct on GD 15.5. On GD 17.5, the Wolffian duct had started to degenerate, and as a result showed a decrease in

cell proliferation. It is conceivable that there may be a critical time point for induction of Wolffian duct degeneration between GD 15.5 and 17.5, initiated in the absence of androgens (Josso & Picard 1986).

In male fetuses, testicular androgens regulate cell proliferation and differentiation of the Wolffian duct (Byskov & Hoyer 1994). The promoting effect of androgens on cell proliferation is mediated by EGF in Wolffian duct organ culture (Gupta *et al.* 1991). In female fetuses, however, primary and essential factors which regulate cell proliferation and differentiation of the Müllerian duct have not yet been identified either *in vitro* or *in vivo*. EGF and

Figure 7 Immunohistochemical staining of ER α in adult rat uterus and ovary. In uterus (A) nuclei stained positive for ER α in luminal epithelium (Le), glandular epithelium (Ge), stroma (St) and myometrium. In ovary (B), ER α nuclear staining was seen in theca cells (Th), interstitial cells (In) and granulosa cells (Gr), but not granulosa cells (Gr). Immunoreactivities of ER α and ER β were abolished by incubation with normal mouse IgG1 in uterus (C) and ovary (D). Bar: 100 μ m.

insulin-like growth factor (IGF)-I stimulate cell proliferation in immature mouse uterine and vaginal epithelial cells *in vitro* (Tomooka *et al.* 1986, Iguchi *et al.* 1987, Shiraga *et al.* 1997, 2000). Because estrogen stimulates uterine epithelial cell proliferation (McCormack & Glasser 1980, Quarmby & Korach 1984) and expression of EGF, IGF-I, heparin-binding EGF-like growth factor, transforming growth factor (TGF)- α and EGF-R in the reproductive tracts of mice and rats, these growth factors may mediate the estrogen-induced proliferative and differentiative responses in rodent reproductive tracts (Murphy *et al.* 1987, DiAugustine *et al.* 1988, Nelson *et al.* 1991, 1992, Beck & Garner 1992, Ignar-Trowbridge *et al.* 1992, Kapur *et al.* 1992, Das *et al.* 1994, Sahlin *et al.* 1994, Zhang *et al.* 1994, 1998, Falck & Forsberg 1996, Hom *et al.* 1998). In the fetal reproductive tract, expression of EGF-R in mice (Bossert *et al.* 1990), and EGF, TGF- β and IGF-I in rats (Koike & Noumura 1993, Kanno *et al.* 1994, Gu *et al.* 1999) was reported. Kanno *et al.* (1994) reported that immunoreactivity of EGF was not-detected, moderate, slight and moderate during GD 15, GD 16–17, GD 18–20 and GD 21 respectively, in the female rat Müllerian duct. They also reported that no immunoreactivity for EGF was detected in the Wolffian duct of fetal female rats. Differences in ontogenetic expression of EGF

between Kanno *et al.* (1994) and the present study may be due to differences in polyclonal antibodies used. In the present study, a parallel decrease between cell proliferation and EGF expression was observed, suggesting that EGF may be a primary factor necessary for regulation of Müllerian duct cell proliferation in fetal female rats.

A variety of reproductive tract abnormalities occurred in young women whose mothers received DES during the first trimester of pregnancy (Herbst *et al.* 1971, Herbst & Bern 1981). Perinatal treatment of mice and rats with DES also causes a variety of abnormalities in the Müllerian derivatives (Boylan 1978, McLachlan *et al.* 1980, Newbold *et al.* 1983a,b, 1984, Rothschild *et al.* 1987/88, Ozawa *et al.* 1991). While the rodent Müllerian duct involves simple straight tubes lined with a single-layered epithelium from proximal to caudal regions during prenatal life, region-specific effects of DES were found in the developing Müllerian duct, suggesting the developmental presence of different functional mechanisms for cell growth and differentiation in the various fetal Müllerian duct regions. In addition, the present study also showed cell-type-specific DES effects on cell proliferation of the Müllerian duct such as a stimulatory effect in epithelium and mesenchymal stroma at the proximal region and an inhibitory effect in epithelium at the caudal region.

Figure 8 Effect of DES on ER α expression in the GD 19.5 Müllerian ducts. In the oil group, nuclear ER α immunostaining was intense mainly in epithelium of the proximal region (A) and mesenchyme of the middle and caudal regions (B, C). DES had no effect on ER α expression in any region (D–F). Arrow: positive nuclear epithelial staining; arrowhead: positive nuclear mesenchymal staining. Me: Müllerian epithelium, Mm: Müllerian mesenchyme. Bar: 50 μ m.

The role of ERs in the effects of DES has been demonstrated (Greco *et al.* 1993, Couse *et al.* 1997). Two subtypes of ERs have been identified in rats, ER α (Koike *et al.* 1987) and ER β (Kuiper *et al.* 1996). The physiological significance of ERs in the development and differentiation of the reproductive tract has not yet been clarified; however, the importance of ERs was suggested from the phenotypes of ER knockout (ERKO) mice, α ERKO, β ERKO and $\alpha\beta$ ERKO (Lubahn *et al.* 1993, Kreye *et al.* 1998, Couse & Korach 1999, Couse *et al.* 1999). Although different expression of ER α and ER β

were demonstrated during mouse embryogenesis in various organs including those of the reproductive tract (Lemmen *et al.* 1999, Jefferson *et al.* 2000, Nielsen *et al.* 2000), mouse reproductive tract development is essentially normal during the prenatal and the neonatal stages in α ERKO, β ERKO and $\alpha\beta$ ERKO mice, suggesting that signaling through the ERs is not necessary for female reproductive tract morphogenesis. Nonetheless, the estrogen stimulatory effect on cell growth was not found in the female reproductive tract of α ERKO mice (Couse & Korach 1999). Thus, prenatally exposed DES could act

Table 2 PCR primers for the detection of various gene expressions used in this study

	Direction	Sequence (5'–3')	Amplified length (bp)	Genbank Accession No.
Gene				
EGF	Forward	AGCCATGCCCACTGTATTTC		
	Reverse	CCACCACACAGACAGACACC	598	U04842
EGF-R	Forward	GTGTGTGCAGATTGCAAAGG		
	Reverse	TAATGCAAGCCACAGTGGAA	701	M37394
ERα	Forward	AAGAGAAGGACCACATCCACC		
	Reverse	GGAATGTGCTGAAGTGGAGC	326	Y00102
ERβ1	Forward	TTCTGGACAGGGATGAGGG		
	Reverse	GGGACCACATTTTGCACCT	376	AF042058
ERβ2	Forward	GTCCTCAGAAGACCCTCACTGG		
	Reverse	GGGACCACATTTTGCACCT	420	AF042059

Figure 9 Competitive RT-PCR of (A) EGF, (B) EGF-R, (C) ERα, (D) ERβ1 and (E) ERβ2 mRNAs. Left panels are oil group, right panels DES group. Reverse-transcribed total RNA isolated from the GD 19.5 reproductive tracts (approximately 20 fetuses per group) were co-amplified with serially diluted competitor. The RT-PCR products were electrophoresed and stained with EtBr. The densities of competitor bands and target bands were quantified and the log ratio of target vs competitor was plotted against copy numbers for competitor DNA. The standard curves were obtained by linear regression. On the standard curve, the point where the log ratio equaled 0 (target/competitor=1) is considered the concentration of the reverse-transcribed mRNA.

through ERs to cause abnormalities in the female reproductive tract. In the present study, ERα, ERβ1 and ERβ2 were detected in the GD 19.5 reproductive tract of female rats by immunohistochemistry and/or competitive RT-PCR. This is the first report showing ERα, ERβ1 and ERβ2 expression in the fetal female rat reproductive tract.

Altered expression of ERα but not ERβ was reported in adult male and female reproductive tracts after neonatal DES treatments in mice and rats (Sato *et al.* 1994, 1996, Atanassova *et al.* 2001). In the present study, inhibitory effects of DES on ERβ1 and ERβ2 mRNA expression in the fetal rat reproductive tract were demonstrated by

Figure 10 DES effects on Müllerian EGF(A), EGF-R (B), ER α (C), ER β 1 (D) and ER β 2 (E) mRNA levels quantified by competitive RT-PCR. Expression levels in the oil group were designated as 100%, and relative expression levels in the DES group are presented. Significant decreases in mRNA levels of EGF, ER β 1 and ER β 2 after DES were observed. No changes in EGF-R or ER α levels were observed after DES. Data are represented as means $\pm$ S.E.M. from three independent experiments. * P < 0.05.

competitive RT-PCR. These inhibitory effects may be a result of down-regulation. DES did not alter ER α mRNA or protein. Therefore, it is hypothesized that DES may act mainly through ER β 1 and/or ER β 2 in the fetal reproductive tract.

To clarify the role of EGF in the fetal female reproductive tract after DES treatment, EGF and EGF-R were

investigated by immunohistochemistry and/or competitive RT-PCR. DES did not alter EGF-R mRNA, but EGF protein and mRNA were inhibited in the Müllerian duct. However, there was no positive correlation between changes in EGF expression and cell proliferation following DES. Therefore, EGF may not mediate DES action and other growth factors should be considered.

Recently, the correlation of *Hox* gene expression with reproductive tract morphogenesis was considered based on insights from studies of knockout mice (Satokata *et al.* 1995, Gendron *et al.* 1997, Warot *et al.* 1997). *Hox* genes are the vertebrate homologues of the *Drosophila* homeotic selector genes. The pattern and timing of genes of the *Hoxa* axis were demonstrated in the development of the Müllerian duct (Taylor *et al.* 1997). Effects of prenatal treatment of DES on *Hoxa* mRNA expression *in vivo* or in organ culture were examined (Ma *et al.* 1998, Block *et al.* 2000). In these studies, DES caused posterior shift and reduced expression in *Hoxa-9*, *-10* and *-11* in the Müllerian duct, suggesting a potential molecular explanation for teratogenic effects of DES in the developing reproductive tract. Studies clarifying the relationship of these genes with reproductive tract abnormalities caused by DES are needed.

In conclusion, the present study demonstrates that there is parallel relationship between cell proliferation and EGF expression in the Müllerian duct during normal fetal development, and that prenatal exposure to DES alters cell proliferation in a region- and cell-type-specific manner while inhibiting EGF, ER β 1 and ER β 2 expressions in the fetal reproductive tract of female rats.

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