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Original Article

# Exposure of neonatal rats to diethylstilbestrol affects the expression of genes involved in ovarian differentiation

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**Exposing neonatal rats with the synthetic estrogen, diethylstilbestrol (DES), induces morphological and functional abnormalities in the adult ovary. We examined the events that lead to this condition using female rats that were exposed to DES for the first five days after birth. The expression of steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage cytochrome P450 (P450scc), which are both required for steroidogenesis in the theca/interstitial region was markedly reduced. The expression of Müllerian inhibiting substance (MIS) was transiently increased in small growing follicles in the ovary of DES-treated rats at postnatal day 7 (P7), and the expression profile in the ovary differed between DES- and vehicle oil-treated rats at P14 and P21. The expression of the transcription factor, steroidogenic factor-1 (SF-1), reduced in theca/interstitial cells, but increased in granulosa cells of primary follicles. These results indicate that altered steroidogenesis and MIS production are mechanisms through which DES induces abnormal ovarian development, and support the notion that androgens and MIS are both critical factors in regulating early ovarian differentiation.**

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## Introduction

Diethylstilbestrol (DES) is a synthetic estrogen that induces abnormalities in the reproductive organs of mice and rats when administered during a specific developmental period[1]. In the Müllerian-derived structures of the female reproductive tract, such as oviduct, uterus, cervix and upper vagina, the developmental expression of several estrogen-regulated genes is altered after exposure to DES. This suggests that the effects of DES are exerted by estrogen receptor (ER)-mediated mechanisms to induce the abnormal development of these organs[2-4]. Although morphological and functional abnormalities of the ovaries in adult animals have been assessed[5], effects of DES on the developing ovary of DES-treated animals have not been addressed.

Steroidogenic factor (SF-1) was originally identified as a transcription factor of steroidogenic enzyme genes[5]. Studies using SF-1-deficient mice have suggested that SF-1 is a key regulator of development and function in the hypothalamic-pituitary-adrenal/gonadal axis[6,7]. In the developing ovary, SF-1 is expressed by both granulosa and theca cells from the earliest stage of differentiation[8,9], and it is thought to play an important role in the development of steroidogenesis and cellular differentiation[10]. We recently analyzed changes in the

expression of genes, including SF-1 and its target genes (StAR, P450scc, and MIS) during development of the ovary of rats exposed to estradiol benzoate (EB), a second synthetic estrogen, as neonates. Morphologically abnormal changes correlated with altered gene expression and a possible action of EB on SF-1 and the ERs[11,12].

In the present study, in order to understand the mechanism through which exogenous estrogens affect the developing ovary and also the mechanisms regulating normal ovarian differentiation, we examine gene expression in the developing ovary of rats exposed to DES during the first three post natal weeks, a period that which is critical for early ovarian differentiation.

## Materials and Methods

### Animal treatment and tissue preparation

Female Sprague-Dawley rats received a subcutaneous injection of DES (5 $\mu$g/0.02 ml) dissolved in olive oil or olive oil alone (controls) for five consecutive days starting within 24 hr of birth. The day of birth was considered postnatal day 0 (P0). Groups of untreated, control, and DES-treated rats were killed on P7, P14, and P21. Ovaries were dissected and either fixed in 4% ice-cold paraformaldehyde in 0.1 M PBS or processed for RNA extraction. The fixed tissues were embedded in paraffin, sectioned at 6 $\mu$m thickness, mounted on silane-coated slides, and processed for *in situ* hybridization. To prepare total RNA, two to three ovaries from each group were lysed in Trizol (GIBCO BRL) and RNA was purified according to the manufacturer's instructions. Tissues were collected from groups of more than five animals from each of the groups at P7, P14 and P21, and experiments were repeated four times. All animals were handled in accordance with the principles and procedures in "Guidelines for Animal Experimentation, Tokyo Medical and Dental University".

### Semi-quantitative RT-PCR

Semi-quantitative RT-PCR proceeded as described with some modifications[11]. To discriminate products of RT-PCR from those derived from contaminating genomic DNA, forward and reverse primers were designed for different exons of each of the tested genes. The oligonucleotide primer sequences for amplification of SF-1, StAR, P450scc, MIS, and GAPDH cDNAs were as described[11]. The predicted sizes of the PCR products were as follows: StAR, 247bp; P450scc, 525 bp; SF-1, 463 bp; MIS, 233 bp; GAPDH, 452bp. First-strand cDNA was synthesized from 2 $\mu$g of total RNA in a final volume of 20 $\mu$l containing 7.5 $\mu$M Oligo $(dT)_{12\text{-}18}$ primer using the SUPERSCRIPT preamplification system (GIBCO BRL) according to the manufacturer's instructions. An aliquot (1 $\mu$l) of the reaction mixture was amplified with AmpliTaq Gold DNA polymerase (Perkin Elmer) as specified by the manufacturer. The PCR conditions were denaturation at 95°C for 5 min followed by 25-37 cycles of denaturation at 95°C for 30 sec, annealing for 30 sec and extension at 72°C for 1 min with a final extension at 72°C for 10 min. The optimal conditions for linearity to permit semiquantitative analysis of signal length were determined and the number of PCR cycles for each gene is indicated in Figure 1. Annealing temperatures were 50°C for SF-1, StAR, and P450scc, and 55°C for MIS and GAPDH. The PCR products were resolved by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining (0.1 mg/ml). The density of each PCR product was determined using an image analysis system (LAS-1000 plus, FUJIFILM), and the values for target genes were normalized with respect to those of GAPDH. Semi-quantitative data are expressed as means$\pm$S.E.M. from three independent experiments. Statistically significant differences between groups were determined by one-way ANOVA, followed by Tukey's test. $P<0.05$ was considered statistically significant. The specificity of PCR products was confirmed by DNA sequencing using a fluorescent dye terminator and an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer).

### Immunohistochemistry

We used an antibody generated in our previous study to detect SF-1 and techniques for immunohistochemistry were performed as described[11].

### *In situ* hybridization

Techniques for *in situ* hybridization were performed using a kit according to the manufacturer's protocol (Novagen). Sense and antisense probes were generated for P450scc[13], StAR[14], SF-1[5,] and MIS[15]. Emulsion-coated slides were exposed for one or two weeks at 4°C. Control studies used the sense probe and signals higher than the background were undetectable.

## Results

We investigated the molecular basis for the effects of DES on ovarian development. Semi-quantitative RT-PCR evaluated expression levels of SF-1 and some of its target genes, including StAR, P450scc and MIS in the ovary of oil-treated rats (oil-ovary) and the ovary of DES-treated rats (DES-ovary) at P 7 and P14 (Fig. 1). Both StAR and P450scc mRNAs were significantly reduced in DES- than in oil-ovaries at all stages investigated. The mRNAs for SF-1 and MIS in DES-ovaries appeared slightly decreased and increased, respectively, compared to oil-ovaries, but these differences were not significant (Fig. 1B).

To further determine whether the localization of these genes changes, we performed *in situ* hybridization. In oil-ovaries, signals for P450scc (Fig. 2A, C, and E) and StAR (Fig. 2G, I, and K) mRNAs were essentially restricted to the interstitial region from P7 through P21, with the exception of weak signals for StAR mRNA within follicles only at P7 (Fig. 2G). Consistent with the RT-PCR analysis, signals for these genes were either faint or absent in DES-ovaries throughout development (Fig. 2B, D, F, H, J, and L).

At P7, signals for MIS mRNA were detected in follicles located within the medulla, but the intensity varied between follicles. Follicles located in the outer region exhibited more intense signals than those in the inner region (Fig. 3A). However, signal intensity did not vary in DES-ovaries, being uniformly intense in almost all MIS-positive follicles (Fig. 3B). At P14 and P21, signals were restricted to follicles located in the periphery of oil-ovaries (Fig. 3C and E), whereas signals in most follicles in DES-ovaries were intense (Fig. 3D and F).

*In situ* hybridization signals for SF-1 mRNA were detected in both follicles and in the interstitial region of oil- and DES-ovaries throughout development, but we did not identify specific differences between the two groups (Fig. 4A-D). However, an immunohistochemical analysis revealed some differences in the cellular level of SF-1 protein expression between the two groups. At P7, primary follicles in DES-ovaries were more SF-1-immunoreactive (SF-1-ir) than those in oil-ovaries (Fig. 4E and F). Expression of SF-1 in primary follicles of DES-ovaries was no longer elevated at P14 and P21 (Fig. 4E and F, and data not shown). At these times, SF-1-ir interstitial cells were fewer in DES-ovaries than in oil-ovaries, indicating underdevelopment of the interstitial region in DES-ovaries.

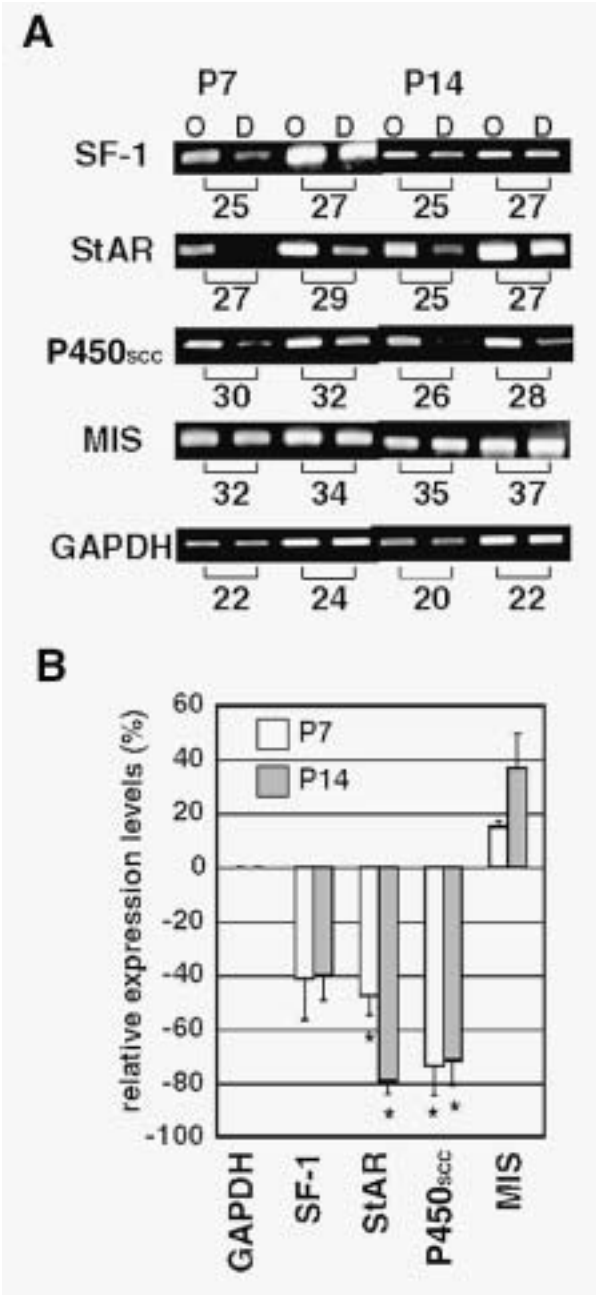


**Fig. 1.** RT-PCR analysis of SF-1 related genes during development of DES-treated rat ovary. RNA was prepared from oil- (O), and DES (D)-treated rat ovaries on the indicated days after birth. A: Photographs of ethidium bromide-stained gels. GAPDH served as internal control. Numbers of PCR cycles are indicated below each picture. B: Relative expression levels of SF-1 related genes in DES-treated rat ovaries. Densitometric values of each PCR product were normalized to those of GAPDH. Expression levels of each gene in DES-treated ovaries compared with controls (oil) are presented as means±S.E.M. from three independent experiments. Asterisks indicate significant difference ($P<0.05$) from control values.

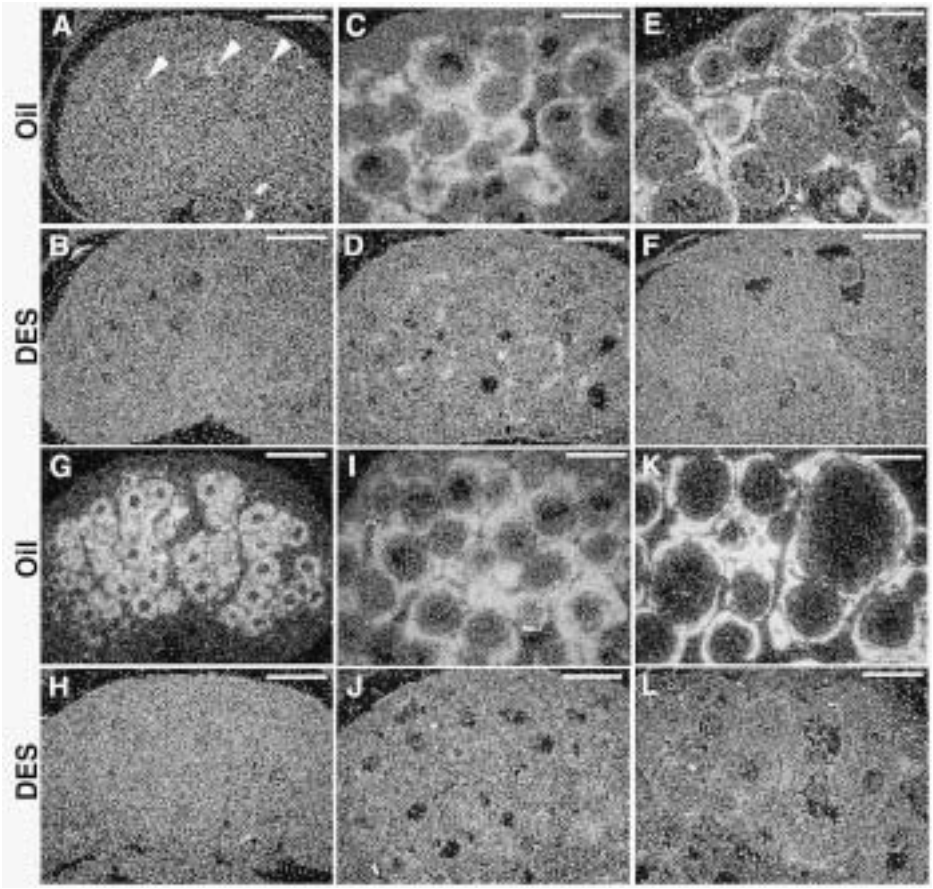


**Fig. 2.** Localization of P450scc and StAR mRNAs in the DES-treated rat ovary during postnatal development. Photomicrographs A-F and G-L are representative dark-field images of signals for P450scc and StAR mRNAs, respectively, in ovarian sections at P7 (A, B, G and H), P14 (C, D, I and J), and P21 (E, F, K and L) from oil- (Oil) and DES-treated (DES) rats. Arrowheads in (A) indicate signals for P450scc mRNA in the oil-ovary at P7. In DES-treated ovaries, no or very-low-intensity signals of P450scc and StAR mRNAs were evident at P7, P14 and P21. Scale bars = 200 $\mu$m.

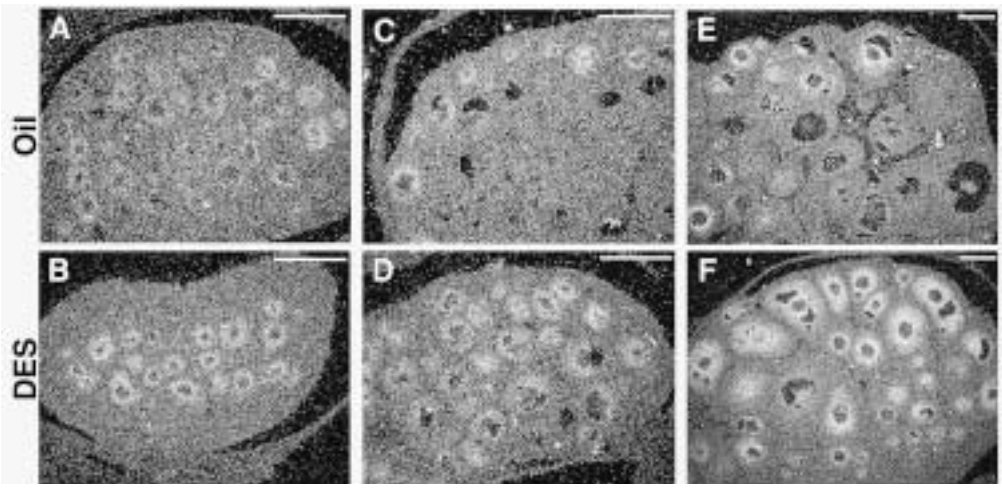


**Fig. 3.** Expression of MIS mRNA in DES-treated rat ovary during postnatal development. Photomicrographs A-F are representative images of expression of MIS mRNA in ovarian sections cut at the central level from oil- (Oil) and DES-treated (DES) rats at P7 (A and B), P14 (C and D), P21 (E and F). Scale bars = 200 $\mu$m.

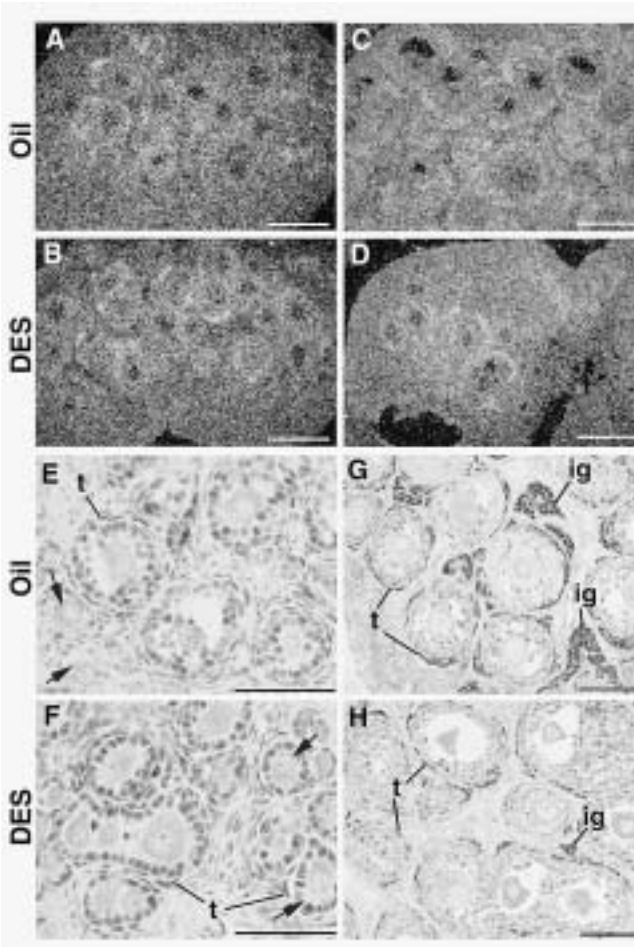


**Fig. 4.** Expression of SF-1 mRNA and protein in DES-treated rat ovary during postnatal development. Photomicrographs A-D and E-H are representative images of SF-1 mRNA and protein expression, respectively, in ovarian sections cut at the central level from oil- (Oil) and DES-treated (DES) rats at P7 (A, B, E, and F) and P14 (C, D, G and H). ig, interstitial gland; t, theca. Arrows in E and F show primary follicles, demonstrating that SF-1 expression in primary follicles is higher in the DES-treated rat ovary than in controls. Scale bars = 200 $\mu$m.

## Discussion

The mechanism that controls early follicle development is unknown. MIS is a member of the TGF$\beta$ superfamily of growth and differentiation factors that is responsible for Müllerian duct regression during male fetal development[16]. This factor is also expressed in the granulosa cells of small growing follicles in the ovary[17,18], and is indicated to act a negative regulator of the recruitment of primordial follicles. The present study showed that expression of MIS is increased in primary follicles in DES-ovaries at P7, which coincided with the increased expression of SF-1. Since SF-1 acts as a transcriptional activator of the MIS gene[15,19], the inhibition of primordial follicle development might be due to abnormally high levels of MIS produced by primary follicles, and MIS up-regulation may be attributed to the estrogen-induced elevation of SF-1 expression. However, the mechanism by which DES induces SF-1 remains to be elucidated.

The decreased expression of both StAR and P450scc genes by DES indicates decreased steroidogenesis, which we also detected in EB-treated rat ovaries[11]. The results from this and previous studies suggest inhibitory actions of estrogens on steroidogenesis[11,20-23]. Despite a significant reduction of the steroidogenic genes, the expression of SF-1, a transcriptional activator of both StAR and P450scc, was not significantly changed between DES- and oil-ovaries. This suggested that down-regulation of the steroidogenic genes may be mediated via a factor(s) other than SF-1, although SF-1 may work in concert with other factors to contribute to this suppression. Alternatively, the reduction of steroidogenesis could be mediated indirectly through granulosa cells. Since MIS might act in a paracrine fashion to inhibit steroidogenesis in interstitial cells by blocking steroidogenic gene expression[24], the increased MIS expression in primary follicles in DES-ovaries might be involved in the inhibition of steroidogenesis. These results indicate that DES affected the functional differentiation of theca cells into steroid (androgen) -producing cells.

Neonatal treatment of rats with either EB or DES induces similar changes in expression of SF-1 and its target genes including StAR, P450scc and MIS, during early ovarian differentiation[11,12]. These findings suggest that altered expression of these genes is a mechanism that induces abnormal ovarian development. The similar effects of these different chemicals on the developing ovary indicate a common mechanism in the induction of abnormal ovarian development by exogenous estrogen. Androgens and MIS are essential for male sexual development. Our results indicate that they also play important roles in ovarian differentiation in females. The data further suggest that autocrine and paracrine actions of factors such as androgens and MIS on granulosa and theca/interstitial cells and the highly coordinated interactions between granulosa and theca/interstitial cells contribute to regulating ovarian differentiation.

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