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Estrogens modulate the gene expression of Wnt-7a in cultured endometrial adenocarcinoma cells

Jörg Wagner and Leane Lehmann

Institute of Applied Biosciences, Section of Food Chemistry and Toxicology, University of Karlsruhe, Kaiserstraße, Karlsruhe, Germany

The glycoprotein Wnt-7a participates in a signaling pathway that transmits information among uterine cell types. Disruption of this pathway by the transplacentally acting carcinogen diethylstilbestrol (DES) is associated with morphological abnormalities of the female reproductive tract (FRT). This raises the question whether estrogens in the diet might also interfere with this pathway. Therefore, this study investigated the influence of the steroid hormone 17β-estradiol (E2), the mycotoxin zearalenone (ZEN), the soy phytoestrogen genistein (GEN), and DES on the expression of Wnt-7a in an endometrial adenocarcinoma cell line (Ishikawa cells) by reverse transcription/competitive PCR. In addition, the enzymatic activity of alkaline phosphatase (ALP) was determined, which is estrogen receptor (ER)-dependently regulated in Ishikawa cells. After treatment of Ishikawa cells with E2, ZEN, GEN, and DES, a decrease in the gene expression of Wnt-7a was observed. Maximum effect (50% reduction) was observed after treatment with concentrations that induced maximum expression of the ALP. Experiments in the presence of the ER antagonist (ICI 182,780) suggested that the ER is involved in the regulation of Wnt-7a in Ishikawa cells. In conclusion, interference with the expression of Wnt genes in the FRT might be a novel mechanism by which estrogens disrupt the function of the FRT.

Keywords: 17β-estradiol / Diethylstilbestrol / Genistein / Ishikawa cells / Zearalenone

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1 Introduction

Genes of the Wnt family are involved in signal transduction cascades important for development and carcinogenesis [1]. Wnt-7a participates in a signaling pathway that transmits information among different uterine cell types, thus mediating uterine architecture during development as well as the function of the adult female reproductive tract (FRT). Disruption of this pathway is associated with morphological abnormalities of the FRT [2]. Mice treated neonatally or *in utero* with the transplacentally acting carcinogen diethylstilbestrol (DES, Fig. 1) exhibit such abnormalities, thus indicating involvement of Wnt signaling in uterine carcinogenesis [3]. Estrogen receptor (ER)-alpha knockout mice are resistant to the developmental effects of neonatal DES exposure on the FRT [4]. This raises the question whether estrogens in the diet might also interfere with this pathway. In particular, the developing FRT of fetuses *in utero* and babies consuming a soy-based diet could be vulnerable.

The aim of the present study was to determine the influence of the endogenous steroid hormone 17β-estradiol (E2, Fig. 1), the estrogenic mycotoxin zearalenone (ZEN, Fig. 1), the soy phytoestrogen genistein (GEN, Fig. 1), and the synthetic estrogen DES on the gene expression of Wnt-7a in an endometrial adenocarcinoma cell line (Ishikawa cells). The role of the ER was also examined by determining the activity of the enzyme alkaline phosphatase (ALP), which is regulated by the ER in Ishikawa cells [5].

Correspondence: Dr. Leane Lehmann, Institute of Applied Biosciences, Section of Food Chemistry and Toxicology, University of Karlsruhe, Kaiserstraße 12, D-76131 Karlsruhe, Germany
E-mail: leane.lehmann@lmc.uni-karlsruhe.de
Fax: +49-721-608-7255

Abbreviations: ALP, alkaline phosphatase; **DES,** diethylstilbestrol; **E2,** 17β-estradiol; **ER,** estrogen receptor; **FRT,** female reproductive tract; **GEN,** genistein, 5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one; **HPRT,** hypoxanthine guanine phosphoribosyltransferase; **ICI 182,780,** 7-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]-estra-1,3,5(10)-triene-3,17-diol; **ZEN,** zearalenone, 3,4,5,6,9,10-hexahydro-14,16-dihydroxy-3-methyl-1H-2-benzoxacyclotetradecin-1,7(8H)-dione

2 Materials and methods

2.1 Chemicals

E2, DES, ZEN, and GEN were obtained from Sigma (Taufkirchen, Germany). 7-[9-[(4,4,5,5,5-pentafluoropentyl)sul-

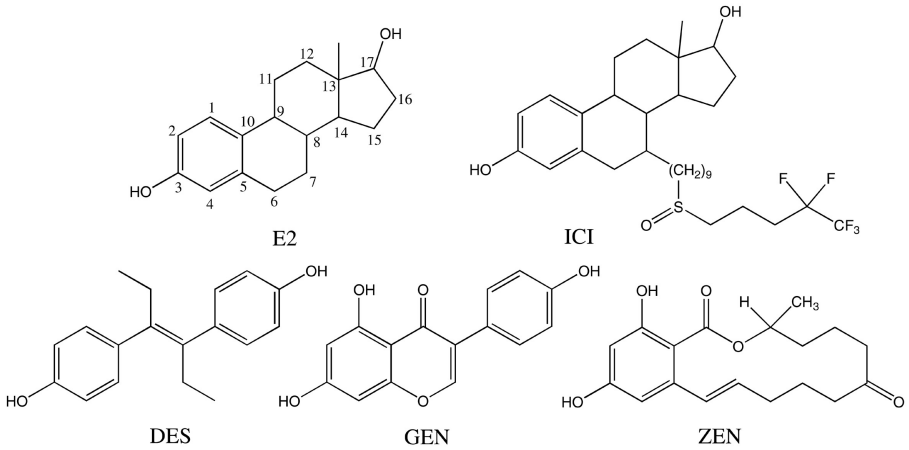

Figure 1. Chemical structures of E2, ICI 182,780, DES, GEN, and ZEN.

finyl]nonyl]-estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) was purchased from Tocris (Bristol, UK). All other chemicals, cell culture media, and medium supplements were obtained from Sigma or Roth (Karlsruhe, Germany) if not specified otherwise.

2.2 Cell culture conditions

Ishikawa cells [6] were kindly provided by Dr. Ken Korach (National Institute of Environmental Health Sciences, USA). Cells were cultured in DMEM: F12 with 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and supplemented with 10% fetal calf serum (FCS, Life Technologies, Karlsruhe, Germany).

2.3 ALP assay

The ALP assay was conducted as described elsewhere [5] with slight modifications. Briefly, Ishikawa cells were seeded in six-well plates (Nunc, Wiesbaden, Germany, 6×10^5 cells *per* well) in medium containing 5% charcoal/dextrane-treated FCS (Perbio Science, Bonn, Germany) 24 h prior to incubation with various concentrations of E2, DES, ZEN, or GEN with and without coincubation with 1 µM ICI 182,780. After 72 h, the activity of ALP was assessed by photometric measurement of the conversion of 4-nitrophenylphosphate to 4-nitrophenol as follows: Plates were washed three times with PBS free of calcium and magnesium (PBS-CMF) pH 7.4 and lysed at –80°C for at least 20 min. Cells were then thawed for 5 min at 20°C, and 1.5 mL of 4-nitrophenylphosphate (5 mM in a buffer containing 1 M diethanolamine and 0.24 mM $MgCl_2$, pH 9.8, 4°C) was added to each well. After 5 min at room temperature, the absorption was measured at 420 nm with a microplate reader (Genios, Tecan, Crailsheim, Germany) every 10 min for 1 h.

2.4 Expression of the Wnt-7a gene

Ishikawa cells were seeded in six-well plates and treated with the compounds as described above. After 48 h, which induced maximum effect according to pilot studies, total RNA was isolated using the GenElute total RNA isolation kit (Sigma). Contaminating traces of DNA were digested with DNase (Sigma), and 1 µg total RNA was reverse transcribed with 200 U M-murine lymphoma virus (MuLV) reverse transcriptase (Fermentas, St. Leon-Rot, Germany) using oligo$(dT)_{18}$ primer (Fermentas) according to the instruction of the manufacturer. In addition to Wnt-7a, the expression of the housekeeping gene hypoxanthine guanine phosphoribosyltransferase (HPRT) was determined as reference in order to compensate for variations in RNA isolation and reverse transcription as described previously [7]. The primers (Hermann GBR, Freiburg, Germany) for the cDNA of Wnt-7a were as follows: Wnt-7a forward 5′-cttcctgaagatcaagaagccactgtc, Wnt-7a reverse 5′-ctgcacgtgttgcacttgacatagcag, Wnt-7a linker 5′-cacttgacatagcaggaggtcacagccgct. Amplification conditions were: 94°C for 3 min; then 30 cycles: 94°C, 30 s; 53°C, 1 min; 72°C, 1 min; then 72°C for 5 min. PCR products were prestained with SYBR®Green and separated on a 3% agarose gel in TRIS acetate–EDTA buffer (TAE). Electrophoresis was conducted at 5 V/cm (Biorad, München, Germany) for 1 h at 20°C. PCR products were visualized after UV-excitation, photographed digitally (DIANA, Raytest, Straubenhardt, Germany) and the fluorescence intensities of the PCR product of the standard (Wnt-7a, 184 bp; HPRT, 176 bp) and of the target (Wnt-7a, 270 bp; HPRT, 214 bp) were quantified using Metaview (Molecular Devices, Sunnyvale, USA). DNA standards were generated by PCR as described by Anderson *et al.* [8] and quantified fluorimetrically using SYBRGreen (Invitrogen, Karlsruhe, Germany).

2.5 Statistical methods

Data are expressed as mean ± SD of at least three individual experiments (new batch of cells, fresh solution of test com-

pound), and the *t*-test of significant differences was conducted using the Origin® program of Microcal™ Software.

3 Results

The experiments were conducted in cultured Ishikawa cells. These human endometrial adenocarcinoma cells are known to express predominantly ERβ [6]. When the mRNA of both ERs were determined by RT-PCR, the mRNA of ERβ was also detected and accounted for about one tenth of the mRNA of ERα as determined by comparative PCR (Lehmann, unpublished data). ALP enzyme activity in Ishikawa cells is specifically stimulated by estrogens; antiestrogens completely block the induction of ALP activity by E2 [5]. For the assessment of the estrogenic potential of the estrogens of interest, Ishikawa cells were treated with various concentrations of E2, DES, ZEN, and GEN, and the activity of ALP was determined after 72 h.

Treatment with physiologic concentrations of the endogenous steroid hormone E2 induced the activity of ALP in a concentration-dependent manner (Fig. 2). After treatment with 10 nM and more E2, ALP activity plateaued at about 16-fold induction compared to solvent control (0.1%

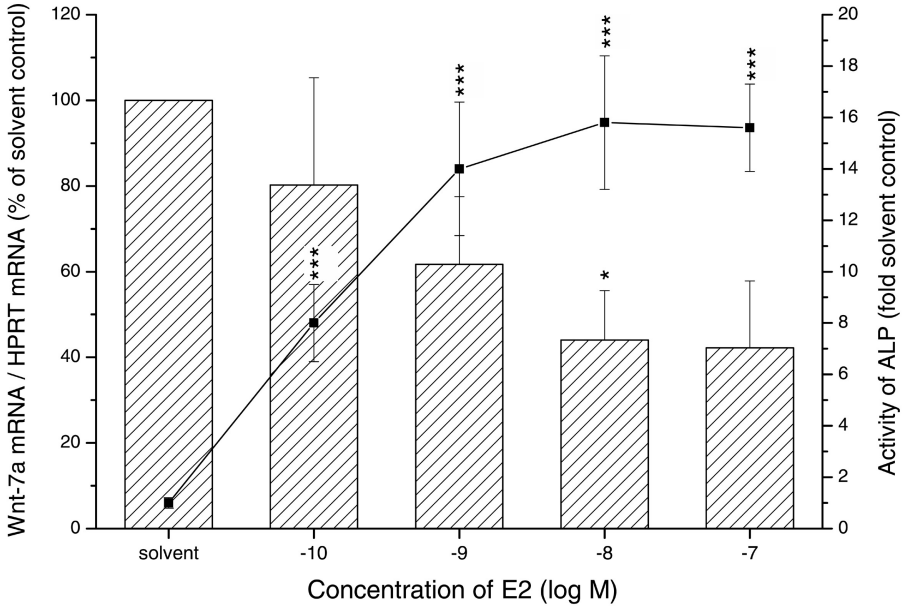

Figure 2. Relative Wnt-7a mRNA levels (columns, left *Y*-axis) and relative activity of ALP (line/symbol, right *Y*-axis) after treatment of Ishikawa cells with solvent alone (0.1% DMSO), or with various concentrations of E2 for 48 h (mRNA) or 72 h (ALP activity). Data represent mean ± SD of at least three independent experiments. Asterisks, significantly different from corresponding solvent control; *, $p = 0.05$; ***, $p = 0.01$; (*t*-test).

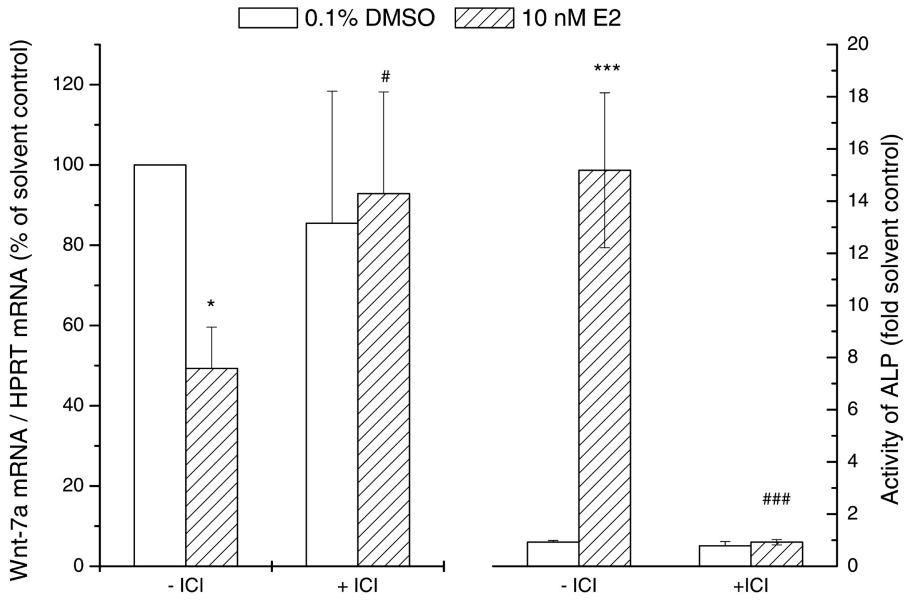

Figure 3. Relative Wnt-7a mRNA levels (left) and relative activity of ALP (right) after treatment of Ishikawa cells with solvent alone (0.1% DMSO), or 10 nM E2 in the absence (–ICI) and presence (+ICI) of 1 μM ICI 182,780 for 48 h (mRNA) or 72 h (ALP activity). Data represent mean ± SD of at least three independent experiments. Asterisks, significantly different from corresponding solvent control; crossbars, significantly different from experiments without ICI (–ICI); *, # $p = 0.05$; ***, ### $p = 0.01$; (*t*-test).

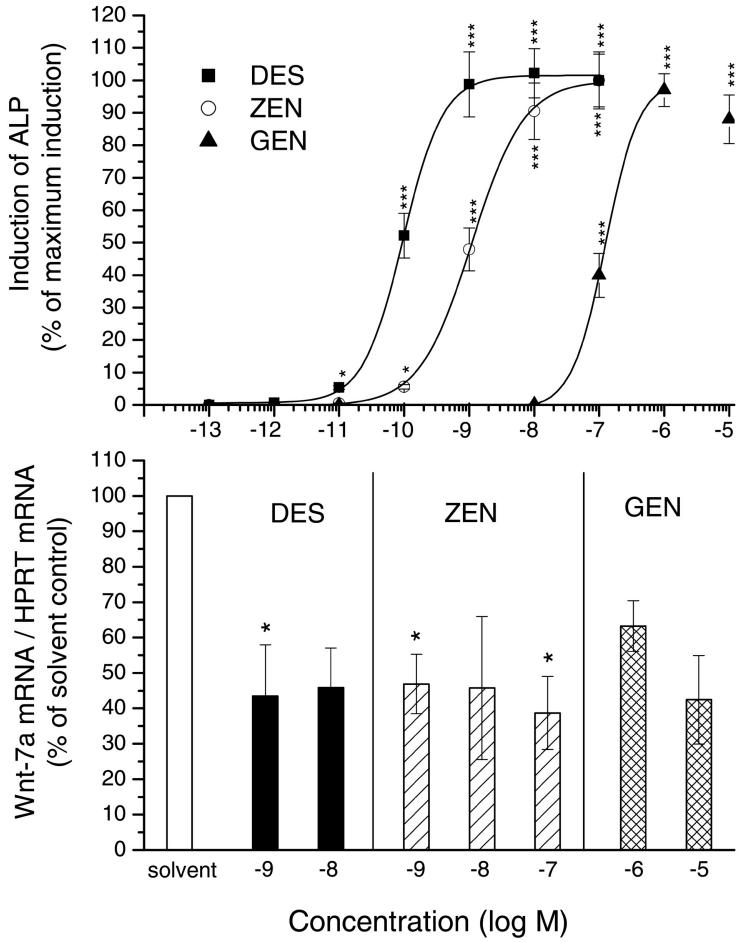

Figure 4. Relative activity of ALP (top chart) and relative Wnt-7a mRNA levels (bottom chart) after treatment of Ishikawa cells with solvent alone (0.1% DMSO), DES, ZEN, or GEN for 48 h (mRNA) or 72 h (ALP activity). Data represent mean ± SD of three independent experiments. *, $p = 0.05$; ***, $p = 0.01$ (*t*-test).

DMSO, Fig. 2). In contrast, the expression of Wnt-7a mRNA was reduced by treatment with concentrations of E2 that induced the expression of ALP (Fig. 2): treatment with 10 nM E2 (maximum induction of ALP) for 48 h reduced the expression of Wnt-7a mRNA to about 50% compared to the solvent control (Fig. 2). The E2-induced increase of the activity of ALP was completely inhibited by the ER antagonist ICI 182,780 (Fig. 3). The E2-induced reduction of Wnt-7a expression was also reversed to some extent by 1 μM ICI 182,780 (Fig. 3), thus indicating that the down-regulation of Wnt-7a in Ishikawa cells is partly mediated by the ER.

Treatment of Ishikawa cells with DES, ZEN, and GEN in estrogen-active concentrations for 48 h also caused a decrease in the gene expression of Wnt-7a: maximum effect (50% reduction) was observed after treatment with concentrations of the compounds that induced maximum expression of ALP (Fig. 4). Thus, the relative Wnt-7a expression in our experiments correlated inversely with ALP induction.

4 Discussion

DES reduced the expression of Wnt-7a in Ishikawa cells. A similar reduction has been reported for the expression of Wnt-7a in the endometrial epithelium of DES-exposed neonatal mice [9]. The uterus of neonatal mice is only susceptible to this effect of DES for a few days, yet the uterine architecture is irreversible disrupted even if no difference in Wnt-7a expression is observed at later time points [9]. This complicates the interpretation of studies concerning the expression of Wnt-7a in the endometrium of adult women and makes the development of a suitable *in vitro* test system difficult. However, in spite of its origin from a uterine adenocarcinoma, the Ishikawa cell line seems to reflect at least the response to the disruption of endometrial signaling processes by DES. Further studies with steroid hormones and selective ER modifiers are needed to evaluate the test system and elucidate whether the reduction of Wnt-7a expression is induced by every estrogen-active compound and generally correlates with estrogenic potential.

Soy food and soy-based nutritional supplements are currently gaining much popularity due to their putative benefi-

cial health effects. In particular, the high content of the isoflavones GEN and daidzein in soy is believed to account for the protection against cancer of the breast and prostate as well as against cardiovascular diseases and osteoporosis [10]. The chemoprotective effect of GEN is restricted to exposure in early life stages [11], so that a soy-based diet of infants is discussed to exert beneficial health effects in adult life. The daily exposure of infants to isoflavones in soy-based infant-formulas is 6- to 11-fold higher on a body-weight basis than the dose that has hormonal effects in adults consuming soy foods [12]. Using an experimental animal model previously reported to result in a high incidence of uterine adenocarcinoma after neonatal DES exposure, it has been shown that GEN is carcinogenic if exposure occurs during critical periods of FRT differentiation [13]. Furthermore, unconjugated GEN crosses the rat placenta at maternal serum levels that are relevant to those observed in humans after ingestion of a soy diet [14]. The mycotoxin ZEN is a potent estrogenic food contaminant, which has been detected predominantly in corn products, but recently also in soy-based infant cereals [15].

The present study demonstrates for the first time that GEN as well as ZEN influence the expression of Wnt-7a in a human endometrial cell line in the same way as the known human carcinogen DES. In concordance with studies in mice [4], the ER seems to play a major role in the regulation of Wnt-7a in human cells as well. Since maintenance of Wnt-7a expression during critical periods in the development of the FRT is crucial for correct architecture and function of the FRT in adult life [3], interference with the expression of Wnt genes in the developing FRT might be a novel mechanism by which estrogens disrupt the function of the FRT. In order to further elucidate the role of the ER, experiments in Ishikawa cells expressing a nonfunctional ER and selective transfection studies with ERα and ERβ are in progress and will yield more information about the cellular processes involved in the regulation of Wnt-7a in endometrial cells.

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