Diethylphthalate, Possible Interactions in Fetal Brain Development

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Abstract: Many natural and synthetic compounds, including a variety of the chemicals used as plasticizers or in the production of cosmetics and therapeutics, have steroid agonist or antagonist activities, altering hormone-regulated gene expression. The phthalate (diethylphthalate, extensively used as a plasticizer and in consumer products, are evaluated using the human renal epithelial cell line 293T/17. Emphasis of the study was on genes essential for central nervous system development or function. Cells were treated with 1, 10 or 100 μM phthalate and gene expression was measured in treated cells, showing significant up- or down-regulation of a large number of genes in treated compared to untreated cells. Of the 19,000 human genes on the DNA array chip utilized, two specific genes, *FGD1* and *NGPF2*, were selected to corroborate mRNA levels using quantitative real time PCR (qrtPCR) data to confirm results obtained from the microarray determinations. *FGD1* (faciogenital dysplasia) and *NGPF2* (neurite growth-promoting factor 2, also called Midkine, *MDK*), showed a significant, possibly estrogen-synergistic, down-regulation of genes essential for fetal brain development. These studies were designed to provide data on the gene expression-altering capacity of a widely distributed chemical, diethylphthalate (DEP), and to show possible associations between the previously reported widespread presence of DEP and the DEP metabolite, MEP, in urine samples from a reference population, the potential for altered gene expression in human cells *in vitro*, and possible neurodevelopmental effects that could be correlated with *in utero* exposure to DEP.

Key Words: Gene expression, DNA microarray analysis, plasticizers, phthalates, qrtPCR.

INTRODUCTION

The phthalates, diesters of phthalic acid, are a group of organic chemicals used in the production of a variety of personal, consumer, and medical products [1]. They are widely used in the manufacture of a number of products made from polyvinylchlorides (plastics) which have been softened for injection or extrusion molding by the inclusion of one or more of a variety of plasticizers, including one of the variety of phthalates. Plastic containers for milk, fruit juice, water, and food packaging materials may leach phthalates, some of which are reported to be reproductive and developmental toxicants [2]. In addition, many cosmetics contain phthalates as stabilizers of scents and color, and a number of medications may contain phthalates as formulants of enteric coatings [3]. Phthalates may leach from solution storage bags and tubing used in blood transfusions, enteral and parenteral nutritional support, and kidney dialysis [4-6]. Phthalates and their metabolites have been found in urine samples from a reference population [4-6], and diethylphthalate has been been reported to be present at variable levels in urine samples from 100% of males evaluated [7]. Phthalates and their monoester metabolites have been reported to be androgen antagonists [8], initiators of tumors in rats [9], to alter the expression of a large number of genes with known estrogenic regulation [10], and to interfere, as a function of phthalate estrogenicity, with chemotherapy-induced apoptosis in human breast cancer cells [11]. While a number of phthalates, including diethylphthalate, are considered to be endocrine interactive, their mechanisms of toxicity are not well understood, but are thought to be functions of the disruption of gene expression by interaction with one or more of the nuclear steroid receptors.

Steroid receptors are a structurally related group of cellular proteins which bind with reasonably high specificity to both the steroid hormones and to specific upstream genetic regulatory elements. Receptor binding of steroid hormones and interaction of homo-or heterodimeric receptor-ligands with specific regulatory elements are essential events for hormone-regulated gene expression necessary for maintaining endocrine-interactive physiological functions in eukaryotic organisms [12]. Humans are chronically exposed to low levels of a variety of plasticizers as formulants of enteric coatings on drugs, cosmetic components, as compounds leeching into food or water, and as environmental contaminants, many of which may disrupt steroid hormone-regulated physiological activities. A number of these chemicals are endocrine-interactive because they bind steroid hormone receptors or in some way interact within the cell to up- or down-regulate expression of endocrine-regulated genes.

Extensive recent research has lead to an understanding of the cause and effect relationship between exposure to chemicals that have endocrine disruptive activities (endocrine disruptive chemicals; EDC) and the initiation of altered endocrine-regulated physiological functions in animals. Chemi-

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cally-initiated changes in gene expression may lead to changes in synthesis of essential enzymes, structural, or regulatory proteins resulting in altered gender determination, neurodevelopmental and/or morphological alterations, and decreased normal immune system responses to infectious agents [13-22]. Morphological and neurodevelopmental changes *in utero* after exposure of pregnant animals to EDC may result in a variety of birth defects related to gene dysfunction which may occur even though concentrations of the offending chemicals may be quite low in the environment [16, 23-32].

In this paper the authors present data from an investigation of DEP (Fig. **1**) alteration of gene expression in the human cell line 293T/17. A large number of the 19,000 genes on the chip used for these studies showed statistically valid dysregulation, that is up-regulation by 100% (to a tetraploid level), or down-regulation by at least 50% (to a haploid level). Two of these genes, *FGD1* (faciogenital dysplasia) and *NGPF2* (neurite growth-promoting factor 2, also called Midkine, *MDK*) were selected for further corroboration from a list of neurodevelopmentally associated genes that were dysregulated (Table **1**). Both of these genes have been classified as being in the group known, in mutant form, to be associated with fetal brain developmental abnormalities.

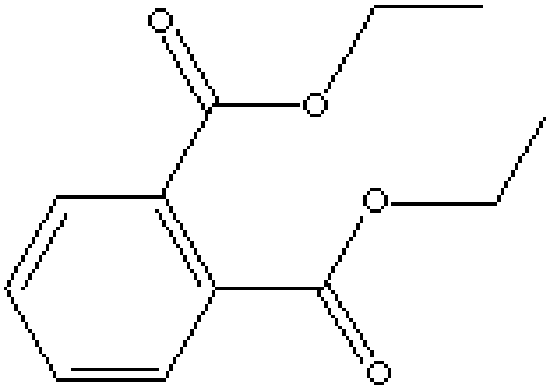

Fig. (1). Structure of diethylphthalate, DEP.

MATERIALS AND METHODS

Tissue Culture

The human renal epithelial cell line, 293T/17, obtained from ATCC (Manassas, VA), was grown in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Laurenceville, GA), 4mM L-glutamine, and 1.5g sodium bicarbonate/liter (both from Sigma, St. Louis, MO). Cells were then switched to DMEM with steroid-stripped FBS for 18hr to reduce exposure to estrogens. This was followed by treatment for another 18hr with or without diethylphthalate (DEP) at 1, 10, or 100 μM concentrations in the presence or absence of $3x10^{-10}$ M 17β-estradiol (E2). This is very close to the growth and development optimum of 17β-estradiol determined for another human cell line, MCF-7, by Miller *et al.*, [33].

Total RNA Extraction and Quantification

Cells were harvested at the end of 36hr of treatment using a lysis solution from Ambion (Austin, TX) and stored at -80º C until RNA was purified. RNA was extracted from the lysate using Ambion's Totally RNA kit, spectrophotometrically quantified, and evaluated on a 1% agarose denaturing gel to check for quality.

cDNA Microarray

Fluorescently labeled cDNA was prepared from total RNA using a SuperScript III kit from Invitrogen (Carlsbad, CA). Control cDNA (SFBS cultured cells) were labeled with cyanine 5 and test cDNA (DEP+E2 treated cells) was labeled with cyanine 3 (both dyes from Amersham, Piscataway, NJ) before combining them in a 1:1 ratio and allowing cDNAs to hybridize to the 19,000 single human gene arrays from the University Health Network Microarray Center (University of Toronto, Toronto, Canada). All arrays were done in quadruplicate. The hybridization reaction was completed at 37ºC for 16hr in a humidified environment. Slides were washed in sodium dodecyl sulfate-saline sodium citrate buffers of increasing stringency to remove unbound labeled cDNA. Slides were then centrifuged in a 50cc conical tube to remove residual buffer.

Array Scanning and Data Analysis

Total RNA recovered after TRIzol isolation was less than 75 μg/sample. Microarray reactions were completed according to the protocol for the MICROMAX^{TM} TSA (Tyramide Signal Amplification) Labeling and Detection Kit (Perkin Elmer Life Sciences, Boston, MA). This kit is specifically designed for amplification and detection of small amounts of RNA. Details of this protocol may be obtained from the Perkin Elmer web site (Ref: MICROMAX TSA Labeling and Detection Kit). Slides were stringently washed x3 at room temperature in 0.5X SDS, 0.06X SSC/0.01% SDS, placed into a 50 mL polypropylene tube, and centrifuged at 500xg for 5 min to remove excess liquid prior to scanning. Microarray slides were scanned in a BioChip Imager (Packard, Meridien, CT). Laser and photomultiplyer tube voltages (PMT) were manually adjusted to maximize signal to noise ratio. Signal intensities were standardized relative to one another by comparing the total signal intensities of all spots in each channel. The scanner output images were quantified using ScanAlyze software (developed by Michael Eisen, University of California, Berkeley, CA). Statistical analysis utilized one-way ANOVA followed by Dunnett's test to analyze differences between control and chemically-treated samples, with $P<0.05$ considered to be statistically significant.

Reverse Transcription

1μg of high quality total RNA was used for reverse transcription. Briefly, for a 25μl RT reaction, random hexamers (16μM final concentration, Roche, Indianapolis, IN), Oligo dT (500ng/μl, Promega, Madison, WI), RNA 1.0μg, and RNAse-free water were combined, heated at 65º C for 5 min, and allowed to cool to room temperature. A cocktail of the following components was added to each sample: 5X 1st strand buffer, 0.1M DTT (both included with SuperScript II), RNase block (Brinkman, Westbury, NY), 10mM dNTPs (Promega, Madison, WI) and SuperScript II RT (Invitrogen, Carlsbad, CA). Cocktail was also made without RT as a control. Each tube was mixed and centrifuged briefly. The RT

Table 1. A Partial List of CNS-Specific Genes Dysregulated by DEP

Gene Name	OMIM Number	Array Ratio
Myosin light chain 4, EMBRYONIC	*160770	0.374
NGPF2, Neurite Growth Promoting Factor, MDK, Midkine	*162096	0.359
Histidine Rich Calcium Binding Protein	*142705	0.356
Ciliary Neurotrophic Receptor Factor	*118946	0.352
DDEF2, Developemental and Enhancement	*603817	0.283
Double Cortex Syndrom, DC50	#300067	0.252
RICS, Rho-GTPASE	*608541	0.315
Thyroid Hormone Receptor 8, TRIP8	*604503	0.357
S100A4, CAPL, Calcium Placental Prot	*114210	0.4
Pregnancy specific glycoprotein, PSG6	*176395	0.331
Glycoprotein M6A, GPM6A, Neuronal membrane 6A	*601275	0.321
Surfactant, pulmonary assot protein B	*178640	0.32
THY-1 T cell antigen, cd90 antigen	*188230	0.306
Acetylglucosylaminotransferase, MGAT1	*160995	0.300
Heart & Neural Crest Derivatives 2,	*602407	0.403
Neogenin 1, NEO1, NGN	*601907	0.416
Pregnancy asssociated plasma protein A	*176385	0.418
High mobility group nucleosome binding protein	*163920	0.423
Nerve Growth Factor Receptor, Tumor Necrosis Factor receptor	*162010	0.424
Glycerophosphodiesterase phosphodiesterase, GDPD5	*609632	0.427
Pregnancy spec beta glycoprotein, psg1	*176390	0.431
Transcription Factor AP-2 Beta	*601601	0.441
Ephrin receptor, EPHa7	*602190	0.449
Protein phosphatase 2a, reg subunit B	*605997	0.457
NeuroFibromin, Schwanomin, Merlin	*607379	0.467
Claudin 11, Oligodendrocyte specific protein, OSP	*601326	0.469
Thyroid Hormone Receptor alpha, CNS	*190120	0.478
Faciogential dysplasia, mental retardation, ADHD, FGD1	305400	0.482
Mitogen activated protein kinase kinase6	*601254	2.382
Integrin, Alpha 1, late activation protein	*192968	2.235
Creatine Neuro transporter, X link MR	*300036	2.144
Glia Maturation Factor Beta, GMFB	*601713	2.030

Table **1** was constructed to provide the reader with OMIM numbers to retrieve data on function of the specific gene.

reaction was continued at 37ºC for 1hr. Reverse transcriptase was inactivated by heating at 90ºC for 5 minutes and samples were cooled on ice or stored at -80ºC.

Quantitative Real Time PCR (qrtPCR)

Twenty-three μL of a cocktail containing Universal PCR master mix, no-UNG, from ABI (Branchburg, New Jersey), pre-made ABI expression assays (primers) specific for the genes of interest, *FGD1* (Hs00171676_m1) or *NGPF2* (Hs00171064_m1**)**, and RNAse/DNAse-free water were combined with 2 μL of each reverse transcription reaction in triplicate, plated into a semi-skirted 96-well PCR plate, and sealed with optically clear mylar film. qrtPCR Employed an Applied Biosystems 7500 Real Time PCR System thermocycler (Foster City, CA) using a 3 step cycling program rec-

ommended by ABI: One cycle at 95ºC for 10 min and forty cycles of (95ºC for 15 sec followed by 60ºC for 1 min).

RESULTS

Human renal epithelial cells were grown in medium containing 10% normal FBS (NFBS), and changed to medium with steroid stripped FBS (SFBS) for 18hr before treatment with DEP (1, 10, or 100μM), with or without 17β-estradiol (E2). Cells were harvested after 36hr, RNA was isolated and microarray slides were labeled to examine the effects of DEP on gene expression. A partial list of genes on the Toronto chip that were dysregulated by DEP, are endocrine interactive, and known to be important in fetal CNS development is given in Table **1**. For verification of microarray data, expression dysregulation, the genes *FGD1* and *NGPF/MDK*, which showed significant down-regulation on the DNA array, with ratios of 0.482 and 0.359 respectively, were chosen for qrtPCR evaluation.

Both *FGD1* and *NGPF2/MDK* showed significant down-regulation by 10 μM DEP as determined by qrtPCR analysis. These data from DEP-treated cells confirmed results of the DNA microarray analysis, with normalized expression levels for *FGD1* computed to cross the threshold line at least 1 complete cycle later than the untreated sample for *FGD1* and 1.5 cycles later for *NGPF2* at 10μM DEP (Fig. **2A**, **2B**). At 100μM DEP the data indicated at least a 5 cycle down-

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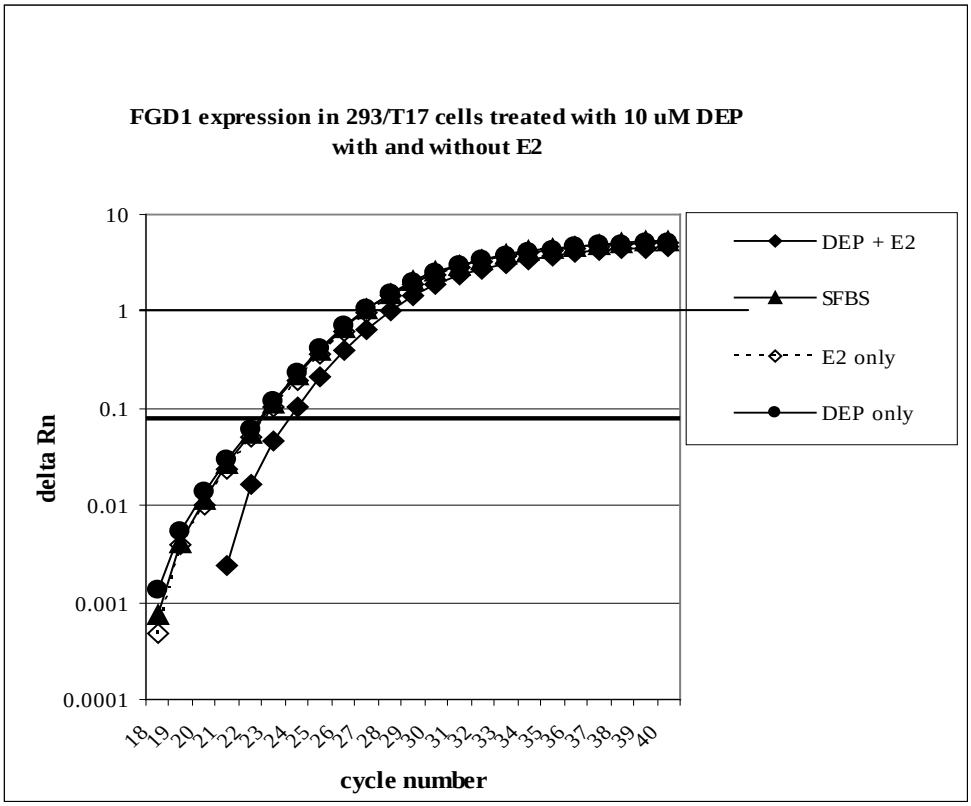

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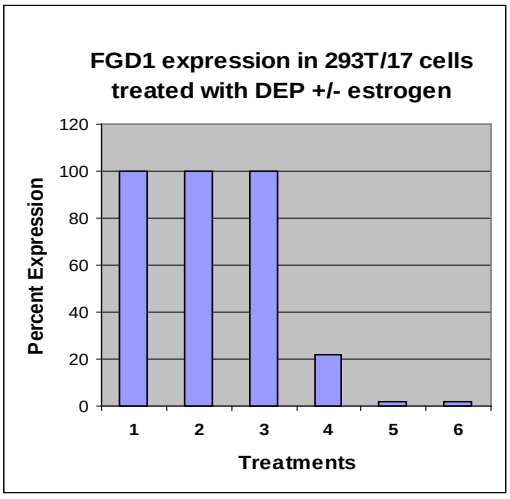

1. estrogen, 2. SFBS, 3. 10 uM DEP, 4. 10 uM DEP + estrogen, 5. 100 uM DEP, 6. 100 uM DEP + estrogen

Fig. (2). qrtPCR Plots provided using specific FGD1 Primers in human cell extracts after varying treatments.

regulation of *FGD1* expression. These data suggest a reduction in expression to a point greater than 25% but less than 50% of expression seen for control cells for both genes at 10μM DEP, and down-regulation of *FGD1* to less than 10% of the control by 100μM DEP. qrtPCR Values for *NGPF2* were below consistently detectable levels in cells treated with 100μM DEP, a functional level just above 0.00. A comparison of DEP with phthalic acid, rather one of the phthalate esters, indicated that phthalic acid itself was not gene expression inhibitory (data not shown).

DISCUSSION

The phthalates are a family of organic chemicals used to stabilize colors and scents in a variety of shampoos and cosmetics [9], as solvents in insect repellant sprays, paint, and glue [34], as components of some drug coatings, and as solubilizers in a wide variety of plastics, including food wraps, water bottles, milk containers, and medical products [35, 36]. The widespread use of phthalates results in exposure of humans through a number of routes, including ingestion, dermal absorption, exposure from medical tubing and infusion sets, and from inhalation [3, 37-41].

With the development of analytical methods to detect and quantify esters of phthalates in urine, phthalate levels have been determined in large numbers of urine samples [3, 5, 6, 38, 39] from both reference and random populations. These studies have lead to the disturbing conclusion that the vast majority of humans have detectable levels of phthalate metabolites in their urine, and that all males in a specific reference population have the primary urinary metabolite of diethylphthalate, MEP, in their urine samples. Very little is known about the mechanisms of DEP, and/or its metabolite MEP, toxicity, but they are generally considered to be estrogen agonists and androgen antagonists [39-44].

Data presented here suggest strongly that cellular exposure to DEP is correlated with dysregulation of a number of genes identified as having an association with development and/or function of the fetal brain. Of the genes shown in Table **1** that are dysregulated by DEP, *FGD1* and *NGPF2* were selected for qrtPCR corroboration. *FGD1* was down-regulated to slightly less than 50% of control and *NGPF2* to between 25% and 50% of control levels by 10μM DEP. At 100μM DEP, *FGD1* was down-regulated almost two full cycles, equivalent to expression at 25% of the control value, and *NGPF2* was downregulated to a level barely detected by qrtPCR.

Faciodigitogenital syndrome, the human disorder associated with *FGD1,* (also called Aarskog-Scott syndrome, AAS, and FGDY), is group of closely related disorders initially described by Aarskog over thirty years ago [45]. Children with this highly variable disorder may have ocular hypertelorism, anteverted nostrils, a broad filtrim, an altered anal-to-genital distance, and a deformed penal and scrotal area that has been called shawl scrotum. AAS children show a frequent occurrence of connective tissue abnormality displayed as hyperextensibility of the fingers, genu recurvatum, and flat feet. Excessive variable laxity in the cervical spine is common, with anomalies of the odontoid region often seen associated with neurologic deficit. A recent paper reported attention deficit hyperactivity disorder, ADHD (44), in Aarskog-Scott syndrome children. Significant mental retardation is not always present in AAS children, and is not necessarily the same even in two families with the same mutation (Orrico *et al*., 2004). However, Fryns [48] estimated that cognitive impairment at some level may be as high as 30% in all persons presenting with Aarskog-Scott syndrome.

AAS mimicry of several other syndromes has confounded the diagnosis and reporting of this disorder, and even within a family group physical and mental differences may be quite variable. Although there is some disagreement on the mode of inheritance, most data support the finding that AAS is X-linked recessive [49] with a map locus of Xq11-12. Males with AAS are reported to be reproductively competent [50, 51], while females can display a variable form of the syndrome and are reproductively unimpaired, but are reported to show a mild form of the disorder [52]. The majority of variant types of AAS appear to be associated with mutation of the *FGD1* gene, and suggest an association between *FGD1* and *FGD2* expression essential for normal fetal morphological and neurological development. When one considers the data correlating *FGD1* mutations and decreased anogenital distance in neonatal males, along with recent reports of decreased anogenital distance in neonatal males exposed to DEP**,** and recent reports that neonates in intensive care facilities are routinely exposed to DEP [3], there appears to be the possibility of a strong correlation between diminished *FGD1* expression, DEP exposure, and the physical characteristics associated with AAS.

NGPF2 is one of a group of highly conserved human genes that are apparently regulated by steroid hormones during development. The gene product has neurite outgrowth promoting activities, and as such, is likely a factor in promoting CNS development *in utero*. *NGPF2* is expressed over a short span during the late second and early third trimesters, when major neural development occurs. The protein encoded by *NGPF2* is a 143 amino acid precursor for a functional protein with 121 amino acids which is partially homologous to a different heparin-binding neuronal outgrowth promoting factor, *NGPF1*. Both *NGPF1* and *2* have neurotrophic activities, but map to different loci [32, 53-55]. A variety of studies have reported that *NGPF2* expression is essential for normal fetal brain development, but its precise mode of action remains unclear.

DNA microarray analysis, followed by qrtPCR corroboration of the microarray data, demonstrated here that exposure of human cells to DEP *in vitro* is consistent with initiation of decreased expression of a large number of genes that have been identified as essential for fetal brain development The analyses indicate that two genes, *FGD1* and *NGPF2*, exhibit decreased expression in human cells exposed to DEP at concentrations as low as 10μM, and suggest the possibility that decreased *in utero* expression of these genes would be likely to occur if the developing fetus was exposed to DEP at pharmacologically relevant concentrations. The degree to which developing embryos or neonatal infants show neurodevelopmental changes associated with decreased expression of *NGPF2* is not clear at this time. In summary, these studies suggest that failure to develop of a normal brain/central nervous system may occur due to *in utero*, and perhaps to early post-natal, exposure to DEP or its metabolite MEP. Existing clinical studies have linked these two genes with a curious variety of morphological anomalies and an equally

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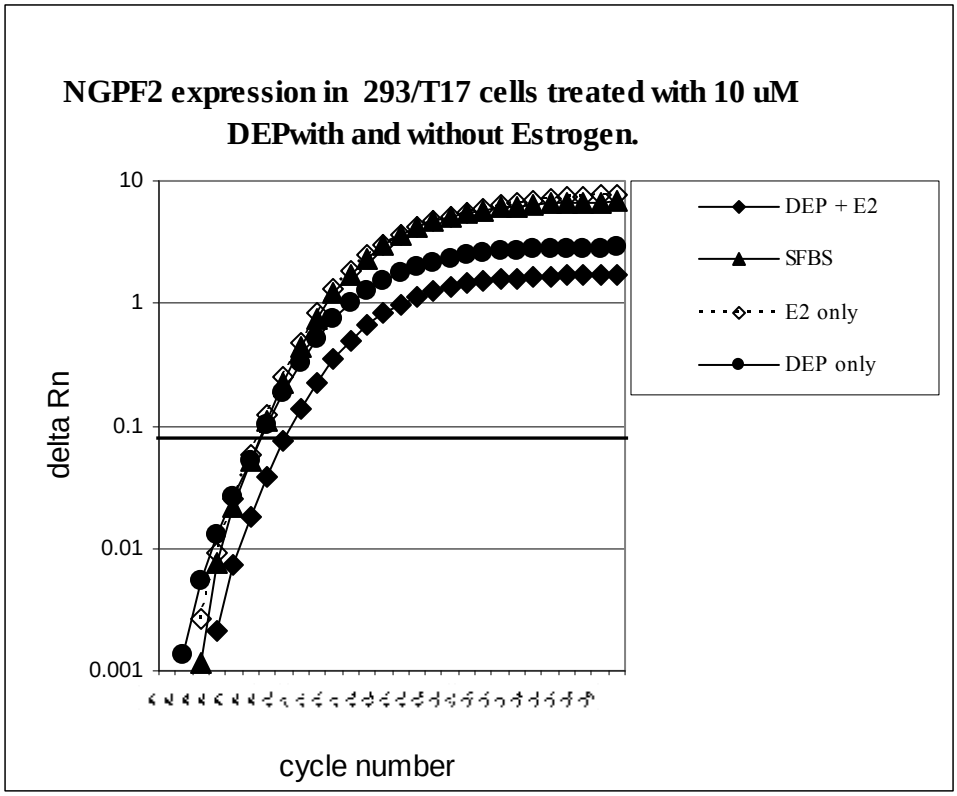

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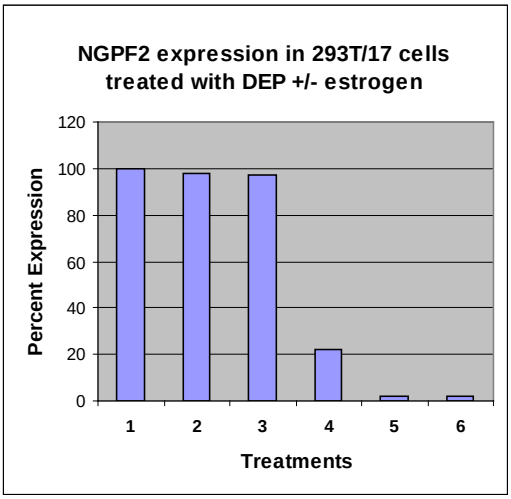

1. estrogen, 2. SFBS, 3. 10 uM DEP, 4. 10 uM DEP + estrogen, 5. 100 uM DEP, 6. 100 uM DEP + estrogen

Fig. (3). qrtPCR Plots provided using specific MDK/NGPF2 Primers in human cell extracts after varying treatments.

curious variety of neurodevelopmental and functional disorders, notably, mental retardation and attention deficit hyperexcitability disorder, ADHD. Considering that essentially all males tested have been shown to have detectable DEP metabolites in their urine, and that 9.3% of children in the United States have been diagnosed as having ADHD, this could be a significant finding. However, at this time it is not known if the endocrine dysfunction and phthalate-altered gene expression presented here in an *in vitro* investigation can be correlated with any instance of *in vivo* phthalate exposure or human disease.

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