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Neonatal diethylstilbestrol exposure alters the metabolic profile of uterine epithelial cells

Yan Yin¹, Congxing Lin¹, G. Michael Veith¹, Hong Chen¹, Maulik Dhandha¹ and Liang Ma^{1,2,*}

SUMMARY

Developmental exposure to diethylstilbestrol (DES) causes reproductive tract malformations, affects fertility and increases the risk of clear cell carcinoma of the vagina and cervix in humans. Previous studies on a well-established mouse DES model demonstrated that it recapitulates many features of the human syndrome, yet the underlying molecular mechanism is far from clear. Using the neonatal DES mouse model, the present study uses global transcript profiling to systematically explore early gene expression changes in individual epithelial and mesenchymal compartments of the neonatal uterus. Over 900 genes show differential expression upon DES treatment in either one or both tissue layers. Interestingly, multiple components of peroxisome proliferator-activated receptor- γ (PPAR γ)-mediated adipogenesis and lipid metabolism, including PPAR γ itself, are targets of DES in the neonatal uterus. Transmission electron microscopy and Oil-Red O staining further demonstrate a dramatic increase in lipid deposition in uterine epithelial cells upon DES exposure. Neonatal DES exposure also perturbs glucose homeostasis in the uterine epithelium. Some of these neonatal DES-induced metabolic changes appear to last into adulthood, suggesting a permanent effect of DES on energy metabolism in uterine epithelial cells. This study extends the list of biological processes that can be regulated by estrogen or DES, and provides a novel perspective for endocrine disruptor-induced reproductive abnormalities.

INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are defined by the US Environmental Protection Agency as a group of substances that “interfere with biosynthesis, secretion, transport, elimination and function of naturally existing hormones in the human body”. Exposure to endocrine disruptors during crucial developmental time windows increases an individual’s risk of developing a variety of diseases, including inborn errors, infertility, obesity and cancer (Crisp et al., 1998). One of the most well-known EDCs is diethylstilbestrol (DES), a synthetic estrogen that was widely prescribed in the 1940s-1970s to prevent miscarriage. During that time period, millions of women and their offspring were exposed to this compound, which was later shown to have teratogenic and oncogenic effects on many organ systems especially the reproductive tract (Ma, 2009). Since then, the mechanisms underpinning DES-related pathogenesis have been intensively studied and several animal models have been established (McLachlan, 1977; McLachlan et al., 1980; Newbold and McLachlan, 1982). The most widely used model for DES research is the neonatal-DES model, which was first described by McLachlan and co-workers in 1982 (Newbold and McLachlan, 1982). Female mice receiving 1 mg/kg body weight/day of DES in the first 5 days of their lives exhibited uterine malformations, including metaplasia

and reduced adenogenesis. These mice were overweight, subfertile and developed uterine adenocarcinoma later in life (McLachlan et al., 1982; Newbold and McLachlan, 1982; Newbold et al., 2007). Although the mouse phenotypes do not fully recapitulate the clinical findings (Hatch et al., 2001; Hoover et al., 2011), this model is nonetheless valuable for understanding how EDCs impact reproductive organ function in general. Notably, most of these DES effects are mediated through the estrogen receptor α (ESR1), as shown by the finding that *Esr1*-null mice are largely resistant to DES-induced phenotypes (Couse et al., 2001).

The effect of DES on the uterus is of particular interest because uterine metaplasia is one of the most common health problems for women exposed to DES before birth (DES-daughters), which could lead to infertility. The neonatal uterus is composed of a simple columnar luminal epithelium, a fibroblast mesenchymal (stromal) layer and smooth muscles. It is well known that stromal cells play an inductive role in the differentiation of the uterine epithelium (UE) during reproductive tract patterning (Cunha, 1972). We and others have shown that the neonatal uterus is prone to DES-mediated teratogenic effects, exhibiting marked morphological and gene expression changes shortly after DES exposure (Huang et al., 2005; Ma et al., 1998; Miller et al., 1998; Yin et al., 2008). A recent study demonstrated that the uterine epithelial ESR1 is dispensable for the proliferative response but is required for suppressing UE apoptosis as well as lactoferrin induction, indicating both cell-autonomous and non-cell-autonomous mechanisms in estrogen signaling (Winuthayanon et al., 2010).

To better understand the dynamic regulation of uterine biology by DES treatment, we performed tissue-specific cDNA microarray analyses and discovered that DES-elicited gene expression changes occurred mostly in the UE. A core group of genes involved in adipogenesis and glucose metabolism, especially the CCAAT-enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptor γ (PPAR γ), were induced by DES in the UE. We

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further demonstrated that activation of PPAR γ was essential for initiation of abnormal adipogenesis in UE cells. This work implicates the adipogenic program as a downstream target of DES in the UE, which leads to the hypothesis that DES and possibly other EDCs might activate similar genetic pathways in other estrogen-responsive tissues, especially the adipocytes, to cause adulthood obesity.

RESULTS

Neonatal DES exposure alters uterine cell metabolism predominantly in the epithelium

The prevailing hypothesis is that the reproductive pathology in DES-daughters is defined by transient molecular changes during the crucial developmental period when DES is administered. To reveal potential mechanisms involved in this complex process, we sought to capture early DES-induced gene expression changes in the UE and uterine mesenchyme (UM) using cDNA microarray analyses. We separated UE from the UM from vehicle (oil)- or DES-treated postnatal day 5 (P5) mice, and prepared biological triplicates of RNA from pooled specimens ($n \geq 3$). Those samples were analyzed on two MouseWG-6 BeadChips, which detects 45,200 transcripts, including more than 26,000 annotated genes in the NCBI RefSeq database. A difference of at least twofold in signal intensity of each given probe set with a P value less than 0.05 was considered statistically significant. As a result of this analysis, 981 transcripts were found differentially expressed in the UE, UM or both between oil- and DES-treated groups (supplementary material Table S1 and the Gene Expression Omnibus, GEO# GSE37969). Hierarchical clustering analysis showed that among these differentially regulated genes (DRGs), the majority (80%) were either changed exclusively in the UE or showed a more prominent dysregulation in the UE (Fig. 1A, red boxes). Around 10% of the DRGs were similarly regulated in both tissue layers, and another 10% showed altered gene expression in the UM alone (Fig. 1A, green and black boxes, respectively). When plotted on a log₂-scaled star glyph, it is obvious that the fold-changes of most DRGs were more pronounced in the UE, either up- or downregulated (Fig. 1B). The number of DRGs is summarized in Fig. 1C, which further demonstrates that the UE contains more unique DRGs compared with the UM. The UE-specific DRGs were further analyzed and categorized by their biological functions following the criteria of the Gene Ontology Consortium. Genes involved in metabolism accounted for the largest proportion of the DRGs (16.7%), followed by those regulating cell growth and maintenance (12.5%), cell communication (10.1%) and signal transduction (8.1%; Fig. 1D; supplementary material Table S2). These results indicated that neonatal DES treatment affected biology of the UE more than it did the UM, even though the UE expresses only marginal levels of estrogen receptor- α , the key mediator of DES action (Korach et al., 1988).

Neonatal DES exposure alters adipogenesis and lipid metabolism in the UE

Further analysis of the 213 DRGs involved in metabolic regulation according to the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database showed that metabolism of complex lipids was the most affected cellular metabolic pathways (supplementary material Table S3) (Kanehisa et al., 2006). Careful

examination of genes involved in fatty acid transport and metabolism showed that many were expressed abnormally in the UE (Fig. 2A, red boxes). *Ppar γ* , a gene encoding the key regulator of adipocyte differentiation, insulin sensitivity, inflammation and the endocrine signaling pathway (Barak et al., 1999; Fischer-Posovszky et al., 2007; Tontonoz et al., 1994), showed marked upregulation in DES-treated UE in our microarray. We further verified this result using quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR), and confirmed a tenfold upregulation in DES-treated UE (Fig. 2B). Furthermore, both western blotting and immunohistochemistry clearly demonstrated elevated PPAR γ protein levels in DES-treated UE (Fig. 2C-E). PPAR γ is a nuclear hormone receptor that belongs to the peroxisome proliferator-activated receptor gene family. Both natural ligands, such as unsaturated fatty acids, and synthetic ligands, such as thiazolidinediones (TZDs), can activate PPAR γ (Lehmann et al., 1995). Once activated, cytoplasmic PPAR γ translocates into the nucleus where it heterodimerizes with retinoid X receptor (RXR), and directly regulates gene transcription by binding to PPAR-response elements (PPREs) through its DNA binding domain. Consistent with the observed upregulation of PPAR γ expression, many known PPAR γ transcriptional targets, including *Gpr109a*, *Acsl1*, *Rarb* and *Arnt1* (Inoue et al., 2005; James et al., 2003; Jeninga et al., 2009; Karnik et al., 2009), also showed marked changes in expression upon DES treatment in the UE (Fig. 2B). Notably, the expressions of these genes were either low or unchanged in the UM, which is consistent with a cell-autonomous role for PPAR γ in mediating transcriptional regulation. Collectively, these data suggest that the PPAR γ signaling cascade is abnormally activated in the UE by neonatal DES treatment in the mouse.

In addition to the PPAR γ pathway, expressions of many other genes implicated in adipocyte differentiation were also perturbed by DES exposure. Besides PPARs, C/EBPs are the other crucial factors regulating preadipocyte differentiation. It is well established that preadipocyte differentiation starts with activation of C/EBP β and C/EBP δ , followed by activation of C/EBP α and PPAR γ (Wu et al., 1996; Yeh et al., 1995). This process is positively regulated by transcription factors Krüppel-like factor 4 (*KLF4*), *KLF6*, early growth response gene 2 (*EGR2*), and negatively regulated by transmembrane proto-oncogene Delta-like 1 (*DLK1*) (Birsoy et al., 2008; Boyle et al., 2009; Li et al., 2005). Interestingly, we detected a significant increase in expression of aforementioned positive regulators, including *Cebpa*, *Cebpb*, *Klf4*, *Klf6* and *Egr2* in the UE by quantitative RT-PCR analyses (Fig. 2A,B). Accumulation of nuclear KLF4 protein was also evident by immunofluorescence (Fig. 2F,G). By contrast, expression of the negative regulator *Dlk1* was decreased by DES in the UE (Fig. 2B). These data suggest that the core transcriptional cascade favoring adipogenesis is ectopically switched on in the luminal epithelial cells of the neonatal uterus by DES treatment.

Possibly as a result of this ectopic activation of the adipogenic signaling pathway, expressions of many genes involved in lipid metabolism were also altered. As shown in Fig. 2A,H, the expression of long-chain fatty acid transport protein 1, encoded by *Slc27a1*, was dramatically increased in the luminal epithelium by DES treatment. Similarly, the expression of *Slc27a2*, which encodes fatty acid transport protein 2, was also induced in the UE by DES

treatment. Moreover, the expression of *Acp11*, a gene encoding an acyltransferase that converts phospholipid lysophosphatidic acid (LPA) into phosphatidic acid (PA), and that of *Dgat2*, a gene encoding diglyceride acyltransferase that catalyzes the formation of triglycerides from diacylglycerol and Acyl-CoA, were both upregulated in the UE (Fig. 2H). On the other hand, the gene encoding phospholipid transfer protein (*Pltp*) was significantly downregulated in the UE. Taken together, these data suggest that, in addition to turning on the lipogenic signaling cascades, neonatal DES treatment also alters expression of genes responsible for lipid transport and metabolism in UE cells.

DES induces PPAR γ -dependent adipogenesis in the UE

Our gene profiling and subsequent analyses indicated that ectopic adipogenesis is induced by DES in UE cells, which prompted us to evaluate the ultrastructure of the UE for evidence of adipogenesis. Transmission electron microscopy (TEM) on ultra-thin sections of P5 uterus revealed many conspicuous, electron-dense droplets located close to the basal side of DES-treated UE cells, which were rarely detected in control UE (Fig. 3A,B, arrows). As the morphology of these droplets resembles lipid droplets observed in many adipocytes, we stained frozen uterine sections with Oil-Red O (ORO), a lysochrome diazo dye that binds neutral lipids. Normal

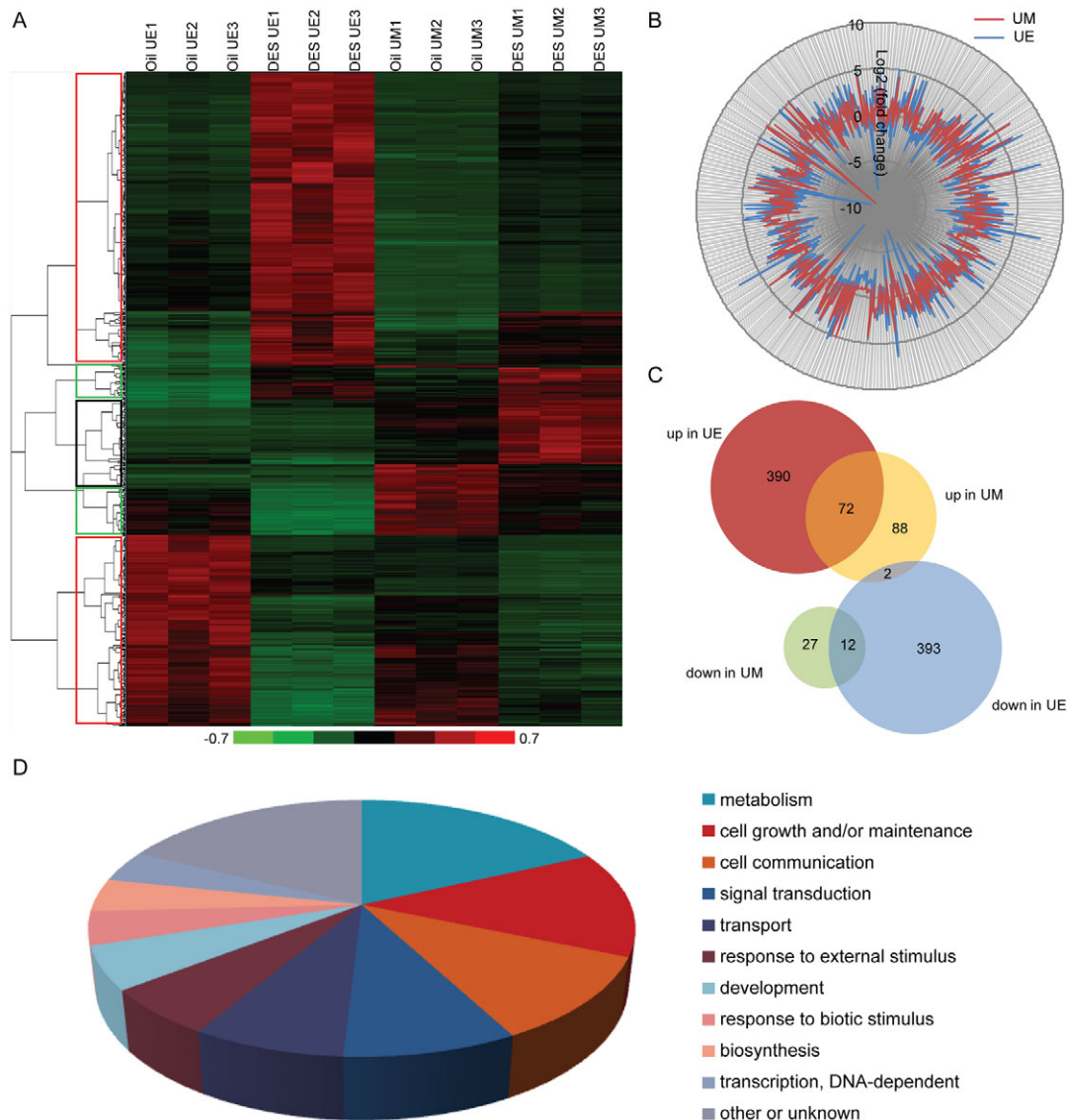


Fig. 1. DES alters uterine gene expression primarily in the epithelium. (A) Hierarchical clustering heatmap of differentially regulated genes by DES in the UE and UM. Green to red, color range gradient of mean abundance (–0.7 to 0.7). Each column represents a pool of more than three animals. Red box, genes whose expression was altered by DES primarily in the UE; green box, genes regulated by DES similarly in the UE and UM; black box, genes altered by DES primarily in the UM. (B) Star glyph distribution of the fold-change of differentially regulated gene in the uterus; log₂ values of the mean fold-change. Red, UM; blue, UE. (C) Venn diagram of UE and UM DRGs. (D) Pie chart showing classification of DRGs based on Gene Ontology Consortium biological processes. Note that some genes might have multiple functions and could be represented in more than one category.

uterus at P5 did not stain for ORO; however, prominent ORO-staining was evident in DES-treated luminal epithelial cells close to the basement membrane, which coincides with the abundant droplets seen by TEM (Fig. 3E,F, arrows). These data demonstrate that in merely 5 days, DES transiently activated the adipogenic program in the UE, rendering them lipogenic.

Because PPAR γ is considered the key modulator of adipogenesis, we tested the hypothesis that DES-induced lipogenic effects are mediated through PPAR γ . We pooled neonatal female mice into four groups which received oil, DES, or DES in combination with PPAR γ inhibitors GW9662 or BADGE, and examined their uteri at P5. Indeed, co-treatment with either inhibitor attenuated DES-induced lipid accumulation in the UE, resulting in a significantly reduced number of lipid droplets (Fig. 3C,D,G,H,I) (oil, 0.25 ± 0.16 ; DES, 6.91 ± 1.15 ; GW+DES, 2.32 ± 0.80 ; BADGE+DES, 1.90 ± 0.57 droplets/ μm^2 ; $*P < 1 \times 10^{-4}$). In addition, combined treatments also

reduced the size of the residual lipid droplets (Fig. 3C,D,I). When neonatal mice received five daily injections of Pioglitazone, a TZD-class PPAR γ agonist that selectively stimulates PPAR γ activity, lipid droplet accumulation was evident in the UE (Fig. 3J). Interestingly, these droplets were present throughout the UE with no subcellular confinement (Fig. 3J, inset, arrowheads). When DES was co-injected with TZD, however, intense ORO staining was detected only on the basal side of the UE (Fig. 3K, arrows, compare with 3F,J). Taken together, these data clearly demonstrated that PPAR γ , induced by neonatal DES exposure, is both necessary and sufficient to cause aggregation of lipid droplets in the UE. Moreover, DES not only upregulates PPAR γ expression but must also increase endogenous PPAR γ ligand expression and accumulation in the UE to ectopically activate this program. On the other hand, genes involved in lipid trafficking must also be affected by DES to elicit the distinct basally-localized lipid droplet phenotype in the UE.

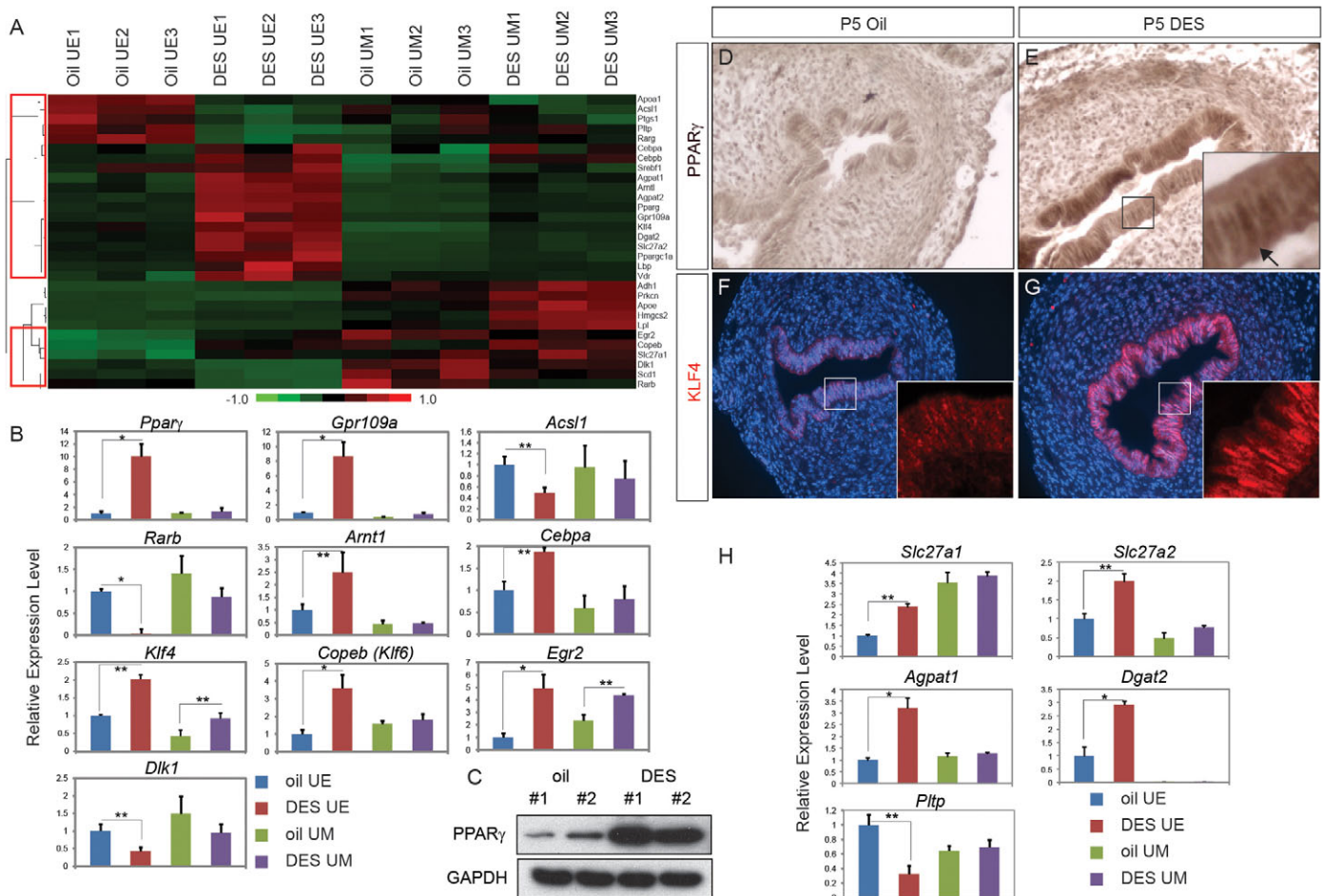


Fig. 2. DES affects lipid metabolism and transport. (A) Hierarchical clustering heatmap of genes involved in lipid trafficking and metabolism. Green to red, color range gradient of mean abundance (−1.0 to 1.0). Red box, genes whose expression was altered by DES primarily in the UE. (B) Real-time RT-PCR survey of genes involved in adipocyte differentiation in the uterus. Expression of each gene was normalized to that of *Rpl7*; normalized expression by oil UE was considered to be 1.0. (C) Western blot on whole uterine lysates showed markedly increased PPAR γ protein in DES-treated uteri. GAPDH served as a loading control. (D,E) Immunohistochemistry of PPAR γ showed increased staining in the DES-treated UE. Inset shows that PPAR γ was predominantly detected in the UE nuclei (arrow). (F,G) Increased KLF4 protein was detected in the UE nuclei by immunofluorescence. Red, KLF4; blue, nuclei. Insets show magnified immunofluorescence signal of the boxed region. (H) Real-time RT-PCR validated genes involved in fatty acid transport and metabolism in oil- or DES-treated UE and UM. Data are presented as mean + s.d. of three samples analyzed in each treatment group from corresponding tissues. $*P < 0.01$, $**P < 0.05$.

Neonatal DES exposure alters glucose transport and metabolism in the UE

In addition to lipid metabolism, carbohydrate metabolism was also strongly affected by DES treatment. The expression of *Slc2a1*, which encodes facilitated glucose transporter 1 (also known as GLUT1), was upregulated 4.5-fold in the UE (supplementary material Table S1). Immunofluorescence revealed that GLUT1 was expressed in the cytoplasm of UE cells at low levels under normal conditions, but was dramatically induced and translocated to the basal-lateral membrane of the UE cells upon DES exposure (Fig. 4A,B, insets). This rapid increase and translocation of GLUT1 could facilitate glucose uptake by the UE cells from the extracellular matrix. To test whether this is the case, we isolated UE from oil- and DES-treated P5 female pups and performed glucose uptake assays on suspended epithelial cells. In support of our hypothesis, DES-treated UE cells showed a marked increase in their ability to transport free D-glucose in solution (Fig. 4G). Enhanced glucose uptake probably leads to increased conversion from glucose to glucose-6-phosphate and stimulates subsequent glycolysis or other alternative pathways. We therefore analyzed the expression of crucial enzymes regulating this metabolic pathway in oil- and DES-treated uterine samples. We found that hexokinase 2 (HK2), a low- K_m glycolytic enzyme converting glucose to glucose-6-phosphate, showed a 3.7-fold upregulation in DES-treated UE by our

microarray (supplementary material Table S1). Immunofluorescence analysis further revealed that HK2 expression in the UE was strongly upregulated by DES, compared with that in controls (Fig. 4C,D). Similarly, our microarray results demonstrated a 2.8-fold increase in transcript level of X-linked G glucose-6-phosphate dehydrogenase (*G6pdx*), an enzyme involved in the first step of pentose phosphate pathway (PPP). This increase was also confirmed by immunofluorescence (Fig. 4E,F; supplementary material Table S1). Elevated protein expression of HK2 and G6PD was further confirmed by western blot (Fig. 4H). Taken together, these data demonstrated an overt effect of DES on carbohydrate homeostasis of the UE cells.

Neonatal DES exposure alters metabolic pathways in adult UE

To determine the long-term effects of neonatal DES exposure, we evaluated UE cell metabolism in adult female mice neonatally exposed to DES. Neonatally treated mice were ovariectomized, received a single injection of either oil, DES or 17 β -estradiol (E2) at 10 weeks of age, and their uteri examined 24 hours later. ORO staining showed sporadic lipid droplets in neonatally DES-treated uteri, whereas none was detected in controls (Fig. 5A,D, arrows). On the other hand, one dose of DES was sufficient to cause lipid droplet accumulation in adult UE after merely 24 hours, regardless of their neonatal exposure status (Fig. 5B,E). It is noteworthy that

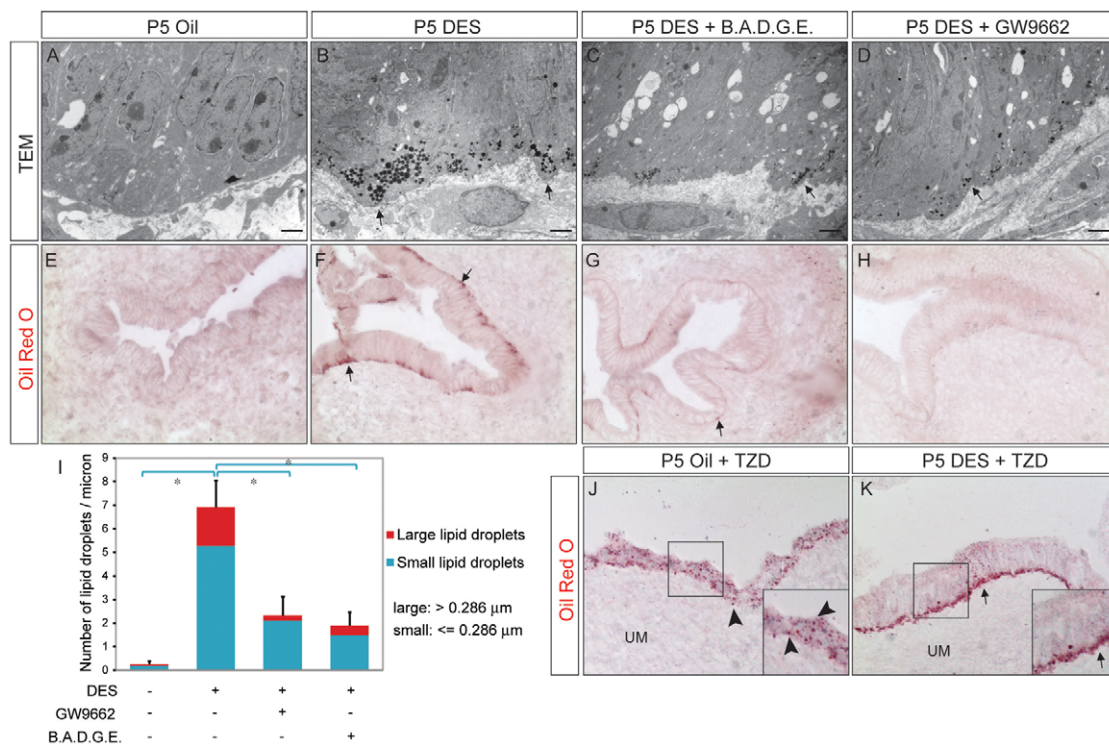


Fig. 3. DES-induced lipogenesis in UE is mediated through PPAR γ . (A-D) TEM of UE at P5 revealed that DES treatment alone caused accumulation of electron-dense droplets in the UE close to the basement membrane (B, arrows, compare with A). Co-injection of PPAR γ inhibitor BADGE (C) or GW9662 (D) dampened this effect, resulting in a lower number of droplets. (E-H) ORO staining identified droplets as neutral lipid droplets (F) and confirmed that co-treatment of DES with PPAR γ inhibitors severely attenuated this effect (G,H). (I) Quantification of lipid droplets from TEM images confirmed DES-induced accumulation of lipid droplets in the UE. Co-treatment of DES with PPAR γ inhibitors resulted in reduced number and size of the droplets. * $P < 1 \times 10^{-4}$. (J) PPAR γ agonist pioglitazone (TZD) induced lipid droplet formation in the UE without specific subcellular localization (arrowheads). (K) Co-injection of TZD and DES resulted in intense ORO staining on the basal side of the UE (arrows).

this change was confined to the luminal epithelium, but was not seen in the glandular epithelium (Fig. 5B, arrowhead). Intriguingly, E2 treatment at physiological concentration (100 ng) readily induced lipid droplet formation in the UE previously exposed to DES but had minimal effect on the control UE (Fig. 5C,F). Concordantly, *Pparg* expression was induced by DES in both neonatally oil- or DES-treated uteri, but its induction by E2 was observed only in the neonatally DES-treated uterus (supplementary material Fig. S1). On the other hand, *G6PD* was expressed at a much higher level in the neonatally DES-treated adult UE than in controls (Fig. 5), compare with 5G). Similarly, *GLUT1* expression was elevated in the UE of adults treated neonatally with DES (supplementary material Fig. S1B,C). DES or E2 treatment in adults did not significantly affect the expression of either gene in the uterus (Fig. 5H,I,K,L and data not shown). Collectively these results

indicate that neonatal DES exposure has a permanent effect on lipid and glucose homeostasis in the adult UE.

DISCUSSION

Ever since the discovery of the adverse effects of DES on human reproductive organs, great efforts have been invested in elucidating the molecular mechanisms leading to these detrimental defects. The neonatal mouse DES model is an excellent model because adult mice exposed to DES at an early developmental stage present many phenotypic resemblances to human DES-sons and DES-daughters. Neonatal DES exposure alters the expression of essential developmental regulators such as *Wnt* and *Hox* genes during the crucial period of uterine differentiation (Ma et al., 1998; Miller et al., 1998). It is our hypothesis that the transient gene expression perturbation caused by neonatal DES treatment can permanently alter uterine cell physiology and thus prime the UE for future pathogenesis. Previously, our group has conducted gene expression profiling experiments on neonatal mouse uterus exposed to DES and uncovered 427 differentially expressed genes, including those involved in cell proliferation, apoptosis and differentiation (Huang et al., 2005). However, mRNA used in this analysis was extracted from the whole uterus, a morphologically heterogeneous tissue, which did not reveal tissue-specific gene regulation. To resolve this issue, we employed a combined enzymatic and mechanical method modified from the protocol introduced by the Cunha group to isolate UE from the underlying UM (Bigsby et al., 1986). This approach allowed us to determine the effect of DES on distinct cell types and to identify target genes in a tissue-specific manner. Our current microarray data revealed 981 differentially regulated genes in the uterus, which is many more than found in our previous study using the whole uterus (Huang et al., 2005). This increase in the number of DRGs could be the result of different array platforms used in the two studies. Alternatively, using the whole uterus could have masked any expression changes that occurred only in one tissue compartment and rendered the changes not statistically significant.

Among the 981 DRGs, the majority of them were either specific to the UE or showed a more prominent fold-change in the UE. It is believed that DES exerts its function mainly through binding to the estrogen receptors (ERs or ESRs), and *Esr1*-null females showed complete resistance to the long-term effects of neonatal DES (Couse et al., 2001). Because *ESR1* is expressed primarily in the UM at postnatal day 4-5 (Korach et al., 1988), it was surprising that the UE is more readily responsive than the UM to DES exposure and showed a myriad of gene expression changes. One possible explanation is that the ER signaling pathway is more robust in the UE than in the UM. For example, the $ER\alpha$ co-activators might be differentially expressed in the two tissue compartments. To support this notion, we examined the expression of known $ER\alpha$ co-activators in our array and found that two co-activators, *Ada* and *PBP*, were expressed at higher levels in the UE than in the UM (GEO# GSE37969). A second possibility is that some epithelial gene expression changes are secondary to altered stromal signaling because the UE is plastic and receives instructive signals from the UM at this stage of development (Cunha, 1976). In this scenario, DES induces primary gene expression changes in the UM, which subsequently causes more dramatic changes in UE gene expression through paracrine signaling. Thirdly, our microarray data indicates

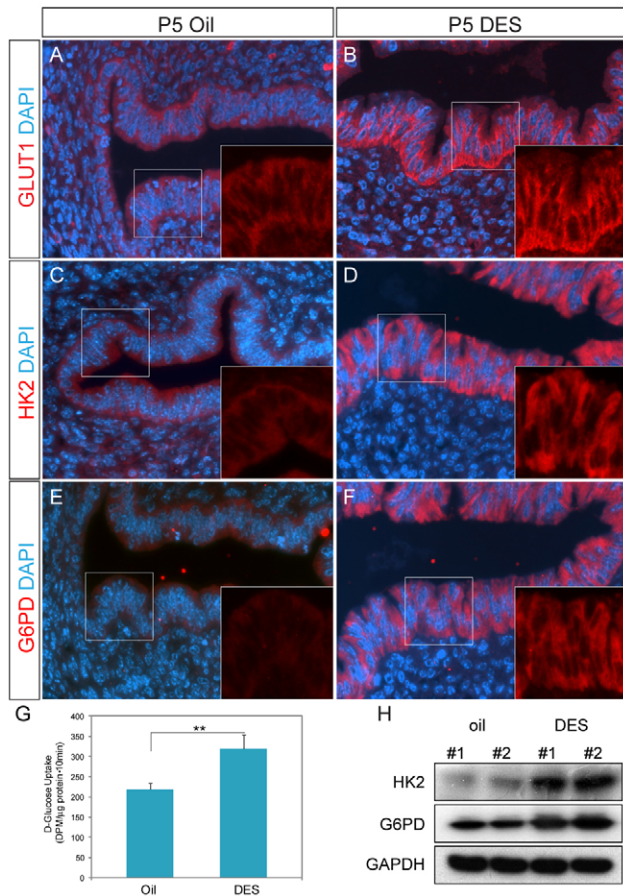


Fig. 4. DES alters glucose transport and metabolism in the UE.

(A,B) Immunofluorescence against GLUT1 showed a dramatic increase and translocation to the basal-lateral membrane of the protein upon DES treatment. (C-F) Immunofluorescence of HK2 (C,D) and G6PD (E,F) revealed that both were expressed at low levels in the UE at P5, but were markedly upregulated by neonatal DES treatment. Insets show magnified immunofluorescence signal of the boxed area in each panel. (G) Glucose uptake assay showed a 1.4-fold increase in isolated DES-treated UE compared with controls. ** $P < 0.025$. (H) Western blot of HK2 and G6PD confirmed their upregulation by DES in P5 whole uterine lysates.

that DES exclusively induces *Esr1* expression in the UE but not in the UM (GEO# GSE37969), which might contribute to increased ESR signaling in the UE. Finally, it is also possible that, in addition to ESRs, other steroid hormone receptor(s) transduce DES signaling in UE cells. One such candidate is the orphan nuclear receptor estrogen-receptor-related receptors ($ERR\alpha$, $ERR\beta$ and $ERR\gamma$), previously reported to interact with DES, which suppresses co-activator binding and affects transcription of downstream targets in trophoblast cells (Tremblay et al., 2001). We found in our microarray that *Esrra*, which encodes $ERR\alpha$, was highly expressed in the UE, and was upregulated 2.8-fold ($P=0.00047$) by DES treatment. On the other hand, its expression was low in the UM and unaffected by DES (supplementary material Table S1 and GEO# GSE37969).

The most interesting finding in our study was the ectopic accumulation of lipid droplets in the UE, as evidenced by both ultrastructural and histological analyses. This abnormal activation of adipogenesis results from aberrant lipid metabolism and

trafficking in the UE upon DES exposure. As a consequence of the altered lipid metabolism, UE cells might use alternative metabolic pathways for energy storage and/or consumption, which could lead to undesired epigenetic changes (Wellen et al., 2009) and cause disease later in life. Thus, this abnormal adipogenesis could also be a potential mechanism that contributes to the etiology of DES-induced uterine metaplasia.

Previous studies have reported that estrogen treatment in adult mice resulted in increased lipid biosynthesis (Bourke et al., 1991; Stacey et al., 1991). In this study we provide additional mechanistic insights into this process by both gene profiling experiments and functional analyses. The program of adipocyte differentiation is tightly regulated through a genetic cascade mainly involving sequential activation of $C/EBP\beta$ and $C/EBP\delta$, followed by $PPAR\gamma$ and $C/EBP\alpha$ (Tontonoz et al., 1994). We found that DES ectopically activated the adipogenic program by affecting the expression of both positive and negative regulators of this pathway. Activation of $PPAR\gamma$ is a crucial event in this process, as clearly demonstrated

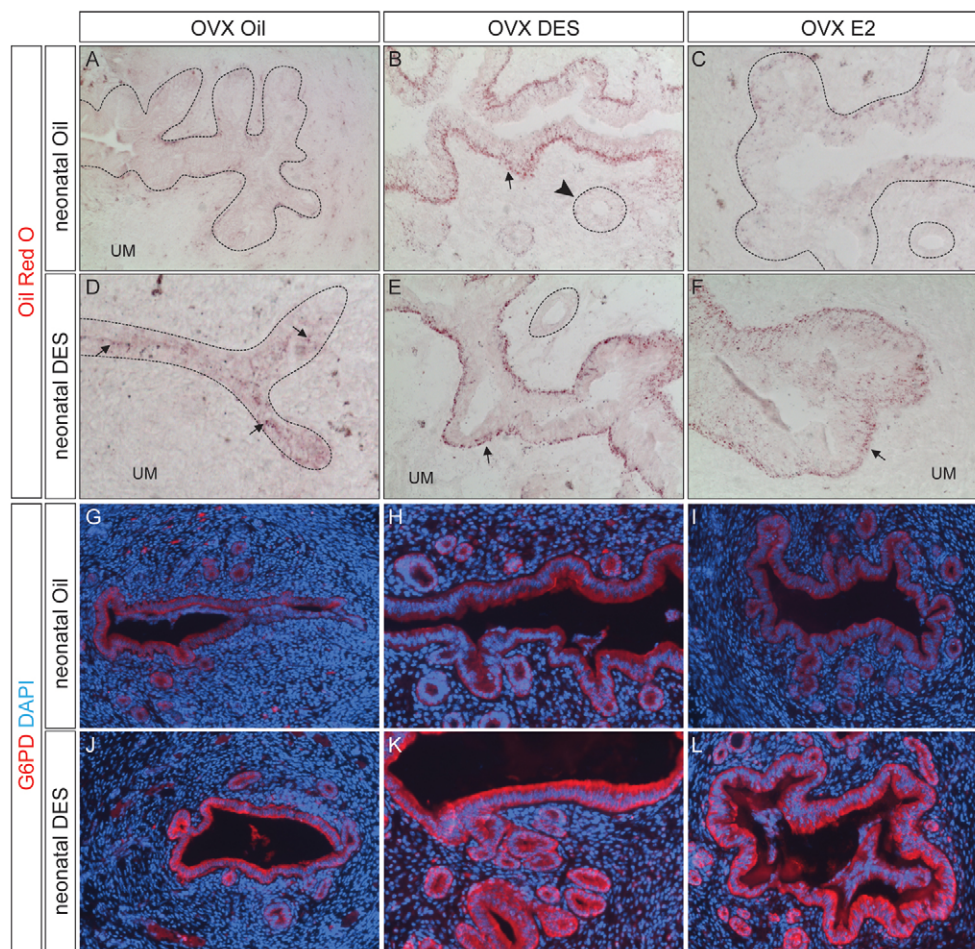


Fig. 5. Neonatal DES treatment alters adult UE metabolic homeostasis. (A-F) ORO staining of uteri of ovariectomized (OVX) adult mice. No staining was detected in the OVX UE of control animals (A), whereas a number of lipid droplets were observed in the OVX UE neonatally exposed to DES (D, arrows). Dashed lines outline the UE. DES induced ORO staining in the luminal UE of both groups (B,E, arrows), without affecting the glandular epithelium (B, arrowhead). E2 treatment induced ORO staining in the UE of animals previously exposed to DES (F, arrow), but had little effect on neonatally oil-treated UE (C). (G-L) Expression of G6PD assessed by immunofluorescence. G6PD was higher in the UE with neonatal DES exposure (J) and remained high when treated with DES or E2 (K,L). Neonatally oil-treated UE had lower G6PD levels regardless of hormone treatment in adulthood (G-I).

by antagonist and agonist experiments showing that it is both necessary and sufficient to induce ectopic lipid droplet formation in the UE. The exact mechanism through which DES activates PPAR γ is still not clear, although a previous study suggested that PPAR γ could be regulated by ovarian hormones during implantation (Li et al., 2004). We found that elevation of *Ppar γ* transcript was not observed until 18 hours after DES treatment (data not shown), suggesting that its regulation by DES might be secondary and mediated by other upstream factors. One such candidate is C/EBP β , which is a known activator of PPAR γ in 3T3-L1 adipocytes and has previously been shown to be a direct target of 17 β -estradiol in the adult mouse uterus (Gellersen et al., 2010). The subcellular localization of DES-induced lipid droplets, however, cannot be achieved by activating PPAR γ alone (Fig. 3)), indicating that other lipid-interacting molecules must also be induced to contribute to this phenotype.

PPAR γ has two isoforms in the mouse resulting from alternative usage of 5' exons (Tontonoz et al., 1994; Zhu et al., 1995). PPAR γ 1 is expressed ubiquitously at low levels whereas PPAR γ 2 is highly expressed in the adipose tissue and large intestine (Mansen et al., 1996; Tontonoz et al., 1994). PPAR γ 1 is induced by DES in neonatal uterus, but is suppressed in abdominal fat pad (supplementary material Fig. S2B). By contrast, PPAR γ 2 is absent in the uterus but induced by DES in the fat pad (supplementary material Fig. S2B). These results indicate tissue-specific regulation of *Ppar γ* isoforms by DES. Recent epidemiological studies indicate that exposure to EDCs during early development is associated with overweight, obesity and type 2 diabetes later on (Gladden et al., 2000; Goncharov et al., 2008; Smink et al., 2008; Vasiliu et al., 2006). Other than its adverse effects on the reproductive tract, neonatal DES exposure also leads to the development of obesity in adult animals as young as 2 months of age and throughout adulthood (Newbold et al., 2007). Newbold et al. clearly showed that all white fat depots weighed more in DES-treated mice than in controls, especially the inguinal and retroperitoneal fat pads, whereas brown fat depot weights were not affected. This DES-induced weight gain is ESR1-dependent (Couse et al., 2000). The fact that ESR1 is only expressed in white but not brown adipocytes (Rodriguez-Cuenca et al., 2005) is sufficient to explain the differential response of white and brown adipose tissues to neonatal DES treatment. We speculate that neonatal DES exposure might also activate the adipogenic program through ESR1 in mesenchymal cells, leading to an increased number of adipocytes at an early age. Indeed, we showed that as early as P5, increased *Ppar γ 2* transcript and PPAR γ protein were evident in the abdominal fat pad (white adipose tissue) of mice exposed neonatally to DES (supplementary material Fig. S2B,C). Future investigations addressing molecular changes in fat tissues in DES-treated mice will further our understandings on how DES, and possibly other EDCs, cause obesity and other related diseases.

Lipogenesis and glycolysis are two closely regulated processes sharing common intermediate metabolites such as dihydroxyacetone phosphate (DHAP), which is generated by glycolysis and serves as a main source of glycerol backbone for lipogenesis. Thus, perturbation of one process often affects the other. We observed altered glucose homeostasis in DES-treated UE, including elevated GLUT1 expression and enhanced glucose transport. These findings are in line with previous reports that estradiol alters glucose transporter expression and glucose uptake

in adult mouse and pre-pubertal rat uteri (Kim and Moley, 2009; Welch and Gorski, 1999). In addition, we found that several key enzymes involved in glycolysis, including HK2, ALDO1 and PGK1, are all upregulated in UE cells by DES (Fig. 4 and data not shown). Abnormal glucose metabolism in the UE is also evidenced by the abnormal expression of enzymes involved in the PPP: G6PD, the enzyme responsible for the first and rate-limiting step of PPP, is strongly activated in the UE (Fig. 4). Notably, utilization of PPP leads to production of NADPH, which provides the reducing power needed in cellular biosynthesis, particularly lipogenesis (Chascione et al., 1987). Whether the PPP is the preferred choice of glucose metabolism in DES-treated UE cells requires further investigation.

Our findings also indicate that neonatal DES exposure has a long-lasting effect on UE metabolism. We observed a higher number of lipid droplets in ovariectomized adult UE neonatally exposed to DES, as well as increased G6PD and GLUT1 expression. These data suggest that DES elicited permanent changes in gene expression, which was independent of ovarian hormones. Moreover, neonatal DES exposure altered adult UE response to estrogens, as evidenced by lipid droplet accumulation as well as molecular markers. Previously Newbold and co-workers reported that a high dose of neonatal DES treatment desensitized uterine responsiveness to E2 in juvenile mice, as measured by uterine weight gains (Newbold et al., 2004). By contrast, we found that early DES exposure renders the adult uterus more sensitive to E2-induced lipid droplet formation as well as to some gene expressions (*PPAR γ*). On the other hand, neonatal DES dampened the E2 regulation of *Arntl* expression (supplementary material Fig. S1A). Therefore, early DES exposure leads to a more complex and pleiotropic estrogenic responsiveness in the adult uterus. It is likely that epigenetic changes caused by DES are the underlying mechanism for the long-term effects of DES.

In summary, we have demonstrated that neonatal DES treatment dramatically alters the metabolic pathways in UE cells. It is not clear at this point whether the mouse DES phenotype is relevant to clinical findings; nevertheless, our observations provide a novel mechanism through which DES, and possibly other EDCs, can affect homeostasis of the female reproductive tract.

METHODS

Mice

All mice were housed in the animal facility at Washington University with controlled light and dark cycles and handled in accordance with National Institutes of Health guidelines. All procedures were approved by the Washington University Institutional Animal and Use Committee. Time-pregnant CD-1 mice were purchased from Charles River Breeding Laboratory (Wilmington, MA). DES was prepared and injected subcutaneously into female pups from P1 to P5 as described previously (Huang et al., 2005). PPAR γ inhibitors bisphenol A diglycidyl ether (BADGE) and GW9662 were purchased from Sigma-Aldrich (St Louis, MO). Stock solution (50 \times) was prepared in DMSO at a concentration of 150 mg/ml for BADGE and 10 mg/ml for GW9662. Working solutions were made fresh in sterile PBS on the day of injection. PPAR γ inhibitor was administered daily intraperitoneally at a concentration of 30 mg/kg for BADGE (Naveiras et al., 2009) or 2 mg/kg for GW9662 (De Backer et al., 2009). PPAR γ agonist pioglitazone (Sigma) was dissolved in either pure corn oil or DES

solution and injected subcutaneously (20 mg/kg) into neonatal female mice from P1 to P5 (Ji et al., 2009). Routine ovariectomy was performed on 8-week-old mice exposed to oil or DES from P1 to P5. The mice were allowed to recover for 2 weeks, followed by one subcutaneous injection of either 100 μ l corn oil, 100 μ l DES (20 μ g) or 100 μ l E2 (100 ng) (Sigma). Uteri were harvested for fixation or RNA extraction 24 hours later.

Separation of UE cells

To isolate UE cells, we followed a protocol described previously with slight modifications (Biggs et al., 1986). Briefly, whole uteri were dissected from P5 mice of the same treatment group and pooled accordingly. Each uterine horn was cut into 3–4 mm pieces and rinsed in Ca^{2+} -free, Mg^{2+} -free Hank's balanced salt solution (HBSS). Tissues were digested in 1% trypsin in HBSS at 4°C for 1 hour on a rotating platform. An equal volume of 5 mg/ml bovine serum albumin (BSA) was added to stop the digestion. Gentle pressure was applied along each piece with tweezers to squeeze out the epithelia, which were transferred to 1.5-ml tubes by pipetting. The epithelial cells were collected by low-speed centrifugation. The remaining uterine tissue containing mostly mesenchymal cells was transferred to 1.5-ml tubes and spun down.

RNA extraction and quantitative RT-PCR

Total RNA was isolated from the cell and tissue pellets with RNA Stat-60 (Tel-Test, Friendswood, TX) following the manufacturer's instructions. Primer design, reverse transcription and real-time RT-PCR were performed as previously described (Yin et al., 2008). Student's *t*-test was performed on biological triplicates and $P < 0.05$ was considered statistically significant.

cDNA microarray and data analysis

Oil- or DES-treated P5 mice were harvested and the epithelia separated from the mesenchyme. UE or UM tissues from three or four animals of the same treatment group were pooled together and RNA isolated to make one sample. Three RNA samples of each tissue per treatment group were used for microarray analysis for statistical purposes. Total RNA was cleaned using RNeasy Kit (Qiagen, Valencia, CA) and 1 μ g purified RNA of each sample was submitted to the Genome Technology Access Center at Washington University School of Medicine. A total of 12 samples were randomly applied to two Illumina MouseWG-6 BeadChips, hybridized and imaged as per the manufacturer's instructions. An average normalization algorithm was used to normalize the signals and is available upon request. Normalized signals were first filtered by intensity to eliminate genes that were not expressed in any of the four groups. Average signal intensity and fold-change for each probe were then calculated (oil UE versus DES UE and oil UM versus DES UM). The remaining data were further filtered to eliminate those significantly changed by no more than twofold ($P < 0.05$ was considered significant) in any of the two comparisons. Hierarchical clustering was performed on DRGs using the program Cluster 3.0 and the output dendrograms and heatmaps were visualized using the program Treeview (Eisen et al., 1998). Star Glyph was plotted in Microsoft Excel 2007. The data discussed in this paper have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO series accession number GSE37969 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37969>).

Western blot, immunohistochemistry, immunofluorescence and Oil-Red O staining

Western blot, immunohistochemistry and immunofluorescence were performed as described previously (Yin et al., 2008; Yin et al., 2011). Antibodies and dilutions used were: 1:100 (immunohistochemistry) and 1:1000 (western blot) for PPAR γ (Cell Signaling); 1:5000 (western blot) for GAPDH (Cell Signaling); 1:200 (immunofluorescence) for KLF4 (Santa Cruz Biotechnology); 1:100 (immunofluorescence) for G6PD and GLUT1 (generous gifts from Kelle Moley, Washington University, St Louis, MO); 1:100 for HK2 (Cell Signaling); 1:1000 for Alexa-Fluor-594-conjugated goat anti-rabbit (Invitrogen) and 1:5000 for HRP-conjugated goat anti-rabbit secondary antibodies. For ORO staining, tissues were fixed in 4% paraformaldehyde briefly, washed through a series of Tissue-Tek optimal cutting temperature compound (OCT)-sucrose solutions, and embedded in OCT Slides of 10- μ m frozen sections were washed in water once, twice in 100% propylene glycol (Sigma) and then stained in 0.7% ORO in propylene glycol for 7 minutes with agitation at 60°C. Slides were then washed in 85% propylene glycol briefly, rinsed in water and mounted with glycerin jelly.

Transmission electron microscopy

Uterus was fixed in 4% PFA and 2.5% glutaraldehyde, contrasted with osmium tetroxide and embedded in resin. Ultrathin sections were cut and examined under a Hitachi H7600 TEM system. To count lipid droplets in each image, a line was drawn along the basement membrane of the UE cells, its length measured. All

TRANSLATIONAL IMPACT

Clinical issue

In utero exposure to endocrine-disrupting chemicals (EDCs) is associated with reproductive tract malformations, cancer, obesity and type 2 diabetes later in life. Diethylstilbestrol (DES), a synthetic estrogen that was prescribed in the 1940s–1970s to prevent miscarriage, is a prototype EDC that causes reproductive tract malformations and cancers in exposed populations. An established mouse model has shown that neonatal DES exposure results in uterine malformations, uterine adenocarcinoma and weight gain in adult mice. However, the molecular mechanisms underlying DES toxicity have been elusive.

Results

The authors use a genetic profiling approach to uncover genes regulated by DES in the mouse uterus and identify several new targets, including genes involved in lipid and glucose metabolism. They show that DES activates the transcriptional program for adipogenesis in the uterine epithelium, and identify peroxisome proliferator-activated receptor- γ (Ppar γ) as a crucial player in this process. Furthermore, they found that DES exposure altered glucose metabolism in the uterine epithelium. Finally, the authors show that neonatal DES treatment elicits permanent metabolic changes in the uterus that are still detectable in adult mice.

Implications and future directions

These findings indicate that DES exposure causes marked metabolic changes in uterine epithelial cells, some of which persist into adulthood, providing new clues regarding how EDCs might cause reproductive problems. In addition to affecting uterine epithelium, DES also induces PPAR γ expression in neonatal adipose tissue; the authors propose that *in utero* exposure to other EDCs might contribute to adult obesity via activation of the adipogenic program through similar molecular mechanisms. Future studies that investigate molecular changes in adipose tissues in DES-exposed mice will further understanding on how DES, and possibly other EDCs, contribute to obesity and related diseases.

droplets in the included UE cells were counted. The average number of lipid droplets per length was calculated from images (five images per sample, $n=3$ per treatment). Student's t -tests were performed for statistical analysis.

Glucose uptake assay

Glucose uptake assays were performed on freshly isolated UE cells from oil- or DES-treated mice on P5 following previously described methods with modifications (Frolova et al., 2009; Keawpradub and Purintrapiban, 2009). Briefly, enriched UE cells were washed and incubated in 1× Krebs-Ringer buffer (125 mM NaCl, 4.7 mM KCl, 2 mM CaCl₂, 2.4 mM MgSO₄, 25 mM HEPES, 0.5% BSA and 1.2 mM K₂HPO₄) containing 1 mM glucose for 10 minutes. Cells were spun down then resuspended in 1 ml 1× Krebs-Ringer buffer containing 1 mM glucose and 0.1 μCi/ml 2-deoxy-D-glucose, [1-¹⁴C] (MP Biomedicals) for exactly 1 minute. Cytochalasin B (5 μl of 50 mM stock solution) was added to the reaction to stop glucose uptake. Cell pellets were washed and lysed in 0.05 N NaOH. Radioactivity taken up by the cells was determined using a scintillation counter. Aliquots from each sample were used to determine the protein concentration using the Bradford assay. Reactions were normalized to total protein, and glucose uptake expressed as DPM/mg protein/10 minutes. Three pools of UE cells receiving control or hormone treatment were used for statistical analysis.

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COMPETING INTERESTS STATEMENT

The authors have nothing to declare.

AUTHOR CONTRIBUTIONS

Y.Y. and L.M. conceived and designed experiments. Y.Y., C.L., G.M.V. and H.C. performed the experiments. Y.Y., C.L., M.D. and L.M. analyzed data. Y.Y., C.L. and L.M. wrote the paper.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at <http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.009076/-/DC1>

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