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Altered Gene Expression Patterns During the Initiation and Promotion Stages of Neonatally Diethylstilbestrol-Induced Hyperplasia/Dysplasia/Neoplasia in the Hamster Uterus

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Abstract

Neonatal treatment of hamsters with diethylstilbestrol (DES) induces uterine hyperplasia/dysplasia/neoplasia (endometrial adenocarcinoma) in adult animals. We subsequently determined that the neonatal DES exposure event directly and permanently disrupts the developing hamster uterus (initiation stage) so that it responds abnormally when it is stimulated with estrogen in adulthood (promotion stage). To identify candidate molecular elements involved in progression of the disruption/neoplastic process, we performed: 1) immunoblot analyses and 2) microarray profiling (Affymetrix Gene Chip System) on sets of uterine protein and RNA extracts, respectively, and 3) immunohistochemical analysis on uterine sections; all from both initiation stage and promotion stage groups of animals. Here we report that: 1) progression of the neonatal DES-induced hyperplasia/dysplasia/neoplasia phenomenon in the hamster uterus involves a wide spectrum of specific gene expression alterations and 2) the gene products involved and their manner of altered expression differ dramatically during the initiation vs. promotion stages of the phenomenon. Particularly interesting changes included members in the functional categories of nuclear receptors (progesterone receptor), cell-cell interactions (E-cadherin, connexins), cytokine action (IRF-1, Stat5A), growth factor action (IRS-1), extracellular matrix component (tenascin-C), transcription factors (Nrf2, Sp1), and multi-functional nuclear protein (SAFB1).

1. Introduction

The medical misadventure commonly known as the “DES Syndrome” resulted from the mistaken belief that treatment during pregnancy with diethylstilbestrol (DES), the first orally

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active estrogen [1], would protect against miscarriage [2]. That treatment regimen began in 1947 and then quickly and greatly expanded worldwide [2] even though evidence questioning its effectiveness appeared as early as 1953 [3]. Unfortunately, it wasn't until 1971 with two independent reports of clear cell vaginal adenocarcinoma in the young daughters of DES-treated mothers that such treatment ceased [2]. Since then, numerous clinical and experimental animal studies of the effects of perinatal DES exposure documented teratogenic and neoplastic lesions throughout both the female and male reproductive tracts and thereby established DES as a transplacental carcinogen and the prototypical endocrine disruptor agent [2, 4].

To study the phenomenon of perinatal DES-induced endocrine disruption, we established a convenient and sensitive model system using Syrian golden hamsters [5]. In that system, we defined the progression and extent of endocrine alterations and morphological lesions in the reproductive tracts of both females and males [5–9]. A particularly striking observation very early in the system was that, in mature (postpubertal) hamsters, 100% of the neonatally DES-exposed uteri developed hyperplasia and a large proportion progressed to neoplasia (endometrial adenocarcinoma) [5]. We subsequently determined that, consistent with the two-stage model of carcinogenesis [10], neonatal DES exposure directly and permanently alters (re-programs) the developing hamster uterus (initiating event) such that it responds abnormally later in life to stimulation (promoting event) with the natural estrogen, estradiol [5, 6]. We are now probing the mechanism of this two-stage phenomenon at the molecular level. Here we report that: 1) progression of the neonatal DES-induced hyperplasia/dysplasia/neoplasia phenomenon in the hamster uterus involves a wide spectrum of specific gene expression alterations and 2) the gene products involved and their manner of altered expression differ dramatically during the initiation vs. promotion stages of the phenomenon.

2. Materials and methods

2.1. General Animal Information

Animals were maintained and treated in an AAALAC-accredited facility as authorized by the Wichita State University Institutional Animal Care and Use Committee (IACUC). All procedures including neonatal treatment, anesthesia, ovariectomy, chronic estrogenic stimulation, sacrificing, and tissue collections followed well-established [5–7] and IACUC-approved protocols.

2.2. Neonatal Animal Treatment

Timed pregnant Syrian golden hamsters (*Mesocricetus auratus*) from Charles River Breeding Laboratories (Wilmington, MA) or Harlan Sprague Dawley, Inc. (Indianapolis, IN) were caged singly under a 14 hr light:10 hr dark photoperiod at 23–25°C with laboratory chow and water provided ad libitum. The food was a 2:1 mixture of #5001 rodent diet and #5015 mouse diet from LabDiet (PMI Nutrition Int. LLC, Brentwood, MO). According to the manufacturer, total isoflavone (diadzein, genistein, glycitein) content of that diet mixture was 426 mg/kg. Within 6 hr of birth (day-0), litter size was adjusted to eight neonates/litter by eliminating males and all animals in a litter received a single s.c. injection of 50 µl corn oil vehicle either alone (control, CON) or containing 100 µg of DES (both from Sigma

Chem. Co., St. Louis, MO). As acknowledged previously [5–7], that dose level is high but not unreasonable considering that DES ingestion levels by women were as much as 150 mg daily and 18.2 g total during their pregnancy [11]; with the median total dose being 10.7 g [12]. It is also the dose level we previously used in hamsters to establish, assess, and compare neonatal DES-induced disruption in various regions of both the female and male reproductive tract [5–7]. Tissues were harvested (see below) from some of these neonatal animals when they were 5 days of age (Initiation Stage or IS).

2.3. Prepubertal Procedures

On day 21 of life (~7 days prior to puberty), groups of control and neonatally DES-treated animals were bilaterally ovariectomized and began chronic estrogen stimulation by the s.c. insertion (between the shoulder blades) of a plugged Silastic (Dow Corning Corp., Midland, MI) tube (open lumen length, 1.0 cm; inner diameter, 1.57 mm; outer diameter, 2.41 mm) filled with crystalline estradiol-17 β (from Sigma Chem. Co.; St. Louis, MO) (O+E2). According to previous determinations [5, 6], that procedure maintains serum E2 levels at approximately 200 pg/ml for at least 5 mo. Tissues were harvested (see below) from the O+E2 animals when they were 2 months of age (Promotion Stage or PS).

2.4 Tissue harvesting and processing

Both IS and PS animals were anesthetized/asphyxiated with CO₂ and then decapitated. For preparation of total protein and RNA extracts, isolated and trimmed uterine horns were snap frozen on dry ice and cryostored at –80°C. For histological processing, mid-region uterine horn segments from freshly killed animals were immediately placed in fixative (4% paraformaldehyde in Dulbecco's phosphate-buffered saline [PBS], pH 7.2) followed by two changes (24 hr each) of fresh fixative, stored in 70% ethanol, and ultimately embedded in paraffin so as to generate transverse (cross) sections.

2.5 Preparation of total protein extracts

Frozen tissues were quickly weighed, received 9 volumes (ml/g) of hypotonic buffer (10 mM Tris base, 1 mM ethylenediaminetetraacetic acid, pH 7.5), homogenized on ice (2 $\times$ 5 sec with a Tekmar Model TR-19 TissueMizer at a power setting of 70), received ¼ volume of 5 $\times$ sample buffer (to provide a final concentration of 0.1 M dithiothreitol, 2% sodium dodecyl sulphate, 80 mM Tris base, pH 6.8, 10% glycerol, 1% saturated bromophenol blue solution), brought to 100°C for 5 min, and cryostored at –20°C. As noted previously [8], this procedure yields groups of denatured total protein extracts that are matched or normalized based on tissue equivalents.

2.6 Preparation of total RNA extracts

Frozen tissues (50 – 100 mg) received 1 ml TRIzol[®] Reagent (Ambion, Grand Island, NY), were homogenized as above (preparation of protein extracts), incubated at 25°C for 5 min, received 0.2 ml chloroform and mixed (shaken by hand for 15 sec), incubated at 25°C for 2–3 min, and then centrifuged (12,000 $\times$ g at 4°C for 15 min). The resulting upper/aqueous phase fractions were transferred to pre-centrifuged Eppendorf Phase Lock Gel tubes, re-mixed, and re-centrifuged. The resulting upper/aqueous phase fractions were transferred to

fresh tubes, received 0.5 ml isopropanol, incubated at 25°C for 10 min, re-centrifuged, supernatants were aspirated, RNA pellets were air-dried, dissolved in 100 µl RNase-free water at 55–60°C for 10 min, and stored at –80°C following spectrometric analysis/quantitation at 230/260/280/320 nm.

2.7 Western blot analysis

The Western blot (WB) procedure was as fully described for our analysis of extracts prepared from male hamster reproductive tract tissues [8]. In brief, sets of uterine extract aliquots (25 µl) prepared from control and neonatally DES-exposed animals (both IS and PS) were electrophoresed under denaturing conditions on 5–15% acrylamide gradient gels. The gels were either Coomassie stained to visualize the overall pattern of resolved proteins or were electro-transferred to nitrocellulose membranes. The membrane blots were probed with antibodies directed against the indicated protein targets (see Table 1 for descriptions of antibody provider, species source, monoclonal or polyclonal type, and dilution or concentration used) and the primary antibody:antigen complexes were detected using a biotin-labeled and species-specific anti-IgG second antibody followed by an avidin-biotin-horseradish peroxidase complex (ABC) reagent (both from Vector Laboratories, Burlingame, CA) and finally a diaminobenzidine (DAB) substrate reagent (SigmaFast™ from Sigma, St. Louis, MO) that generates deposition of an insoluble, dark-brown product. Densitometric scans of the blots were analyzed using Quantity One® quantitation software from Bio-Rad Laboratories (Chicago, IL). The quantitative data was analyzed by Student's t-tests to determine if differences between mean values for triplicate sets of extracts from control versus DES-exposed uteri were statistically significant ($p < 0.05$).

2.8 DNA microarray analysis

Gene expression profiling using sets of total RNA prepared from control and neonatally DES-exposed uteri (both IS and PS) was carried out with the Affymetrix GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA). This array consists of over 45k probe sets covering over 39k transcripts and variants from over 34k well-characterized mouse genes. Probe intensities from the arrays were RMA-background corrected, quantile-normalized and gene-level summarized using the Median Polish algorithm [13]. The resulting log (base 2) transformed signal intensities (expression values) were used for ascertaining differentially expressed genes between the two treatment groups. Fold-change statistics for individual genes were calculated by taking the linear contrast between the least square means of the (log) DES-exposed and (log) control groups and back transforming the result to a linear scale (this is the ratio of the geometric mean of the DES-exposed samples to the geometric mean of the control samples). Corresponding significance scores (p-values) were assigned based on the t-statistic of the linear contrast. The false discovery rate (FDR) was calculated using the Benjamini and Hochberg procedure. Expression measurement replicates were three each from control and DES-exposed groups of IS animals and six from control and five from DES-exposed groups of PS animals. All computations were performed in Matlab (R2009b, The MathWorks Inc, Natick, MA) and the Partek Genomic suite (v 6.5, Partek Inc., St. Louis, MO).

2.9 Histology and immunohistochemistry

Uterine tube cross sections (4.5 μm) were deparaffinized in xylene and then rehydrated. For histology, sections underwent standard hematoxylin and eosin (H&E) staining. For immunohistochemistry (IHC), antigen retrieval was performed in 10 mM trisodium citrate, pH 6.0 at 95°C for 30 min and then endogenous peroxidase activity was quenched with 3% H_2O_2 in MeOH at 37°C for 30 min. After rinsing (here and between subsequent steps) in PBST (PBS plus 0.05% Tween 20, pH 7.5), sections were blocked first with 10% serum (from the same species used to raise the relevant second antibody) at 37°C for 20 min and then with avidin/biotin solutions (SP2002 kit from Vector Laboratories, Burlingame, CA) according to kit instructions. After incubation with primary antibody (see Table 1 for descriptions of antibody provider, species source, monoclonal or polyclonal type, and see photomicrograph figure legends for dilution or concentration used) for either 35 min at 37°C or overnight at 4°C, sections were incubated with appropriate species-specific anti-IgG antibody:biotin conjugate (15 $\mu\text{g}/\text{ml}$) and then with Vectastain® Elite ABC reagent (both from Vector Laboratories, Burlingame, CA); both at 37°C for 20 min and all reagents diluted in PTM (PBST plus 5% nonfat dry milk). Finally, sections were reacted with DAB substrate reagent (same as for WB protocol) at 37°C for a maximum of 15 min, rinsed with H_2O , counterstained with Methyl Green, and coverslipped using Permount™. All photomicrographs were captured using a Nikon Eclipse E800 microscope fitted with CFI Plan Apochromat objectives and a Nikon Digital/DSFi1 color camera linked to a NIS-Elements image analysis system.

3. Results

3.1 Uterine histomorphology

Representative histomorphology of the uteri used for this study is shown in Fig. 1. Our choice of age and treatment conditions to represent IS and PS animal sample groups is based on an extensive assessment of how the neonatal DES-induced disruption phenomenon progresses in the hamster uterus [5]. Some of the disruption effects that peak on day 5 of life in pre-pubertal animals (IS) include: 1) the DES-exposed uteri are considerably larger, 2) their endometrial epithelium is more folded but does not yet form true glands, and 3) their endometrial epithelial cells are much taller (hypertrophy) compared to control uteri. In adult (2-mo old) E_2 -stimulated animals (PS), cystic glandular structures develop in all uteri but the DES-exposed uteri remain much larger and they are more hypertrophic and exhibit more severely disrupted histomorphology compared to control uteri. Specifically, the luminal and glandular epithelium in DES-exposed uteri are markedly hyperplastic to dysplastic because the cells are extremely tall, disorganized, overcrowded, and poorly demarcated from the underlying stroma. Furthermore, that epithelium appears “foamy” because it is riddled with infiltrating leukocytes and cavities that contain degenerating (apoptotic) cells [5].

3.2 Western blot analysis

The proteomic profiling findings reported here are part of our long-term research program to: 1) define how the neonatal DES-induced disruption phenomenon progresses and 2) elucidate the molecular mechanisms responsible for it in various regions of both the female and male hamster reproductive tract. Prior results of that program plus clues from the

literature about gene products implicated in both normal and pathological development and function of reproductive tract tissues/organs guided our choice of antibodies and protein targets to test here. Of the more than 200 antibodies tested, Table 1 lists: 1) those (24) from the indicated sources and of the indicated type (species, polyclonal or monoclonal) that generated detectable, specific, and reproducible signal bands on immunoblots; 2) molecular weight of the detected target band according to migration position relative to that of standard proteins; 3) whether the target bands were detected using extracts from IS and/or PS animals; 4) densitometric quantitation of the bands when analyzed in triplicate sets of sample extracts from control and DES-exposed uteri; and 5) whether differences in the mean densitometric values between the control vs. DES-exposed groups for each target band were statistically significant.

Evidence that tissue total protein loading was equivalent among the electrophoretically resolved sample lanes prior to WB is shown in the left panels of Fig. 2 for IS animals and Fig. 3 for PS animals. Those panels also demonstrate that the pattern and density of all the detectably Coomassie-stained proteins were quite similar in all six sample lanes for both IS and PS animals. The right panels of Fig. 2 (IS) and Fig. 3 (PS) show actual immunoblot results for: 1) all those target proteins for which detectable bands were present at 2-fold and at significantly different levels in uterine extracts from control vs. DES-exposed animals and 2) two targets (different specific proteins [PCNA and ER α] and a pair of non-specific bands [NSB] consistently seen in IS and PS extracts) that were present at similar levels in the uterine extracts from control and DES-exposed animals (top two signal band profiles in both right panels). For many of the immunopositive target proteins listed in Table 1, the relative differences in mean band intensity between the triplicate sets of extracts from control vs. DES-exposed uteri are shown in Fig. 4 for IS animals and in Fig. 5 for PS animals.

Of the 24 proteins detected with some of the antibodies tested in this study, levels of 9 (β -catenin, ER α , FAK, laminin B1, NES1, p100, PCNA, and vimentin) were not significantly different in extracts from control compared to DES-exposed uteri at either stage. Those immunoblotting results plus the total protein staining patterns (left panels of Figs. 2 and 3) confirm that neonatal DES-exposure did not disrupt the overall protein makeup of the uterus. However, neonatal DES-exposure did affect the total organ level of a wide range of specific proteins (or isoforms thereof) in uteri from IS and PS animals as described below (expressed as up-regulated or down-regulated in extracts from DES-exposed vs. control uteri).

3.2.1 Western blotting / IS differences (Figs. 2 and 4)—Individual connexin molecules join to make hexameric hemichannels (connexons) that dock to connexons on neighboring cells and thereby form gap junction pores [14]. The expression level, cellular localization, and function of those intercellular communication moieties are implicated in both normal and dysplastic/neoplastic development in various physiological systems [15]. Both the anti-connexin-26 and anti-connexin-43 antibodies we used generated bands that were up-regulated (23.0 $\times$ and 3.6 $\times$, respectively). While the set of isoform bands generated by the anti-connexin-43 antibody were of the expected molecular weight (~43 kDa), the

anti-connexin-26 antibody generated single isoform bands of a higher molecular weight (46 kDa) than expected.

The progesterone receptor (PR) is a member of the nuclear receptor superfamily of ligand-activated transcription factors, is expressed as two predominant isoforms (PR-A and PR-B), and mediates action of the ovarian steroid progesterone in multiple tissues and organs including the brain, breast, uterus, ovary, and cervix [16]. A dramatic effect that we observed early and very consistently in our analyses was precocious expression (up-regulation) of both PR-A and PR-B isoforms and thus also their total levels (64.1×, 70.1×, and 67.6×, respectively).

The specificity protein transcription factors (Sp1 and Sp3) are expressed in all mammalian cells, regulate the expression of numerous genes involved in cell proliferation, apoptosis, and differentiation, and they are dysregulated in many diseases including cancer [17]. Furthermore, the Sp1 family of proteins are implicated as important mediators of steroid hormone action in the endometrium [18]. While the triad of higher molecular weight isoforms (103–86 kDa) of Sp1 was not affected, the lower molecular weight isoform (51 kDa) was up-regulated (2.6×).

The Stat (signal transducers and activators of transcription) proteins are activated in response to various cytokines, growth factors, and hormones [19]. The Stat5a and Stat5b family members can modify chromatin organization and also play important roles in cell proliferation, apoptosis, and differentiation [20]. We observed up-regulation (2.7×) of the Stat5a family member.

Tenascin-C is a matricellular protein implicated in intercellular cell adhesion, tissue repair, and in neoplastic processes including angiogenesis, epithelial/mesenchymal transition (EMT), and metastasis [21]. In particular, altered tenascin-C expression is linked to endometrial carcinoma expression both in vivo [22, 23] and in vitro [24]. We detected a number of signal bands for the large tenascin-C protein monomer (>200–148 kDa), all of which were up-regulated (27.1×).

3.2.2 Western blotting / PS differences (Figs. 3 and 5)—While all the significant proteomic expression differences described above for the uterus in IS animals were up-regulated in nature, we detected both up-regulated and down-regulated effects in the uterus of PS animals. They included some of the protein targets affected in IS animals but the overall spectrum of effects was considerably wider.

The androgen receptor (AR), another member of the nuclear receptor superfamily of ligand-activated transcription factors, is expressed and involved in the development and regulated function of the mammalian uterus [25, 26]. As found in the neonatally DES-exposed seminal vesicle of adult hamsters [8], AR protein expression also was down-regulated (89%) in PS uteri.

The E-cadherin/β-catenin protein complex plays pivotal roles in maintaining epithelial cell/tissue integrity [27] and altered expression of E-cadherin, both up and down-regulation, is implicated in various stages of carcinogenesis [28]. Furthermore, the normal and neoplastic

functions of E-cadherin are influenced by complex posttranslational processing events including endocytosis, proteolysis, and recycling [28, 29]. We found that the total level of immunodetected E-cadherin protein forms was up-regulated (3.4×). Contributing to that were up-regulated levels of the full-length (119 kDa) protein (22.9×) and a slightly smaller (103 kDa) but much fainter form (8.6×). In contrast, the level of an intermediate form (35 kDa) was not affected while that of the smallest form (23 kDa) was down-regulated (95%).

As observed in IS uteri (see above), the anti-connexin-26 antibody generated single isoform bands that were up-regulated (12.5×) in PS uteri. Also as noted above, the molecular weight of those bands (46 kDa) was higher than expected. Whether this observation represents an antibody cross-reaction with another specific connexin isoform particular to the hamster remains undetermined.

Interferon regulatory factor-1 (IRF-1) is a transcriptional activator that regulates immune responses and exhibits marked functional diversity in the regulation of carcinogenesis [30]. We found that expression of this protein was up-regulated (5.9×).

The family of insulin receptor substrate proteins (IRS-1 – IRS-4) serve as intermediates in the action of cell surface receptors that, in addition to their metabolic and growth regulation functions, are implicated in malignant transformation [31, 32]. We found that the IRS-1 family member protein was down-regulated (67%).

The proto-oncogene/stem cell receptor c-Kit is a transmembrane protein with tyrosine kinase activity, was early identified as the product of a “transforming gene” [33], and is now an active target for new cancer therapies [34]. We found that expression of the c-Kit protein was up-regulated (7.3×).

The active NFκB transcription factor complex consists of two subunits (NFκB1 and RelA or p50 and p65, respectively) that regulate a wide variety of cellular and viral genes involved in both normal and tumor development [35]. We found that the full-length NFκB, p50 protein was not affected but a smaller form (38 kDa) was down-regulated (74%).

The Nrf2-Keap (nuclear factor [erythroid-derived 2]-like - Kelch-like ECH-associated protein 1) pathway is: 1) a master regulator of the anti-oxidative stress response and anabolic metabolism; 2) co-regulated with the p53 tumor suppressor gene product; and 3) highly active in a variety of human cancers and associated with tumor aggressiveness [36]. We found that expression of the Nrf2 protein was up-regulated (7.1×).

The integral membrane linker protein occludin participates in the formation of intercellular tight junctions and their dysregulation is a hallmark of many diseases including cancer [37]. We found that the full-length (57 kDa) occludin protein was not affected but a smaller form (49 kDa) was up-regulated (3.5×).

A protein whose isoform expression was dramatically altered in both IS and PS uteri is the progesterone receptor (PR). However, whereas in IS uteri both PR-A and PR-B isoforms were precociously expressed (up-regulated), expression of both of them and thus their sum were down-regulated (71%, 82%, and 74%, respectively) in PS uteri.

Scaffold attachment factor B1 (SAFB1) is another multifunctional protein involved in the stress response and in cancer [38]. For that protein, we found that levels of the smallest form (38 kDa) were not affected but that of three larger forms (182/158 and 65 kDa) were down-regulated (52% and 65%, respectively) such that their sum was also down-regulated (50%).

Another protein whose isoform expression was dramatically altered in both IS and PS uteri was Sp1. However, whereas only the smallest isoform was up-regulated in IS uteri, expression of all isoforms (103-86 and 51 kDa) and thus their sum was up-regulated (10.3×, 3.8×, and 6.9×, respectively) in PS uteri.

3.3 DNA microarray analysis

The mouse genome-based microarray analysis detected multiple genes whose expression level at the mRNA level in whole organ extracts were significantly and 2-fold different in control vs. DES-exposed uteri from both IS and PS animals (Supplemental Tables). However, only a few of them were consistent with the differences noted at the proteomic level according to our WB analyses (see above). In IS uteri, those differences included 2.5× up-regulation of connexin-43 mRNA (*Gja1*) and 2.0× up-regulation of PR mRNA (*Pgr*). In PS uteri, they included only 47% down-regulation of IRS-1 mRNA (*Irs1*).

3.4 Immunohistochemistry analysis

First, Fig. 6 illustrates the typical negative control immunohistochemistry (IHC) results (uniform blue counterstain but no brown reaction product) observed in control and DES-exposed uterine tissue sections from both IS and PS animals. Of those antibodies that generated specific signal bands in our WB analyses of total protein extracts prepared from IS and PS uteri, not all of them generated clear and specific staining of tissue sections prepared from the same organs. Representative results for those that did (ordered alphabetically as in Table 1) are shown in Figs 7 – 18 and they depict some interesting patterns at the tissue and cell-specific level.

As expected, staining for the intercellular adhesion protein E-cadherin (Fig. 7) localized primarily to the plasma membrane of luminal epithelial cells in all tissue sections. While the signal intensity there was similar between control and DES-exposed uteri from IS animals, it was clearly stronger in DES-exposed uteri from PS animals. Also in the latter, staining was enhanced in the stromal/mesenchymal tissue compartment. Thus these IHC results match our WB analysis results (up-regulated total isoforms in PS).

The β -catenin protein was initially identified as a structural adaptor linking cadherins to the actin cytoskeleton in cell:cell adhesion but is now also known as a transcription cofactor with the T cell factor/lymphoid enhancer factor (TCF/LEF) in the Wnt pathway that regulates cell proliferation and differentiation [27, 39]. Staining for this protein (Fig. 8) was plasma membrane/cytoplasm-localized in the luminal epithelial cell compartment of all tissue sections and somewhat more intense there in the sections from PS vs. IS animals. Also consistent with our WB analyses, overall staining levels were similar in control and DES-exposed uteri from both IS and PS animals.

Regarding gap junction channel structure elements, staining for the antigen recognized by the anti-connexin-26 antibody (Fig. 9) was primarily plasma membrane/cytoplasm-localized in luminal epithelial cells and up-regulated in uteri from both IS and PS animals. In contrast, staining for the connexin-43 protein (Fig. 10) was up-regulated in both the luminal epithelial and stromal/mesenchymal uterine tissue compartments of IS animals whereas in PS animals, overall staining levels in the uterus were similar but the intensity of staining specifically in the luminal epithelial compartment was down-regulated. Still, we can conclude that staining intensities at the overall organ level match our WB analysis results (connexin-26: up-regulated in both IS and PS; connexin-43: up-regulated only in IS).

Another member of the nuclear receptor superfamily of ligand-activated transcription factors, the alpha form of the estrogen receptor (ER α), is a well established mediator of normal and neoplastic development of the mammalian uterus [26, 40–42]. Staining for this protein (Fig. 11) was nuclear-specific in stromal/mesenchymal cells but both cytoplasmic and nuclear in luminal epithelial cells of uteri from both IS and PS animals. Also consistent with our WB analysis results, overall IHC staining levels for ER α were similar in the control and DES-exposed uteri of both IS and PS animals.

Focal adhesion kinase (FAK) is a tyrosine kinase that is linked to cell:extracellular matrix (ECM) signaling [43, 44] and may also exert nuclear functions [45] in both normal and neoplastic cells. We observed that FAK staining (Fig. 12) was: 1) distinctly membrane/cytoplasm-localized in the luminal epithelial cells of all uterine sections, 2) present but less distinctly localized in the stromal/mesenchymal tissue compartment, and 3) the latter staining pattern was more pronounced in sections from PS than IS animals. However, and again consistent with our WB analysis results, overall IHC staining levels were similar in control and DES-exposed uteri from both IS and PS animals.

Staining of the IRF-1 transcriptional activator protein (Fig. 13) was relatively uniform but indistinctly localized in the stromal/mesenchymal tissue compartment of all uterine sections. In the luminal epithelial cells, staining was: 1) more intense at the apical membrane/cytoplasm region and similar in the control and DES-exposed uteri from IS animals but 2) uniformly membrane/cytoplasm-localized and much more intense in the DES-exposed uteri from PS animals. The latter observation is also consistent with our WB analysis results (up-regulated in PS).

Staining patterns for the IRS-1 protein (Fig. 14) were quite interesting. In IS animals: 1) staining in the luminal epithelial compartment was intensely cytoplasmic in control uteri but undetectable in DES-exposed uteri and 2) staining in the stromal/mesenchymal tissue compartment was also cytoplasmic but somewhat more intense in DES-exposed than in control uteri such that 3) overall staining levels were similar in control and DES-exposed uteri. In PS animals, staining was detected only in the stromal/mesenchymal tissue compartment of both control and DES-exposed uteri but was: 1) distributed both cytoplasmically and in the ECM and 2) less intense in DES-exposed than in control uteri. The overall staining levels thus were consistent with our WB analysis results (down-regulated in PS).

Another member of the nuclear receptor superfamily of ligand-activated transcription factors for which IHC staining patterns were distinctive was the progesterone receptor (PR) protein (Fig. 15). For IS animals, staining was nuclear only in some of the luminal epithelial cells in uteri from control animals but was uniformly nuclear in all luminal epithelial and stromal/mesenchymal cells in DES-exposed uteri. In contrast for PS animals, nuclear staining was detected in both luminal epithelial cells and stromal/mesenchymal cells of control uteri but was not detected in the luminal epithelial cells of DES-exposed uteri. Consequently, the overall staining levels matched the divergent differences (up-regulated in IS; down-regulated in PS) noted in our WB analyses.

Proliferating cell nuclear antigen (PCNA) functions as a eukaryotic sliding clamp that coordinates various DNA interactions involved in DNA replication, repair, chromatin dynamics, and cell cycle regulation and is one of several nuclear proteins for which post-translational modifications are implicated in both normal and neoplastic development [46]. In all tissue sections, staining for PCNA (Fig. 16) was nuclear-specific in both luminal epithelial and stromal/mesenchymal compartments. While cell staining was less uniform in uteri from PS than from IS animals, overall staining levels were similar in control and DES-exposed and thus also consistent with our WB analysis results.

Staining of the Stat5A protein (Fig. 17) was more intense in both the luminal epithelial and stromal/mesenchymal compartments of the DES-exposed uteri from IS animals. Also, the staining pattern there was cytoplasmic in the luminal epithelial compartment but was more nuclear-localized in the stromal/mesenchymal compartment. The same localization pattern but similar staining levels were detected in control and DES-exposed uteri from PS animals such that the overall staining levels were consistent with our WB analysis results (up-regulation in IS).

As expected, detectable staining for the tenascin-C protein (Fig. 18) was present exclusively in the ECM of the stromal/mesenchymal compartment. While that staining pattern was undetectable in control uteri from IS animals, its level was similar among DES-exposed uteri from IS animals and both control and DES-exposed uteri from PS animals such that the overall staining levels were consistent with our WB analysis results (up-regulation in IS).

4. Discussion

Although considerable clinical [2] and experimental [4] evidence establishes DES as the prototypical endocrine disruptor agent, elucidation of its mechanism of action at the molecular level remains incomplete [12, 47, 48]. Furthermore, we found in the hamster that neonatal DES exposure elicits distinct disruption profiles in various male and female reproductive tract regions that include both direct and indirect mechanisms [5–9]. Here we focused on the hamster uterus and assessed the stage-specific pattern of altered gene expression at the mRNA and protein levels. Our findings regarding altered expression of specific gene products are grouped and discussed below according to functional categories.

4.1 Nuclear receptors

Members of this receptor class that play key roles in both development and adult function of the uterus include the AR, ER, and PR [25, 26]. In the adult mouse and human uterus, IHC analysis detected primarily nuclear-localized AR protein in both epithelial and stromal/mesenchymal tissue compartments [49]. While our IHC analyses were non-productive, our WB analysis did detect down-regulated total AR protein expression in the much larger and hyperplastic/dysplastic uteri from PS animals. However, the relevance of that effect is unclear because, in AR knockout mice, uteri are smaller than normal and exhibit reduced estrous-dependent and gonadotropin-induced growth [25].

In rodents, ER and particularly its alpha form (ER α) clearly mediates reproductive tract disruption following neonatal DES exposure [40, 50]. Furthermore, ERs are directly involved in various forms of estrogen-dependent cancer [41, 42]. However, our earlier studies detected no alterations in the ontogeny or the physicochemical and functional properties of the uterine ER system in neonatally DES-exposed hamsters [5, 51] and our current ER α -specific WB and IHC analyses confirm those results.

It is well established that PR-mediated progesterone action coordinates normal uterine physiology and protects against estrogen-dependent uterine cancer [16, 42]. It is also well established that estrogen up-regulates both ER and PR while progestins down-regulate both receptors in the mature uterus [16, 42, 52–54]. Early on, we observed altered PR ontogeny in the neonatally DES-exposed hamster uterus [5, 51] and our current analyses confirm and extend those observations. At the whole organ level, we observed precocious expression (up-regulation) of the PR protein (both A and B forms) as well as the PR mRNA in IS animals. This appears to represent a selective estrogenic effect in that the early developmental exposure to DES enhanced PR but not ER protein expression. In contrast, whole organ levels of the PR protein (again both A and B forms) were down-regulated in the chronically estrogen-stimulated PS animals. This suggests that part of the uterine re-programming induced by neonatal DES exposure is subsequent reduction of estrogen-enhanced PR protein expression and thus possible modification of progesterone's protective action against estrogen-dependent uterine cancer [16, 42]. Relevant to the above plus our PR IHC observations is the topic of epithelial-stromal/mesenchymal paracrine interactions for both ER- and PR-mediated actions in the uterus [16]. For instance, it was striking in IS animals that PR nuclear staining in the stromal/mesenchymal compartment was undetectable in control uteri but uniformly strong in both the epithelial and stromal/mesenchymal compartments of DES-exposed uteri. Also striking was the contrasting finding in PS animals that the overall PR down-regulation phenomenon involved virtual elimination of nuclear staining in the luminal epithelial cell compartment. However, the implications of the latter observation with regard to the actions of estrogens and progestins in uterine dysplasia/neoplasia is unclear in view of the findings that progestins inhibit estrogen-induced endometrial epithelial cell proliferation via PR in stromal/mesenchymal cells [55, 56].

4.2 Cell-cell interactions

Intercellular junctions are complex at both the structural and molecular level [57]. The relevant proteomic components we could immunologically probe in our experimental

system (E-cadherin, β -catenin, connexin-26, connexin-43, and occludin) are discussed below.

Since downregulation of E-cadherin expression is commonly linked to the EMT phenomenon and thus also to the neoplastic process [29, 58], our observation of E-cadherin upregulation in the uterus of PS animals was unexpected. However, evidence does exist for a promoting role of E-cadherin in some aspects of carcinogenesis [28, 59]. Furthermore, extracellular “shedding” of E-cadherin proteolytic fragments does occur and evidence indicates that the soluble fragments can influence various cell dynamics including junctional integrity, migration, invasion, and signal initiation [60]. Whether altered levels of such fragments, for instance the 103 and 23 kDa forms we detected in our WB analyses, are involved in the uterine dysplasia/neoplasia phenomenon deserves further attention. For instance, they may be part of the explanation for the enhanced IHC staining signal level we found in the stromal/mesenchymal tissue compartment of neonatally DES-exposed uteri from PS animals. Despite such alterations in E-cadherin protein levels and its localization in the uterus of PS animals, levels and localization of its important intracellular partner protein, β -catenin [27, 39], were not altered in either IS or PS animals. Thus, there is no clear indication if and/or how the multitasking β -catenin protein [39, 61] is involved in the uterine dysplasia/neoplasia phenomenon.

Gap junctions and hemichannels formed by the protein subunits connexins play diverse roles in various organs and tissues [62]. Of the multiple known connexin gene products [14, 15, 57], our proteomic analyses detected altered uterine expression patterns for proteins recognized by anti-connexin-26 and anti-connexin-43 antibodies. At the whole-organ level, WB analysis found that connexin-26 (or possibly another cross-reactive connexin isoform particular to the hamster) was up-regulated in both IS and PS animals while connexin-43 mRNA and protein was up-regulated only in IS animals. Furthermore, IHC analysis showed that connexin-26 up-regulation occurred in the endometrial epithelial compartment for both IS and PS animals. While these observations seem contrary to the more generally held view that down-regulated connexin expression is linked with cellular transformation and carcinogenesis [63], evidence does exist linking up-regulated connexin expression and gap junction function with later stages of tumor progression [64]. On the other hand, IHC analysis detected connexin-43 down-regulation in the endometrial epithelial compartment of PS animals. That latter finding is consistent with other observations in endometrial normal and carcinoma cells [65]. Thus, there is no simple resolution for the role of gap junction/hemichannel structure and function in the uterine dysplasia/neoplasia phenomenon.

Occludin is the key trans-membrane protein component of intercellular tight junctions [57] and its function is influenced by a spectrum of specific post-transcriptional and post-translational modifications that include alternative precursor mRNA splicing and proteolysis [37]. While either of those processes could generate the up-regulated smaller isoform bands (49 kDa) detected by WB analysis of uterine extracts from PS animals, the exact mechanism and the functional relevance of that isoform are not known.

4.3 Cytokine action

IRF-1 is generally considered a transcription factor that acts in the cell nucleus to regulate interferon α and β expression and thereby serve as a tumor suppressor [30]. While that view seems to conflict with our finding of cytoplasmic IRF-1 up-regulation specifically in the endometrial epithelial compartment of PS animals, other evidence does not. For instance, a spectrum of regulatory inputs can influence IRF-1's intracellular localization and function [66, 67] and cytoplasmic IRF-1 localization is reported in the human endometrial epithelial compartment [68, 69].

In contrast, another transcription factor (Stat5a) involved in cytokine-mediated responses [19, 20] but also with oncogenic action [70] was up-regulated in the uterus of IS animals in both the endometrial and stromal/mesenchymal compartments and its localization was primarily cytoplasmic in the former but nuclear in the latter compartment. Since activated ER α directly induces Stat5a gene expression [71], our IHC observations of up-regulated Stat5a in uterine stromal cell nuclei of IS animals is somewhat consistent with the paradigm that stromal estrogen receptors mediate the mitogenic effects of estrogen on uterine epithelium [72]. Clearly, the cellular dynamics of both proteins and their roles in the uterine dysplasia/neoplasia phenomenon deserve further investigation.

4.4 Growth factor action

The IRS-1 member of the insulin receptor substrate protein family is most generally viewed as a cytoplasmic adaptor protein downstream of an activated cell surface receptor, particularly for the binding of insulin-like growth factor-1 to its receptor (IGF-1R), and its expression is reported up-regulated in human tumors [31, 73] and in the hyperplastic endometrial epithelium of neonatally DES-treated rats [74]. However, discrepancies from this view, including evidence of nuclear IRS-1 localization/action [32] and its down-regulation in some tumors [75] do exist. Our IRS-1 expression observations do not easily align with such disparate reports. In IS animals for instance, our WB analyses detected no difference in IRS-1 expression at the whole-organ level but our IHC analyses revealed a dramatic expression pattern at the cell/tissue-specific level. That is, when present, IRS-1 was cytoplasmically localized and: 1) dramatically down-regulated in the endometrial epithelial compartment but 2) up-regulated in the stromal/mesenchymal compartment. In contrast for PS animals, our WB analyses detected down-regulated IRS-1 expression at the whole organ level (also down-regulated at the mRNA level) that, according to our IHC analyses, involved: 1) lack of signal in the endometrial epithelial compartment of both treatment groups and 2) down-regulated cytoplasm/ECM signal in the stromal/mesenchymal compartment. Further investigations are needed in order to reconcile these observations with the general regulatory and tumor biology considerations listed above and with the complex biochemical pathways with which IRS-1 is implicated in estrogen-induced cell proliferation in the uterus [76].

4.5 Oncogene

We previously reported that neonatal DES exposure alters the expression of both cell proliferation (c-jun, c-fos, and c-myc) and apoptosis-related (bax, bcl-2, and bcl-x) oncogenes in the uterus of PS animals [5]. Here we report that, at the whole organ level, the

c-Kit protein also is up-regulated in PS animals. The implications of this finding are not clear in part because enhanced expression and/or gain-of-function changes in the protein, also known as stem cell factor receptor, are primarily linked with stromal/mesenchymal-derived tumors [33, 34]. However, when it was detected and comparatively analyzed in benign, hyperplastic, and malignant human endometrial epithelium tissues, the reported c-Kit expression changes include no clear trend [77], up-regulation [78], or a biphasic trend [79] associated with malignant progression. Thus, the role of c-Kit in uterine hyperplasia/dysplasia/neoplasia remains unresolved.

4.6 ECM components

We found that tenascin-C protein levels were dramatically up-regulated in the uterine stromal/mesenchymal ECM compartment of IS but not PS animals. This finding was somewhat surprising since altered tenascin-C expression is generally linked with later stages (tumor angiogenesis and metastasis) of the neoplastic process [21]. Perhaps future investigation should focus on more subtle changes in tenascin-C structure/function such as those resulting from alternatively spliced regions of the protein [24, 43].

Uterine levels of another ECM component, the laminin B1 protein that forms part of the basement membrane [80], were not affected in either IS or PS animals. This was also somewhat surprising since we observed extensive disruption of the sub-endometrial epithelial cell basement membrane in PS animals [5]. Again, further analysis of the levels and localization of these and other ECM components is needed.

4.7 Transcription factors

The NF κ B transcription factor complex is a pleiotropic regulator of processes including carcinogenesis and inflammation [35, 81]. The NF κ B signaling proteins are expressed in the normal human endometrium [82] and interactions between estrogen receptor and NF κ B signaling pathways are likely involved in the blastocyst implantation event [83]. The NF κ B system alteration we detected was subtle and consisted only of down-regulation of a small isoform (38 kDa) of the NF κ B1 or p50 protein.

The Nrf2-Keap signaling pathway is somewhat enigmatic in that it exhibits both cytoprotective and pro-carcinogenic effects [36, 84] and can contribute to both intrinsic and acquired resistance to cancer chemotherapies [85]. Our observation that the Nrf2 protein was up-regulated in PS animals suggests that it may promote later stages of the uterine dysplasia/neoplasia phenomenon.

Our WB analyses detected stage-specific uterine Sp1 protein differences. In PS animals, all isoforms (103-86 and 51 kDa) of the protein were up-regulated. That finding of overall Sp1 up-regulation is consistent with observations in many cancer types [86] and suggests it also may promote later stages of the uterine dysplasia/neoplasia phenomenon. In contrast, we detected up-regulation only of the low-molecular weight isoform (51 kDa) in IS animals. The biological relevance of that finding is unclear because, while information about the mechanism and functional role for many types of Sp1 post-transcriptional modifications is extensive [17, 87], that regarding its proteolytic processing is not.

4.8 Proliferation-associated factors

Our WB analyses detected no differences in the levels of p100, described as a proliferation-associated nuclear protein specifically restricted to cell cycle phases S, G₂, and M [88]; nor that of PCNA, another nuclear protein involved with cellular proliferation and associated with neoplastic transformation [89] and so frequently used as a cell cycle marker [90, 91]. However, while our PCNA-targeted IHC analysis did demonstrate nuclear-specific staining of both epithelial and stromal/mesenchymal uterine cells, we observed no differences in staining pattern or intensity between control and DES-exposed groups of uteri from either IS or PS animals. Whether that observation is a hamster-specific phenomenon and/or due to the possibility that our analyses fail to recognize any of the known spectrum of PCNA post-translational modifications [46, 92] deserves further investigation.

4.9 Multi-functional nuclear protein

Our WB analyses detected down-regulation of most SAFB1 protein isoforms in the uterus of PS animals. That finding of overall SAFB1 down-regulation is consistent with the facts that the protein is generally considered a tumor suppressor [38] and also functions as an ER α co-repressor [93, 94]. Again, information regarding the mechanism and functional role of SAFB1 proteolytic processing is not available so the biological relevance of the various resolved protein isoforms is not known.

4.10 Comparisons among gene expression studies

When we compared studies regarding differences in uterine gene expression related to perinatal DES exposure, we found the following limited correlations among them and with our analyses. In mice treated with DES on days 1–5 of life and killed 6 hr later [95], Stat5a was upregulated at the mRNA level (upregulated at the protein level in our IS uteri). In rats exposed to DES during late gestation and killed on postnatal day 5 [96], IRF-1 was upregulated at the mRNA level (upregulated at the protein level in our PS uteri). In mice treated with three different doses of DES on days 1–5 of life and killed on postnatal day 19 (prior to puberty) [97], connexin-43 was upregulated only in the highest dose group (upregulated at the mRNA and protein level in our IS uteri). In mice also treated with DES on days 1–5 of life but killed at 10 weeks of age [98], upregulation at the mRNA level was detected for connexin-43 (upregulated at the mRNA and protein level in our IS uteri) and for PR (upregulated at the mRNA and protein level in our IS uteri but downregulated at the protein level in our PS uteri). Consequently, such comparisons fail to provide clear insights into the key biological pathways that drive the phenomenon of endocrine disruption.

5. Summary and conclusions

The observations presented here demonstrate that: 1) progression of the neonatal DES-induced hyperplasia/dysplasia/neoplasia phenomenon in the hamster uterus involves a wide spectrum of specific gene expression alterations at both the mRNA and protein levels and 2) the gene products involved and their manner of altered expression differ dramatically during the initiation vs. promotion stages of the phenomenon. Particularly interesting changes included members in the functional categories of nuclear receptors (progesterone receptor), cell-cell interactions (E-cadherin, connexins), cytokine action (IRF-1, Stat5A), growth factor

action (IRS-1), extracellular matrix component (tenascin-C), transcription factors (Nrf2, Sp1), and multi-functional nuclear protein (SAFB1). Rather unexpected was the very limited matches in gene expression differences detected at both the mRNA and protein levels. Part of the explanation may be technical and due to the fact that commercially available hamster genome-based microarrays were not available. Thus, despite positive claims for the strategy [99], our default use of the mouse genome-based microarray likely resulted in missed detection of some relevant hamster mRNA targets because of interspecies differences at the genomic DNA level. Of course, a remaining fundamental question is how is it that perinatal exposure to DES and other suspected endocrine disruptor agents exerts pathophysiological consequences into adulthood. An emerging and increasingly compelling answer to that question is the paradigm of epigenetics [50, 100]. In fact, we are now assessing epigenetic endpoints in the neonatally DES-exposed hamster uterus and have observed alterations at the DNA methylation and micro RNA levels (manuscripts in preparation). Lastly, we and other investigators still need to define which specific molecular alterations, genetic and/or epigenetic, actually drive a given endocrine disruption phenomenon and precisely how it does so.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ABC	avidin-biotin-horseradish peroxidase complex
AR	androgen receptor
CON	control
DAB	diaminobenzidine
DES	diethylstilbestrol
E2	estradiol-17 β
EMT	epithelial/mesenchymal transition
ERα	estrogen receptor alpha
ECM	extracellular matrix
FDR	false discovery rate

H&E	hematoxylin and eosin
IHC	immunohistochemistry
IRF-1	interferon regulatory factor 1
IRS-1	insulin receptor substrate 1
IS	Initiation Stage
Keap	Kelch-like ECH-associated protein
LEF	lymphoid enhancer factor
NES1	normal epithelial-cell specific 1
NFκB	nuclear factor kappa B
Nrf2	nuclear factor (erythroid 2-related) factor 2
NSB	non-specific bands
O	ovariectomized
PBS	phosphate-buffered saline
PBST	PBS plus 0.05% Tween 20
PCNA	proliferating cell nuclear antigen
PR	progesterone receptor
PS	Promotion Stage
PTM	PBST plus 5% nonfat dry milk
SAFB1	scaffold attachment factor B1
s.c	subcutaneous Sp1, specificity protein 1
Stat5A	signal transducer and activator of transcription 5a
TCF	T cell factor
WB	Western blot/blotting

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Highlights

- Neonatal DES exposure directly disrupts the hamster uterus (initiation stage).
- It then responds abnormally to estrogen in adulthood (promotion stage).
- We analyzed specific protein and RNA expression in uteri from both stages.
- The disruption process involves many specific gene expression alterations.
- They differed dramatically during the initiation vs. promotion stages.

Figure 1.

Effects of neonatal DES exposure on histomorphology of the hamster uterus. Shown are representative H&E stained cross sections of control (CON, left panels) and neonatally DES-exposed (right panels) uteri from both IS (upper four panels) and PS animals (lower four panels). Indicated in the photomicrographs taken at both low (2x) and high (40x) magnification are the endometrial epithelial (E) and stromal (S) tissue compartments plus glandular cysts (Cy). Scale bars in each panel represent 100 μm.

Figure 2.

Effect of neonatal DES treatment on total protein pattern and specific immunodetected proteins in the uterus of IS hamsters. Total protein extracts from control (CON) and DES-exposed uteri of 5-day old animals were resolved by denaturing gel electrophoresis/ Coomassie staining (left panel) and analyzed by WB (right panel). Indicated on the left side of the left-hand panel are the migration positions of standard proteins with the indicated molecular weights (kDa). For the right-hand panel, multiple immunodetected isoforms of a given protein are identified by their molecular weight (kDa) in parentheses. Also indicated are B and A isoforms for PR and high (H) and low (L) molecular weight isoforms for Sp1.

Figure 3.

Effect of neonatal DES treatment on total protein pattern and specific immunodetected proteins in the uterus of PS hamsters. Same as for Fig. 2 except using total protein extracts from control (CON) and DES-exposed uteri of 2-month old/O+E2 hamsters. Also indicated are high, (H) intermediate (I), and low (L) molecular weight isoforms for some target proteins.

Figure 4.

Effect of neonatal DES treatment on relative levels of immunodetected proteins in the uterus of IS hamsters. Total protein extracts from control (CON) and DES-exposed uteri of 5-day old hamsters underwent WB (see Fig. 2) and densitometric analysis. Shown are the values for mean band density from DES-exposed tissues / mean band density from control tissues (DES/CON) as listed in Table 1. Asterisks indicate instances where differences between the group means were statistically significant as indicated in Table 1.

Figure 5.

Effect of neonatal DES treatment on relative levels of immunodetected proteins in the uterus of PS hamsters. Same as for Fig. 4 except using total protein extracts from control (CON) and DES-exposed uteri of 2-month old/O+E2 hamsters (see Fig. 3).

Figure 6.

Negative control IHC. Shown here and in the following micrographs are representative results for cross sections of control (CON) or neonatally DES-exposed uteri from IS and PS animals following IHC processing without (this figure) or with a primary/1st antibody (dilution specified in the following figure legends) targeting a specific protein (listed and detailed in Table 1) and then counterstaining with Methyl green. Scale bars in each panel represent 100 μ m.

Figure 7.
E-cadherin IHC. At 1:250 antibody dilution.

β -Catenin

Figure 8.
 β -catenin IHC. At 1:5,000 antibody dilution.

Connexin-26

Figure 9.
Connexin-26 IHC. At 1:200 antibody dilution.

Figure 10.
Connexin-43 IHC. At 1:500 antibody dilution.

Figure 11.
ER α IHC. At 1:2,500 antibody (MC20) dilution.

Figure 12.
FAK IHC. At 1:50 antibody dilution.

Figure 13.
IRF-1 IHC. At 1:200 antibody dilution.

Figure 14.
IRS-1 IHC. At 1:100 antibody dilution.

Figure 15.
PR IHC. At 1:200 antibody dilution.

Figure 16.
PCNA IHC. At 1:200 antibody dilution.

Figure 17.
Stat5A IHC. At 1:100 antibody dilution.

Figure 18.
Tenascin-C IHC. At 1:500 antibody dilution.

TABLE 1

Summary of Western blot analyses and antibody information^a.

Protein ^b	kDa ^c	Stage ^d	CON Dens. ^e	DES Dens. ^e	P value ^f
Androgen Receptor (AR)	113	P	104 ± 27	11 ± 5	0.03
(C-19) Santa Cruz Biotechnology; Santa Cruz, CA; rabbit pAb; 1:500					
Cadherin, E-	119	P	65 ± 19	1,487 ± 142	0.0006
	103	P	20 ± 4	172 ± 8	<0.0001
	35	P	368 ± 45	395 ± 61	0.7
	23	P	152 ± 18	8 ± 4	0.001
	(all isoforms)	P	604 ± 79	2,062 ± 101	0.0003
(Clone 36) BD Transduction Laboratories; San Diego, CA; mouse mAb; 1:2,500					
Catenin, β	97	I&P	NA	NA	NA
(H-102) Santa Cruz Biotechnology; Santa Cruz, CA; rabbit pAb; 1:500					
Connexin-26	46	I	46 ± 6	1,057 ± 5	<0.0001
	46	P	22 ± 18	274 ± 36	0.003
Zymed Laboratories; San Francisco, CA; rabbit pAb; 1:250					
Connexin-43	43–41	I	372 ± 11	1,324 ± 31	<0.0001
W. Eckhart; The Salk Institute for Biological Studies; San Diego, CA; rabbit pAb; 1:5,000 [101]					
Estrogen Receptor Alpha (ER _α)	65	I&P	NA	NA	NA
(C1355) Upstate Biotechnology; Lake Placid, NY; rabbit pAb; 1:1,000					
Estrogen Receptor Alpha (ER _α)	65	P	212 ± 71	194 ± 23	0.8
(MC20) Santa Cruz Biotechnology; Santa Cruz, CA; rabbit pAb; 1:500					
Focal Adhesion Kinase (FAK)	110	I&P	NA	NA	NA
W. G. Cance; University of North Carolina School of Medicine; Chapel Hill, NC; mouse mAb; 1:500 [44]					
Interferon Regulatory Factor 1 (IRF-1)	53	P	28 ± 5	166 ± 37	0.02
(C-20) Santa Cruz Biotechnology; Santa Cruz, CA; rabbit pAb; 1:500					

Protein ^b	kDa ^c	Stage ^d	CON Dens. ^e	DES Dens. ^e	P value ^f
Insulin Receptor Substrate 1 (IRS-1) (Clone 6) BD Transduction Laboratories; San Diego, CA; mouse mAb; 1:500	155	P	721 ± 74	235 ± 135	0.03
Kit, c- (M-14) Santa Cruz Biotechnology; Santa Cruz, CA; goat pAb; 1:500	110	P	44 ± 8	321 ± 28	0.0006
Laminin B1 (Clone LT3) NeoMarkers/Lab Vision; Union City, CA; rat mAb; 1:1,000	205	I&P	NA	NA	NA
Normal Epithelial Cell-Specific 1 (NES1) E. P. Diamandis; Mount Sinai Hospital; Toronto, Ontario, Canada rabbit pAb; 1:1,000 [102]	43	I&P	NA	NA	NA
Non-Specific Bands (NSB)	77, 71	I P	296 ± 17 226 ± 11	357 ± 35 217 ± 10	0.2 0.6
Nuclear Factor Kappa B (NFκB) p50 StressGen Biotechnologies; Victoria, BC, Canada; rabbit pAb; 1:200	50 38 (both isoforms)	P P P	549 ± 7 183 ± 26 839 ± 129	554 ± 40 47 ± 8 601 ± 56	0.9 0.007 0.2
Nuclear Factor (Erythroid 2-Related) Factor 2 (Nrf2) (C-20) Santa Cruz Biotechnology; Santa Cruz, CA; rabbit pAb; 1:500	80	P	57 ± 7	403 ± 60	0.005
Occludin Zymed Laboratories; San Francisco, CA; rabbit pAb; 1:250	57 49 (both isoforms)	P P P	630 ± 58 43 ± 5 672 ± 62	600 ± 17 152 ± 17 752 ± 26	0.7 0.004 0.3
p100 (repp 86) H. J. Heidebrecht; University of Kiel; Kiel, Germany; mouse mAb; hybridoma supernatant [88]	100	NA	NA	NA	NA
Progesterone Receptor (PR)	(B) 111 (A) 87 (both isoforms) (B) 111 (A) 87	I I I P P	18 ± 5 26 ± 14 44 ± 17 969 ± 109 3,096 ± 197	1,153 ± 49 1,823 ± 9 2,976 ± 58 170 ± 35 886 ± 294	<0.0001 <0.0001 <0.0001 0.002 0.003

Protein ^b	kDa ^c	Stage ^d	CON Dens. ^e	DES Dens. ^e	P value ^f
(410) Affinity BioReagents; Golden, CO; mouse mAb; 1:500					
Proliferating Cell Nuclear Antigen (PCNA)	36	I	1,317 ± 145	1,361 ± 106	0.8
Biomeda, Foster City, CA; mouse mAb; 1:500					
Scaffold Attachment Factor B1 (SAFB1)	182,158	P	1,568 ± 63	759 ± 145	0.006
	65	P	677 ± 133	238 ± 74	0.04
	38	P	330 ± 64	298 ± 64	0.7
	(all isoforms)	P	2,574 ± 209	1,296 ± 195	0.01
S. Oesterreich; Baylor College of Medicine and Methodist Hospital; Houston, TX; rabbit pAb; 1:1,000 [103]					
Specificity Protein 1 (Sp1)	103-86	I	2,237 ± 142	2,267 ± 48	0.9
	51	I	69 ± 15	179 ± 1	0.002
	(both isoforms)	I	2,306 ± 157	2,446 ± 49	0.4
	103-86	P	97 ± 40	1,002 ± 109	0.001
	51	P	104 ± 13	393 ± 30	0.0009
	(both isoforms)	P	201 ± 53	1,396 ± 139	0.001
(PEP 2) Santa Cruz Biotechnology; Santa Cruz, CA; rabbit pAb; 1:500					
Signal Transducer and Activator of Transcription 5a (Stat5a)	95	I	761 ± 50	2,066 ± 74	0.0001
(Clone 51) BD Transduction Laboratories; San Diego, CA; mouse mAb; 1:250					
Tenascin-C	>200-148	I	71 ± 36	1,921 ± 95	0.0001
(HxB-2873) H. P. Erickson; Duke University Medical Center, Durham, NC; rabbit pAb; 1:1,000 [43]					
Vimentin	57	I&P	NA	NA	NA
(Clone V9) NeoMarkers/Lab Vision; Union City, CA; mouse mAb; 1:2,000					

Animals were injected on the day of birth with vehicle either alone (control, CON) or containing 100 µg of DES. For the comparisons between triplicate groups of samples, immunodetected protein band levels in total protein extracts from the uteri of Initiation (I) and Promotion (P)-Stage animals (see below) were measured by densitometric analysis. For the other immunodetected but not statistically analyzed protein bands, some column entries are indicated as not applicable (NA).

^a Antibody information includes identifying details in parentheses, donor or commercial source, whether it was a mouse or rat monoclonal (mAb) or a rabbit or goat polyclonal (pAb) antibody, either the dilution or IgG concentration at which it was used, and for donated antibodies, a reference citing use of that antibody.

^b Name of the target protein and its common abbreviation.

^c Size of the target protein in kilodaltons (kDa).

^d Refers to extracts from 5-day old hamsters (Initiation or I stage) or from 2-month old hamsters (Promotion or P stage) that were ovariectomized and received estradiol-releasing pellets on day-21 of life.

^e Signal band density expressed as mean $\pm$ standard error, n = 3.

^f Difference probability between the extracts from control vs. DES-exposed organs according to Student's t-test.