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Developmental reprogramming of IGF signaling and susceptibility to endometrial hyperplasia in the rat

Adrienne S McCampbell[1], Cheryl L Walker[2], Russell R Broaddus[1], Jennifer D Cook[2] and Peter JA Davies[3]

In rodents, a brief neonatal exposure of the developing reproductive tract to the xenoestrogen, diethylstilbestrol (DES) reprograms developing tissues to increase susceptibility to tumorigenesis in adult animals, including uterine adenocarcinoma. Progression from a normal endometrium to carcinoma occurs via the intermediate stage of endometrial hyperplasia. We previously reported that endometrial hyperplasia in postmenopausal women is linked to abnormal insulin-like growth factor-I (IGF-I) signaling. To identify early events involved in the development of hyperplasia in the endometrium, we examined expression and activation of IGF-I pathway components in endometrium of rats exposed to DES. By 5 months of age, 36/60 (60%) of rats exposed to DES on days 3–5 after birth developed endometrial hyperplasia compared to 0% of vehicle-treated controls. Consistent with activation of a mitogenic signaling pathway, Ki67-positive cells increased in DES-exposed endometrium despite compromised ovarian function and hypoestrogenic milieu characteristic of DES-exposed animals. The endometrium of DES-exposed rats overexpressed IGF-II and insulin receptor substrate-1 (IRS-1) and exhibited elevated Akt expression and activation (as judged by phosphorylation) and mTOR signaling (phosphorylation of S6) compared to vehicle-treated endometrium. In contrast to vehicle-treated endometrium, in which negative feedback to IRS-1 was observed (phosphorylation of S636/639), negative feedback to IRS-1 was absent in DES-exposed endometrium. These data support a central role for IGF-I signaling in the development of both human and rodent endometrial hyperplasia. Furthermore, both global activation of IGF-IR signaling and abrogation of negative feedback to IRS-1 appear to be reprogrammed by DES in endometrial hyperplasia, implicating for the first time loss of negative feedback to IRS-1 in development of a preneoplastic lesion.
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Insulin-like growth factor-I (IGF-I) and IGF-II expression and signaling play important roles in regulating the cyclic growth and differentiation of the endometrium in the human and rodent.[1–5] Estrogen stimulates IGF-I gene expression in the uterus and induces endometrial proliferation of mouse uterine epithelial cells.[4,6] IGF-I is produced in a cell cycle-dependent manner. IGF-I expression is elevated during proestrus and estrus phases of the rodent estrus cycle, when serum levels of estrogen are high, relative to the metestrus and diestrus phases, when serum levels of estrogen are low.[1] IGF-II expression in the uterus is not directly regulated by ovarian steroids, and its mRNA levels are consistently expressed throughout the estrus cycle.[7] IGF-I and IGF-II are produced by endometrial stromal cells and act in an autocrine or paracrine manner to regulate proliferation and differentiation.[8,9]

The effects of IGF-I and IGF-II are mediated primarily by activation of the IGF-I receptor (IGF-IR), a tyrosine kinase receptor expressed in endometrial stromal and epithelial cells that signals via activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.[4,8] IGF-IR is not known to be regulated by estrogen.[4] Ligand activation of IGF-IR by IGF-I and IGF-II induces a phosphorylation cascade of the downstream components insulin receptor substrate-1 (IRS-1) and Akt to promote cell proliferation and survival.[10,11] In endometrial cells, the activity of the PI3K/Akt pathway is negatively regulated by the activity of PTEN (phosphatase and tensin), a lipid phosphatase that dephosphorylates phosphoinositol (3,4,5) triphosphate.[12] Conversely, the insulin-like growth factor-II receptor (IGF-IIR) does not have intrinsic tyrosine kinase activity and functions to regulate the

[1]Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; [2]Science Park Research Division, Department of Carcinogenesis, University of Texas MD Anderson Cancer Center, Smithville, TX, USA and [3]Department of Integrative Biology and Pharmacology, The University of Texas Health Science Center, Houston, TX, USA

Correspondence: Dr Peter JA Davies, MD, PhD, Department of Integrative Biology and Pharmacology, MSB 5.104, University of Texas Houston Health Science Center, 6431 Fannin Street, Houston, TX 77030, USA. E-mail: Peter.J.Davies@uth.tmc.edu

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bioavailability of IGF-II. The IGF-IIR binds IGF-II selectively and transports the ligand to the lysosome for degradation.[13]

Human endometrial carcinoma can be divided into two categories. Type I, the low-grade endometrioid carcinomas, is associated with estrogen exposure and overall good prognosis. Type II, the non-endometrioid carcinomas, is not associated with estrogen exposure and have a poor prognosis.[14–18] The progression from normal endometrium to type I endometrial adenocarcinoma (EC) involves an intermediary state of abnormal proliferation, complex atypical hyperplasia (CAH).[19] PTEN activity is suppressed in 55–64% of CAH and 83–100% of type I EC.[20,21] Decreased PTEN expression has been proposed to facilitate an increase in activation of Akt, thereby stimulating survival signaling in the endometrium and contributing to the development of CAH and EC.[22] In contrast, the type II non-endometrioid endometrial cancers arise in atrophic endometrium and are associated with mutations of p53 and ERBB-2(Her-2/neu).[15]

Despite the well-known relationship between exposure to unopposed estrogen and the development of endometrial lesions, our recent studies have demonstrated that endometrial CAH is not highly 'estrogenized', as indicated by the lack of significant change in the expression of genes known to be induced by estrogen.[20] Instead, we have shown that overexpression and activation of the IGF-IR and activation of Akt are important in endometrial CAH and EC. In these studies, loss of expression of PTEN did not correlate with increased activation of IGF-IR. However, the simultaneous loss of PTEN expression and increased IGF-IR activation in hyperplasia was associated with an increased incidence of concurrent endometrial cancer.[20] These results suggest that upregulation of IGF-IR and loss of PTEN may be independent events that give rise to complementary activation of the IGF-I pathway and increase the probability of the development of EC from the precursor lesion CAH.[20]

Neonatal exposure of the developing rodent reproductive tract to xenoestrogens is a well-characterized model of hormone-dependent tumorigenesis in the uterus.[23–26] The xenoestrogen diethylstilbestrol (DES) acts by ERα-dependent mechanisms to cause changes in uterine morphology and gene transcription.[23,24,26,27] DES was previously administered to women at high risk of miscarriage and was subsequently found to increase the incidence of cancers of the cervix and vagina in their offspring.[28] In the rodent, neonatal DES exposure has been demonstrated to induce morphological abnormalities of the uterus and cause changes in the expression of estrogen target genes that persist into adulthood, including c-fos and lactoferrin.[27,29–32] In the Eker rat model, neonatal DES exposure of female Eker rats (*Tsc-2*$^{Ek/+}$) imparts a permanent estrogen imprint that alters reproductive tract morphology, increases susceptibility to develop uterine leiomyoma and induces endometrial hyperproliferative lesions in adult animals.[23,26,33] However, while the ability of neonatal xenoestrogen exposure to promote the development of tumors of the female reproductive tract is well established, the molecular mechanism by which this occurs is unclear.

To begin to understand the relationship between development of endometrial hyperplasia in this rodent model and the human disease and to define the early events in the molecular pathogenesis of endometrial carcinoma, we focused on the impact of neonatal xenoestrogen exposures on well-characterized estrogen-regulated genes in the endometrium (progesterone receptor (PR), and c-fos) and genes involved in IGF-I signaling, including the downstream signaling components, IRS-1 and Akt,[34–36] While estrogen-responsive genes were expressed equivalently in both vehicle- and DES-exposed animals, we found clear evidence for upregulation of IGF-I signaling in adult uteri exposed neonatally to DES, with concomitant loss of negative feedback to IRS-1. Thus, aberrant signaling via the IGF-I pathway appears to be a common, early event associated with the development of hyperplasia in both rodent and human endometrium.

MATERIALS AND METHODS

Animals

The care and handling of rats were in accord with National Institutes of Health guidelines and Association for the Accreditation of Laboratory Animal Care-accredited facilities. All protocols involving the use of these animals were approved by the MD Anderson Animal Care and Use Committee. Neonatal female wild-type (*Tsc-2*$^{+/+}$) and Eker (*Tsc-2*$^{Ek/+}$) rats received injections of either 10 μg of DES (Sigma) per rat per day or 50 μl of sesame seed oil (vehicle control) on neonatal days 3–5. For our studies, neonatal DES had similar morphological and biomarker effects on the wild-type (*Tsc-2*$^{+/+}$) and Eker (*Tsc-2*$^{Ek/+}$) rat endometrium. We therefore combined wild-type and Eker rat data for these studies. Animals were killed at 5 months of age. The right and left uterine horns, ovaries and vagina were formalin-fixed and paraffin embedded (FFPE) for light microscopic examination ($n = 85$). The reproductive stage of the estrous cycle (proestrus, estrus, metestrus, diestrus or DES-treated persistent estrus) was determined by microscopic examination of the vagina and ovaries.[1] H&E-stained slides of left and right uterine horns collected from all animals were microscopically evaluated to characterize the endometrium. On the basis of microscopic evaluation, uteri of vehicle- or DES-treated rats were divided into the following groups: vehicle proliferative phase (proestrus and estrus, $n = 20$), vehicle secretory phase (metestrus and diestrus, $n = 5$) and DES persistent estrus (DES normal, $n = 25$ and DES hyperplastic, $n = 35$; $n = 60$ total). To examine gene expression by quantitative real-time PCR in the endometrium for a subset of animals ($n = 30$), the endometrium of the uterine body (excluding the right and left uterine horns) was dissected from the myometrium by scraping with a razor blade into Trizol reagent and frozen at -80°C. Using the FFPE right and left uterine horns from the same rodents, the endometrium was characterized by light

microscopy. Of the 30 animals in the subset from which RNA was isolated there were 7 secretory-phase endometria, 7 proliferative phase-endometria, 6 DES-exposed endometria with normal histology (DES 'normal') and 10 DES-exposed endometria with the presence of endometrial hyperplastic foci (DES 'hyperplastic').

RNA Isolation from Frozen Tissues

At the time of killing, TriReagent (Molecular Research Center Inc., Cincinnati, OH) was added directly to the rat uterus, and the endometrium was scraped using a razor blade. Tissues were frozen at −80°C. Tissue was homogenized and nucleic acids were isolated by phenol–chloroform extraction. RNA was precipitated with isopropanol and purified by RNeasy columns (Qiagen, Valencia, CA). RNA was resuspended in RNAse-free water. DNA was digested by incubation with RNase-free DNase I (Roche Diagnostics Corp., Indianapolis, IN) with RNase Inhibitor (Roche Diagnostics Corp.) for 15 min at 37°C. DNase I was heat inactivated at 75°C for 10 min.

Quantitative RT-PCR

Aliquots (40 ng) of each RNA were reverse transcribed in quadruplicate (including a no reverse transcriptase control) with 300 nm of assay-specific reverse primer, 10 × Stratascript Reverse Transcriptase buffer (Stratagene, La Jolla, CA), 4 mM $MgCl_2$, 500 μM deoxynucleotide triphosphates, 10 U Stratascript Reverse Transcriptase (Stratagene) at 50°C for 30 min, followed by 72°C for 5 min. PCR mix (40 μl) containing 10 × PCR buffer, 300 nM specific forward and reverse primers, 4 mM $MgCl_2$, *Taq* DNA polymerase and 100 nM fluorogenic probe were added to each 10 μl reverse transcription reaction. Amplification was performed by use of the ABI-Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) performing 40 cycles at 95°C for 12 s and 60°C for 1 min. Data analysis was performed using Sequence Detector Software (Perkin Elmer, Foster City, CA). Single-strand DNA amplicon standards were serially diluted in water. Values of transcripts were obtained by interpolating their PCR cycle threshold values based upon the standard curve established by the single-strand DNA amplicon. Transcript levels were normalized to the 18S ribosomal RNA. Sequences of forward and reverse primers and probes are listed in Table 1.

Immunohistochemistry

Uterine horns from vehicle- and DES-treated rats were sectioned, and fixed in formalin and paraffin-embedded. Tissue sections were deparaffinized and endogenous peroxidases were quenched by incubation in 1% H_2O_2. Antigen retrieval was performed by microwave in 10 mM citrate buffer (pH 6.0). Slides were incubated with primary antibody (1:50) in phosphate-buffered saline (PBS) containing 10% normal goat or horse serum overnight at 4°C. The following primary antibodies were purchased from Cell Signaling Technology, Beverly, MA: phosphorylated (tyrosine 1135/1136) IGF-IR (no. 3024), the β subunit of the IGF-IR (no. 3027), phosphorylated (serine 473) Akt (no. 3787), Akt (no. 9272), PTEN (no. 9559), IRS-1 (no. 2382) and phosphorylated (serine 636/639) IRS-1 (no. 2388). Primary antibody for phosphorylated (tyrosine 632) IRS-1 (sc-17196-R) was purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Primary antibody for Ki67 (no. M7248) was purchased from Dako North America Inc., Carpentaria, CA. A biotin-labeled secondary antibody was conjugated for 30 min at 37°C. Sections were stained using avidin-biotinylated horseradish peroxidase complex from the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Diaminobenzidine (DAB) reagent (Vector Laboratories) was incubated with sections for up to 30 min. Sections were counterstained with hematoxylin or methyl green, dehydrated and mounted. Controls that lacked primary antibody were incubated in 1 × PBS with 10% goat serum in each experiment. Immunostained sections were examined by light microscopy and scored semiquantitatively according to the intensity of staining on a scale of 0 (no staining) to 3+ (strong staining). Tissues with 2+ or 3+ staining in greater than 10% of cells were considered positive for protein expression. Ki67 labeling index was scored by assessing the percentage of cells positive for Ki67 nuclear antigen in 100 epithelial cells for vehicle- and neonatal DES-treated endometria.

Statistical Analysis

Differences in means were calculated by unpaired Student's *t*-tests, analysis of variance or non-parametric Mann–Whitney test. The χ^2 test was used to evaluate the frequency of immunostaining by group. Statistical significance was defined as a *P*-value less than 0.05.

RESULTS

Endometrium Exposed Neonatally to DES is Hyperproliferative

Neonatal exposure of female rats to DES causes functional and morphological changes in the adult reproductive tract, including the ovary, which fails to cycle normally and produce estrogen and progesterone.[26,33] In both wild-type and Eker rats exposed to vehicle on neonatal days 3–5, normal epithelial cells of the endometrium were microscopically characterized by luminal and glandular epithelial cells, cuboidal in shape with a nucleus oriented in the center of the cell (Figure 1a). In wild-type and Eker animals exposed neonatally to DES, at 5 months of age endometrial hyperplastic foci occurred in 60% (36/60) of exposed females, while none (0/25) of the vehicle-treated females developed these preneoplastic lesions (Figure 1). In contrast to normal epithelial cells (Figure 1a and b), the hyperplastic endometrium that developed in neonatally DES-exposed females was characterized by the presence of taller columnar epithelial cells with excess cytoplasm and uterine glands with

Table 1 Rodent qRT-PCR primer/probe sequences for IGF-I pathway components and estrogen-regulated genes

Gene name	Primer/Taqman probe sequences	GenBank accession no.
IGF-I	984(+) 5′-GCATTGTGGATGAGTGTTGCT	NM_178866
	1050(−) 5′-CAGCGGACACAGTACATCTCC	
	1010(+) 5′-(FAM)CCGGAGCTGTGATCTGAGGAGGCT(TAMRA)	
IGF-II	1416(+) 5′-TACCTCTCAGGCCGTACTTCC	NM_031511
	1489(−) 5′-TCCAGGTGTCGAATTTGAAGA	
	1446(+) 5′-(FAM)CCCCAGATACCCCGTGGGCAA(TAMRA)	
IGF-IR	3601(+) 5′-AAGGATGGCGTCTTCACCA	NM_052807
	3670(−) 5′-GAGTGGCGATCTCCCAGAG	
	3621(+) 5′-(FAM)TCATTCCGATGTCTGGTCCTTTGGG(TAMRA)	
IGF-IIR	1620(+) 5′-GTGCTGCAGGCGGGAAAG	NM_012756
	1669(−) 5′-GTGCTGTGGATAAGAGTGGAAGTA	
	1640(+) 5′-(FAM)CAAGAACTGTCCTGAAGGTGCTGCCGTG(TAMRA)	
IGFBP-3	482(+) 5′-CTGATTCCAAGTTCCATCCACTC	NM_012588
	572(−) 5′-CATAGTCAACTTTGTAGCGCTGG	
	509(+) 5′-(FAM)TCAAAGATGGAGGTCATCATAAAAGGCCA(TAMRA)	
IGFBP-5	1225(+)5′-GAGAAAGCAGTGCAAGCCTTC	NM_012817
	1286(−)5′-ACTTGTCCACACACCAGCAGA	
	1249(+)-(FAM)TGGCCGCAAACGTGGCATC(TAMRA)	
IRS-1	2665(+) 5′-CCATGGACAAACGGAGTAG	NM_012969
	2732(−) 5′-CTCTCAACAGGAGGTTTGG	
	2692(+) 5′-(FAM)ATGGGGAAGGGCATGGGTATG(TAMRA)	
Estrogen receptor α	638(+) 5′-CTACGCTGTACGCGACACC	NM_012689
	706(−) 5′-CCATTCTGGCGTCGATTG	
	659(+) 5′-(FAM)CCCTCCCGCCTTCTACAGGTCCA(TAMRA)	
Progesterone receptor[a]	2068(+) 5′-TATGCAGGGCATGACAACAC	NM_022847
	2142(−) 5′-CCTCTCGCCTAGTTGGTTGAG	
	2094(+) 5′-(FAM)CGACACTTCCAGCTCTTTGCTGACCA(TAMRA)	
RALDH2	327(+) 5′-AAGCTTGCAGACTTGGTGGA	NM_053896
	420(−) 5′-CGATGTAAAAAGCTTGCAGGA	
	348(+) 5′-(FAM)CGGGACAGGGCAACTCTTGCAACTA(TAMRA)	
p27Kip1	755(+) 5′-GGACCAAATGCCTGACTCG	NM_031762
	818(−) 5′-CCTCATCCCTGGACACTGC	
	775(+) 5′-(FAM)CAGACAGTCCGGCTGGGTTAGCG(TAMRA)	
p21Cip1	75(+) 5′-AACGGTGGAACTTTGACTTCG	NM_080782
	140(−) 5′-GAACACGCTCCCAGACGTAG	
	98(+) 5′-(FAM)ACTGAGACGCCACTGGAGGGCA(TAMRA)	
Cyclin D1	814(+) 5′-CGCACTTTCTTTCCAGAGTCA	NM_171992
	887(−) 5′-AAGGGCTTCAATCTGTTCCTG	
	846(+) 5′-(FAM)CCGGACTGCCTCCGTGCCT(TAMRA)	
c-fos	220(+) 5′-ACTACCATTCCCCAGCCG	NM_022197
	291(−) 5′-GATCTGCGCAAAAGTCCTGT	
	264(+) 5′-(FAM)TCCAGCATGGGCTCtCCTGTCA(TAMRA)	
Mammalian 18S ribosomal RNA	535(+) 5′-GAGGGAGCCTGAGAAACGG	NM_10098
	602(−) 5′-GTCGGGAGTGGGTAATTTGC	
	555(+) 5′-(FAM)TACCACATCCAAGGAAGGCAGCAGG(TAMRA)	

[a]Accounts for transcripts of PRA and PRB isoforms.

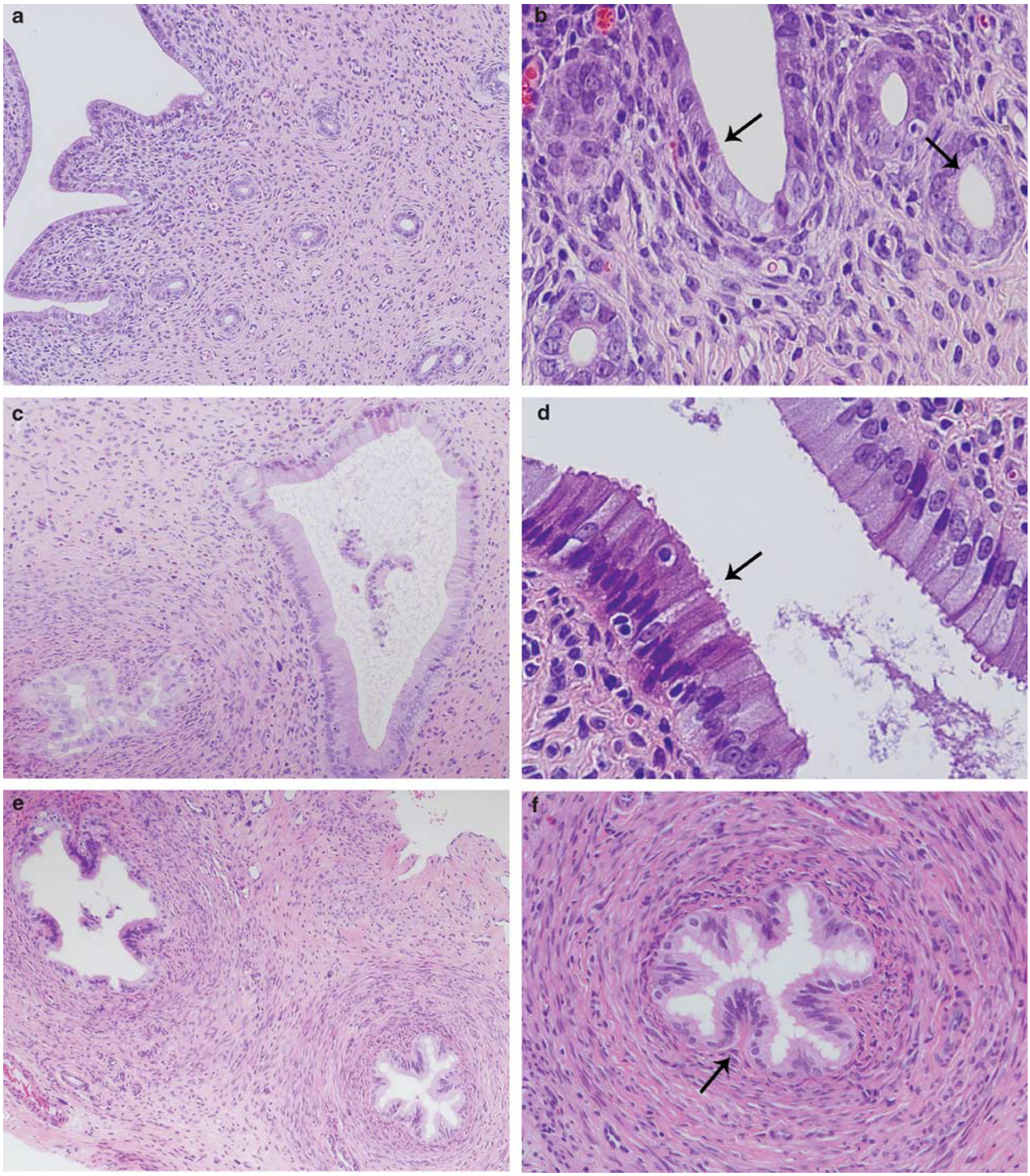

Figure 1 Microscopic endometrial changes induced by DES. The endometrium of vehicle-treated rats is characterized by small cuboidal-shaped epithelial cells lining simple glands (**a**, **b**). Approximately 60% of rats with neonatal DES exposure develop endometrial hyperplasia, characterized by taller columnar cells lining enlarged glands that are more complex with papillary architecture (**c**–**f**). Arrows in (**b**) indicate normal luminal (left) and glandular (right) epithelial cells, in (**d**) indicate columnar luminal epithelial cells with increased cytoplasm, and in (**f**) indicate papillary architecture. H&E $\times$ 100 (**a**, **c**, **e**) and H&E $\times$ 200 (**b**, **d**, **f**).

papillary-shaped projections and nuclear stratification (Figure 1c–f). At this early stage of endometrial hyperplasia, we do not observe the glandular proliferation and crowding associated with endometrial hyperplasia in humans. The hyperplasias occurred with an equal frequency in wild-type and Eker rats. Microscopically, the hyperplastic foci were

identical. Therefore, for all of the following experiments, results for wild-type and Eker rats are pooled.

Proliferation in the epithelial compartment of the endometrium is driven by estrogen, being highest in proestrus and estrus (proliferative phase) relative to metestrus and diestrus (secretory phase) of the cycle.[37] As anticipated, in vehicle-treated females that were cycling normally (data not shown), proliferation as assessed by Ki67 nuclear immunoreactivity was indeed highest during this proliferative phase of the cycle (Figure 2a). In contrast to vehicle-treated rats that cycle normally, females exposed neonatally to DES have compromised ovarian morphology and function resulting in reduced levels of circulating ovarian hormones and a reproductively senescent phenotype characterized as persistent estrus.[26,33] However, the proliferative index of the endometrium of 5-month-old rats exposed neonatally to DES was equal or greater than the endometrium of vehicle-treated controls during their peak proliferative phase of the cycle (Figure 2a). Thus, despite having compromised ovarian function, in rats exposed neonatally to DES, the normal endometrium was proliferating to the same degree as the normal endometrium of vehicle-treated rats in proestrus and estrus.

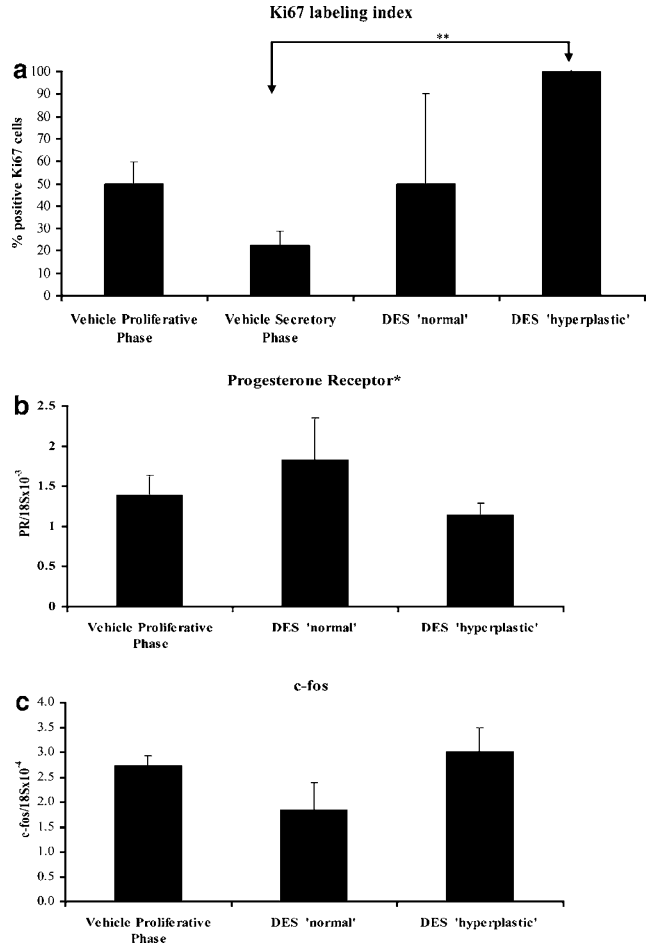

Figure 2 Endometrial proliferation (**a**) in vehicle-treated and neonatal DES-exposed rats with normal morphology or endometrial hyperplastic foci. Expression of estrogen-regulated genes PR (**b**) and c-fos (**c**) in the vehicle-treated proliferative-phase endometrium and the neonatal DES-exposed normal and hyperplastic endometrium. Cell proliferation was scored by assessing the percentage of cells positive for Ki67 nuclear antigen in 100 epithelial cells for vehicle-exposed endometrium in the proliferative phase (vehicle proliferative phase, $n=7$), secretory phase (vehicle secretory phase, $n=7$) of the cycle and the neonatal DES-exposed normal endometrium (DES 'normal', $n=6$) and DES-exposed hyperplastic endometrium (DES 'hyperplastic', $n=10$). The levels of transcripts of the estrogen-regulated genes PR and c-fos were measured in RNA isolated from neonatal vehicle-treated endometrium in the proliferative phase and neonatal DES-exposed normal or hyperplastic endometria. Transcript levels were normalized to 18S rRNA. Values are the mean + s.e. **Significant at the $P<0.025$ level. *Accounts for transcripts of PRA and PRB isoforms.

To further investigate the potential contribution of estrogen in stimulating endometrial proliferation, we examined the expression of genes well known to be regulated by estrogen in the endometrium of vehicle- and DES-exposed females. In the endometrium, PR and c-fos are well-characterized estrogen-regulated genes.[38,39] No differences were observed between expression of PR (Figure 2b) and c-fos (Figure 2c) in the endometrium of adult females exposed neonatally to vehicle or DES. Several other estrogen-induced genes (RALDH2 (retinaldehyde dehydrogenase-2), ERα, p27Kip1, p21Cip1 and cyclin D1) were also similarly expressed in the endometrium of vehicle-treated and neonatal DES-exposed endometrium (data not shown). There were no significant differences in the transcript levels of estrogen-induced genes between DES-exposed normal endometrium and DES-exposed hyperplastic endometrium (Figure 2b and c). These data were reminiscent of results from our previous studies[20] where the endometrium of women that developed CAH did not show evidence of 'hyper-estrogenization' as indicated by the lack of global induction of genes regulated by estrogen. This suggested the possibility that a compensating mitogenic pathway had become activated as a result of DES exposure that persisted in adult DES-exposed females and promoted epithelial proliferation.

Neonatal DES Exposure Results in Increased IRS-1 and IGF-II Expression in Adult Endometrium

The IGF-IR participates in an important mitogenic signaling pathway in the endometrium, and overexpression and activation of the IGF-IR are major contributors to the abnormal proliferation of endometrial epithelial cells in women with endometrial CAH.[20] To examine the potential contribution of this signaling pathway to proliferation in endometrium of DES-exposed rats, we quantified the expression of several components of the IGF-I pathway (IGF-I, IGF-II, IGF-binding protein3 (IGFBP3), IGFBP5, IGF-IR, IGF-IIR and IRS-1) in adult (5 months) endometrium of rats exposed neonatally to vehicle or DES. In contrast to our previous findings in women with endometrial CAH, there was no significant difference in the transcript levels of IGF-IR between proliferative (proestrus and estrus) vehicle-treated endometrium and DES-exposed endometrium with either normal or hyperplastic morphology (Figure 3a). Similar data

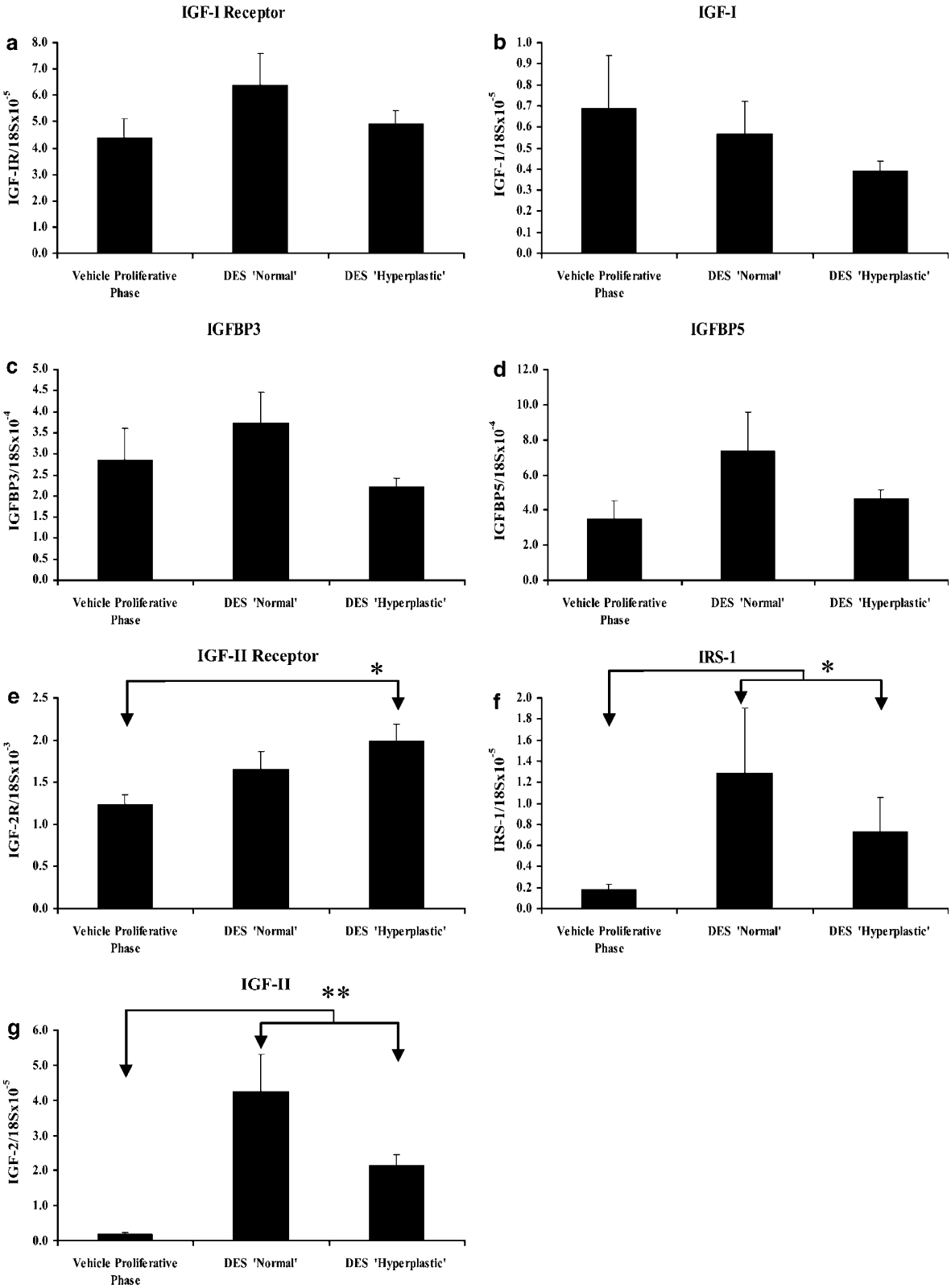

Figure 3 Expression of IGF-I pathway components in the neonatal DES-exposed normal endometrium and DES-exposed hyperplastic endometrium as compared to the vehicle-treated proliferative-phase endometrium. The levels of transcripts of IGF-I pathway components IGF-IR (**a**), IGF-I (**b**), IGFBP3 (**c**), IGFBP5 (**d**), IGF-IIR (**e**), IRS-1 (**f**) and IGF-II (**g**) were measured in RNA isolated from neonatal vehicle-treated endometrium in the proliferative phase ($n=7$) and neonatal DES-exposed normal endometrium (DES 'normal', $n=6$), and neonatal DES-exposed hyperplastic endometrium (DES 'hyperplastic', $n=10$). Transcript levels were normalized to 18S rRNA. Values are the mean + s.e. *Significant at the $P<0.05$ level. **Significant at the $P<0.025$ level.

were obtained for transcript levels for IGF-I, IGFBP3 or IGFBP5, with no significant differences between vehicle- or DES-exposed animals (Figure 3b–d). IGF-IIR showed a modest 1.5-fold increase in the DES-exposed hyperplastic endometrium as compared to the proliferative phase vehicle-treated endometrium (Figure 3e). Transcript levels of the IRS-1, a transducer of IGF-I signaling, increased 11-fold in neonatal DES-exposed normal endometrium and 5-fold in DES-exposed hyperplastic endometrium (Figure 3f). Additionally, transcript levels of IGF-II were 24-fold higher in the neonatal DES-exposed normal endometrium and 12-fold higher in DES-exposed hyperplastic endometrium compared to proliferative phase vehicle-treated endometrium (Figure 3g).

Activation of the IGF-I Signaling Pathway in Neonatally Exposed DES Endometrium

To determine if the increased expression of IGF-II and IRS-1 in the neonatal DES-exposed endometrium resulted in increased mitogenic signaling via the IGF-IR pathway, we evaluated expression and activation of the IGF-IR pathway components IRS-1, IGF-IR, Akt and PTEN by immunohistochemistry. For the panel of IGF pathway components examined, we found no significant difference in expression between DES-exposed normal endometrium and DES-exposed hyperplastic endometrium (data not shown). We therefore combined IHC data for DES-exposed normal and hyperplastic endometria and compared to vehicle-treated endometria. Loss of PTEN, a negative regulator of the IGF-I/Akt pathway, is an early event in endometrial carcinogenesis that occurs in human endometrial hyperplasia.[21] We investigated PTEN expression in the normal vehicle-treated endometrium, and endometrium of DES-exposed animals with and without hyperplasia. PTEN expression was equivalently high (3+) in the cytosol of the glandular epithelium of the normal proliferative phase vehicle-treated endometrium and in the DES-exposed normal and hyperplastic endometrium (Figure 4a and b, and Table 2).

The highest levels of IGF-IR expression (3+) were detected in the cytosol of the stroma and epithelium of the vehicle-treated endometrium in the proliferative phase of the cycle and in the cytosol of the stroma and epithelium of the DES-exposed endometrium (Figure 4c and d). To determine whether increased expression of IGF-II and IRS-1 (see above) was associated with increased activation of IGF-IR signaling, we evaluated IGF-IR phosphorylation at tyrosine 1131 within the kinase domain, which is autophosphorylated in response to ligand activation.[40] The level of phosphorylated IGF-IR was low (1+) in the vehicle-treated endometrium in the proliferative phase, where proliferation is primarily estrogen-driven (Figure 4e). In contrast, the level of phosphorylated IGF-IR was high (3+) in the cytosol and nucleus of the stroma and epithelium in DES-exposed endometrium (Figure 4f and Table 2), suggesting increased mitogenic signaling via the IGF-IR in neonatal DES-exposed endometrium expressing elevated IRS-1 and IGF-II (Figure 3f and g).

Consistent with activated IGF-IR, signaling to PI3K/Akt, a key downstream signaling pathway for the IGF-IR, was increased. Total Akt and activated Akt (phosphorylated on serine 473) were highly expressed in the endometrial epithelial cells and stromal cells of DES-exposed rats compared to proliferative-phase vehicle-treated animals (Figure 4g–j). Here, 98% of DES-exposed rats had 3+ endometrial expression of Akt, and 80% had 3+ expression of phosphorylated Akt (Figure 4h and j, and Table 2). Consistent with their proliferative phenotype (ie estrogen-induced proliferation in cycling vehicle-treated animals and IGF-II-induced proliferation in DES-exposed animals), the expression of phosphorylated S6 ribosomal protein (Ser 235) was high (3+) in the cytosol of the luminal epithelium of all vehicle- and DES-exposed rat endometria (Figure 4k and l).

Loss of Negative Feedback to IRS-1 in DES-Exposed Endometrium

IGF-IR signaling is regulated by both positive (ligand-mediated activation) and negative (phosphorylation of IRS-1 by S6 kinase (S6K) signals. Ligand binding results in activation of IGF-IR and phosphorylation of tyrosine 632 of IRS-1.[41] Negative feedback occurs downstream of Akt as a result of S6K (a downstream effector of mTOR) phosphorylation of IRS-1 at serine 636/639, which inhibits IRS-1 function.[42,43] Therefore, we next evaluated activity of IRS-1 using antibodies specific for phospho-tyrosine (632) (activating), and phospho-serine (636/639) (inactivating) IRS-1 residues. Consistent with the qRT-PCR data (Figure 3f), IRS-1 expression was quite low (1+) in proliferative-phase endometrium of vehicle-treated rats (Figure 4m). Phospho-Tyr-IRS-1 expression was similarly low (1+) in the glandular epithelium of proliferative-phase endometrium (Figure 4o).

Figure 4 Immunostaining of PTEN (**a**, **b**), IGF-IR (**c**, **d**), phosphorylated IGF-IR (**e**, **f**), Akt (**g**, **h**), phosphorylated Akt (**i**, **j**), phosphorylated S6 ribosomal protein (**k**, **l**), IRS-1 (**m**, **n**), tyrosine phosphorylation of IRS-1 (**o**, **p**) and serine phosphorylation of IRS-1 (**q**, **r**) in the vehicle-treated proliferative-phase endometrium as compared to neonatal DES-exposed endometrium. PTEN immunoreactivity in vehicle-treated proliferative-phase endometrium (**a**), and DES-treated endometrium (**b**). IGF-IR and phosphorylated IGF-IR (Tyr 1131) immunoreactivity in vehicle-treated endometrium (**c**, **e**) and DES-treated endometrium (**d**, **f**). Akt and phosphorylated Akt (Ser 473) immunoreactivity in the vehicle-treated endometrium (**g**, **i**) and DES-treated endometrium (**h**, **j**). Phosphorylated S6 ribosomal protein (Ser 235) in the vehicle-treated proliferative phase endometrium (**k**) and neonatal DES-exposed endometrium (**l**). IRS-1 and tyrosine phosphorylated IRS-1 (Tyr 632) immunoreactivity in the vehicle-treated endometrium (**m**, **n**) and DES-treated persistent estrus endometrium (**o**, **p**). Serine phosphorylation of IRS-1 (Ser 636/639) in the vehicle-treated endometrium (**q**) and DES-treated endometrium (**r**). Tissues with 2+ or 3+ staining in greater than 10% of cells were considered positive for protein expression. DAB ×200.

However, in DES-exposed rats, IRS-1 expression was high (3+) in the cytoplasm of endometrial stromal and glandular epithelial cells (Figure 4n and Table 2). Phospho-Tyr-IRS-1 expression was also high (3+) in the cytosol of the stroma and epithelium of 80% of the DES-exposed rats (Figure 4p and Table 2), indicative of IRS-1 activity.

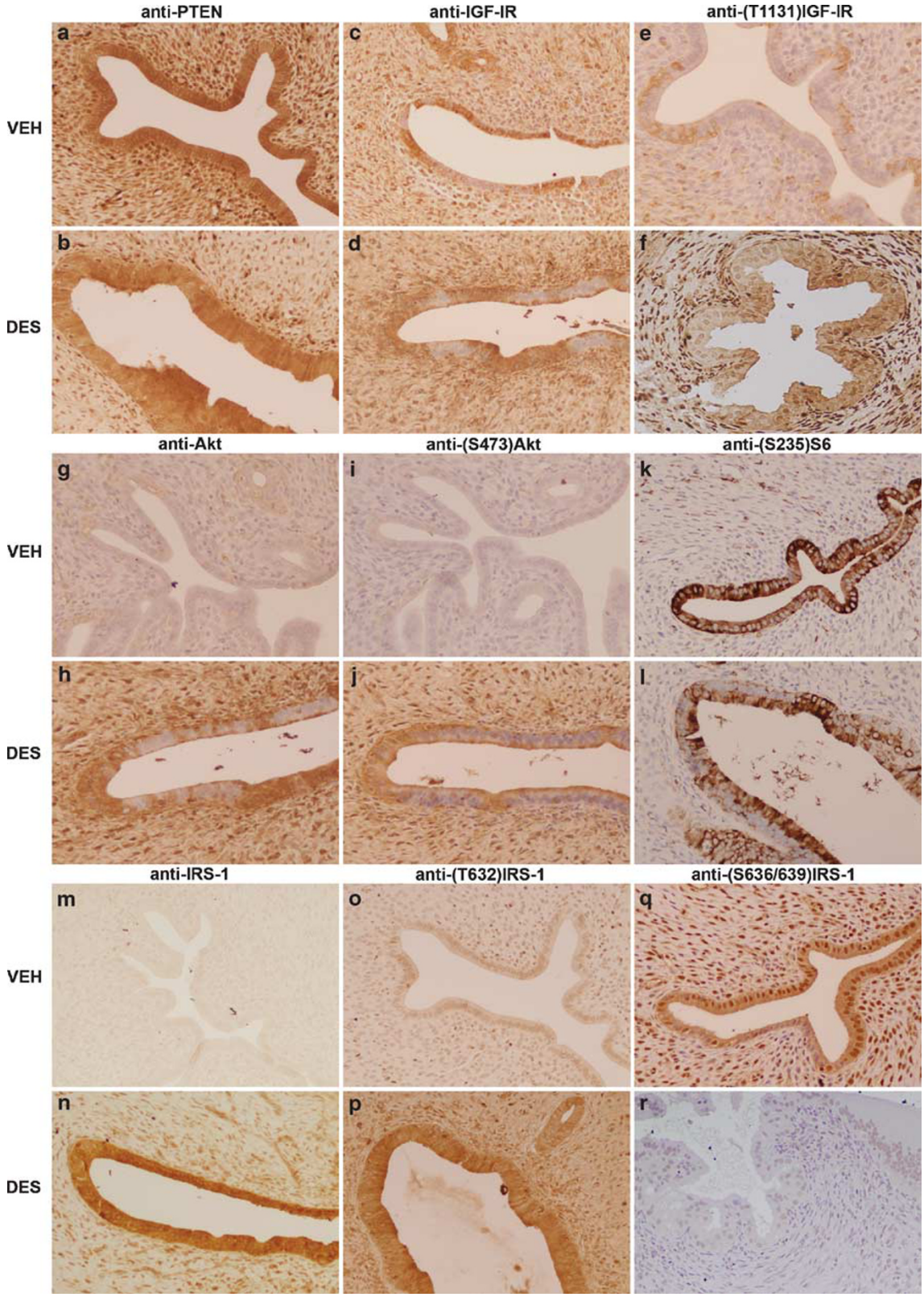

Table 2 Immunohistochemical expression of IGF-IR and downstream signaling components in the vehicle-treated and neonatal DES-exposed rat endometrium[a]

Protein	Vehicle proliferative phase (%)	DES persistent estrus (%)	*P*-value
PTEN	100	100	NS
IGF-I receptor	80	100	NS
Phospho (T1131) IGF-IR	25	70	<0.001
Akt	35	98	<0.001
Phospho (S473) Akt	15	80	<0.001
Phospho (S235) S6	100	100	NS
IRS-1	0	88	<0.001
Phospho (T632) IRS-1	25	80	<0.025
Phospho (S636/639)IRS-1	95	25	<0.001

[a]Results are expressed as the percentage of cases with 2+ or 3+ staining. Here, 60 of DES-exposed rats and 20 of vehicle-treated rats were evaluated. Significance was evaluated between groups (vehicle- and DES-exposed endometrium) by χ^2 analysis.

In contrast to activated IRS-1, the expression of phospho-Ser-IRS-1 was high $(3+)$ in the cytosol and nucleus of the stroma and epithelium of 95% of the vehicle-treated endometrium (Figure 4q and Table 2). Importantly, phospho-Ser-IRS-1 levels were low $(1+)$ in 75% of DES-exposed rat endometria (Figure 4r and Table 2), suggesting that the combined effect of reprogrammed IRS-1 activation and loss of negative feedback inhibition of IRS-1 participated in susceptibility to develop hyperplasia in neonatally DES-exposed animals.

DISCUSSION

Neonatal exposure of the developing reproductive tract to DES results in an increased incidence of endometrial hyperplastic foci in the adult rat. In our analysis of endometrial hyperplasia resulting from neonatal DES exposure, we found overexpression of IRS-1 (5-fold) and IGF-II (12-fold), activation of the IGF-IR and IRS-1 and a commensurate proliferative phenotype. DES-exposed rat endometrium also had a slight increase in IGF-IIR (1.5-fold), a mannose-6-phosphate receptor that lacks detectable kinase activity.[13] Therefore, it appears that the principle molecular abnormality in DES-exposed rat endometrium is increased IGF-II ligand. IGF-IIR can bind IGF-II resulting in subsequent degradation of the ligand and suppression of mitogenesis by reducing IGF-II availability for binding the IGF-IR.[13] The observed modest 1.5-fold increased IGF-IIR expression may represent an inhibitory compensatory mechanism in an attempt to counteract the greater overexpression of IGF-II and IRS-1. This compensation is clearly inadequate, based on the fact that we detected activation of Akt and IRS-1 and loss of negative feedback to IRS-1 in the neonatal DES-exposed endometrium as compared to vehicle-treated endometrium. Additionally, IRS-1 activation and concomitant loss of negative feedback inhibition to IRS-1 was associated with the development of endometrial hyperplasia in the neonatal DES-exposed endometrium, consistent with increased Akt phosphorylation in DES-exposed relative to vehicle-treated endometrium. Loss of negative feedback to IRS-1 in hyperplasias of DES-exposed animals makes this the first reported example of loss of negative feedback inhibition of IRS-1 in a precancerous lesion.

We previously reported that activation of IGF-IR signaling via overexpression of IGF-IR, activation of Akt and frequent loss of expression of PTEN was a major factor in the pathogenesis of human endometrial complex hyperplasia with atypia.[20] In this rodent model of endometrial hyperplasia, we have shown that hyperproliferation of the endometrium secondary to neonatal exposure to the xenoestrogen DES is also characterized by increased IGF-IR signaling. However, in contrast to human lesions, activation of IGF-IR appears to be due not to overexpression of the receptor, but to ligand activation of the receptor by increased expression of IGF-II, coupled with increased IRS-1 expression. Increased IGF-IR signaling leads to increased activation of Akt, and likely contributes to the hyperproliferative phenotype of DES-exposed endometrium and susceptibility to develop endometrial hyperplasia. Thus, IGF-IR signaling appears to play an important role in the development of endometrial hyperplasia, albeit by different mechanisms, in both the rodent and human disease.

A number of studies have observed that plasma levels of IGF-II are significantly increased in women with endometrial cancer compared to case controls.[44,45] Within the endometrium, IGF-I and IGF-II are produced mainly in stromal cells and act in an autocrine or paracrine manner to stimulate IGF-IR in stromal and epithelial cells to promote cell proliferation.[8,9] The expression of IGF-I is controlled by growth hormone and estrogen, whereas IGF-II expression is stable and does not depend upon growth hormone or sex steroids.[7] While circulating levels of IGF-II have not been examined in rats exposed neonatally to DES, we observed that local expression of IGF-II transcripts in the endometrium was increased and associated with IGF-IR activation in endometrial epithelial and stromal cells.

It is thought that perinatal DES exposure can reprogram gene expression via epigenetic mechanisms, such as inducing methylation of gene promoters.[46] A study comparing gene expression patterns in mouse uterus treated with estradiol or DES identified that 45% of target genes examined were shared by both estrogens, but many distinct target genes were identified for DES and estradiol.[47] In the mouse, neonatal DES exposure has been demonstrated to induce persistent expression of estrogen-regulated genes including c-fos and lactoferrin.[30,31,46] McLachlan *et al*[46] demonstrated that neonatal DES exposure causes changes in the methylation pattern of specific sites in the promoter regions upstream of the estrogen response elements of multiple genes in the mouse

genome. In the neonatal DES-exposed rat endometrium, expression of the estrogen-regulated genes including PR and c-fos were equivalent to normal endometrium. However, IGF-II and IRS-1 transcripts were significantly overexpressed in neonatal DES-exposed rat endometrium compared to vehicle-treated controls. Interestingly, IGF-II, which is not estrogen responsive, is one of many genes that is a target of epigenetic gene silencing.[48,49] IGF-II is normally expressed from the paternal allele; the expression from the maternal allele is silenced. Loss of imprinting (LOI) leads to the biallelic expression of IGF-II. LOI of IGF-II occurs in endometrial carcinosarcoma, uterine leiomyosarcoma, breast cancer, cervical cancer, prostate cancer and in normal colonic epithelium from patients at risk for colorectal cancer.[48,49] It is possible that LOI of IGF-II resulting in its overexpression may contribute to abnormal endometrial proliferation characteristic of endometrial hyperplasia in the rat and human.

At the molecular level, we identified global changes in gene expression associated with DES exposure that correlated with susceptibility to progression to hyperplasia. Elevated expression of IGF-II and IRS-1 and loss of the negative feedback of S6K phosphorylation of IRS-1 at S636/639 occurred in DES-exposed endometrium regardless of the presence or absence of hyperplasia, suggesting that additional event(s) are required to progress to these lesions. However, given the dramatic difference in the incidence of hyperplasias in DES-exposed *vs* vehicle-treated animals (60 *vs* 0%), it is likely that the global changes in gene expression that occurred in the endometrium are early events in the genesis of endometrial hyperplasia, which act via increasing endometrial cell proliferation. The rat endometrium can be affected by several possible stimuli for proliferation, including estrogen and mitogenic growth factors such as IGF-I/-II. However, in the case of the vehicle-exposed endometrium, we speculate that proliferation is still restrained by the negative feedback from S6K to IRS-1, which is lost in neonatally DES-exposed endometrium.

Similar to the human endometrium, IGF pathway dysregulation and activation of Akt are characteristic of endometrial hyperplasia in the DES-exposed rat. In humans, the primary drivers of such dysregulation include overexpression of IGF-IR and loss of expression of PTEN.[20,21] In DES-exposed rats, however, IGF-IR expression is not altered. Rather, we observed an increase in endometrial IGF-II expression, activation of IGF-IR and increased expression of IRS-1 with subsequent activation. Although the mechanisms of dysregulation of the IGF pathway differ between humans and neonatal DES-exposed rats, the downstream consequences are identical, resulting in activation of the Akt pathway.[50] We therefore propose that the neonatal DES-exposed rat is a good model of endometrial hyperplasia, the precursor lesion to endometrioid endometrial carcinoma in humans, and may be suitable as an experimental model for pharmacological manipulation of the IGF pathway as a target for the therapeutic treatment of endometrial hyperplasia. In addition, given the high frequency with which DES reprogramming leads to development of hyperplasia, this may also be a useful animal model to identify additional event(s) involved in progression from a proliferative to a hyperplastic phenotype.

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