Response of adult mouse uterus to early disruption of estrogen receptor- α signaling is influenced by Krüppel-like factor 9

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Abstract

Inappropriate early exposure of the hormone-responsive uterus to estrogenic compounds is associated with increased risk for adult reproductive diseases including endometrial cancers. While the dysregulation of estrogen receptor- α (ESR1) signaling is well acknowledged to mediate early events in tumor initiation, mechanisms contributing to sustained ESR1 activity later in life and leading to induction of oncogenic pathways remain poorly understood. We had shown previously that the transcription factor Krüppel-like factor 9 (KLF9) represses ESR1 expression and activity in Ishikawa endometrial glandular epithelial cells. We hypothesized that KLF9 functions as a tumor suppressor, and that loss of its expression enhances ESR1 signaling. Here, we evaluated the contribution of KLF9 to early perturbations in uterine ESR1 signaling pathways elicited by the administration of synthetic estrogen diethylstilbestrol (DES) to wild-type (WT) and *Klf9* null (KO) mice on postnatal days

(PNDs) 1–5. Uterine tissues collected at PND84 were subjected to histological, immunological, and molecular analyses. Compared with WT mice, KO mice demonstrated larger endometrial glands and lower endometrial gland numbers; DES exposure exacerbated these differences. Loss of KLF9 expression resulted in increased glandular ESR1 immunoreactivity with DES, without effects on serum estradiol levels. Quantitative RT-PCR analyses indicated altered expression of uterine genes commonly dysregulated in endometrial cancers (*Akt1*, *Mmp9*, *Slpi*, and *Tgfb1*) and of those involved in growth regulation (*Fos*, *Myc*, *Tert*, and *Syk*), with loss of *Klf9*, alone or in concert with DES. Our data support a molecular network between KLF9 and ESR1 in the uterus, and suggest that silencing of KLF9 may contribute to endometrial dysfunctions initiated by aberrant estrogen action.

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Introduction

The steroid hormone 17 β -estradiol (E₂) regulates numerous physiological and developmental processes in target tissues by binding to its cognate receptors estrogen receptor- α (ESR1) and - β (ESR2) in the presence of numerous co-regulators (Heldring *et al.* 2007). In the uterus, ESR1 is the predominant isoform, and its presence is necessary for reproductive functions (Couse & Korach 1999, Matthews & Gustafsson 2003). Indeed, mice null for *Esr1* are infertile in contrast to *Esr2* KO mice (Hewitt & Korach 2003) due in part to the failure of the uteri to develop properly (Couse *et al.* 1995). Conversely, the unopposed activity of uterine ESR1 promotes the development and progression of endometrial cancers in humans and animal models (Di Cristofano & Ellenson 2007). Multiple mechanisms underlie the control of ESR1 expression and activity; these include regulation at the levels of transcription involving multiple promoters (Kos *et al.* 2001); alternative splicing (Heldring *et al.* 2007); posttranslational modifications via phosphorylation,

acetylation, and polyubiquitination (Fu *et al.* 2004, Masuyama & Hiramatsu 2004, Atriku *et al.* 2009); and posttranscriptional silencing by micro-RNA targeting (Castellano *et al.* 2009). The aforementioned complex regulation of ESR1 occurring at many levels predicts that perturbations in any or all of these mechanisms will lead to reproductive dysfunctions and diseases.

Endocrine-disrupting chemicals (EDCs) mimic or antagonize the effects of naturally occurring substances that elicit physiological responses in target cells (Newbold *et al.* 2006, 2007). Numerous studies are aimed at elucidating the mechanism(s) by which such chemicals exert potentially devastating health consequences in subjects upon environmental exposure (Diamanti-Kandarakis *et al.* 2009). Diethylstilbestrol (DES), a synthetic estrogen, earned its notoriety as an EDC based on the documented increased breast cancer risk in exposed mothers (Greenberg *et al.* 1984, Colton *et al.* 1993, Palmer *et al.* 2006) and vaginal clear cell adenocarcinoma in daughters exposed *in utero* (Herbst *et al.* 1971). In mice, the decreased fecundity and higher incidence

of structural abnormalities of the female reproductive tract with prenatal exposure to DES (Newbold 1995, Kaufman *et al.* 2000) were recapitulated by postnatal DES administration (postnatal days (PNDs) 1–5), a critical window of reproductive tract differentiation (Newbold *et al.* 1990). The majority of the uterine defects observed with DES had been attributed to its ability to alter the expression, co-regulatory protein recruitment, and downstream signaling events of ESR1 (Newbold 1995, Couse *et al.* 2001, Yamashita 2004). Indeed, the overexpression of ESR1 enhanced the progression of DES-induced uterine adenocarcinoma (Couse *et al.* 1997). While these findings implicate perturbations in ESR1 signaling to play a pivotal role in DES-mediated reproductive dysfunctions, the precise mechanisms and participating molecules remain poorly understood.

Krüppel-like factor (KLF) 9, a member of the Sp/KLF family of transcription factors (Suske *et al.* 2005), is a 244-amino acid protein characterized by three C-terminal zinc finger motifs that are important for binding to GC-rich sequences in DNA (Imataka *et al.* 1992). By using *Klf9* null mice and endometrial cell lines with *KLF9* knockdown *in vitro*, our laboratory has shown that KLF9 is a progesterone receptor (PGR) co-regulator (Zhang *et al.* 2002a, 2003), and depending on cell context, a negative (Velarde *et al.* 2007) or positive (Pabona *et al.* 2009) regulator of ESR1 signaling. In mice, *Klf9* null mutation was associated with uterine hypoplasia and reduced uterine progesterone sensitivity, leading to a subfertility phenotype (Simmen *et al.* 2004), and with delayed parturition due to dysregulated PGR-A expression (Zeng *et al.* 2008). *Klf9* mRNA expression was recently shown to be attenuated in abnormal uterine conditions such as in a mouse model of endometriosis and in human leiomyoma (Rackow & Taylor 2008, Lee *et al.* 2009). These findings indicate that *Klf9* plays an important role with ESR1 and PGR in uterine growth, function, and pathology.

In the present study, we evaluated the existence of a uterine ESR1/KLF9 crosstalk in the context of perturbed ESR1 signaling elicited by postnatal (days 1–5) DES exposure of wild-type (WT) and *Klf9* null mice. We have shown that relative to those of WT mice, adult uteri of *Klf9* null mice had reduced luminal epithelial (LE) height, larger endometrial glands, and lower endometrial gland numbers, and that the DES effects on these indices were dependent on *Klf9* expression. We have further shown that uterine *Klf9* expression temporally lagged behind ESR1 expression (Yamashita *et al.* 1989, Sato *et al.* 1992, Newbold 1995), and was insensitive to early DES exposure. We have demonstrated that while loss of *Klf9* expression had no effect on basal ESR1 expression in endometrial stromal and glandular epithelial compartments of adult uteri, early DES-exposed *Klf9* null mutants exhibited higher glandular ESR1 expression. Finally, we found altered expression of a subset of genes frequently dysregulated in endometrial cancers (*Akt1*, *Mmp9*, and *Slpi*) and of pro-proliferative

genes (*Fos*, *Myc*, *Tert*, and *Syk*), with loss of *Klf9* alone or in concert with DES exposure. These findings provide support for KLF9's participation in endometrial ESR1-sensitive developmental events, and suggest that silencing of KLF9 may contribute to the etiology of endometrial cancers initiated by aberrant ESR1 signaling.

Materials and Methods

Animals and treatments

Animal studies were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences. C57BL/6J *Klf9* WT and mutant mice were propagated as published previously (Morita *et al.* 2003, Simmen *et al.* 2004). To eliminate confounding effects of maternal phenotype on pup viability (Simmen *et al.* 2004), WT and mutant offspring were generated from male *Klf9* (+/–) × female *Klf9* (+/–) matings. At birth (PND1) until PND5, female pups were injected daily with 2 µg of DES dissolved in sesame oil (vehicle) or sesame oil alone (Oil; control) (Newbold *et al.* 1990). At PND21, the pups were weaned and genotyped. For purposes of this study, only WT and KO (–/–) mice were compared. At PND84, body weight and stage of estrus (by vaginal smears) were determined, and the females were killed by CO₂ asphyxiation. For each mouse, the ovary pairs and uterine horns were weighed. One uterine horn was placed in 10% neutral buffered formalin and processed for histomorphometry, while the other horn was processed for RNA isolation. A total of 16 WT (9 and 7 respectively for Oil and DES) and 16 KO (8 and 8 respectively for Oil and DES) mice were used for tissue collection and analyses (Fig. 1).

Figure 1 Experimental design using *Klf9* WT and null mutant mice according to the DES model of endometrial carcinoma. PND, postnatal day.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from uterine tissues with TRIzol reagent (Invitrogen) following the manufacturer's protocol. RNA concentrations were determined using the Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). cDNA was synthesized from 1 µg of total RNA using the Taqman Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA, USA), and analyzed by quantitative RT-PCR (QPCR) using SYBR Green (Bio-Rad) and the Bio-Rad MyiQ optical module and iCycler thermal cycler as described previously (Velarde *et al.* 2006). Intron-flanking primers were designed to eliminate the amplification of genomic DNA using the Primer Express software (Applied Biosystems), and were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA). The primer sequences (sense and antisense respectively) and the corresponding PCR product sizes are given in Table 1. A standard curve was generated by serially diluting pooled cDNAs from all animals beginning with the most concentrated cDNA pool designated as 10 000 arbitrary units. Focused QPCR array (Mouse Cancer PathwayFinder, SABiosciences, Frederick, MD, USA) analyses followed protocols described by the manufacturer, using cDNAs prepared from pooled RNAs (of same amount) isolated from DES-treated WT ($n=5$) and *Klf9* mutant ($n=4$) mice.

Immunohistochemistry, uterine morphometry, and X-Gal staining

Whole uteri were fixed in 10% neutral buffered formalin as described previously (Simmen *et al.* 2004). Sections (5 µm) were mounted on poly(lysine)-coated slides (Fisher Scientific, Pittsburgh, PA, USA), and used for uterine morphometry or immunohistochemistry. For morphometrical analyses, the sections were deparaffinized in xylene, rehydrated in a series of alcohols, and stained with hematoxylin and eosin following

standard protocols. LE height and gland numbers were quantified using the Carl Zeiss Axiovert 200M microscope equipped with a Zeiss Axiocam HRc camera and Axiovision software (Carl Zeiss, Inc., Thornwood, NY, USA). Measurements were taken from five distinct fields (100×) per section, and data from 2 to 3 serial sections from each *Klf9* WT and KO mouse ($n=3$ mice per treatment per genotype) were analyzed. For detection and quantification of ESR1-immunostaining cells, sections were treated with 3% hydrogen peroxide for 30 min to quench endogenous peroxidase activity and with Citra Plus (Biogenex, San Ramon, CA, USA) in a microwave set at power 10 for 105 s and at power 1 for 10 min to unmask antigen. Sections were incubated in a blocking solution (VectaStain ABC kits; Vector Laboratories, Burlingame, CA, USA) overnight to reduce non-specific staining prior to incubation for 16–24 h with rabbit anti-mouse polyclonal ESR1 antibody (1:250 dilution; sc-542, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a humidity chamber at 4 °C. Incubation with biotinylated anti-rabbit secondary antibody (1:200 dilution; VectaStain ABC kits) was carried out for 30 min at room temperature. Sections were stained with DAB Chromogen (3,3'-diaminobenzidine, Dako, Carpinteria, CA, USA), counterstained with hematoxylin, dehydrated, cleared, and coverslipped for examination under a microscope. Immunostained cells were counted from four randomly chosen fields (200× magnification) per slide per mouse ($n=3-4$ mice per genotype per treatment). KLF9 expression was quantified by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining because *Klf9* mutant mice were generated by knock-in of the *LacZ* gene into the open reading frame of *Klf9* exon 1 (Morita *et al.* 2003), and because in-house and commercially available anti-KLF9 antibodies give high background by immunohistochemistry. Tissue sections from *Klf9* heterozygous (+/−) mice were prepared and processed for X-Gal staining as described previously (Simmen *et al.* 2004, Zeng *et al.* 2008).

Table 1 Mouse primer sequences used for quantitative RT-PCR

	Sense	Antisense	Product size (bp)
Gene			
<i>Rn18s</i>	5'-TGCTCTTAGCTGAGTGCCCG-3'	5'-TCATGGCCTCAGTTCGAAA-3'	153
<i>Gapdh</i>	5'-TGTGTCCGTCGTGGATCTGA-3'	5'-GCATCGAAGGTGGAAGAGTGG-3'	173
<i>Klf13</i>	5'-ACACAGGTGAGAGGCCTTTCG-3'	5'-AGCATGCCTGGGTGGAAGT-3'	199
<i>Slpi</i>	5'-AAGTCCTGCGGCTTTACCT-3'	5'-GGCATTGTGGCTTCTCAAGCT-3'	154
<i>Pten</i>	5'-CCCACCACAGCTAGAACTTATCAA-3'	5'-TCCGTCCCTTCCAGCTTTAC-3'	110
<i>Myc</i>	5'-TACTTGGAGGAGACATGGTGAATC-3'	5'-AGAAGCCGCTCCACATACAGTC-3'	102
<i>Akt1</i>	5'-GACTGACACCAGGTATTTTCGATGA-3'	5'-CTCCGCTCACTGTCCACACA-3'	102
<i>Fos</i>	5'-TGAAGAGGAAGAGAAACGGAGAAT-3'	5'-TCCGCTTGGAGTGTATCTGTCA-3'	102
<i>Syk</i>	5'-AGGGTCAAAGACCAATGGAAA-3'	5'-CTGTCAATGCGGTAGTGCAATACT-3'	108
<i>Tert</i>	5'-CCTCCAGACAGTCTGCATCAATAT-3'	5'-TCTTCTTAACACGCTGGTCAAA-3'	104
<i>Tgfb1</i>	5'-GCCTGAGTGGCTGTCTTTTGAC-3'	5'-CACAAAGCAGTGAGCGCTG-3'	101
<i>Mmp9</i>	5'-TCGCGTGGATAAGGAGTTCTC-3'	5'-AACTCACACGCCAGAAGAATTTG-3'	107

Western blots

Western blot analyses for KLF9 protein levels in Oil (control)- and DES-treated WT mouse uteri were performed as described previously (Zhang *et al.* 2002a). Protein extracts were prepared from TRIzol reagent (Invitrogen) following the manufacturer's protocol. The protein extracts were dissolved in 1% SDS, diluted 1:10 in water, and quantified using the BCA Protein Assay Kit (Fisher Scientific), with BSA diluted in 0.1% SDS as the standard. Blots were probed with rabbit anti-KLF9 antibody (1:1000; generated in-house; Zhang *et al.* 2002a), followed by HRP-conjugated goat anti-rabbit IgG (1:1500; Bio-Rad) secondary antibody. Expression was detected with Amersham ECL Plus reagent (GE Healthcare, Piscataway, NJ, USA), and band intensities were quantified using Quantity One 1-D Analysis Software (version 4.6.5; Bio-Rad).

RIA

Approximately 500 µl of whole blood were collected by closed cardiac puncture from PND84 female *Klf9* WT and KO mice. Serum was separated from whole blood by centrifugation at 4600 g for 1 h, and was stored at -20 °C prior to analysis. Serum E₂ levels were measured using the Ultrasensitive Estradiol kit (Diagnostic Systems Laboratories, Webster, TX, USA) following the manufacturer's protocol.

Data analysis

Data (mean ± S.E.M.) were analyzed for statistical significance by two-way ANOVA followed by all pairwise multiple comparison procedures (Tukey's test). Where applicable, Student's *t*-test was performed to determine the effect of treatment within a particular genotype. All statistical analyses were performed using the SigmaStat software (Systat Software, Chicago, IL, USA) version 3.5. Differences were considered significant at $P < 0.05$.

Results

DES-associated growth effects with *Klf9* mutation

WT and KO mice administered either Oil (vehicle) or DES daily beginning at birth for 5 days (PND1–5) were examined for body weights at weaning (PND21) and at PND84. At PND21, KO mice were numerically lighter than WT mice, although the difference did not reach statistical significance. With DES treatment, WT mice were significantly lighter, while KO mice were unaffected, relative to respective Oil-treated controls (Fig. 2A). At PND84, WT mice were heavier with DES administration (Fig. 2B), and this was accompanied by a numerical (albeit not significant) increase in gonadal fat pad weight (Fig. 2C). *Klf9* mutant mice showed no differences in body or fat pad weights with DES relative to the corresponding Oil group.

Figure 2 Body and gonadal fat pad weights of control (Oil) and DES-treated *Klf9* WT and null mutant mice at postnatal days (PNDs) 21 (A) and 84 (B and C). Data are mean ± S.E.M. Means with different superscripts differed at $P < 0.05$ by two-way ANOVA followed by Tukey's test.

DES-associated alterations in uterine parameters with *Klf9* mutation

We had shown previously that KLF9 negatively regulates *ESR1* signaling in endometrial glandular epithelial cells (Velarde *et al.* 2007). To investigate DES effects on the uterus with loss of *Klf9* expression, we evaluated a number of uterine parameters (wet weight, LE height, gland size, and numbers), which are well-recognized indicators of dysfunctional estrogenic stimulation, in PND84 WT and KO mice. Uterine wet weights relative to body weight did not differ with genotype in control and DES treatment groups, with both WT and KO mice showing reduced uterine weights from corresponding Oil control with DES treatment (Fig. 3A). LE height varied as a function of *Klf9* mutation, with Oil-treated KO mice showing reduced LE heights than WT mice, comparable to those of DES-treated WT mice (Fig. 3B). Uterine gland numbers were decreased with loss of *Klf9*, and further reductions were observed for both genotypes with DES treatment (Fig. 3C). Loss of *Klf9* expression was associated with a significant increase in uterine gland size (Fig. 3D), and while both genotypes showed increased gland areas with DES treatment, the significant differences between WT and KO mice were maintained (Fig. 3D). Serum E₂ levels did not differ with *Klf9* status among the Oil-treated groups, and DES suppressed E₂ levels only for WT mice (Fig. 3E). Ovarian weights normalized to body weight were unaffected by genotype or treatment (Fig. 3F).

Figure 3 Uterine and ovarian indices of control (Oil) and DES-treated *Klf9* WT and null mutant mice at PND84. Data are mean $\pm$ S.E.M. Means with different superscripts differed at $P < 0.05$ by two-way ANOVA followed by Tukey's test.

Developmental expression of uterine KLF9

Disruption of molecular and structural parameters in the mouse uterus with early DES exposure was lost with *ESR1* null mutation, suggesting DES dysregulation of ESR1-dependent events as an underlying cause (Couse *et al.* 2001). Moreover, our previous studies suggested the existence of KLF9/ESR1 crosstalk under distinct cellular contexts (Velarde *et al.* 2007, Pabona *et al.* 2009). We determined whether KLF9 expression was manifested in the uterus during the period of DES exposure to evaluate the possibility of KLF9 contributing to later uterine phenotype with early perturbations in ESR1 signaling by DES. The temporal expression of *Klf9* gene was evaluated in uterine cryosections of *Klf9* heterozygous mice at PND5, 11, 21, and 28 by X-Gal staining (Fig. 4A). Uterine *Klf9* expression was undetectable at PND5 and PND11, and was highly visible at PND21 and PND28 (Fig. 4A). Consistent with previous findings (Simmen *et al.* 2004), *Klf9* gene was expressed predominantly in endometrial stroma, sporadically in glands, and was undetectable in luminal epithelium. QPCR (Fig. 4B) and western blot (Fig. 4C) analyses showed robust expression of both KLF9 mRNA and protein in uteri of PND84 WT mice, which were unaffected by early DES treatment.

KLF9 regulation of ESR1 expression with DES treatment

Because the effects of DES, which signals through ESR1, on estrogen-responsive uterine parameters (e.g. LE height, gland number, and size) differed with *Klf9* genotype at PND84 (Fig. 3), the expression of uterine ESR1 as a function of KLF9 expression status and DES treatment was assessed by

immunohistochemistry (Fig. 5A). Immunoreactive ESR1 protein was detectable in all endometrial cellular compartments. Notably, glands showed the highest staining, with most cells in each gland showing strong nuclear immunoreactivity. The numbers of nuclear stromal ESR1 (quantified as percentage of ESR1-immunopositive cells relative to the total cells counted) were similar in Oil-treated WT and KO mice, with a significant increase noted for WT mice but not for KO mice with early DES exposure (Fig. 5B). In the glandular compartment, nuclear ESR1-immunopositive cells were significantly higher only in DES-treated KO mice; Oil- and DES-treated WT and control KO mice showed comparable numbers of nuclear ESR1-immunopositive cells (Fig. 5B).

Altered uterine gene expression in WT and *Klf9* KO mice with DES treatment

To further evaluate the contribution of KLF9 to ESR1 signaling, we next analyzed the expression of genes previously shown to be regulated by KLF9 (Simmen *et al.* 2004) and/or DES treatment (Hong *et al.* 2006, Newbold *et al.* 2007) in the mouse uterus. KLF13, a member of the Sp/KLF family of transcription factors, exhibits the highest similarity in primary structure to KLF9 (Kaczynski *et al.* 2003). We had shown previously that *Klf13* expression is increased in uteri of *Klf9* null mice (Simmen *et al.* 2004). PTEN is a tumor suppressor whose loss of expression is associated with the development of endometrial carcinoma in mice (Daikoku *et al.* 2008), and is considered a prognostic marker for the disease in humans (Lian *et al.* 2006). Secretory leukocyte protease inhibitor

Figure 4 Temporal expression of uterine *Klf9*. (A) Uterine X-Gal staining demonstrating the absence of *Klf9* gene expression at PND5 and PND11 and predominantly stromal *Klf9* expression (arrow) at PND21 and PND28, using *Klf9* (+/–) mice. Images were taken at 100× (upper panels; scale bar, 40 μm) and 400× (lower panels; scale bar, 10 μm). (B) Uterine *Klf9* mRNA expression was quantified by QPCR and normalized to 18S mRNA for Oil- and DES-treated *Klf9* WT mice at PND84. Data (mean ± S.E.M.) are expressed as fold change relative to WT Oil treatment group ($n=4–8$ mice per treatment per genotype). (C) Western blot analyses for KLF9 protein levels in Oil- and DES-treated *Klf9* WT mice at PND84. Data (mean ± S.E.M.; $n=2$ independent analyses) are expressed relative to loading control, a non-specific (<17 kDa) protein detected in the same blot.

(SLPI) is a glandular epithelial protein whose increased expression is linked to a highly proliferative uterine state (Zhang *et al.* 2002b) and *Klf9* expression (Simmen *et al.* 2008). In control (Oil-treated) mice, *Klf13* mRNA expression was elevated significantly with *Klf9* mutation (Fig. 6A), and DES treatment did not alter this pattern of expression with genotype. *Pten* mRNA expression was numerically reduced with loss of *Klf9* expression in the Oil groups, albeit the decrease was not statistically significant (Fig. 6B). DES treatment decreased *Pten* expression ($P=0.047$) in WT mice, and maintained the low levels of *Pten* in KO mice (Fig. 6B). By contrast, uterine *Slpi* mRNA expression, while comparable between Oil-treated WT and *Klf9* null mice, was increased by DES exposure but only in *Klf9* null mice ($P=0.028$; Fig. 6C).

To expand the repertoire of genes whose expression may be further disrupted by *Klf9* mutation in the background of early DES exposure, we performed additional studies using the Mouse Cancer PathwayFinder PCR Array. Analyses of the uterine genes altered between DES-treated WT and *Klf9* mutant mice (from the 84 genes representing six biological pathways involved in transformation and tumorigenesis and

present on the array) identified a total of 23 genes whose expression was either upregulated or downregulated by at least twofold (data not shown). In order to validate the results from the microarrays, a subset of these upregulated genes were examined for differential expression by QPCR using cDNAs prepared from uterine RNAs of Oil- and DES-treated WT and KO mice (Fig. 7A). Interestingly, with the exception of *Fos*, whose transcript levels were only altered with DES in KO mice, the majority of the genes (*Akt1*, *Myc*, *Tert*, and *Syk*) demonstrated expression patterns that were increased with loss of *Klf9* expression, independent of DES treatment. *Mmp9* and *Tgfb1* showed comparable expression patterns, with higher levels in *Klf9* KO mice relative to WT mice and which tended to decrease with early DES exposure for KO mice but not for WT mice (Fig. 7A).

Early effects of KLF9 mutations on gene expression

The gene expression data from the above analyses (Figs 6 and 7A) identified *Akt1*, *Fos*, and *Slpi* as genes that are differentially regulated by early DES exposure in the background of *Klf9* null mutation at PND84. To evaluate

Figure 5 Endometrial stromal and glandular epithelial ESR1 expression levels as a function of *Klf9* genotype and DES exposure. (A) Representative images of ESR1 immunostaining in stromal and glandular epithelial cells of Oil-treated PND84 *Klf9* WT and KO mice. Images are at 400× magnification; scale bar, 10 μm. Control (NTC) represents tissue section from *Klf9* mutant mice in the absence of primary antibody. Arrows indicate nuclear staining. PND84 stromal (B) and glandular epithelial (C) ESR1 quantification (mean ± S.E.M.) for $n=3-4$ mice per genotype per treatment (Oil or DES). Means with different superscripts differed at $P<0.05$ by two-way ANOVA followed by Tukey's test.

whether these genes may function as early markers of disrupted ESR1 signaling influenced by loss of *Klf9* expression, their expression levels were evaluated in uteri of prepubertal (PND28) WT and *Klf9* null mice. As shown in Fig. 7B, only the *Slpi* expression pattern duplicated that found at PND84. While *Akt1* transcript levels were affected by *Klf9* null mutation similar to that at PND84 ($P=0.059$ for WT Oil versus KO Oil), *Akt1* response to DES exposure at PND28 was different from that at PND84. *Fos* expression at PND28 remained unchanged with *Klf9* genotype and DES treatment, which contrasted with the findings for PND84 uteri (Fig. 7A).

In silico analysis of selected genes in endocarcinoma

To correlate the changes in gene expression identified in the present study with previously reported alterations in gene expression for endometrial tumors (Mutter *et al.* 2001), an online cancer data-mining resource (www.oncomine.org) was used as described previously (Simmen *et al.* 2010). Table 2 lists those genes that were commonly altered between the findings reported here and the published dataset (Mutter *et al.* 2001). The changes in expression of *KLF9*, *AKT1*, *MMP9*, and *SLPI*, but not of *TGFB1*, were in the same direction for endometrial tumors as that found in uterine tissues of *Klf9* null mice relative to WT mice. By contrast, the uterine expression of *MYC*, *SYK*, and *TERT* genes, which were altered in *Klf9* null mice relative to WT mice, did not differ in endometrial tumors from corresponding non-diseased uteri (Mutter *et al.* 2001).

Discussion

In this study, we established a central role for *KLF9* in the response of uterine endometrial cells to early perturbations in ESR1 signaling. Using the well-characterized DES-induced model for dysregulation of ESR1 function leading to pathology (Newbold *et al.* 1990, 2007), we showed that loss of *Klf9* expression in the absence of DES altered uterine morphological parameters (LE height, gland numbers, and gland size) comparable to those for DES-treated WT mice, and increased endometrial gland sensitivity to DES. We demonstrated that *Klf9* null mutation enhanced glandular ESR1 expression with DES, and this was positively associated with increased expression of *Slpi*, a gene encoding an endometrial gland-secreted protein whose overexpression is implicated in the pathogenesis of many cancers, including that of the endometrium, due to its pro-proliferative actions (Zhang *et al.* 2002b, Chen *et al.* 2004). Finally, we demonstrated that unlike ESR1, whose uterine expression was found to be an early target of DES, occurring within days of DES administration (Sato *et al.* 1992) and which remained dysregulated later in life, *KLF9* expression was undetectable in the developing uterus (PND5 and PND11) and was unaffected by DES later in life. Given that endometrial

Figure 6 Uterine expression of selected genes as a function of *Klf9* genotype and DES exposure. mRNA levels were quantified by QPCR and normalized to GAPDH (for *Klf13*) or 18S mRNA (for *Pten* and *Slpi*) for Oil- and DES-treated *Klf9* WT and null mutant mice at PND84. Data (mean ± S.E.M.) are expressed as fold change relative to WT Oil treatment group ($n=4-8$ mice per treatment per genotype). Means with different superscripts differed at $P<0.05$ by two-way ANOVA followed by Tukey's test.

Figure 7 Differential expression of uterine genes identified from Focused QPCR Array. Uterine mRNA expression at PND84 (A) and PND28 (B) was quantified by QPCR and normalized to 18S mRNA for Oil- and DES-treated *Klf9* WT and null mice. Data (mean $\pm$ S.E.M.) are expressed as fold change relative to WT Oil treatment group ($n=4-8$ mice per treatment per genotype). Means with different superscripts differed at $P<0.05$ by two-way ANOVA followed by Tukey's test. For *Myc* (A) and *Akt1* (B), additional analyses by Student's *t*-test between bracketed groups indicated significance difference (asterisk).

cancer originates from endometrial glands (Di Cristofano & Ellenson 2007) and consistent with our previous report of increased ESR1 expression with E₂ treatment upon siKLF9 knockdown in Ishikawa (glandular epithelial) cells (Velarde *et al.* 2007), our results suggest that KLF9 constitutes an important regulator of ESR1 signaling in endometrial glands, and that loss of this regulation may predispose to increased risk for endometrial cancers.

Our findings that early (postnatal) DES exposure alters adult uterine phenotype (PND84) are consistent with previously reported long-term consequences of DES-induced early developmental perturbations for this tissue in mice (Couse *et al.* 2001). The precise mechanism by which early disruption of ESR1 signaling leads to abnormalities in uterine function of adults remains poorly understood. Epigenetic changes involving modifications to chromatin structure due to aberrant histone methylation, histone acetylation, and DNA methylation are increasingly considered to underlie the onset and progression of cancers (Ellis *et al.* 2009). Consistent with this, neonatal DES exposure has been reported to alter genomic DNA methylation due to changes in the expression of uterine DNA methyltransferases (Sato *et al.* 2009). Our data

showing the lack of co-expression of KLF9 with ESR1 in uteri of mice in the first two weeks of life (Yamashita *et al.* 1989, Sato *et al.* 1992, Newbold 1995) suggest that KLF9 mediates glandular ESR1 expression in adult uteri subsequent to and/or independent of early events arising from epigenetic modifications of ESR1. KLF9 may alter nuclear ESR1

Table 2 A subset of differentially expressed genes in human endocarcinoma associated with *KLF9* expression status^a

	Endocarcinoma ^b	P value ^c
Gene		
AKT1	+	0.00018
KLF9	-	0.035
MMP9	+	0.00019
SLPI	+	0.08
TGFB1	-	0.014
MYC	NC	0.408
SYK	NC	0.548
TERT	NC	0.904

^aIn silico analysis of Mutter *et al.* (2001).

^b(+), increased with carcinoma; (-), decreased with carcinoma; NC, no change with carcinoma.

^cP value relative to normal endometrium.

expression through effects on ESR1 stability, nuclear localization, and/or promoter activity in the adult uteri; the latter is consistent with our previous findings that reduced KLF9 expression prevented the recruitment of ESR1 to its own promoter (Velarde *et al.* 2007) to elicit ESR1-negative autoregulation. Alternatively or in addition, given that KLF9 functionally interacts with PGR-B (Zhang *et al.* 2003), the loss of PGR-B/KLF9 interaction in KO mouse uteri may have resulted in loss of PGR antagonism of ESR1 expression, thereby leading to sustained ESR1 signaling. We found that PGR-B transcript levels in PND84 mouse uteri were not altered as a function of genotype and early DES exposure in this study (data not shown), consistent with KLF9 effects at the level of PGR transactivity. While our data do not address the stage in uterine development wherein loss of *Klf9* expression on ESR1 signaling is manifested, we suggest that this probably occurs as early as PND28 based on the comparable patterns of uterine *Slpi* expression in PND28 and PND84 DES-exposed *Klf9* null mice when compared with the corresponding aged WT mice, and given that *Slpi* is an ESR1-induced gene (Chen *et al.* 2004). Further studies will address ESR1 expression in *Klf9* null uteri of younger (prepubertal) mice. Taken together, our data suggest that while the contribution of epigenetic mechanisms to dysregulated ESR1 expression with DES exposure (Li *et al.* 2003, Bromer *et al.* 2009) cannot be excluded, KLF9 involvement in the regulation of ESR1 and *Slpi* expression is probably a consequence of its action at the level of the ESR1 promoter, albeit this may not involve direct physical interactions between KLF9 and ESR1 (Velarde *et al.* 2007).

To determine if changes in estrogen-responsive parameters were attributable to altered ovarian function, we measured serum E₂ levels and ovarian tissue weights of adult WT and *Klf9* null mice with and without early DES exposure. The comparable E₂ levels in both genotypes as well as the lack of significant differences in ovarian wet weights as a function of genotype and DES administration predict a similar lack of effects on serum P levels, consistent with our previous reports of the absence of major ovarian outcomes with *Klf9* null mutation (Simmen *et al.* 2004, Zeng *et al.* 2008). Thus, ESR1/KLF9-dependent effects noted in glandular epithelial cells are largely ovarian independent, and are probably a consequence of direct uterine targeting by KLF9.

We used a combination of candidate gene and focused gene array approaches to identify genes whose expression was differentially altered in adult (PND84) WT and *Klf9* mutant uteri, based on their relationships to KLF9, ESR1, and/or cancer risk. Of the uterine genes confirmed to be regulated with loss of *Klf9* expression, two major categories are proposed: category 1 includes genes that are regulated by KLF9, independent of ESR1 (DES) signaling (*Klf13*, *Myc*, *Tert*, *Syk*, and *Akt1*), while category 2 includes genes that are influenced by KLF9/ESR1 cross-regulation (*Fos* and *Slpi*). The increased expression of pro-proliferative/cell survival, ESR1-regulated genes *Akt1* (Altomare & Testa 2005, Vilgelm *et al.* 2006), *Myc* (Cole 1986), *Syk* (Coopman *et al.* 2000),

and *Tert* (Boggett *et al.* 2006) with loss of *Klf9* expression is consistent with the potential tumor suppressor function of KLF9, as suggested by the reported loss of KLF9 expression in endometrial tumors (Mutter *et al.* 2001, Simmen *et al.* 2008). Moreover, the coincident elevated expression of *Myc* and *Tert* mRNAs is consistent with the observation by Wu *et al.* (1999) that MYC upregulates *Tert* promoter activity. *Fos* and *Slpi* are estrogen-regulated genes, whose gene products exhibit pro-proliferative activities (Zhang *et al.* 2002b, Li *et al.* 2003, Chen *et al.* 2004); hence, their increased expression with *Klf9* mutation coincident with increased ESR1 expression (with DES exposure) suggests a pathway linking the loss of KLF9-mediated ESR1-negative regulation to enhanced proliferation, and provides further support to potential KLF9 tumor suppressor activity. The increased expression of *Klf13* with *Klf9* null mutation implicates a regulatory loop between these highly related family members, as suggested from our previous studies (Simmen *et al.* 2004). *Pten* mutations are among the earliest identifiable aberrations in endometrial carcinoma (Lian *et al.* 2006); hence, our findings of a robust decrease in *Pten* transcript levels in DES-exposed WT uteri relative to control WT uteri are consistent with increased incidence of endometrial carcinoma in perinatal DES-exposed mice (Newbold *et al.* 2006, 2007). While we demonstrated only a trend for decreased uterine *Pten* expression in *Klf9* null mutants without and with DES exposure relative to WT mice, *Pten* transcript levels of *Klf9* null mutants without DES exposure were numerically comparable to those of DES-exposed WT mice, suggesting that dysregulation of *Pten* expression by early DES exposure is partially mimicked by *Klf9* null mutation. In this regard, recent data showing *Slpi* upregulation in the uteri of *Pten* heterozygous mice which develop atypical hyperplasias and carcinomas in situ (Lian *et al.* 2006), and that conditional deletion of endometrial *Pten* resulted in the rapid development of endometrial carcinoma in mice (Daikoku *et al.* 2008) are consistent with linkages between the expression of *Klf9*, *Slpi*, and *Pten* and uterine tumorigenesis, as inferred from the present results. Studies ongoing in our laboratory to evaluate uterine tumor development in *Klf9* null mice with and without postnatal DES exposure will be critical to address the link between KLF9 and uterine endocarcinoma.

In summary, data presented herein suggest a novel role for the transcription factor KLF9 in modulating uterine responses to ESR1-dependent DES actions, the latter leading to a dysfunctional uterus. Our *in vivo* model superimposing *Klf9* null mutation to early (postnatal) DES exposure has revealed a molecular network between KLF9 and ESR1 that is operative in the uterus involving key estrogen-regulated genes that may constitute early predictors of uterine tumor development, and may also underlie infertility associated with EDC exposure. The direct evidence for KLF9 as a tumor suppressor awaits further studies; nonetheless, our results raise the intriguing possibility that the silencing of KLF9 by either genetic and/or epigenetic mechanisms, as demonstrated for many tumor

suppressors and oncogenes (Di Cristofano & Ellenson 2007), may contribute to increased susceptibility for developing endometrial cancers initiated by aberrant estrogen action.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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