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Nuclear receptor co-regulator Krüppel-like factor 9 and prohibitin 2 expression in oestrogen-induced epithelial cell proliferation in the mouse uterus

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Abstract

Oestrogen, acting through its cognate receptor oestrogen receptor-α (ERα), is a critical regulator of uterine endometrial epithelial proliferation. Although the dynamic communication between endometrial stromal and epithelial cells is considered to be an important component in this process, key molecular players in particular compartments remain poorly defined. Here, we used mice null for Krüppel-like factor 9 (KLF9) to evaluate the contribution of this nuclear protein in stromal-epithelial interactions underlying proliferative effects of oestrogen. We find that in ovariectomized mice administered estradiol-17β (E_2) for 24 h, *Klf9* null mutation resulted in lack of E_2-induced proliferative response in all endometrial compartments. We demonstrate a negative association between *Klf9* expression and nuclear levels of ERα transcriptional corepressor prohibitin (PHB) 2 in uterine stromal and epithelial cells of E_2-treated wildtype (WT) and *Klf9* null mice. In early pregnancy uteri of WT mice, the temporal pattern of *Klf9* transcript levels was inversely associated with that of *Phb2*. Deletion of *Klf9* up-regulated uterine *Phb2* expression and increased PHB2 nuclear localization in endometrial stromal and epithelial cells, with no effects on the expression of the related *Phb1*. In the human endometrial stromal cell line HESC treated with E_2 for 24 h, *KLF9* siRNA targeting augmented *PHB2* transcript and increased nuclear PHB2 protein levels, albeit this effect was not to the extent seen *in vivo* with *Klf9* null mutants. Our findings suggest a novel mechanism for control of oestrogen-induced luminal epithelial proliferation involving stromal KLF9 regulation of paracrine factor(s) to repress epithelial expression of corepressor PHB2.

Keywords

oestrogen; oestrogen receptor; Krüppel-like factor 9; prohibitin; endometrial proliferation

Introduction

Oestrogen (E) control of cell proliferation is a complex process that is subject to regulation at many levels. The nuclear receptor/transcription factor estrogen receptor-α (ERα) is the key regulatory participant, transducing E action by binding the ligand to form a complex that upon

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homodimerization, interacts with various co-regulators to target E-responsive gene promoters, leading to transcriptional activation, and the synthesis of gene products that modify cellular phenotype and behavior (Tsai and O'Malley, 1994; Hall *et al.* 2001). Nuclear receptor coregulator proteins, acting as coactivators or corepressors contribute to ERα transactivity and exert their effects by direct or indirect interactions with ERα (McKenna and O'Malley, 2002; Lonard and O'Malley, 2006; Green and Carroll, 2007). Examples of co-factors that directly interact with ligand-bound ERα at its activation function domains include the forkhead protein FOXA1 and the p160 protein family members SRC1/ERAP140/ERA160/NcoA1, SRC2/GRIP1/TIF2/NcoA2, and SRC3/AIB1/Rac3/NcoA3 (Yahata *et al.* 2001; Hu *et al.* 2002; Duterte and Smith, 2003; Wang *et al*. 2007). Coregulators which influence ERα transactivation of target genes indirectly alter chromatin structure by facilitating histone acetylation/deacetylation and methylation/demethylation, and by post-translational modifications of other coregulators and transcription factors within the transcriptional complex (McKenna and O'Malley, 2002; Stenoien *et al.* 2001; Green and Carroll, 2007). The reports on the formation of an extranuclear complex between ligand-bound ERα and SRC-3/AIB1/NcoA3 (Zheng *et al*. 2005) and the increased transcription of ERα target genes upon MAPK phosphorylation of SRC-3 (Font de Mora and Brown, 2000) provide evidence for the increasingly complex and less predictable mechanisms underlying coregulator participation in ERα signaling.

We previously reported that the nuclear protein Krüppel-like factor 9 (KLF9), previously designated Basic Transcription Element Binding Protein-1 (BTEB1) (Imataka *et al.* 1992) and a member of the Sp-family of transcription factors (Suske *et al*. 2005) may function as a coregulator of steroid hormone receptor signaling in the uterine endometrium (Zhang *et al*. 2002; Zhang *et al*. 2003). We found that KLF9 exerts its effects on progesterone receptor (PGR) and ERα actions by distinct mechanisms. KLF9 promotes PGR transactivation through functional and physical interactions with PGR-B and to a lesser extent, PGR-A at progesterone (P)-responsive gene promoters (Zhang *et al*. 2003; Velarde *et al*. 2006). By contrast, KLF9 can inhibit ERα transactivity *in vitro* in a high estrogen environment by promoting estradiol-dependent down-regulation of ERα expression through enhancement of the association of ERα to GC-rich motifs within its promoter (Velarde *et al*. 2007). Based on these studies, we concluded that KLF9 may influence both PGR and ERα genomic pathways to favor P-induced cell differentiation (Velarde *et al*. 2007). Nonetheless, while the latter presented an attractive model integrating KLF9 in the opposing actions of E and P in the uterus, it did not account for our earlier findings that in the endogenous E-dominated environment of early pregnancy, uterine endometrial cells of *Klf9* null mice exhibited delayed (by 24 h) and attenuated proliferation relative to WT counterparts (Velarde *et al*. 2005). The decreased numbers of implanting embryos in *Klf9* null mutants suggested that the altered pattern of proliferation of endometrial cells with *Klf9* ablation resulted in developmental asynchrony between the uterine luminal epithelium and the implantation-ready embryo, leading to subfertility (Simmen *et al*. 2004; Velarde *et al.* 2005). Thus, KLF9 may function as a positive regulator of ERα signaling to influence cell proliferation, with important consequences on pregnancy outcome.

Prohibitin (PHB) 2, also designated as Repressor of Estrogen Receptor Activity (REA) is a 37 kDa protein exhibiting high homology to the putative tumor suppressor protein prohibitin (Phb1) (Montano *et al*. 1999). PHB1 and PHB2 are highly conserved proteins in eukaryotic cells, with similar functions as inhibitors of cellular proliferation (Mishra *et al*. 2006). Unlike PHB1, however, which represses signaling of numerous steroid hormone receptors (Wang *et al*. 2004; Mussi *et al.* 2006; Gamble *et al.* 2007; He *et al.* 2007), PHB2 demonstrates selective repression of ER activity (Montano *et al*. 1999), possibly in a cell-specific context (Wang *et al*. 2004), and cooperates with the Chicken Ovalbumin Upstream Promoter binding transcription factors I and II to decrease transcription (Kurtev *et al.* 2004). PHB2 inhibits ERα transcriptional activity by competing with p160 coregulators such as SRC-1/NcoA1 and SRC-3/NcoA3 for binding to ERα in the presence of E (Montano *et al.* 1999; Delage-Morroux

et al. 2000; Wang *et al*. 2004), and by recruiting class I and class II histone deacetylases (Kurtev *et al.* 2004). Whereas genetic deletion of both *Phb2* alleles resulted in embryonic lethality, heterozygous mice displayed phenotypes that are characteristic of overactivated ERα signaling including uterine epithelial hyperplasia coincident with higher expression levels of E-responsive genes, enhanced mammary gland morphogenesis, and delayed mammary gland involution (Park *et al.* 2005; Mussi *et al*. 2006). These collective results indicate that PHB2 functions as an important mediator of E action *in vivo.*

In the present study, we show that endometrial epithelial cells of ovariectomized (Ovx) mice with *Klf9* null mutation were unresponsive to E_2-induced proliferation. We hypothesized that stromal KLF9 may mediate E_2 effects on uterine epithelial cell proliferation by influencing the expression of specific ERα coregulators. We demonstrate that the KLF9-mediated increase in proliferation with E_2 was negatively associated with uterine *Phb2* but not *Phb1,* expression and with nuclear localization of PHB2 in all endometrial compartments. We further show that this negative linkage between KLF9 and nuclear PHB2 also occurs during the physiological condition of early pregnancy and in an E_2-treated human endometrial stromal cell line HESC. Our results suggest that the repression of epithelial PHB2 expression involving stromal KLF9 signaling may be a necessary component in the paracrine regulation of uterine epithelial proliferation by oestrogen.

Materials and Methods

Animals and treatments

Experiments were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences. WT and *Klf9* mutant (KO) mice lines were propagated and genotyped as previously described (Morita *et al.* 2003; Simmen *et al.* 2004). For steroid hormone treatment studies, approximately 8-wk-old WT and KO female littermates were subjected to bilateral ovariectomy. Two weeks later, mice were treated with vehicle [sesame oil; 0.25 ml] or 17β-estradiol (E_2, 125 ng in 0.25 ml sesame oil), and uteri were collected after 24 h. Uterine tissues from WT and KO mice at day post-coitum (dpc) 2.5, 3.5, and 4.5 were isolated as previously described (Velarde *et al.* 2005). The presence of vaginal plug was considered 0.5dpc.

Immunohistochemistry

Paraffin-embedded uteri from ovariectomized (Ovx) WT and KO females (n=3-5 mice per genotype per treatment) were serially sectioned, dewaxed in xylene and rehydrated through a graded alcohol series. Tissue sections were treated with 3% H_2O_2 to quench endogenous peroxidase activity. Antigen unmasking was performed by boiling the sections in Citra Plus (Biogenex, San Ramon, CA) in a microwave oven for 105 sec at power 10 and then for 10 min at power 1. After blocking with horse IgG (Vectastain *Elite* ABC kit, Vector Laboratories, Inc., Burlingame, CA) for 30 min, sections were incubated with the following antibodies to detect expression of proteins: (a) mouse monoclonal antibody to PCNA (PC-10, Dako) at 1:500 dilution; (b) rabbit polyclonal antibody to ERα (MC-20, Santa Cruz Biotechnology, Inc) at 1:500 dilution; and c) rabbit polyclonal antibody to Phb2 (Bethyl Laboratories Inc.) at 1:250 dilution. Incubations with anti-PCNA and ERα were carried out for 1 h at room temperature, whereas incubation with anti-PHB2 antibody was performed overnight at room temperature. Following incubation with secondary antibody (horse anti-mouse IgG at 1:1600 dilution or goat anti-rabbit IgG at 1:2000 dilution) for 30 min, sections were stained with 3,3'-diaminobenzidine tetra-hydrochloride (Dako) and counter-stained with hematoxylin. Control sections were processed similarly with the omission of the primary antibody. A total of 1000 stromal, 300 luminal epithelial, and 200 glandular epithelial cells were counted on average from at least three randomly selected fields (200X magnification) per slide; one to two slides/

mouse with n=3-5 mice per treatment group per genotype, were evaluated. Results are expressed as % nuclear-immunopositive cells ([number of nuclear positively-staining cells/ number of total cells counted] x 100).

RNA isolation and quantitative RT-PCR

Total cellular RNA was prepared from whole uteri or cells by TriZol reagent (Invitrogen). Integrity of isolated RNAs was confirmed using the RNA 6000 NanoLabChip kit (Agilent Biotechnologies, Palo Alto, CA). RNA samples were reverse-transcribed using random primers and a cDNA synthesis kit (Applied Biosystems, Foster City, CA). SYBR Green quantitative RT-PCR was performed as previously described (Velarde *et al*. 2006). Primer sets were designed to flank an intron to prevent amplification of genomic DNA, using PrimerExpress (Applied Biosystems). Synthetic oligonucleotides were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). The sense and antisense primers for the mouse genes, and the resultant PCR product sizes (in parentheses) were: *Klf9*, 5'-CGT TGC CCA CTG TGT GAG AA-3' and 5'-TTG ATC ATG CTG GGA TGG AA-3' (92 bp); *Phb2/ Rea*, 5'-AGC AGG AAC AGC AC AGA AGA-3' and 5'-CGG AGC TTG ATA TAG CCA GGA T-3' (103 bp); and *Phb1*, 5'-TCC CTT GGG TAC AGA AAC CAA TTA-3' and 5'-TGTGAT ATT GAC GTT CTG CAA GTC T-3 (101 bp). For each sample, gene expression was normalized to that of cyclophilin A (*Ppia*) (sense: 5'-AGA TGC CAG GAC CTG TAT GCT T-3'; antisense: 5'-TGT GCC AGG GTG GTG ACT TTA-3') as internal reference. Human primers (sense and antisense, respectively) for *KLF9*, *PHB2/REA*, and the internal reference gene *RPL7* were as follows: *KLF9*, 5'-TGG CTG TGG GAA AGT CTA TGG-3' and 5'-CTC GTC TGA GCG GGA GAAA CT-3' (124 bp); *PHB2/REA*, 5'-GAA CAG CGG CAG AAA ATT GTG-3' and 5'-CGA ATC TTG CGA AGT TTG ATG T-3' (105 bp), and *RPL7*, 5'-TGCTGT GCC AGA AAC CCT TAA-3' and 5'-GCT TCC TCC TTG CCT TTC G-3' (110 bp).

Cell culture, transfection with siRNAs, immunofluorescence, and Western blots

The human endometrial stromal cell line HESC was a generous gift of Dr. Graziella Krikun (Yale University, New Haven, CT). The cell line was maintained in phenol-red free DMEM and Ham's F12 (1:1 vol/vol) medium supplemented with 10% charcoal-stripped calf serum and 1% antibiotic/antimycotic solution in an atmosphere of 5% CO_2, as previously described (Krikun *et al.* 2004). Transfection with *Klf9* or *scrambled* (non-specific) siRNAs (Dharmacon) at a final concentration of 50 nM was performed using Lipofectamine 2000 according to the manufacturer's protocol when cells were ~60% confluent. After 6 h, the transfection mix was replaced with medium containing 2% charcoal-stripped calf serum. Cells were incubated for an additional 24 h in 2% charcoal-stripped calf serum with added 17β-estradiol (E_2, 10 nM) and collected for RNA analyses. Immunofluorescence was performed essentially as previously described (Velarde *et al*. 2007). In brief, cells were seeded on sterile 22-mm glass cover slides at a density of 1.8 $x10^5$ cells per well and grown overnight. After transfection with siRNAs, cells were incubated in medium containing E_2 (10 nM) in DMSO for 24 h. Cells were fixed in 4% paraformaldehyde for 10 min and permeabilized in 0.1% Triton-PBS for 45 min, with 1 X PBS washes after each step. Cells were sequentially incubated in goat serum blocking solution (Vectastain *Elite* ABC kit) for 30 min and with anti-PHB2 polyclonal antibody (1:250 dilution) overnight. After incubation with biotinylated secondary antibody (Vectastain *Elite* ABC kit), fluorescence signals were developed, visualized, and quantified for percentage of nuclear-staining cells (relative to total number of cells; ~250 per treatment group) (Velarde *et al*. 2007). Western immunoblots were performed (Velarde *et al.* 2007) using anti-rat KLF9 antibody generated in-house (Zhang D *et al*. 2002) and anti-α-actinin antibody (Santa Cruz Biotechnology) for loading control.

Data analysis

Values are presented as mean ± Standard Error of the Mean (SEM). Data were analyzed using SigmaStat (SPSS Science, Chicago, IL) and evaluated for differences between groups by Student's *t* test or two-way ANOVA, followed by Tukey's test. *P* values ≤ 0.05 were considered statistically significant.

Results

Uterine endometrial cells are unresponsive to E_2-induced proliferation with Klf9 null mutation

To evaluate the proliferative response of uterine endometrial cells to E as a function of KLF9 expression status, age-matched Ovx WT and *Klf9* KO littermates were subcutaneously administered E_2 in sesame oil or sesame oil alone (control), and corresponding uteri were isolated after 24 h. Tissue sections from WT and KO mice were immunostained for the proliferation marker PCNA (Fig. 1A), and the numbers of immunopositive cells in each endometrial compartment were counted. In WT mice, E_2 administration increased PCNA expression in LE, GE, and ST cells by at least four-fold over those of corresponding control cells (Fig. 1B). Basal and E_2-induced PCNA expression was significantly greater for LE than for GE and ST. By contrast, PCNA immunoreactivity in all endometrial compartments of E_2-treated *Klf9* null mice did not rise above those of corresponding control cells.

Since E_2 predominantly signals through ERα to exert its proliferative effects in the uterus (Dupont *et al*. 2000), the nuclear levels of this protein in endometrial compartments were evaluated by immunohistochemistry as a function of KLF9 status. Ovx oil-treated WT mice showed robust ERα expression in the uterine endometrium. E_2 treatment had no effect on nuclear levels of this protein for all cell types, in the presence or absence of KLF9. The percentages of cells positive for nuclear ERα in LE from control (15.10±7.43%) and E_2-treated (22.91±5.91%) WT mice did not differ from those of LE in corresponding control (10.67 ±2.25%) and E_2-treated *Klf9* null mice (11.86±2.34%). Similarly, ST cells from WT (control: 45.02±10.38%; E_2-treated: 38.51±6.19%) and *Klf9* null (control: 35.86±11.59%; E_2-treated: 37.78±6.27%) mice and GE cells from WT (control: 29.21±11.42%; E_2-treated: 33.04±3.23%) and *Klf9* null (control: 21.44±6.65%; E_2-treated: 36.23±13.56%) mice showed no differences in percentages of cells positive for nuclear ERα.

KLF9 is negatively associated with uterine PHB2 expression and nuclear localization

To understand the mechanism by which ablation of *Klf9* expression (predominantly in the stroma; Simmen *et al.* 2004) resulted in the lack of E_2-mediated proliferative response in all endometrial compartments, we examined the possibility that KLF9 negatively regulates the expression of the selective ERα transcriptional corepressor PHB2 (Montano et al. *1999*). Uterine tissues from control (oil) and E_2-treated Ovx WT and KO mice were quantified for *Phb2* mRNA abundance by QPCR. Control (oil-treated) WT and KO mice showed comparable levels of *Phb2* transcripts (Fig. 2A). E_2 administration appeared to decrease (*P*=0.10) *Phb2* expression in WT mice, relative to corresponding control, and this trend was reversed with loss of *Klf9* (Fig. 2A). The expression of the related gene *Phb1* was not affected by E_2 or by KLF9 expression status (Fig. 2B). Similarly, E_2 had no effect on *Klf9* transcript levels (Fig. 2C).

We evaluated the presence of PHB2 protein in uterine tissue sections by immunohistochemistry to determine its spatial distribution pattern, as a function of E_2 treatment and KLF9 expression. Using a previously characterized anti-PHB2 antibody (Montano *et al*. 1999), we found immunoreactive PHB2 in both cytoplasmic and nuclear compartments of GE, LE, and ST cells (Fig. 3A), consistent with the reported localization of this protein in the mitochondria and nucleus (Fusaro *et al*. 2003; Mishra *et al*. 2006). Since E-dependent interactions between PHB2

and ERα occur predominantly in the nucleus to inhibit ERα transactivity, nuclear PHB2 expression in endometrial GE, LE, and ST cells was determined in control and E_2-treated mice. In oil-treated Ovx WT and KO mouse uteri, the amounts of nuclear PHB2 (measured as % Phb2-immunopositive cells) were significantly greater in LE (~20%) than in GE (10%) and ST (1%) cells (Fig. 3B). With E_2-treatment, the levels of nuclear PHB2 in GE and ST cells of WT uteri did not differ from those of oil-treated controls whereas in LE cells, nuclear PHB2 levels showed a trend to decrease (Fig. 3B), following that seen for corresponding mRNA in whole uteri (Fig. 2A). Loss of *Klf9* reversed the possible inhibitory effect of E_2 on nuclear PHB2 expression in LE cells to the level of control (non-E_2 treated) cells, and increased the percent of nuclear PHB2-immunopositive GE and ST cells, with the latter demonstrating amost dramatic (~40-fold) enhancement (Fig. 3B).

Uterine Phb2 and Klf9 expression are inversely associated in early pregnancy

We have previously shown that during the E-dominated condition of early pregnancy at dpc2.5, uterine endometrial cells of *Klf9* null mice had negligible BrdU labeling relative to those of WT mice; however, KO cells displayed peak BrdU labeling one-day later at dpc3.5, similar to the level of WT cells at dpc2.5 (Velarde *et al*. 2005). To determine whether the delayed proliferative response to E_2 in *Klf9* null uteri was associated with a transient increase in *Phb2* expression, the same WT and KO uterine tissues previously analyzed for proliferation status (Velarde *et al*. 2005) were evaluated for *Phb2* mRNA abundance. In WT mice, *Phb2* expression was low at dpc2.5, significantly increased at dpc 3.5, and remained at that level at dpc 4.5 (Fig. 4A). By contrast, *Phb1* mRNA abundance in WT mice (relative to WT dpc 2.5) progressively decreased at early pregnancy (Fig. 4B). The large variations in *Phb1* abundance reflect its very low levels of expression, relative to *Phb2*. The pattern of *Phb2* mRNA expression was inversely associated with that of *Klf9* (Fig. 4C), the latter concordant with the peak of proliferation of luminal epithelial cells at dpc 2.5 and subsequently decreasing at dpc 3.5 and dpc 4.5 (Velarde *et al.* 2005). In KO mice, *Phb2* gene expression was significantly higher than for WT mice at dpc 2.5 and dpc 4.5, while at dpc 3.5, no difference in *Phb2* expression levels was noted between genotypes (Fig. 4A). *Phb1* expression was not affected by loss of *Klf9* expression (Fig. 4B).

Next, immunoreactive PHB2 was localized in uteri of WT and KO mice at dpc2.5, 3.5, and 4.5. Representative immunostained uterine sections from WT and KO mice at dpc 2.5 are shown (Fig. 5A). In WT mice, immunoreactive PHB2 was present in all endometrial cell types and in the myometrium (not shown), and was localized to both cytoplasmic and nuclear compartments (Fig. 5B). The percentage of nuclear PHB2-positive cells in WT mice was higher in GE and ST cells at dpc 3.5 and dpc 4.5 than at dpc 2.5 (Fig. 5C), following the temporal pattern observed for corresponding mRNA in whole uteri; by contrast, the percentage of nuclear PHB2-positive LE cells did not change across these pregnancy days (Fig. 4A). At dpc 2.5, there was a greater level (by 2-3-fold) in the percentage of nuclear PHB2-immunostaining cells for all endometrial cell types of *Klf9* null mice, relative to WT counterparts (Fig. 5C). At dpc 3.5, LE and ST, but not GE cells showed similar significant increases in nuclear PHB2 levels with *Klf9* ablation, whereas at dpc 4.5, nuclear PHB2 expression for all cell types was independent of *Klf9* (Fig. 5C).

Phb2 expression in human endometrial stromal cells with Klf9 knock-down

To evaluate if KLF9 directly inhibits PHB2 expression in an E_2-dominated environment as suggested *in vivo* (above), a human endometrial stromal cell line HESC was used in siRNA targeting of human *KLF9* mRNA. *PHB2* expression in *siKLF9* mRNA-transfected cells, relative to those transfected with non-specific (*scrambled*) siRNAs, was subsequently determined at the transcript and nuclear protein levels. Transfection of HESC with a pool of *KLF9* siRNAs (Dharmacon) followed by E_2-treatment diminished *KLF9* gene expression by

at least 70%, relative to similarly treated cells transfected with *scrambled* siRNAs (Fig. 6A); this level of knock-down at the transcript level resulted in a comparable decrease in KLF9 protein levels, as determined by Western blots (Fig. 6B). The decrease in *KLF9* expression significantly (P=0.009), albeit modestly increased (by 20%) *PHB2* transcript levels (Fig. 6A). Immunofluorescence studies with anti-PHB2 antibody confirmed the up-regulated expression of PHB2 at the level of the protein (Fig. 6C). Relative to *scrambled* siRNA, *KLF9* siRNA increased the percentage of nuclear-localized PHB2 in E_2-treated HESC (Fig. 6D). Parallel studies using anti-ERα antibody showed no effect of KLF9 status on the nuclear levels of this protein (data not shown), consistent with the findings for uterine ST cells of Ovx, E_2-treated *Klf9* null mice (described in text, above).

Discussion

Results from the present study suggest the participation of the transcription factor KLF9 in paracrine signaling to regulate E_2-induced proliferation of uterine endometrial epithelial cells. Using Ovx, E_2-treated *Klf9* null mutant mice and corresponding WT littermates as controls, we showed that the stromal KLF9 regulation of the proliferative response of endometrial LE cells toE_2 is negatively associated with uterine *Phb2* expression and nuclear localization of PHB2 in endometrial epithelial cells. We confirmed the physiological relevance of the negative linkage between KLF9 and PHB2 by demonstrating that the transiently lower proliferative response of uterine endometrial epithelial cells of *Klf9* null mutants relative to WT counterparts at dpc 2.5 (Velarde *et al*. 2005) corresponded with increased expression of nuclear PHB2 expression in these cells. Finally, we demonstrated that in the E_2-treated human endometrial stromal cell line HESC, a reduction in KLF9 expression resulted in increased transcript and nuclear protein levels for PHB2. Taken together, our results suggest a working model whereby E_2 control of epithelial cell proliferation can be dynamically influenced by stromal KLF9 (Fig. 7). By attenuating expression of stromal ERα corepressor PHB2 which can interact with ERα coactivators such as SRC-1 (Montano *et al.* 1999) and histone deacetylases (Kurtev *et al.* 2004), KLF9 may promote ERα-mediated transactivation of yet unknown paracrine factor (s) to repress epithelial expression of PHB2, leading to increased proliferative responsiveness to E_2.

The current study provides the first direct evidence for KLF9 involvement in E_2-induced proliferation of uterine endometrial cells. Our previous studies using early pregnant mouse uteri hinted at this possibility, since maximal BrdU-labeling of endometrial epithelial cells occurred at the E-dominated uterine environment of dpc 2.5 (Velarde *et al*. 2005), coincident with highest uterine stromal *Klf9* expression (this study). Since uterine expression of *Klf9* is predominantly stromal and is lacking in luminal epithelial cells (Simmen *et al*., 2004), the absence of proliferative response to E_2 of the latter endometrial compartment in Ovx, E_2-treated *Klf9* null mice suggests paracrine control by an E_2-induced ST-derived growth-regulatory factor(s) whose synthesis may involve functional Klf9/ERα interactions. The latter is in agreement with previous reports that ST-localized ERα mediates the mitogenic effects of E_2 on neighboring LE cells (Cooke *et al.* 1997) and that KLF9 can influence ERα transactivity in endometrial epithelial cells (Velarde *et al.* 2007). Although the identity of this putative KLF9/ERα-regulated paracrine factor(s) is currently unknown, our results suggest that this factor(s) may be involved in the inhibition of PHB2 expression in luminal epithelial cells to allow these cells, which express ERα, to optimally respond to E_2. Interestingly, the negative relationship between PHB2 and KLF9 was not extended to the related *Phb1* gene, whose gene product also functions as a repressor of ERα signaling (Mishra *et al*. 2006; He *et al*. 2007). However, we cannot exclude the possibility that an association between KLF9 and PHB1 expression may be manifest at the level of PHB1 protein.

To further investigate the molecular interplay between ERα and KLF9 in stromal cells, we evaluated the effect of *KLF9* knockdown as a function of E_2 treatment on PHB2 expression using the human stromal cell line HESC. Since loss of KLF9 expression *in vivo* was associated with enhanced expression of nuclear PHB2 in ST (by 40-fold), more so than in GE and LE (by 3-fold) cells, it is possible that KLF9 regulation of PHB2 levels in ST may be distinct from that in LE. Our findings that abrogation of *KLF9* gene expression resulted in increased *PHB2* transcript and nuclear PHB2 protein levels confirm the negative association of KLF9 and PHB2 *in vivo*. However, the *in vitro* effects were modest and did not recapitulate the 2-3-fold (pregnancy) and 40-fold (Ovx/E_2 model) increases in PHB2 expression seen *in vivo*. Albeit this difference may be a function of the limited time of exposure to E_2 *in vitro*, the *in vivo* data was also observed 24 h post-E_2, raising the strong possibility of a requirement for ST and LE communication (paracrine signaling) in this E_2-mediated process.

Our studies indicate that the regulation of uterine PHB2 expression involving KLF9 is complex and could occur at the levels of RNA, protein, and cellular localization. Indeed, the lack of a clear-cut association between nuclear PHB2 and KLF9 expression (this study) with LE proliferation status (Velarde *et al.* 2005) at dpc 3.5 and dpc 4.5, in contrast to that seen at dpc 2.5, and the loss of the inverse association of KLF9 and nuclear PHB2 levels at dpc 4.5 in LE as well as in GE and ST, suggests the additional contributions of progesterone, other KLF9-related proteins which may compensate for the loss of KLF9 function (Simmen *et al.* 2004) and possibly, the attaching embryo in the control of LE proliferation at early pregnancy. Since control of PHB2 levels may constitute part of a homeostatic mechanism to allow for the appropriate proliferative response of uterine cells to E_2, further investigations into the function and molecular regulation of PHB2 in the uterine endometrium by KLF9 in concert with other nuclear steroid receptor co-regulators should constitute an important area for future research.

In summary, our studies define a potential mechanism whereby control of E-induced endometrial epithelial cell proliferation can be dynamically regulated by stromal KLF9 through its indirect inhibition of nuclear PHB2 expression in epithelial cells. Results suggest the participation of KLF9 in E_2-dependent synthesis of an ST-derived paracrine factor(s) that mediates this epithelial mitogenic response to oestrogen. While the global significance of KLF9 regulation of ERα signaling mediated by PHB2 in luminal epithelial cells is currently unclear, the sub-fertility phenotype of *Klf9* null mice (Simmen *et al.* 2004) suggests physiological relevance of KLF9 during the E_2-dominated period of early pregnancy prior to implantation (i.e., pre-decidual stroma) and underscores the delicate control of nuclear coactivator and corepressor expression to achieve the requisite uterine response to oestrogen for successful pregnancy.

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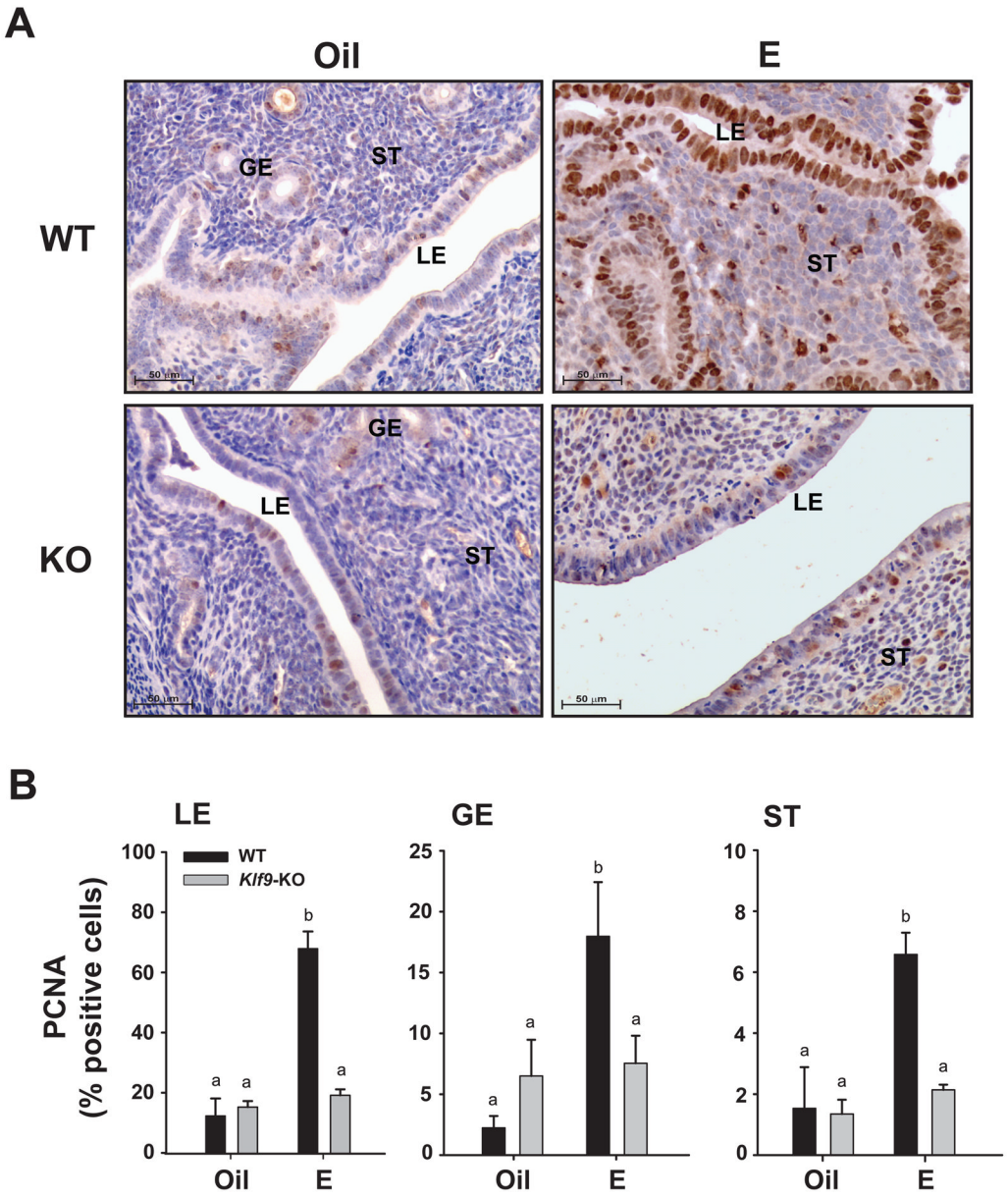

Figure 1.

Nuclear PCNA levels in uterine endometrial cells of Oil (vehicle) and E_2-treated ovariectomized WT and *Klf9* null mice. (A) Representative PCNA immunostaining of glandular epithelium (GE), luminal epithelium (LE), and stromal (ST) compartments are shown at 200X magnification. (B) The percentages of nuclear-staining cells are presented as mean ± SEM (n=3–5 mice per treatment per genotype). Significant differences were identified by two-way ANOVA, followed by Tukey's test. Means with different superscripts differed at $P<0.05$.

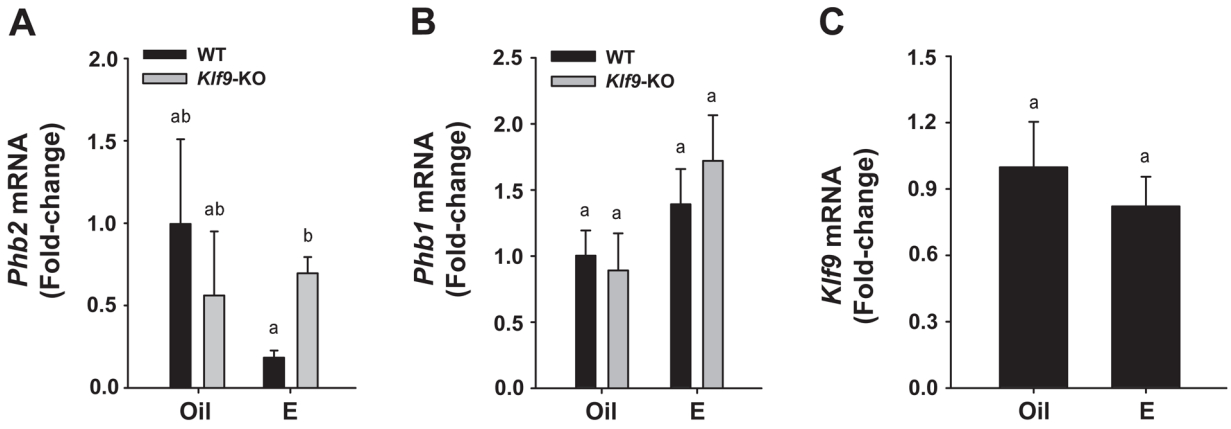

Figure 2.

Transcript levels of (A) *Phb2*, (B) *Phb1*, and (C) *Klf9* in uteri of Oil (vehicle) and E_2-treated ovariectomized WT and/or *Klf9* null mice. mRNA expression was quantified by QPCR and normalized to that of the control gene *Ppia*. Transcript levels (mean ± SEM) are expressed as fold-change relative to WT oil treatment group (n=3–5 mice per treatment per genotype). Means with different superscripts differed at $P<0.05$.

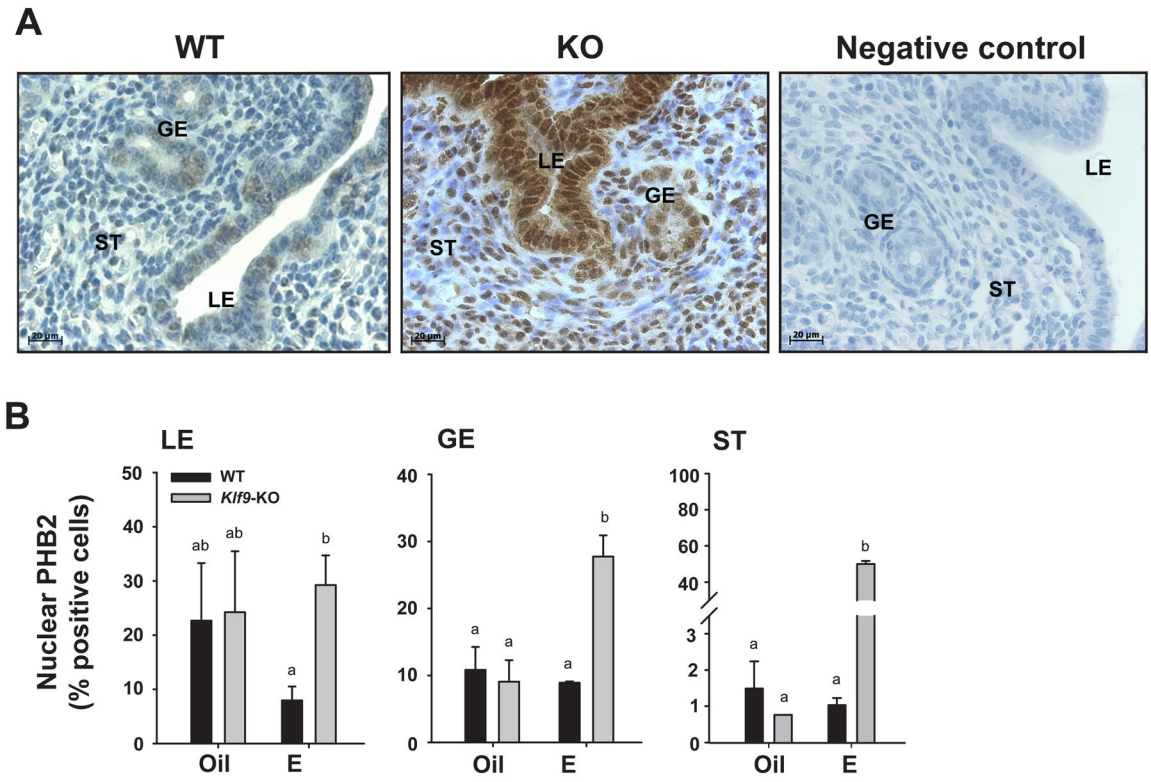

Figure 3.

PHB2 levels in uterine endometrial cells of Oil (vehicle) and E_2-treated ovariectomized WT and *Klf9* null mice. (A) Representative PHB2 immunostaining of GE, LE, and ST cells of E_2-treated WT and *Klf9* null (KO) mice are shown at 400X magnification to demonstrate nuclear localization of immunoreactive PHB2. Negative control was tissue from E_2-treated WT mice processed similarly except for omission of primary antibody. (B) The percentages of PHB2 nuclear-staining cells are presented as mean ± SEM (n=3–5 mice per treatment per genotype). Significant differences were identified by two-way ANOVA, followed by Tukey's test. Means with different superscripts differed at $P<0.05$.

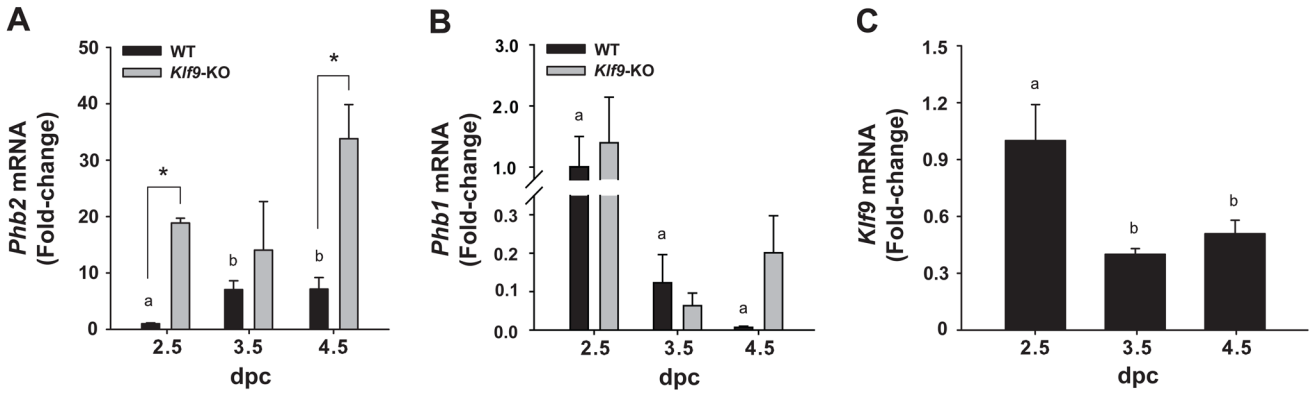

Figure 4.

Uterine transcript levels for *Phb2* (A) and *Phb1* (B) in WT and *Klf9* null mice and for *Klf9* in WT mice (C) during early pregnancy. mRNA expression was quantified by QPCR and normalized to that of the control gene *Ppia*. Transcript levels (mean ± SEM) were determined at dpc 2.5, 3.5 and 4.5 in WT (*Phb2; Phb1; Klf9*) and *Klf9* KO (*Phb2; Phb1*) mice and expressed as fold-change relative to WT (dpc 2.5) group. Significant difference (*) due to genotype at $P<0.05$ was identified by Student's *t*-test for dpc 2.5 and 4.5. One-way ANOVA was used to compare *Phb2* and *Phb1* mRNA levels of WT among pregnancy days. Means with different superscripts differed at $P<0.05$.

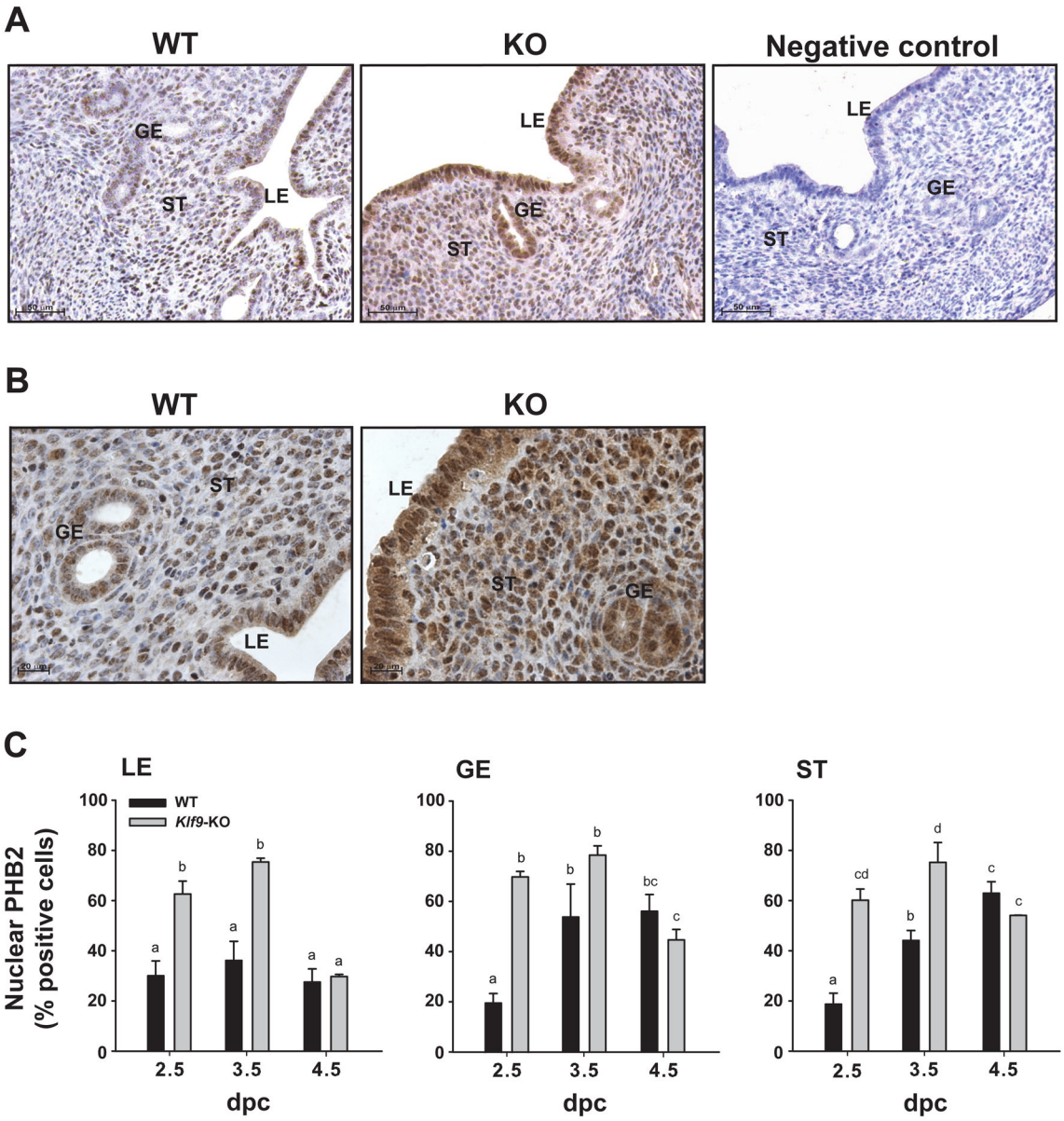

Figure 5.

Nuclear PHB2 levels in uterine endometrial cells of WT and *Klf9* null mice during early pregnancy. (A) Representative immunostaining for uterine PHB2 in pregnant WT and *Klf9* null mice at dpc 2.5. GE, glandular epithelium; LE, luminal epithelium; ST, stroma. Panels are shown at 200X magnification. Negative control was tissue from *Klf9* KO mice processed similarly except for omission of primary antibody. (B) Representative PHB2 immunostaining of GE, LE, and ST cells of pregnant WT and *Klf9* null (KO) mice at dpc 2.5 are shown at 400X magnification to demonstrate nuclear localization of immunoreactive PHB2. (C) The percentages of nuclear-immunostained cells are presented as mean ± SEM (n=3-5 mice per treatment per genotype). Significant differences were identified by two-way ANOVA, followed by Tukey's test. Means with different superscripts differed at $P<0.05$.

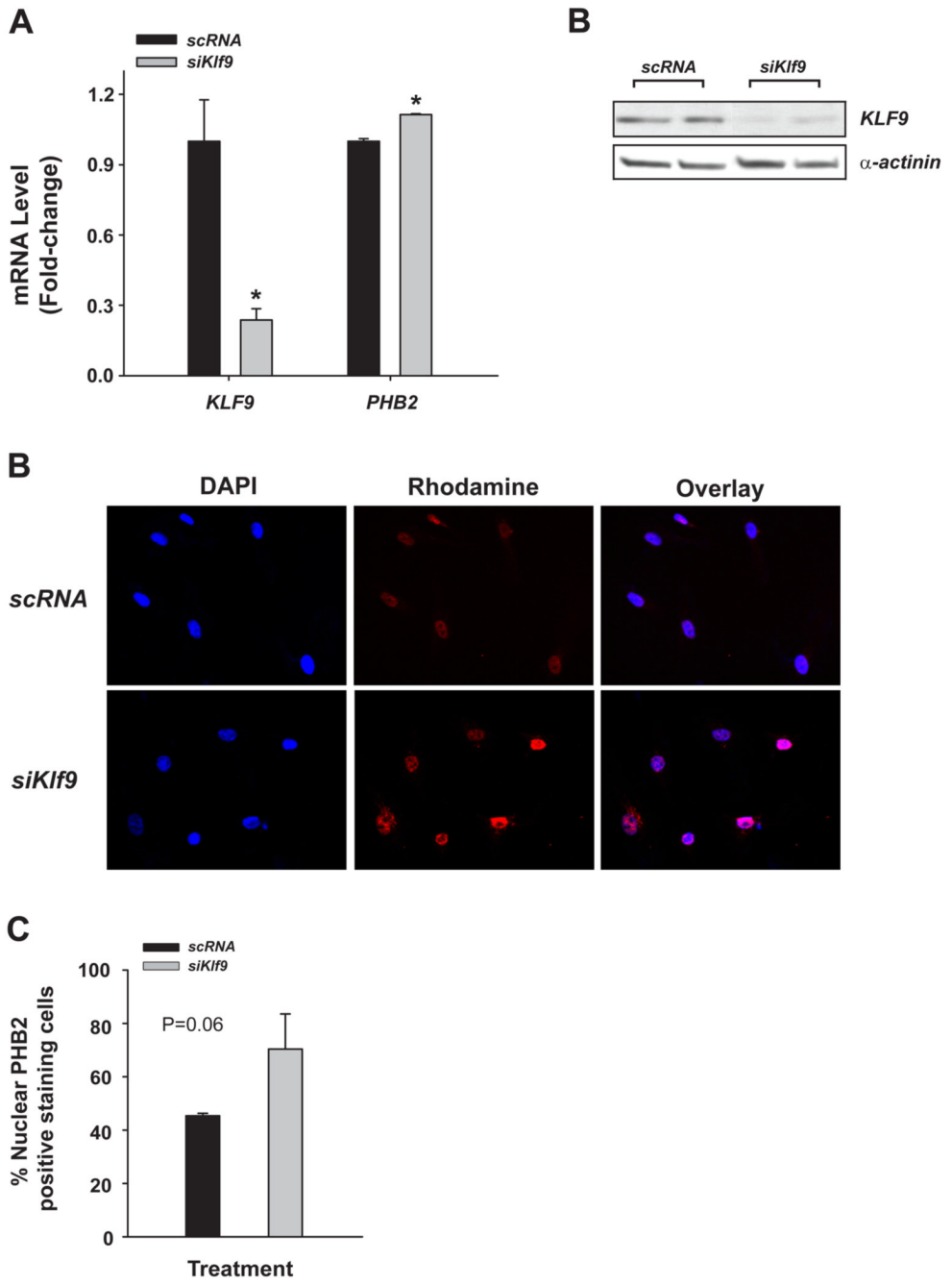

Figure 6.

PHB2 levels in human endometrial stromal cells as a function of KLF9 expression status. HESC were incubated in phenol-red free DMEM/Ham's F12 medium containing 10% charcoal-stripped serum and E_2 (10 nM) in the presence of siRNA to *scrambled* (negative control) mRNA or siRNA to *KLF9* (50 nM). (A) Harvested cells were analyzed for *KLF9 and PHB2* transcripts by QPCR and normalized to control gene *RPL7*. Results (means ± SEM; relative to cells treated with *scrambled* siRNA) shown are representative of 2–3 independent experiments, with each experiment conducted in triplicates. (B) Western blots of whole cell lysates prepared from HESC cells as a function of *Klf9* knockdown. Cells transfected with siRNA to *scrambled* sequence (negative control) or to *Klf9* were subjected to Western blots

using anti-KLF9 or anti-α-actinin antibodies. Each lane represents an independent experiment. (C) HESC treated with E_2 for 24 h were immunostained for PHB2/REA and counterstained for DAPI. Immunopositive cells were visualized using fluorescent antibodies. Overlay of anti-PHB2 (red) and DAPI (blue)-staining cells showed nuclear localization of PHB2. (D) The percentages of nuclear PHB2-staining cells were expressed relative to the total number of cells counted. Results (mean ± SEM) were normalized to those of *scrambled* siRNA-treated cells and were from two independent experiments, with each experiment performed in triplicates. Differences between groups were determined by *t*-test (P=0.06)

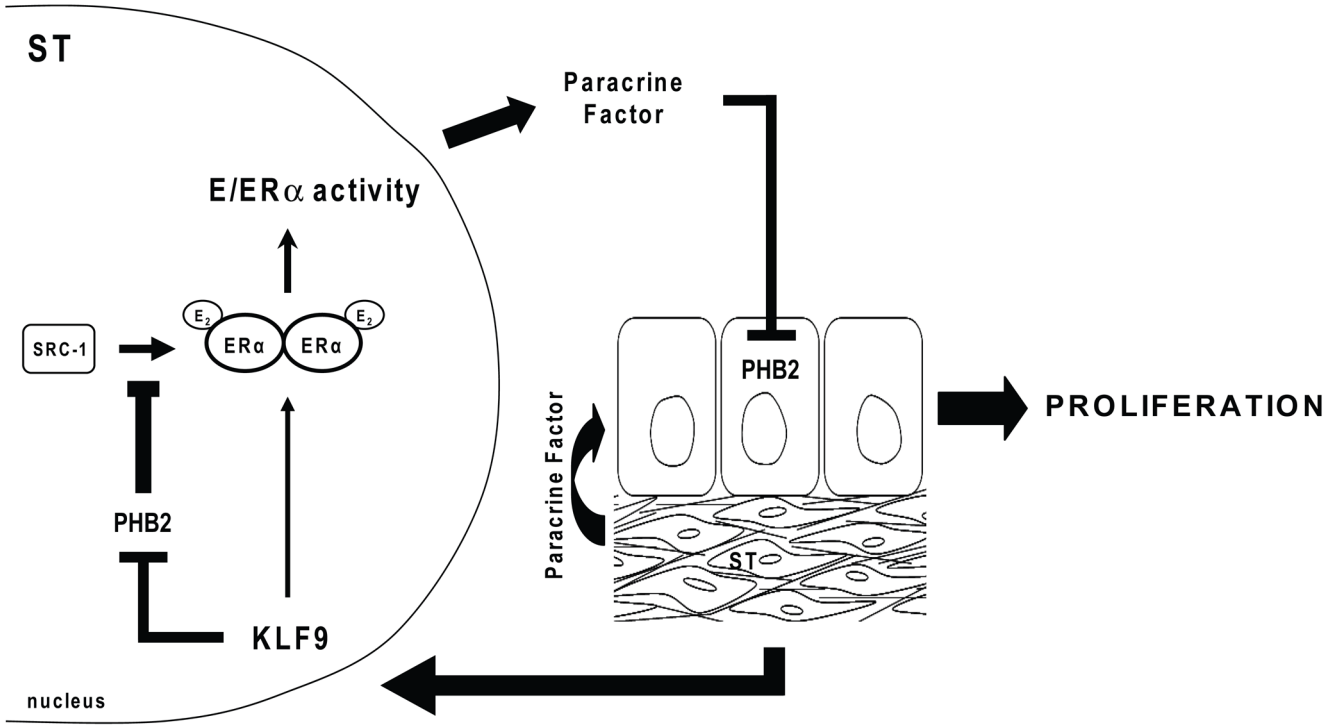

Figure 7.

Postulated model for E_2 control of uterine epithelial cell proliferation involving stromal KLF9. By attenuating expression of stromal corepressor PHB2 which can inhibit the interaction of ERα coactivators such as SRC-1 with ERα, KLF9 may promote ligand-dependent ERα-mediated transactivation of paracrine factor(s) yet unknown to repress epithelial expression of PHB2, leading to cell proliferation.