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Krüppel-Like Factor 13 Deficiency in Uterine Endometrial Cells Contributes to Defective Steroid Hormone Receptor Signaling but Not Lesion Establishment in a Mouse Model of Endometriosis[1]

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ABSTRACT

Krüppel-like Factor (KLF) 13 and the closely related KLF9 are members of the Sp/KLF family of transcription factors that have collectively emerged as essential regulators of tissue development, differentiation, proliferation, and programmed cell death. Steroid hormone-responsive tissues express multiple KLFs that are linked to progesterone receptor (PGR) and estrogen receptor (ESR) actions either as integrators or as coregulators. Endometriosis is a chronic disease characterized by progesterone resistance and dysregulated estradiol signaling; nevertheless, distinct KLF members' contributions to endometriosis remain largely undefined. We previously demonstrated promotion of ectopic lesion establishment by *Klf9* null endometrium in a mouse model of endometriosis. Here we evaluated whether KLF13 loss of expression in endometrial cells may equally contribute to lesion formation. KLF13 transcript levels were lower in the eutopic endometria of women with versus women without endometriosis at menstrual midsecretory phase. In wild-type (WT) mouse recipients intraperitoneally administered WT or *Klf13* null endometrial fragments, lesion incidence did not differ with donor genotype. No differences were noted for lesion volume, number, proliferation status, and apoptotic index as well. *Klf13* null lesions displayed reduced total PGR and ESR1 (RNA and immunoreactive protein) and altered expression of several PGR and ESR1 target genes, relative to WT lesions. Unlike for *Klf9* null lesions, changes in transcript levels for PGR-A, ESR1, and Notch/Hedgehog-associated pathway components were not observed for *Klf13* null lesions. Results demonstrate lack of a causative relationship between endometrial KLF13 deficiency and lesion establishment in mice, and they support the broader participation of multiple signaling pathways, besides those mediated by steroid receptors, in the pathology of endometriosis.

endometriosis, estrogen receptor, Krüppel-like factor 9, Krüppel-like factor 13, mouse model, progesterone receptor

INTRODUCTION

Endometriosis is a benign estrogen (E_2)-dependent disease that is characterized by the presence of endometrial tissues outside of the uterus. The disease affects 10%–15% of reproductive-age women and up to 50% of women with subfertility or infertility [1]. Endometriosis is commonly associated with chronic inflammation, pelvic pain, and early menarche, and it causes a significant emotional, economic, and physical burden in affected women [2]. Although several theories on the origin of endometriosis have been proposed, the most prevalent relates to retrograde menstruation, in which endometrial fragments reflux back to the peritoneal cavity during menstruation [3]. The latter is supported by findings that the ovary, which is anatomically located in the direct path of the retrograde outflow, is the most common site of endometriosis [4]. Nevertheless, because retrograde menstruation occurs in >90% of women [5], yet only 10% have endometriosis, other causative factors likely play a role in the development and progression of the disease.

A central element underlying endometriosis is endometrial progesterone (P_4) resistance, which leads to deregulated P_4 responsive target gene and protein expression [6, 7]. Numerous studies have reported the loss of expression of progesterone receptor (PGR) and PGR coregulators to be associated with disease establishment. In particular, the levels of PGR isoforms PGR-A and PGR-B were found to be comparably lower in the eutopic endometria of women with the disease than those without it. Moreover, in ectopic lesions the preferential loss of PGR-B relative to PGR-A, resulting in a higher PGR-A:PGR-B expression ratio, has been observed [8–10]. Reduced expression levels of PGR coregulators Hic-5, steroid receptor coactivator-1 (SRC-1), and FKBP52 have also been described in the eutopic endometria of women with endometriosis, and the direct contributions of SRC-1 and FKBP52 to disease pathology have been elucidated recently in mouse models of the disease [11–13].

Our laboratory has previously described the regulatory role of the transcription factor Krüppel-like factor 9 (KLF9) in uterine homeostasis, which is important for pregnancy and parturition using mice null for *Klf9* [14–16]. Its relevance to the pathology of uterine diseases has been supported in another mouse model [17], and more importantly in women, wherein loss of its endometrial and myometrial expression was associated with endometrial cancer, endometriosis, myoma, and prolonged labor [18–22]. To investigate a causal relationship between endometriosis and loss of KLF9, we recently developed a mouse model using a syngeneic immunocompetent mouse as a recipient of donor endometria from *Klf9* null and corresponding wild-type (WT) mice [23].

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We found that loss of endometrial KLF9-coordinated regulation of PGR, estrogen receptor (ESR), Notch, Hedgehog (Hh), and inflammatory pathways promoted ectopic lesion development in mice [23]. Among the 17 members of the Sp/KLF family [24], KLF13 is the most structurally related to KLF9. Similar to KLF9 [25], KLF13 functions as a PGR-B-specific coregulator in human endometrial cells [26], and the loss of its expression in the cycling (nonpregnant) uterus in mice resulted in diminished PGR-B expression [27]. Surprisingly, unlike *Klf9* knockout female mice, which are subfertile [14], female mice null for *Klf13* exhibit no reproductive phenotype, demonstrating litter sizes, numbers of implanting embryos, uterine morphology, and ovarian steroid hormone production comparable with those of their WT counterparts [27]. The latter suggests compensatory functions between KLF9 and KLF13; this is supported by our earlier findings of increased KLF13 expression in the peri-implantation endometrium of *Klf9* null mice [14] and of KLF9 and KLF13 cross-regulation of the expression of Bone Morphogenetic Protein-2, a critical component for human endometrial stromal decidualization [28].

Given that loss of PGR-B expression characterizes the eutopic endometria of women with endometriosis relative to women without disease [8–10, 20], and the possibility of overlapping gene targets between KLF9 and KLF13 [28], we hypothesized that loss of endometrial KLF13 may equally contribute to endometriosis establishment and progression and that this may occur through mechanisms similar to those reported for endometrial KLF9 loss of expression. To address this question, we compared the transcript levels of KLF13 in the eutopic endometria of women with and those without endometriosis and evaluated the potential contribution of loss of endometrial KLF13 and underlying mechanisms in the establishment of endometriosis in an immunocompetent mouse model.

MATERIALS AND METHODS

Human Tissues

Endometrial tissue samples were obtained from women without (NN) and with (EE) diagnosed endometriosis who were undergoing endometrial biopsy, following protocols approved by the University of California San Francisco Committee on Human Research. Participants were documented to be nonpregnant, to have not undergone hormone treatments for at least 3 mo before surgery, and to have signed informed consent to participate. The demographic data on the study participants were previously reported [20]. Sample numbers evaluated for each menstrual cycle stage were: early secretory (ES) and late secretory (LS), n = 4 each for NN and EE; proliferative endometrium (PE), n = 6 and n = 5, respectively, for NN and EE; and midsecretory (MS), n = 6 and n = 7, respectively, for NN and EE.

Animals

All animal experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, following protocols approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee. Induction of endometriosis was performed following previously published protocols using *Klf9* null mutant endometria [23]. Briefly, donor uteri isolated from C57BL/6J WT or *Klf13* null mice (ages 10–12 wk; in estrus) were subjected to a full-length midline incision, the myometrium was scraped from the endometrium by light upward and lateral traction, and the intact endometrium was collected, weighed, and finely minced using closely apposed scalpel blades oriented 180° apart. Minced endometrial tissue (~40 μg) was reconstituted in 400 μl of PBS and injected into the peritoneal cavity of recipient WT mice (age 8 wk; in estrus) through the abdominal wall, using a syringe with a 20-gauge needle (Fisher Scientific). Recipient mice were killed at 8 wk after injection for comparison of generated lesion parameters with those reported for *Klf9* null endometria [23].

Imaging and Collection of Ectopic Lesions

Lesions were visualized using a SteREODiscoveryV8 stereomicroscope (Carl Zeiss) equipped with a Canon EOS1000D camera (Canon Inc.). All lesions were photographed, and their sizes were measured using Axiovision software (Carl Zeiss). Lesion volume was calculated as described previously [29]. Collected lesions were snap frozen in liquid nitrogen or placed in 10% neutral-buffered formalin for further processing (as described below).

RNA Isolation, Quantitative RT-PCR, and Focused Quantitative PCR Arrays

Total RNA was extracted from human endometrium and mouse endometriotic lesions using TRIzol reagent (Invitrogen) following the manufacturer's protocol. RNA concentrations were determined using an ND-1000 spectrophotometer (NanoDrop). Complementary DNA was synthesized from 1 μg of total RNA using an iScript cDNA Synthesis Kit (Bio-Rad) and was analyzed by quantitative PCR (QPCR) using iTaq Universal SYBRGreen Supermix (Bio-Rad) and the Bio-Rad CFX96 Real Time System module and c1000 Touch thermal cycler. Intron-flanking primers were designed to eliminate the amplification of genomic DNA using Primer Express software (Applied Biosystems) and were obtained from Integrated DNA Technologies Inc. The primer sequences (sense and antisense, respectively) and the corresponding PCR product sizes are presented in Supplemental Table S1 (available online at www.biolreprod.org). A standard curve was generated by serially diluting cDNAs (pooled at equal volumes from all samples) beginning with the most concentrated cDNA pool, designated as 10 000 arbitrary units. Target mRNA abundance in human eutopic endometria was normalized to that of 18S RNA, whereas abundance values for endometriotic lesions in mice were normalized to a factor derived from the geometric mean of expression values for cyclophilin A (*Ppia*) and TATA box-binding protein (*Tbp*), calculated using the GeNorm program [30]. Focused PCR Profiler array (Mouse Notch Signaling Pathway PCR Array; SABiosciences) analyses followed protocols described by the manufacturer, using cDNAs prepared from RNAs pooled in equal amounts from ectopic endometrial lesions of four individual donor WT and *Klf13* null mice, respectively.

Lesion Morphometry and Immunohistochemistry

Endometriotic lesions were fixed in 10% neutral-buffered formalin and processed for morphometric and immunohistochemical analyses following previously described protocols [15, 23]. Antibodies were obtained from the following sources and used at the indicated dilutions: 1) rabbit anti-mouse ESR1 (sc-542; 1:200; Santa Cruz Biotechnology); 2) rabbit anti-mouse PGR (sc-7208; 1:200; Santa Cruz Biotechnology); and 3) rabbit anti-mouse Ki-67 (ab16667; 1:100; Abcam). Incubation with biotinylated, anti-rabbit secondary antibody (VectaStain ABC kits; 1:200; Vector Laboratories) was carried out for 30 min at room temperature. Control sections were processed similarly, but with the omission of the primary antibody. Sections were stained with 3,3-diaminobenzidine (DAB Chromogen; Dako), counterstained with hematoxylin, dehydrated, cleared, and coverslipped for examination under a microscope. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was performed using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore Corp.), following the manufacturer's instructions. Approximately 1000 stromal cells were counted from four to five randomly chosen fields (400× magnification) per slide from a total of three to four slides, with each slide representing an endometriotic lesion from an individual donor mouse per genotype, using an Axiovert 200M microscope equipped with an Axiocam HRc camera and Axiovision software (Carl Zeiss). A total of 11–21 endometrial glands from lesions formed from WT and *Klf13* null endometrium (n = 3 to 4 mice per donor lesion group), representing >200 glandular epithelial cells, were quantified for nuclear ESR1 and PGR immunostaining. Results are expressed as the percentage of nuclear immunopositive cells ([number of nuclear positively staining cells/number of total cells counted] × 100).

Peritoneal Fluid Collection and ELISA

Peritoneal fluids were collected by injecting 1 ml of cold PBS containing protease inhibitors (Halt Protease Inhibitor Cocktail; Thermo Scientific) into the peritoneum of recipient mouse using a 27-gauge needle, immediately before lesion isolation (Fisher Scientific). Fluid was retrieved using the same syringe after gentle massaging of the peritoneum, it was centrifuged at 1500 rpm for 10 min at 4°C to remove peritoneal cells, and supernatant was stored at −80°C. Peritoneal fluid cytokines were quantified for tumor necrosis factor α (TNFα) and soluble TNF receptor 1 (sTNFR1) levels (n = 9–11 individual mice per

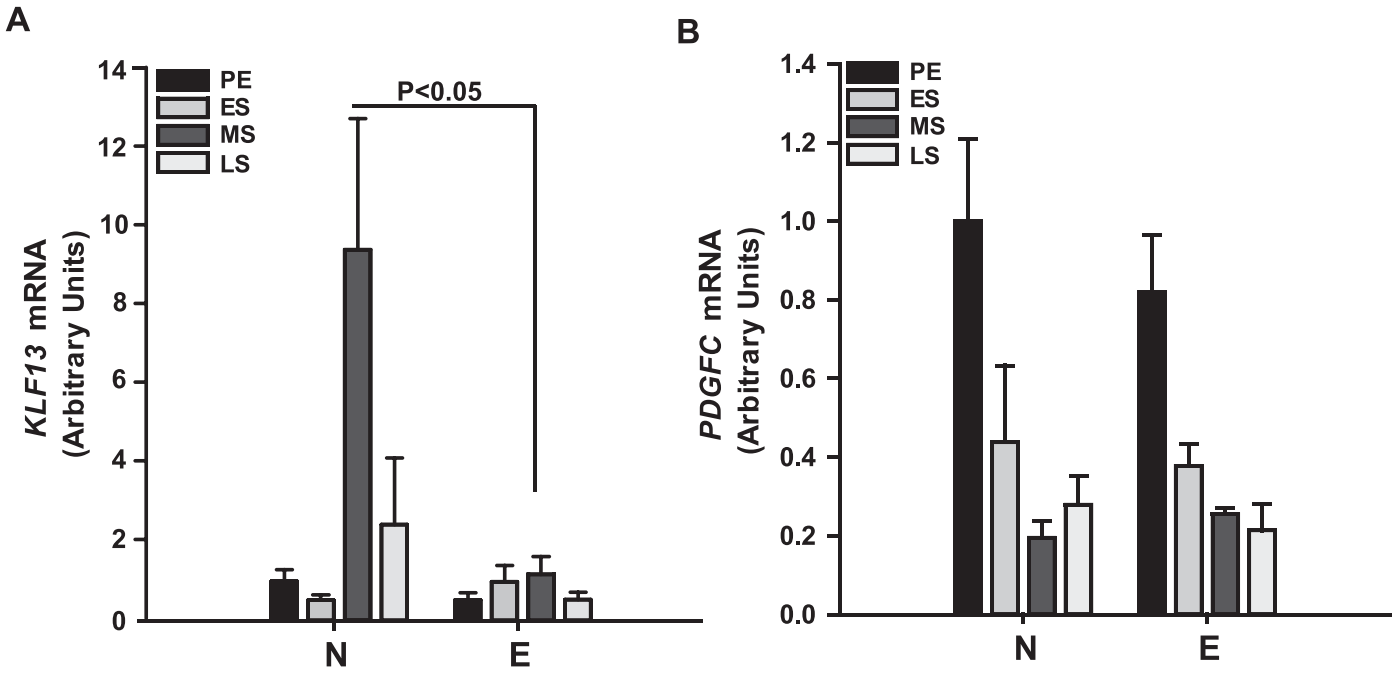

FIG. 1. KLF13 expression is diminished in the eutopic endometria of women with endometriosis. Transcript levels of *KLF13* (**A**) and the control gene *PDGFC* (**B**) in the eutopic endometria of patients with (E) and without (N) endometriosis at different phases of the menstrual cycle were quantified by QPCR and normalized to that of 18S RNA. Each bar represents the mean ± SEM of four to seven samples per group (as described in *Materials and Methods*) per menstrual cycle phase. Significant differences ($P \leq 0.05$) between N and E were determined using two-way ANOVA followed by Tukey test. $P \leq 0.05$ between N and E for MS phase, by Student *t*-test.

donor lesion group) using mouse TNFα and mouse sTNFR1 Quantikine ELISAs (R&D Systems).

Serum RIA and ELISA

Approximately 500 μl of whole blood was collected by closed cardiac puncture from recipient mice. Serum was separated by centrifugation of whole blood at 4600 × *g* for 1 h and stored at −20°C before analysis. Serum E_2 and P_4 levels were measured using an Ultrasensitive Estradiol Kit (Beckman Coulter) and a Progesterone EIA Kit (Cayman Chemical), respectively.

Statistical Analysis

Statistical analysis was performed using SigmaStat software (version 3.5; Systat Software). For comparisons of ectopic lesion volume and number, RNA transcript levels, percentage of immunostaining cells, serum levels of steroid hormones, and peritoneal TNFα and sTNFR1 levels as a function of donor genotype, data (expressed as mean ± SEM) were evaluated for statistical differences by Student *t*- test. Lesion incidence in recipient mice between donor genotypes was analyzed by the Fisher exact test. Differences were considered to be statistically significant at $P \leq 0.05$. Tendencies for differences were considered for $0.05 \leq P \leq 0.10$.

RESULTS

Diminished KLF13 *Expression in MS Eutopic Endometria of Women with Endometriosis*

Eutopic endometria from women with (EE) and without (NN) endometriosis at different menstrual cycle phases [PE, ES, MS, and LS] were evaluated for KLF13 expression by QPCR. Levels of *KLF13* transcripts were lower ($P < 0.05$) in the eutopic endometria of women with versus those without endometriosis only at the MS phase (Fig. 1A). At the other menstrual cycle stages, *KLF13* transcript levels did not differ ($P > 0.05$) between EE and NN endometria. Transcript levels for platelet-derived growth factor C (*PDGFC*), an unrelated gene, were comparable for EE and NN endometria at the same menstrual cycle stage (Fig. 1B). Transcript levels for *KLF13* and *PDGFC* were normalized to that of 18S RNA.

KLF13 Loss of Expression Did Not Contribute to Ectopic Lesion Development in Mice

To determine whether loss of endometrial KLF13 promotes ectopic lesion establishment, we used our recently described mouse model of endometriosis wherein immunocompetent mice served as recipients of donor endometrial tissue [23]. Briefly, endometrial tissues derived from WT or *Klf13* null animals were injected into the peritoneum of WT recipients of the same estrus cycle stage. At 8 wk after induction, ectopic lesions from recipient mice were collected and analyzed for lesion incidence, number, and volume (Fig. 2A). Representative images of ectopic lesions are presented in Figure 2B, and Table 1 summarizes the lesion parameters generated in the WT recipients of endometrial tissues with distinct genotypes. Most (10 of 11; 91%) WT mice receiving *Klf13* null endometrium developed ectopic lesions characterized by adherent tissue containing stroma and glands as confirmed by hematoxylin-eosin staining (Fig. 2C); nevertheless, the incidence of ectopic lesion establishment with *Klf13* null endometria did not differ ($P = 0.19$) from that of those with WT endometria (11 of 17; 64.7%). One recipient of *Klf13* null endometria displayed only nonadherent lesions (designated as ''floaters'' in a previous study [31]), which were easily distinguished from adherent lesions because of their opaqueness and lack of vascularity. Another recipient of *Klf13* null endometria also showed ''floaters'' in addition to viable ectopic lesions. No ''floaters'' were observed in those that received WT endometria (Table 1) or *Klf9* null endometria [23]. Ectopic lesions generated from WT and *Klf13* null endometria also showed no differences in numbers and volumes (Table 1) and in percentages of stromal

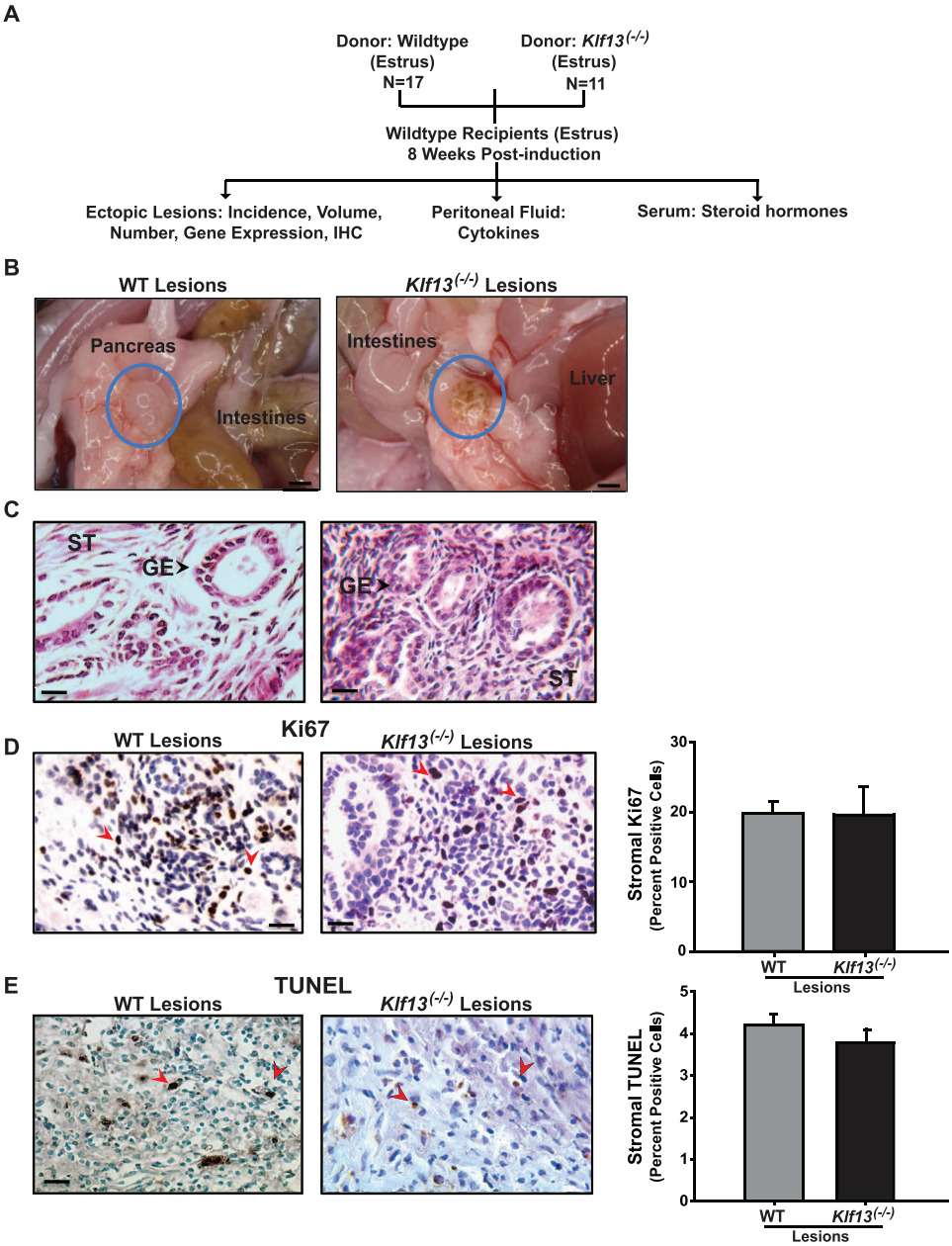

FIG. 2. Schematic of experimental design and characterization of endometrial-like lesions generated from WT and *Klf13* null endometria in WT recipient mice. **A**) Schematic representation of endometrial ectopic lesion generation and procedures for lesion analyses. The WT mice were used as recipients of endometrial fragments from WT and *Klf13*$^{(-/-)}$ mice (donors). N indicates the number of mice used for each experimental paradigm. **B**) Representative images of ectopic lesions isolated from WT recipients 8 wk after intraperitoneal administration of WT or *Klf13* null endometrium. Bar = 1 mm. **C**) Hematoxylin-eosin-stained sections of representative WT or *Klf13*$^{(-/-)}$ lesions at ×400 magnification. Bar = 20 μm. GE, glandular epithelium; ST, stroma. **D**) Stromal cells of WT and *Klf13*$^{(-/-)}$ ectopic lesions show comparable percentages of nuclear immunostaining for the proliferation marker Ki-67. Representative pictures of stromal (ST) and glandular epithelial (GE) cells for lesions of distinct genotype are shown. Bar = 20 μm. **E**) Stromal cells of WT and *Klf13*$^{(-/-)}$ ectopic lesions show comparable apoptotic status, based on the percentages of TUNEL-staining cells. Representative pictures of TUNEL-stained stromal cells are shown. Bar = 20 μm. For **D** and **E**, the percentage of stromal staining cells (percentage mean ± SEM) was determined by counting the number of stained nuclei over the total number of stromal cells per field. Three to four lesions per genotype were analyzed. Red arrowheads indicate representative Ki-67-positive (**D**) and TUNEL-staining (**E**) stromal cells, respectively.

FIG. 3. Endometriotic lesions from WT and *Klf13* null endometria display differential expression levels of PGR and ESR isoforms. **A**) Transcript levels of *Pgr and Esr* isoforms were determined in WT and *Klf13*$^{(-/-)}$ lesions by QPCR and normalized to those of housekeeping genes *Ppia* and *Tbp*, following the GeNorm program [30]. Data are mean ± SEM of fold change in normalized expression (relative to WT lesions) and were obtained from n = 4–5 lesion RNAs, with each lesion isolated from an individual mouse per donor genotype. **B** and **C**) Representative pictures of WT and *Klf13*$^{(-/-)}$ ectopic lesions immunostained for PGR (**B**) and ESR1 (**C**). The percentages of immunostained stromal or glandular epithelial cells (expressed as mean ± SEM) were determined by counting the number of immunopositive nuclei over the total number of cells per field. Red arrowheads indicate representative nuclear staining. Three to four lesions per genotype were analyzed. Bar = 20 μm. **D**) Transcript levels of PGR- and ESR1-responsive genes were quantified in WT and *Klf13*$^{(-/-)}$ lesions by QPCR and normalized to those of housekeeping genes *Ppia* and *Tbp*, following the GeNorm program [30]. Data are mean ± SEM of fold change in normalized expression (relative to WT lesions) and were obtained from n = 4–5 lesion RNAs, with each lesion isolated from an individual mouse per donor genotype. *$P < 005$ by Student *t*-test. **E**) Serum E_2 and P_4 levels in recipient WT mice with WT or *Klf13* null ectopic lesions.

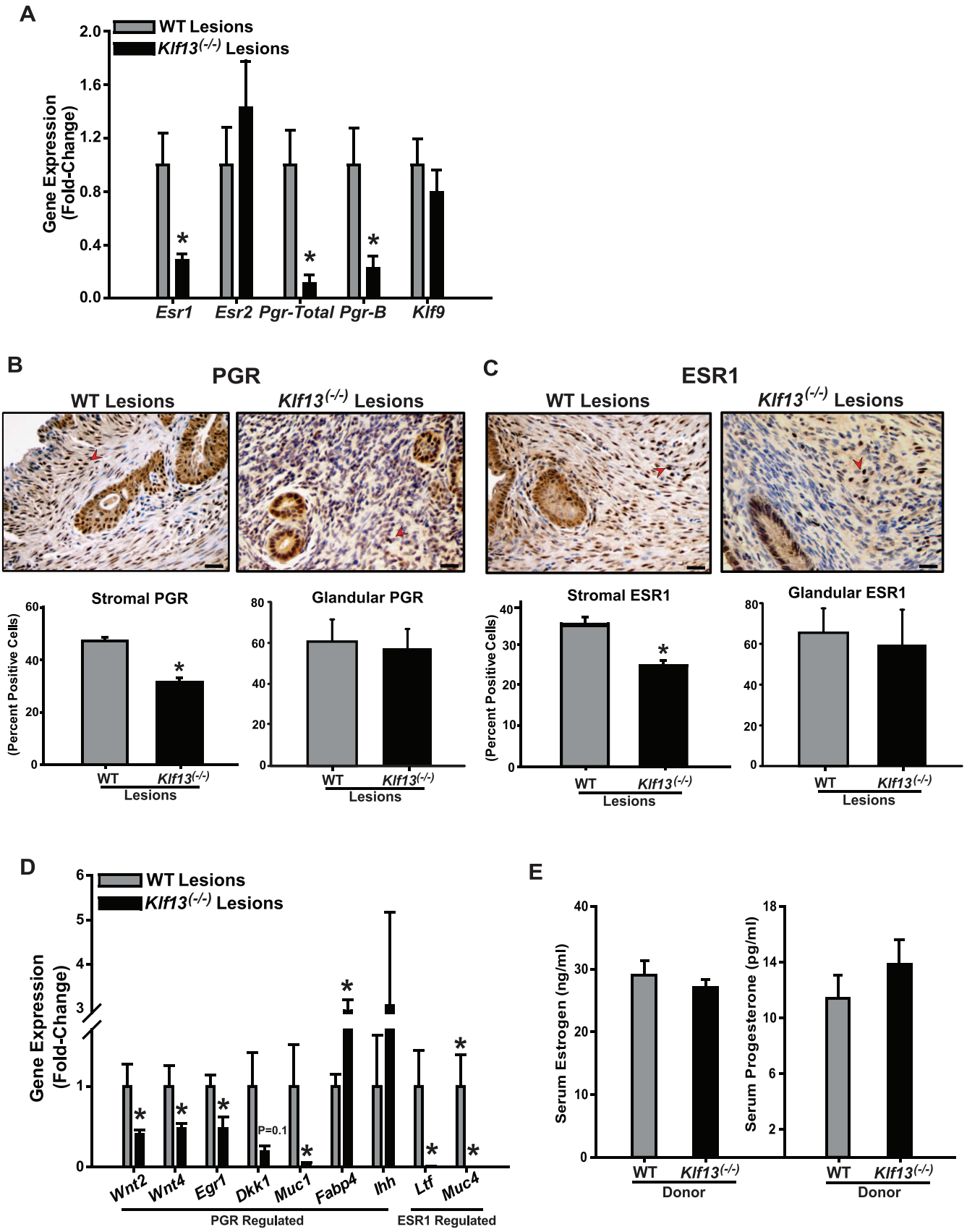
A
WT Lesions
Klf13(-/-) Lesions
Gene Expression (Fold-Change)
2.0
1.6
1.2
0.8
0.4
0.0
Esr1
Esr2
Pgr-Total
Pgr-B
Klf9
B
PGR
WT Lesions
Klf13(-/-) Lesions
Stromal PGR
(Percent Positive Cells)
Glandular PGR
WT
Klf13(-/-)
Lesions
C
ESR1
WT Lesions
Klf13(-/-) Lesions
Stromal ESR1
Glandular ESR1
D
WT Lesions
Klf13(-/-) Lesions
Gene Expression (Fold-Change)
P=0.1
Wnt2
Wnt4
Egr1
Dkk1
Muc1
Fabp4
Ihh
Ltf
Muc4
PGR Regulated
ESR1 Regulated
E
Serum Estrogen (ng/ml)
Serum Progesterone (pg/ml)
WT
Klf13(-/-)
Donor

TABLE 1. Parameters of lesions derived from WT and *Klf13* null endometrium.[a]

Lesion parameters	Donor genotype	
	Wild type	*Klf13*$^{(-/-)}$
No. per mouse	2.1 ± 0.37	1.7 ± 0.20
Volume per mouse, mm^3	27.82 ± 10.53	22.7 ± 10.04
Incidence, % (n)	64.7 (11/17)	91 (10/11)[b]

[a] Lesions were collected at 8 wk after induction.
[b] $P = 0.19$, Fisher exact test.

cells that were Ki-67-immunopositive (Fig. 2D) and apoptotic (by TUNEL; Fig. 2E), respectively.

Klf13 *Null Lesions Displayed Altered Steroid Hormone Receptor Expression and Signaling*

Differential expression of PGR isoforms is considered to be a critical component of the pathogenesis of endometriosis. Given that KLF13 is a PGR coregulator in endometrial cells [26], we evaluated the expression levels of both PGR isoforms in ectopic lesions generated from WT and *Klf13* null endometria. *Klf13* null lesions showed a significant decrease in total PGR transcript levels, which was specific for the *Pgr-B* isoform (Fig. 3A). The decrease in total PGR expression in *Klf13* null lesions was confined to stromal cells, as determined by immunohistochemistry (Fig. 3B). Specifically, *Klf13* null lesions showed a lower percentage of PGR-positive stromal cells compared with WT lesions, whereas the percentage of PGR-positive glandular epithelial cells did not differ with lesion genotype (Fig. 3B). The limited amounts of lesions precluded their further analyses for PGR expression by Western blotting. To further assess the extent of dysregulated PGR signaling in *Klf13* null lesions, we also examined the expression of a subset of PGR-regulated genes that changed in *Klf9* null lesions relative to WT lesions [23]. Analysis by QPCR showed significantly lower levels of *Wnt2*, *Wnt4*, early growth response (*Egr1*), and Mucin 1 (*Muc1*) gene transcripts, as well as a tendency for decreased Dickkopf homolog 1 (*Dkk1*) expression, in *Klf13* null lesions relative to WT lesions (Fig. 3D). *Klf13* null lesions also had higher fatty acid-binding protein 4 (*Fabp4*) and showed a modest, albeit nonsignificant, elevation in Indian Hedgehog (*Ihh*) transcript levels, relative to WT lesions (Fig. 3D).

It is currently unknown whether KLF13 is a context-dependent regulator of ESR signaling, as is suggested for KLF9 [32, 33]. Given the importance of ESR signaling in endometriosis pathology [31], we examined ESR isoform expression in ectopic lesions as a function of genotype. *Klf13* null lesions had lower *Esr1* but comparable *Esr2* transcript levels (Fig. 3A) and showed a reduced percentage of ESR1-immunopositive stromal cells compared with WT lesions (Fig. 3C). By contrast, no differences in the percentage of nuclear ESR1-positive cells were noted in endometrial glands between lesions types (Fig. 3C). To confirm deregulation of ESR1 signaling with attenuated ESR1 expression as a function of lesion genotype, we evaluated the transcript levels of two known estrogen-responsive genes [31] by QPCR. Relative to those of WT lesions, lactoferrin *(Ltf)* and mucin 4 (*Muc4*) transcript levels were diminished in *Klf13* null lesions (Fig. 3D). Interestingly, *Klf9* mRNA levels were comparable in WT and *Klf13* null lesions (Fig. 3A). Recipient animals of both groups also had similar serum levels of E_2 and P_4 at killing (Fig. 3E).

KLF13 Loss Had No Effect on Notch or Hedgehog Signaling Component Expression in Ectopic Lesions

We previously reported that ectopic lesions generated with *Klf9* null endometria in mice showed increased expression of select components of the Notch and Hh signaling pathways [23], both of which constitute critical regulators of cell proliferation and survival [34, 35]. To ascertain whether *Klf13* null lesions are similarly associated with altered expression of these pathway components, we used a Notch signaling-focused PCR array to compare the relative expression of Notch and Hh pathway-associated genes in WT and *Klf13* null lesions. From a total of 84 genes included in the array, 9 were up-regulated and 8 were down-regulated by at least 1.5-fold in *Klf13* null lesions relative to WT lesions. However, further analyses of these genes by QPCR using four to seven individual lesions per endometrial donor genotype did not confirm their differential expression (data not shown). We next evaluated *Klf13* null lesions for expression of a subset of Notch and Hh pathway-associated genes that were increased in *Klf9* null lesions relative to WT lesions [23]. The expression of these select genes also did not differ between *Klf13* null and WT lesions (Fig. 4A). These results suggest that endometrial KLF13 loss does not substantially promote dysregulation of Notch or Hh pathway component expression in lesions.

Peritoneal Fluid and Serum Levels of Cytokines in Mice with Klf13 *Null Versus WT Ectopic Lesions*

Numerous studies have demonstrated the contribution of inflammation to the progression of endometriosis [36]. Because mice with *Klf9* null ectopic lesions exhibited lower TNFα and higher sTNFR1 levels than corresponding mice with WT lesions [23], we evaluated the levels of these cytokines in the serum and peritoneal fluids of recipient WT mice with WT or *Klf13* null lesions. Mice with lesions of either genotype did not differ in peritoneal fluid TNFα or sTNFR1 levels (Fig. 4B). However, recipient mice with *Klf13* null lesions displayed lower serum levels of TNFα, with no comparable change in serum sTNFRS1, relative to recipient mice with WT lesions (Fig. 4C).

DISCUSSION

In the present study, we evaluated the hypothesis that KLF13 deficiency in endometrial cells may promote ectopic lesion establishment, given KLF13's demonstrated ability to regulate PGR-B signaling, which is dysregulated in endometriosis [8–10, 20]. To address this premise, we first evaluated *KLF13* expression in the eutopic endometria of women with and those without endometriosis. We show here that *KLF13* transcript levels were reduced exclusively in the MS endometria of women with endometriosis relative to those without disease, similar to what has been found for *KLF9* transcript [20]. Second, we used an immunocompetent mouse model of endometriosis that was recently established in our laboratory [23] to evaluate whether loss of endometrial *Klf13* expression directly contributes to ectopic lesion establishment. We report here that lesion incidence with *Klf13* null endometria in recipient mice, although numerically higher, did not significantly differ from that found for donor WT endometria, in part because of the formation of nonadherent (''floater'') lesions. Moreover, no appreciable differences in lesion volume and number and in stromal proliferative (Ki-67) and apoptotic (TUNEL) indices were observed between endometrial WT or *Klf13* null donor genotype. The latter contrasted with the

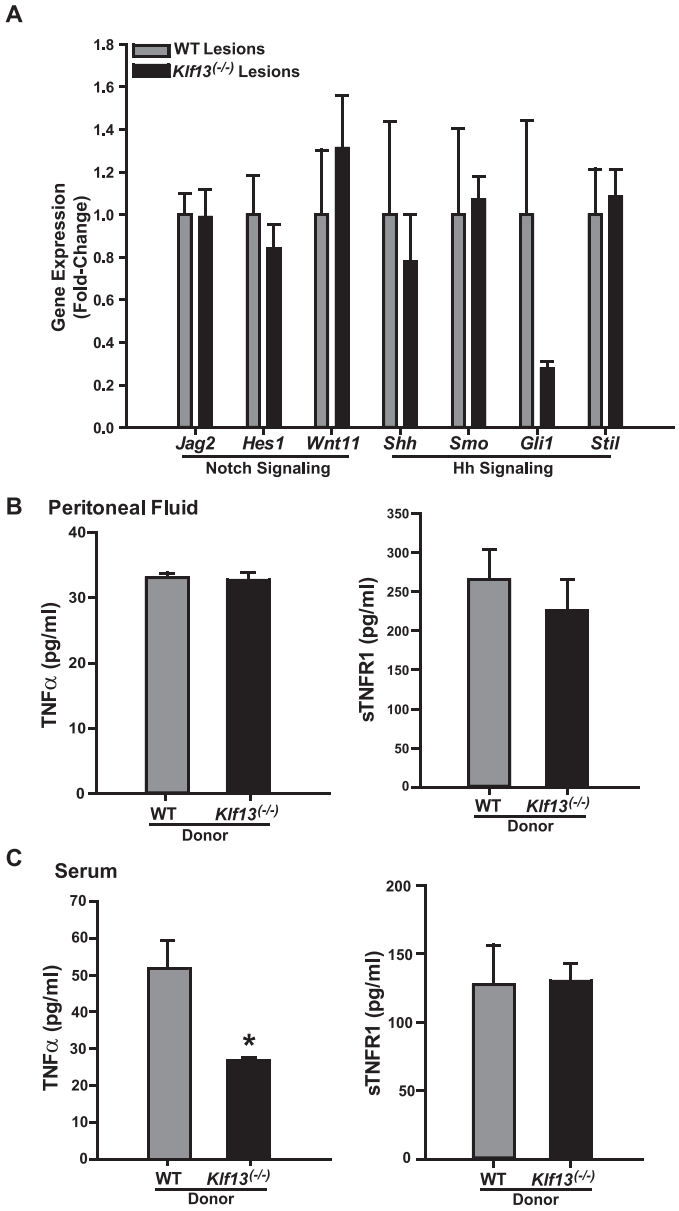

FIG. 4. Loss of *Klf13* expression in endometriotic lesions is not associated with altered Notch/Hedgehog signaling but correlates with changes in serum TNFα levels. **A**) Expression of select Notch and Hedgehog signaling component genes (shown previously to be dysregulated in *Klf9* null lesions [23]) in ectopic lesions from WT and *Klf13* null endometria, measured by QPCR and normalized to those of housekeeping genes *Ppia* and *Tbp*, following the GeNorm program [30]. Data are mean ± SEM in normalized expression (relative to WT lesions) and were obtained from n = 4–7 lesion RNAs, with each lesion isolated from an individual mouse per experimental group. **B**) Levels of TNFα and sTNFR1 in the peritoneal fluids of recipient WT mice with endometriotic lesions formed from WT or $Klf13^{(-/-)}$ donor endometrium. **C**) Serum levels of TNFα and sTNFR1 in recipient WT mice with endometriotic lesions formed from WT or $Klf13^{(-/-)}$ donor endometrium. For **B** and **C**, data (mean ± SEM) are from n = 8–11 individual mice per donor lesion group. *$P < 0.01$ by Student *t*-test.

ability of endometrial *Klf9* null endometrium to effectively promote lesion incidence and proliferative index and to attenuate apoptotic status in WT recipients [23]. These collective results provide support for the lack of a causative linkage between endometrial KLF13 deficiency and the establishment of endometriosis-like lesions in mice.

TABLE 2. Characteristics of *Klf9* null and *Klf13* null ectopic lesions.[a]

$Klf9^{(-/-)}$ lesions[b]	$Klf13^{(-/-)}$ lesions[c]
Significant increase in lesion incidence	Nonsignificant increase in lesion incidence
Decreased stromal *Pgr-a*	Decreased stromal *Pgr-b*
Decreased stromal ESR1	Decreased stromal ESR1
Increased stromal ESR2	No change in stromal *Esr2*
Increased stromal Ki-67	No change in stromal Ki-67
Decreased stromal TUNEL	No change in stromal TUNEL
Activation of Notch/Hh signaling	No activation of Notch/Hh signaling
Decreased systemic TNFα	Decreased systemic TNFα

[a] Relative to WT lesions.
[b] Heard et al. [23].
[c] This study.

The notable difference in the contribution of endometrial *Klf9* loss [23] relative to *Klf13* loss (the present study) in lesion establishment in mice is likely a consequence of the coordinated regulation of Notch, Hh, and steroid receptor signaling pathway components by KLF9, which was not recapitulated by KLF13 (Table 2). Moreover, although loss of KLF13 and of KLF9 altered several features of PGR and ESR signaling in common—as shown by their parallel reductions of ESR1 and total PGR transcript and protein levels—KLF9 loss elicited additional perturbations in *Esr2* transcript levels, resulting in the marked rise in *Esr2*:*Esr1* expression ratio, and selectively reduced *Pgr-a* over *Pgr-b* transcript levels [23]. In contrast, KLF13 loss did not affect *Esr2* expression and preferentially diminished *Pgr-b* rather than *Pgr-a* expression, the latter based on the comparable reductions in total *Pgr* and *Pgr-b* transcripts. The functional consequence of the distinct changes in *Pgr-a* versus *Pgr-b* in *Klf13* null versus *Klf9* null lesions is convincingly illustrated by the contrasting effects elicited by *Klf9* and *Klf13* null mutations (relative to WT) on the expression of the same PGR target genes (*Wnt1*, *Wnt4*, *Dkk1*, and *Egr1*) [37–40], and is consistent with the opposing transactivities of PGR-A and PGR-B isoforms [41]. However, because P_4-regulated genes may also be direct or indirect E_2 target genes (e.g., *Wnt4*, *Egr1*) [42, 43], further studies will be required to dissect the mechanistic contribution of KLF9 relative to KLF13 in regulating these genes' expression. The reductions in *Lif1* and *Muc4* transcript levels with decreased ESR1 consequent to loss of KLF13 expression is in agreement with the identification of these genes as predominantly ESR1 gene targets and dysregulated in endometriosis [44, 45]. Based on these results, we suggest that the coordinate dysregulation of Notch, Hh, PGR-A, PGR-B, ESR1, and ESR2 signaling pathways is likely a key feature of ectopic lesion establishment and that the limited KLF13 regulation of these collective pathways may reflect its restricted contribution to promoting lesion incidence.

A common feature of recipient mice with *Klf9* or *Klf13* null lesions is their reduced levels of systemic TNFα relative to mice with WT lesions. Women with more severe endometriosis show lower levels of this cytokine relative to those with less pathology, suggesting loss of immunogenic response to the implants with disease progression [46, 47]. Moreover, women with endometriosis also exhibited higher levels of sTNFR1 [48], a natural antagonist of TNFα. Given that mice with *Klf13* null lesions have decreased serum levels of TNFα but no corresponding elevations in serum sTNFR1, in contrast to the coincident decrease and increase, respectively, of TNFα and sTNFR1 serum levels in recipient mice with *Klf9* null lesions [23], the less efficacious ectopic lesion establishment with

Klf13 loss may also be attributed to the lesser magnitude of KLF13 effect on TNFα signaling.

In a previous study, Eyster and colleagues [49] identified numerous differentially expressed genes between ectopic lesion and eutopic endometrium from women with endometriosis. Because the distinct gene profiles of eutopic tissue may be due to direct or indirect effects of established ectopic lesions [50], it is not possible to discern whether one or more of the gene changes in eutopic endometrium identified in that study are causal to lesion establishment. Interestingly, *KLF9* and *KLF13* were not reported to be differentially expressed in ectopic lesions and eutopic endometria in the study by Eyster et al. [49], whereas those for *PGR* and *ESR1* (ectopic < eutopic) and *FABP4* (ectopic > eutopic) were found to differ. Because *Klf13* null endometria generated lesions with similarly lower (*Pgr*, *Esr1*) and higher (*Fabp4*) levels of these transcripts relative to WT lesions, these results suggest that loss of endometrial KLF13 expression may be an early event that precedes lesion establishment but is not required for lesion maintenance or progression to a more severe phenotype.

In summary, our findings suggest that loss of endometrial KLF13 expression does not promote endometriosis development in mice to the extent observed with endometrial KLF9 deficiency. Our results further indicate that the presence of KLF9 in *Klf13* null ectopic lesions may be sufficient to properly regulate critical signaling pathways (e.g., Notch, Hh, ESR2, PGR-A) involved in lesion survival. Nevertheless, because the expression of both KLFs is diminished in the eutopic endometria of women with endometriosis, it remains possible that the co-loss of these KLFs may be causal to the disease and/or underlie a more severe phenotype. The latter will be addressed in future studies by the comprehensive analyses of eutopic and ectopic endometrial samples from women with increasing severity or recurrence of the disease. Further investigations using mice with targeted knockout of both *Klf9* and *Klf13* in endometria; primary cultures of human endometriotic cells; and stromal cells with targeted knockdowns of both KLFs will provide additional mechanistic insight into the importance of these transcription factors in mediating endometriosis development in the context of other PGR coregulators. Importantly, such studies may help define the therapeutic potential of KLFs and associated signaling components in the targeting and clinical management of endometriosis.

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