

Ablation of Indian Hedgehog in the Murine Uterus Results in Decreased Cell Cycle Progression, Aberrant Epidermal Growth Factor Signaling, and Increased Estrogen Signaling¹

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ABSTRACT

Conditional ablation of Indian hedgehog (*Ihh*) in the murine uterus results in mice that are sterile because of defects in embryo implantation. We performed microarray analysis on these mice at the time point at which the *Ihh* target genes are induced by the administration of exogenous hormone to mimic Day 3.5 of pregnancy. This analysis identified 863 genes altered by the conditional ablation of *Ihh*. Of these, genes that regulated the cell cycle were overrepresented. In addition, genes involved in epidermal growth factor (EGF) and estrogen (E2) signaling were found to be deregulated upon *Ihh* ablation. Furthermore, upon conditional ablation of *Ihh*, 15-mo-old mice exhibited hallmarks of estrogenized uteri, such as cystically dilated glands and hyalinized stroma. Thus, *Ihh* regulates embryo implantation by having an impact on the cell cycle, EGF signaling, and E2 signaling.

estrogen, implantation, Indian Hedgehog, mouse, uterus

INTRODUCTION

A critical event in the establishment of a successful pregnancy is embryo implantation in which the blastocyst attaches to and invades through the luminal epithelium of the uterus and into the stroma [1]. The ovarian steroid hormones, estrogen (E2) and progesterone (P4), acting through their cognate receptors, the estrogen receptor (ESR1) and the

progesterone receptor (PGR), are necessary for these events in early pregnancy [2]. On Day 0.5 (d0.5; 0.5 = vaginal plug) of pregnancy in mice, a preovulatory surge of E2 stimulates uterine epithelial cell proliferation. Upon formation of the corpus luteum, there is an increase in P4 levels, resulting in uterine stromal cell proliferation on d2.5. On d3.5, there is an acute spike of E2, which in combination with the luteal P4, maintains uterine stromal cell proliferation, which renders the uterus receptive to the implanting embryo. Implantation can only occur if the uterus is receptive to the incoming blastocyst and there is a defined “window of receptivity” during which E2 is the primary determinant [3]. Embryo implantation in the mouse occurs on d4.5. Female *Pgr* knockout (*Pgr*KO) mice are sterile due to a failure in embryo implantation, demonstrating the critical role of P4 signaling in this process [4]. To mechanistically understand how P4 exerts its effect during embryo implantation, downstream target genes of uterine PGR need to be identified.

Indian hedgehog (*Ihh*), which is a member of the Hedgehog family of ligands, was identified as a P4-regulated gene in the uterus [5, 6]. Hedgehog signaling has been shown to be important for the development of multiple tissues, including (but not limited to) the limbs, cerebellum, bone, cartilage, gonads, and heart [7]. Deregulation of hedgehog signaling has also been implicated in cancers, such as basal cell carcinoma, medulloblastoma, pancreatic cancer, prostate cancer, and lung cancer [8]. *Ihh* is expressed in the mouse uterine luminal epithelium in the preimplantation period, with its highest expression on d2.5, whereas its downstream target genes, patched 1 (*Ptch1*) and chicken ovalbumin upstream promoter transcription factor II (*COUP-TFII*, official symbol *Nr2f2*), are expressed in the uterine stroma, with their highest expression on d3.5 during the “window of receptivity” [5, 6, 9]. In the human endometrium, *IHH* expression significantly decreases during the transition from the early to the mid secretory phase, which is associated with a downregulation of cellular division [10].

Ihh has been shown to be critical for uterine function because conditional ablation of *Ihh* (*PR^{cre/+}Ihh^{fl/fl}*, official allele symbols *Pgr^{tm2(cre)Lydl/+}Ihh^{tm1Blan}*) in the mouse uterus results in infertility due to a failure of embryo implantation [11]. Rather than successful apposition and attachment of the blastocyst to the uterine luminal epithelium, embryos in the *Pgr^{tm2(cre)Lydl/+}Ihh^{tm1Blan}* uterus were found to be floating in the uterine lumen. This was partly due to increased expression of mucin 1 (*Muc1*) in the *Pgr^{tm2(cre)Lydl/+}Ihh^{tm1Blan}* uterus, the loss of which is necessary for successful embryo implantation [12]. In addition to the attachment defect, the *Pgr^{tm2(cre)Lydl/+}*

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Ihh^{tm1Blan} uterus failed to undergo the decidualization reaction due to deficient preparation in the preimplantation period, as seen by decreased stromal cell proliferation and decreased vascularization of the stromal compartment [11]. Examination of genes known to be involved in embryo implantation, such as homeobox A10 (*Hoxa10*) and leukemia inhibitory factor (*Lif*), revealed them to be unaltered by *Ihh* ablation. Interestingly, whereas the epidermal growth factor (EGF) ligands heparin-binding epidermal growth factor (*Hbegf*) and amphiregulin (*Areg*) were not altered in the *Pgr^{tm2(cre)Lydl+} Ihh^{tm1Blan}* uterus, one of their receptors, epidermal growth factor receptor (*Egfr*), was significantly reduced in the *Pgr^{tm2(cre)Lydl+} Ihh^{tm1Blan}* subepithelial stroma. These data demonstrate a critical role for *Ihh* in the preimplantation period and suggest that *Ihh* regulates multiple pathways during this critical time point.

Further demonstration of the critical role of *Ihh* in the preimplantation period was the female infertility defect displayed by female mice with *Nr2f2* ablation (either as heterozygotes or conditional uterine ablation) [13, 14]. These mice phenocopied the *Pgr^{tm2(cre)Lydl+} Ihh^{tm1Blan}* mice in that they exhibited defective embryo implantation with failed embryo attachment and a reduced decidual response. Again, this defect was due to improper preparation of the uterus, as there were increased *Muc-1* levels, decreased stromal cell proliferation, and incomplete vascularization of the stroma. In addition, ablation of *Nr2f2* increased ESR1 levels in the luminal epithelium, resulting in increased ESR1 activity in the uterus, suggesting that Hedgehog signaling may be a critical regulator of E2 signaling in the uterus.

Here, we have performed microarray analysis on the *Pgr^{tm2(cre)Lydl+} Ihh^{tm1Blan}* uteri to identify the pathways regulated by *Ihh* during embryo implantation [11, 15]. From this analysis, we have identified multiple pathways as being *Ihh* regulated in the uterus during the preimplantation period. Among these, we observed a decrease in genes necessary for cell cycle progression and a deregulation of the EGF signaling pathway. Furthermore, we observed increased E2 signaling in the *Pgr^{tm2(cre)Lydl+} Ihh^{tm1Blan}* uterus, which resulted in a pathological phenotype. Thus, we have demonstrated a critical role for *Ihh* in the preparation of the uterus for the incoming embryo in the preimplantation period as a mediator of cell cycle progression as well as EGF and E2 signaling.

MATERIALS AND METHODS

Animals and Hormone Treatments

Mice were maintained in the designated animal care facility at Baylor College of Medicine according to the institutional guidelines for the care and use of laboratory animals. Mice were treated with an abbreviated protocol used to elicit an artificial decidual response, as previously described [16]. Briefly, 6-wk-old female wild-type, *Pgr^{tm2(cre)Lydl+}*, *Pgr^{+/+}Ihh^{tm1Blan}*, and *Pgr^{tm2(cre)Lydl+}Ihh^{tm1Blan}* mice were ovariectomized and rested for 2 wk before treatment with three s.c. daily injections of 100 ng of E2 per mouse. After 2 days of rest, mice were then treated with daily injections of 1 mg of P4 and 6.7 ng of E2 per mouse s.c. for 2 days. Mice were killed 6 h after the last E2 plus P4 injection by cervical dislocation while under anesthetic (Avertin [2,2-tribromoethyl alcohol]; Sigma-Aldrich, St. Louis, MO). After tissue dissection, uterine tissues were placed in 4% paraformaldehyde or flash frozen and stored at -80°C . Serum testosterone levels were measured in 15-mo-old mice by radioimmunoassay by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. Serum estradiol levels were measured in 3-mo-old mice using the Estradiol Kit (no. 33540) for the Beckman Coulter Access 2 System (Beckman Coulter Inc., Fullerton, CA).

RNA Isolation and Microarray Hybridization

Total RNA was extracted from uterine tissues using the Qiagen RNeasy total RNA isolation kit (Qiagen, Valencia, CA). The RNA was pooled from the

uteri of three mice per genotype. All RNA samples were analyzed with a Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE) before microarray hybridization. Microarray analysis was performed by the Baylor College of Medicine Microarray Core Facility using Affymetrix murine genome 430 2.0 mouse oligonucleotide arrays (Affymetrix, Santa Clara, CA), as previously described [17]. All experiments were performed in triplicate with independent pools of RNA.

Data Analysis

Microarray data analysis was performed as previously described [17, 18]. DNA chip analyzer dChip was used to adjust arrays to a common baseline using invariant set normalization [19]. To estimate expression, the PM-only model developed by Li and Wong [20] and Li and Hung Wong [21] was used. We selected differentially expressed genes in the *Pgr^{+/+}Ihh^{tm1Blan}* and *Pgr^{tm2(cre)Lydl+} Ihh^{tm1Blan}* mice using a two-sample comparison according to the following criteria: lower boundary of 90% confidence interval of fold-change greater than 1.2 and an absolute value of difference between group means greater than 50. Differentially expressed genes were classified according to Gene Ontology function using Affymetrix annotation (NetAffx; <http://affymetrix.com/index.affx>), and pathway analysis was performed using DAVID Analysis [22, 23] and Ingenuity Systems Software (Ingenuity Systems Inc., Redwood City, CA).

Real-Time RT-PCR Analysis

Total RNA was extracted from uterine tissues ($n = 7$ per genotype) using the Trizol reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Total RNA (1 μg) was reverse transcribed into cDNA with M-MLV (Invitrogen) in a 20- μl volume. Expression levels of mRNA were measured by real-time RT-PCR TaqMan analysis using the ABI Prism 7700 Sequence Detector System according to the manufacturer's instructions (PE Applied Biosystems, Foster City, CA). Real-time probes and primers were purchased from Applied Biosystems; for a complete list, see Supplemental Table S1 (all supplemental files for this article are available online at www.biolreprod.org). All real-time RT-PCR was performed using independent RNA sets. All mRNA quantities were normalized against 18S RNA using ABI rRNA control reagents (Applied Biosystems). Statistical analyses used one-way ANOVA followed by Tukey posthoc multiple range test with the Instat package from GraphPad (San Diego, CA).

Immunohistochemistry

Uteri were fixed overnight in 4% paraformaldehyde (vol/vol), followed by thorough washing in 70% ethanol, and tissues were processed, embedded in paraffin, and sectioned. Uterine sections from paraffin-embedded tissue were cut at 5 μm and mounted on silane-coated slides, deparaffinized, and rehydrated in a graded alcohol series. Sections were preincubated with 10% normal goat serum in PBS (pH 7.5) and then incubated with anti-CCND1 (1:500; NeoMarkers, Fremont, CA), anti-MCM3 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-ER α (ESR1; 1:200; DAKO, Carpinteria, CA) in 10% normal serum in PBS (pH 7.5). On the following day, sections were washed in PBS and incubated with biotinylated secondary antibody (5 $\mu\text{l}/\text{ml}$; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Immunoreactivity was detected using the DAB Substrate kit (Vector Laboratories); immunoreactivity was visualized as intense brown staining. Masson trichrome staining was performed by the Baylor College of Medicine Center for Comparative Medicine Comparative Pathology Laboratory using standard protocols.

RESULTS

Identification of Indian Hedgehog-Regulated Genes During Embryo Implantation

Previously, we generated mice in which *Ihh* was conditionally ablated in the murine uterus using the *Pgr^{cre}* mouse model (*Pgr^{tm2(cre)Lydl+} Ihh^{tm1Blan}*) [11, 15]. These mice were found to be infertile, demonstrating an inability of the uterus to undergo embryo attachment and decidualization. To identify the pathways that *Ihh* regulates during these processes, we performed high-density DNA microarray analysis on the uteri from these mice. *Ihh* expression is highest on d2.5 of pregnancy, whereas the expression of its downstream target

genes *Ptch1* and *Nr2f2* is highest on d3.5 of pregnancy, which is just prior to embryo implantation [5, 6]. For this reason, our microarray analysis was conducted at this time point. However, to avoid variability due to the presence of a dormant blastocyst [24] or variations in staging of pregnancy due to differing times of coitus, we simulated d3.5 of pregnancy by administering ovariectomized mice with exogenous steroid hormones using a modification of the protocol used to induce a decidual response (see *Materials and Methods*) [16]. We had previously determined that 30 h after the first E2 plus P4 injection used in the artificially induced decidual response gave full activation of the *Ihh* signaling pathway (Fig. 1) [9]. Total RNA extracts were subjected to microarray analysis using the Affymetrix mouse genome 430 2.0 arrays. This analysis revealed 429 and 434 transcripts whose abundance was significantly increased or decreased, respectively, in the *Pgr^{tm2(cre)}Lyd⁺/Ihh^{tm1Blan}* uterus compared with *Pgr^{+/+}/Ihh^{tm1Blan}* controls. A complete list of the genes whose transcripts increase or decrease in abundance can be found in Supplemental Tables S2 and S3, respectively. To determine which pathways are regulated by *Ihh* at this time point, we performed pathway analysis using DAVID Analysis and Ingenuity Systems Software [22, 23]. A complete list of the significantly regulated pathways can be found in Supplemental Table S4. The altered pathways included (but were not limited to) those involved in the cell cycle, WNT signaling, MAPK signaling, TGFB signaling, JNK signaling and EGF signaling (Supplemental Table S4). These pathways have all been implicated in embryo implantation, suggesting that *Ihh* is a critical mediator of multiple aspects of making the uterus receptive to an incoming embryo [1, 2]. To begin to dissect out these pathways, we first examined those shown to be previously involved in the phenotypes of the *Pgr^{tm2(cre)}Lyd⁺/Ihh^{tm1Blan}* uterus [11].

Cell Cycle Genes Are Deregulated by Ablation of *Ihh*

Analysis of the differentially regulated genes demonstrated that 23 genes that regulate cell cycle progression were significantly decreased in the *Pgr^{tm2(cre)}Lyd⁺/Ihh^{tm1Blan}* uterus. Among these altered genes were the components of the mitosis-promoting factor, cyclin B1 (*Ccnb1*); cell division cycle 2 homolog A (*Cdc2a/CDK1*); the minichromosome maintenance (MCM) family of proteins that are involved in DNA replication; and cyclin D1 (CCND1), a protein necessary for uterine cell proliferation [25–27]. Both *Ccnb1* and *Cdc2a* were reduced in the *Pgr^{tm2(cre)}Lyd⁺/Ihh^{tm1Blan}* uterus (Fig. 2A). The mRNA expression level of *Mcm5*, a member of the MCM family of proteins, was also significantly reduced, as was that of *Ccnd1* (Fig. 2A). These changes in gene expression of the cell cycle genes were not affected by the presence of Cre in the *Pgr^{tm2(cre)}Lyd⁺/Ihh^{tm1Blan}* mice, because *Pgr^{tm2(cre)}Lyd⁺* mice displayed the same expression of *Ccnb1*, *Cdc2a*, *Ccnd1*, and *Mcm5* as wild-type mice (Supplemental Fig. S1A). CCND1 protein was primarily located in the uterine stroma, with some staining in the glandular epithelium of the *Pgr^{+/+}/Ihh^{tm1Blan}* uterus (Fig. 2B). Although CCND1 levels were comparable in the epithelial cells of the *Pgr^{tm2(cre)}Lyd⁺/Ihh^{tm1Blan}* uterus, they were reduced in the uterine stroma when compared to *Pgr^{+/+}/Ihh^{tm1Blan}* uteri (Fig. 2B). In addition, another member of the MCM family of proteins, MCM3, was reduced in the *Pgr^{tm2(cre)}Lyd⁺/Ihh^{tm1Blan}* stroma compared with *Pgr^{+/+}/Ihh^{tm1Blan}* mice (Fig. 2C). MCM3 exhibited robust expression in the luminal and glandular epithelium of both genotypes. This deregulation of genes involved in cell cycle progression may explain the stromal cell proliferation defect observed in the *Pgr^{tm2(cre)}Lyd⁺/Ihh^{tm1Blan}* uterus [11].

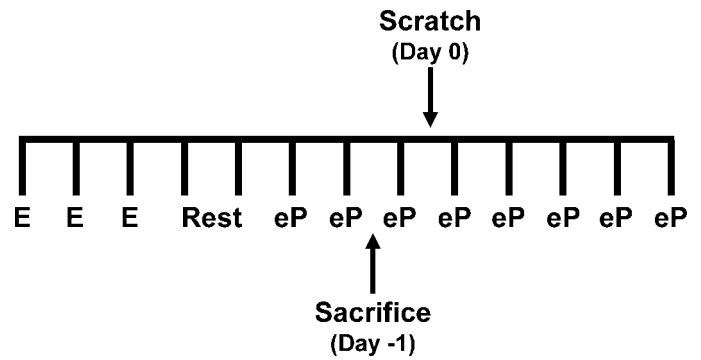


FIG. 1. Schematic of microarray analysis experimental design. Microarray analysis was performed using an abbreviated artificial decidual response protocol in which the uteri were harvested 30 h after the first E2 plus P4 injection. Each vertical bar marks 1 day. E, estrogen injection (100 ng); Rest, no injection; eP, estrogen plus progesterone injection (6.7 ng and 1 mg, respectively).

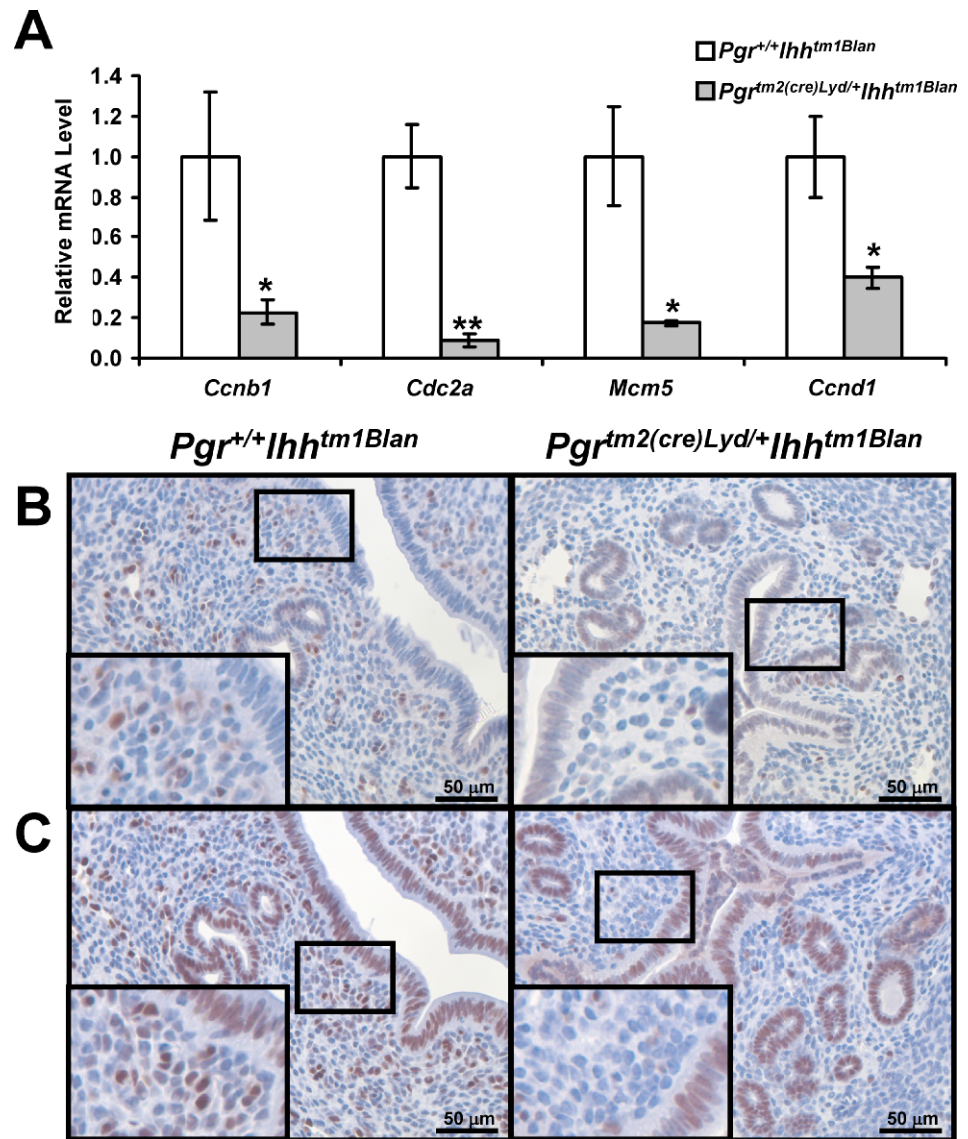
EGF Signaling Is Altered by *Ihh* Ablation

Previously, we demonstrated that the EGF ligands, amphiregulin (*Areg*) and heparin-binding epidermal growth factor (*Hbegf*), were unaltered by *Ihh* ablation, but that the epidermal growth factor receptor (EGFR/ERBB1) was lost in the subepithelial stroma [11]. In addition, we now show that *Ihh* ablation alters the expression of the other members of the ERBB receptor family, with V-ERB-B2 avian erythroblastic leukemia viral oncogene homolog 2 (*ErbB2/Her2*) and V-ERB-B2 avian erythroblastic leukemia viral oncogene homolog 3 (*ErbB3/Her3*) being significantly reduced and V-ERB-B2 avian erythroblastic leukemia viral oncogene homolog 4 (*ErbB4/Her4*) significantly increased (Fig. 3). In addition, a disintegrin and metalloproteinase domain 12 (*Adam12*) expression was significantly reduced. ADAM12 has been shown to be involved in the processing of HBEGF to generate its active form [28]. The expression of *Egfr*, *ErbB2*, *ErbB3*, *ErbB4*, and *Adam12* was unaffected in the *Pgr^{tm2(cre)}Lyd⁺* uteri compared with wild-type controls (Supplemental Fig. S1B). Therefore, *Ihh* regulates the EGF signaling pathway not by regulating the expression of the ligands but by regulating the expression of the receptors that bind the ligands and the enzymes responsible for processing the ligands.

Ihh Acts to Repress E2 Signaling in the Uterus

From the microarray analysis, we observed an increase in *Esr1* expression which was validated by real-time RT-PCR analysis (Fig. 4A). Therefore, we wanted to determine whether there was increased ESR1 activity in the *Pgr^{tm2(cre)}Lyd⁺/Ihh^{tm1Blan}* uterus. Therefore, we compared the list of differentially regulated genes in the *Pgr^{tm2(cre)}Lyd⁺/Ihh^{tm1Blan}* uterus to the genes identified as being E2 regulated in wild-type mice by Hewitt et al. [29]. This analysis revealed that 20 (2.3%) of the 863 genes altered in the *Pgr^{tm2(cre)}Lyd⁺/Ihh^{tm1Blan}* array are in common with genes identified as being altered in the uteri of wild-type mice treated with E2 (Supplemental Table S5). A selection of these E2-regulated genes was validated by real-time RT-PCR analysis (Fig. 4A). In this independent sample set, we again observed increased expression of the E2 target gene *Muc1*, confirming our previous finding. Analysis of these genes in the *Pgr^{tm2(cre)}Lyd⁺* mouse compared with controls revealed no alteration in the mRNA expression level for the majority of genes; however, the expression of *Dcaf6* was

FIG. 2. Cell cycle genes are deregulated by *Ihh* ablation. **A**) Real-time RT-PCR analysis of cell cycle genes (*Ccnb1*, *Cdc2a*, *Mcm5*, *Ccnd1*). The results represent the mean \pm SEM. * $P < 0.05$; ** $P < 0.01$. **B**) Immunohistochemical analysis of CCND1 in *Pgr*^{+/+} *Ihh*^{tm1Blan} (left) and *Pgr*^{tm2(cre)Lyd/+} *Ihh*^{tm1Blan} (right) mice. Positive staining is seen by a brown signal. Nuclei were counterstained with hematoxylin. **C**) Immunohistochemical analysis of MCM3 in *Pgr*^{+/+} *Ihh*^{tm1Blan} (left) and *Pgr*^{tm2(cre)Lyd/+} *Ihh*^{tm1Blan} (right) mice. Positive staining is seen by a brown signal. Nuclei were counterstained with hematoxylin. Original magnification of insets $\times 40$.



significantly increased, albeit not to the magnitude of the *Pgr*^{tm2(cre)Lyd/+} *Ihh*^{tm1Blan} mice (1.4-fold in the *Pgr*^{tm2(cre)Lyd/+} uteri vs. 3.2-fold in the *Pgr*^{tm2(cre)Lyd/+} *Ihh*^{tm1Blan} uteri), and the expression of *Prlr* was significantly reduced, which is opposite to that of the *Pgr*^{tm2(cre)Lyd/+} *Ihh*^{tm1Blan} mice (Supplemental Fig. S1C). Because ESR1 is located in both the epithelium and stroma of the uterus, we wanted to

determine whether there was a compartment-specific effect of *Ihh* ablation on E2 signaling. ESR1 protein remained unchanged in the uterine stroma or glandular epithelium, but it was markedly increased in the uterine luminal epithelium of the *Pgr*^{tm2(cre)Lyd/+} *Ihh*^{tm1Blan} uterus compared with controls (Fig. 4B).

Because unopposed E2 signaling is a hallmark of uterine diseases, we wanted to determine whether the increased E2 signaling observed in the *Pgr*^{tm2(cre)Lyd/+} *Ihh*^{tm1Blan} mice resulted in a pathological condition. Therefore, we examined the histology of untreated, intact *Pgr*^{+/+} *Ihh*^{tm1Blan} and *Pgr*^{tm2(cre)Lyd/+} *Ihh*^{tm1Blan} mice at 15 mo of age and observed that 50% of the *Pgr*^{tm2(cre)Lyd/+} *Ihh*^{tm1Blan} mice ($n = 12$) displayed an abnormal appearance. Microscopically, the *Pgr*^{tm2(cre)Lyd/+} *Ihh*^{tm1Blan} uteri had increased numbers of cystically dilated endometrial glands compared with the control *Pgr*^{+/+} *Ihh*^{tm1Blan} mice (Fig. 5A). These dilated endometrial glands were typically lined by flattened epithelial cells and contained cellular debris within the glandular lumens. In addition, the endometrial stroma from the *Pgr*^{tm2(cre)Lyd/+} *Ihh*^{tm1Blan} uteri contained foci of homogeneous eosinophilic staining, which is indicative of stromal hyalinization. Staining

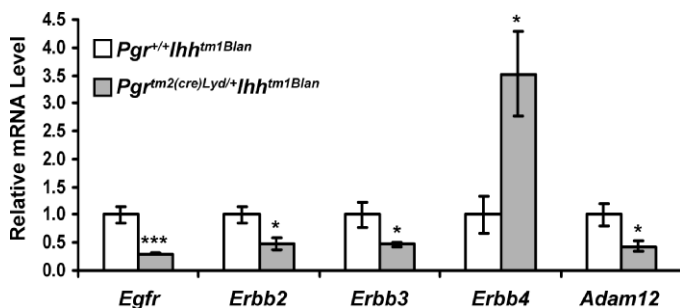


FIG. 3. EGF signaling is altered by ablation of *Ihh*. Real-time RT-PCR analysis of the EGF signaling genes *Egfr*, *ErbB2*, *ErbB3*, *ErbB4*, and *Adam12*. The results represent the mean \pm SEM. * $P < 0.05$; *** $P < 0.001$.

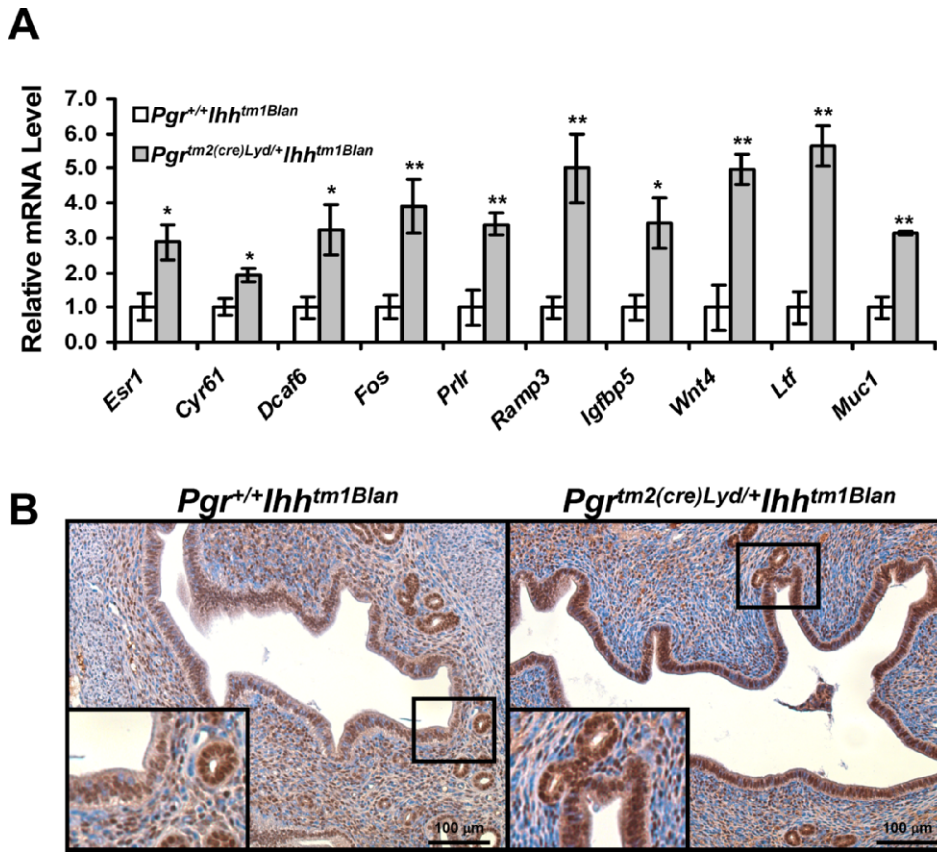


FIG. 4. Estrogen signaling is increased by *Ihh* ablation. **A**) Identification of E2- and *Ihh*-regulated genes during embryo implantation. Genes differentially regulated by *Ihh* were compared to genes regulated by E2 in wild-type mice identified by Hewitt et al. [29]. **B**) Real-time RT-PCR analysis of E2-regulated genes. The results represent the mean \pm SEM. * $P < 0.05$; ** $P < 0.01$. **B**) Immunohistochemical analysis of ESR1 in *Pgr*^{+/+} *Ihh*^{tm1Blan} (left) and *Pgr*^{tm2(cre)}*Lyd*^{+/+} *Ihh*^{tm1Blan} (right) mice. Positive staining is seen by a brown signal. Nuclei were counterstained with hematoxylin. Original magnification of insets $\times 40$.

for Masson trichrome indicated increased collagen deposition in the endometrial stroma of the *Pgr*^{tm2(cre)}*Lyd*^{+/+} *Ihh*^{tm1Blan} uteri (Fig. 5B). These results are consistent with previous findings on chronic treatment of E2 in mice [30]. This phenotype was not due to altered ovarian function as the mice exhibited normal serum testosterone (63.93 ± 6.37 ng/dl [*Pgr*^{+/+}

Ihh^{tm1Blan}]) and 60.01 ± 3.86 ng/dl [*Pgr*^{tm2(cre)}*Lyd*^{+/+} *Ihh*^{tm1Blan}]) and estradiol (2.22 ± 0.55 ng/dl [*Pgr*^{+/+}*Ihh*^{tm1Blan}]) and 1.91 ± 0.48 ng/dl [*Pgr*^{tm2(cre)}*Lyd*^{+/+} *Ihh*^{tm1Blan}]) levels. Thus, *Ihh* acts as a critical mediator of E2 signaling such that its loss results not only in altered uterine receptivity but also a pathological condition.

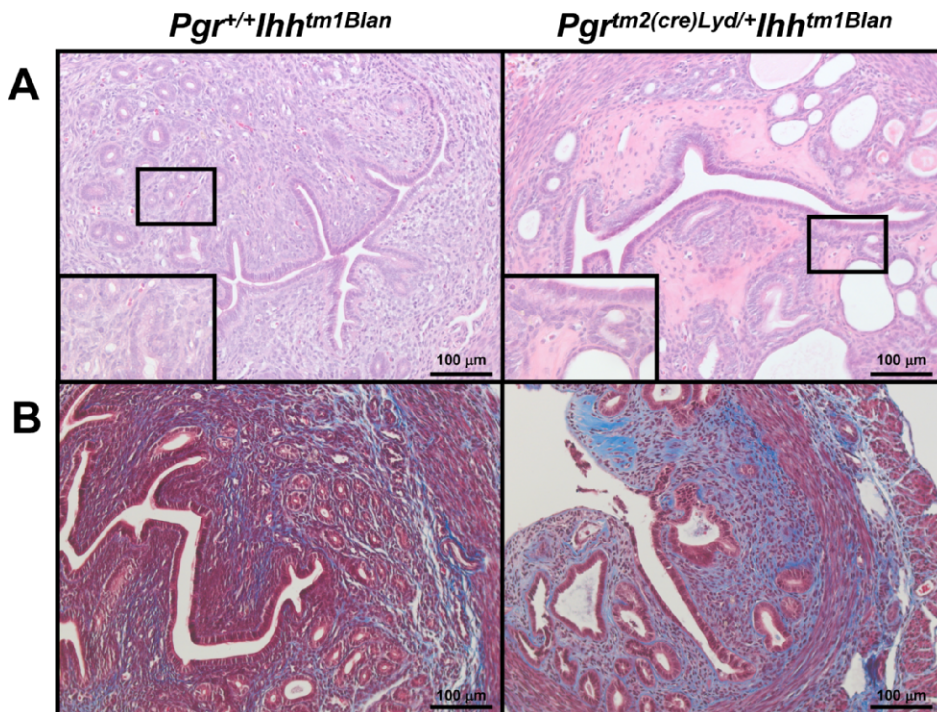


FIG. 5. Examination of 15-mo-old mice. **A**) Hematoxylin-eosin staining of 15-mo-old *Pgr*^{+/+} *Ihh*^{tm1Blan} (left) and *Pgr*^{tm2(cre)}*Lyd*^{+/+} *Ihh*^{tm1Blan} (right) mice. **B**) Masson trichrome staining of 15-mo-old *Pgr*^{+/+} *Ihh*^{tm1Blan} (left) and *Pgr*^{tm2(cre)}*Lyd*^{+/+} *Ihh*^{tm1Blan} (right) mice. Blue signal indicates collagen. Red signal indicates muscle. Black signal indicates nuclei. Original magnification of insets $\times 40$.

Epithelium

Stroma

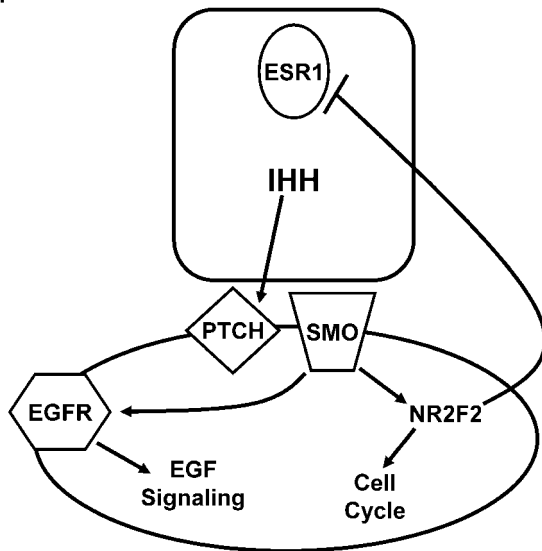


FIG. 6. Model of *Ihh* signaling in the uterus during preimplantation. *Ihh* acts as a mediator of epithelial-stromal communication by mediating multiple signaling pathways in the preimplantation period, such as cell cycle progression, EGF signaling, and E2 signaling.

DISCUSSION

Ihh has been shown to be critical for normal adult uterine function as conditional ablation of *Ihh* in the uterus renders mice infertile due to defective embryo attachment and decidualization [11]. The highest expression of *Ihh* is on d2.5 of pregnancy in the uterine epithelium, whereas that of its target genes *Ptch1* and *Nr2f2* is highest on d3.5 in the uterine stroma [5, 6]. Previously, we determined that these gene expression changes could be mimicked by the administration of exogenous hormones such that the highest expression of the *Ihh* target genes correlated with 30 h after the first E2 plus P4 injection in the artificially induced decidual reaction [9, 16]. We performed microarray analysis at this time point to determine which pathways are regulated by *Ihh* at the time of embryo implantation and identified 863 *Ihh*-regulated genes. In the analysis, we used whole uterus to extract the RNA, which may have limited the number of genes identified because *Ihh* signaling is focally activated in the subepithelial stroma, which makes up a relatively small proportion of the total cell population. Nonetheless, we observed an alteration in multiple pathways, including cell cycle progression, EGF signaling, and E2 signaling (Fig. 6). Although the *Pgr^{tm2}(cre)Lydl⁺ Ihh^{tm1Blan}* uterus exhibited decreased vascularization, we did not observe a significant alteration in genes involved in this process, possibly because of the relative abundance of the vasculature in comparison with the epithelial or stroma cells in the uterus. For the majority of genes examined, these changes in gene expression were not due to the presence of Cre, because wild-type and *Pgr^{tm2}(cre)Lydl⁺* mice exhibited similar gene expression patterns (Supplemental Fig. S1). However, for *Dcaf6* and *Prlr* the expression levels differed in the *Pgr^{tm2}(cre)Lydl⁺* uteri compared with controls, although not in the same magnitude or direction. Despite the loss of one *Pgr* allele, the *Pgr^{tm2}(cre)Lydl⁺* mouse is phenotypically normal, and no changes in P4-regulated gene expression have been observed. In addition, neither *Dcaf6* nor *Prlr* has been shown to be P4 regulated in the uterus by microarray analysis [17]. Therefore, these changes in gene expression in the

Pgr^{tm2}(cre)Lydl⁺ mouse model may be due to those particular genes having increased sensitivity to Cre expression [31].

Stromal cell proliferation is a P4-driven process critical for embryo implantation and subsequent decidualization, and *Ihh* has been shown to be critical for this proliferation, as the *Pgr^{tm2}(cre)Lydl⁺ Ihh^{tm1Blan}* uterus exhibited decreased stromal cell proliferation [11, 32]. In the human endometrium, expression of *IHH* is correlated with cell division as its expression decreases from the early to mid secretory phase, when cellular division is also decreased [10]. Conversely, the Hedgehog signaling axis has been shown to be overexpressed in endometrial cancer and to act as a critical mediator of growth in endometrial carcinoma cells [33]. CCNB1 and CDC2A together form the mitosis-promoting factor, which phosphorylates numerous proteins necessary for entry into mitosis [25]. PTCH1 has been shown to physically interact with CCNB1, resulting in the inhibition of its nuclear localization, thereby inhibiting entry into mitosis [34, 35]. In addition, we now show that *Ihh* regulates the expression of these two critical proteins, demonstrating an additional mechanism by which Hedgehog signaling regulates entry into mitosis (Fig. 2A). *Ccnd1* has been shown to be critical for cell proliferation in the uterus as a target of P4 inhibition of E2-induced proliferation [27, 36, 37]. Here, we show that *Ihh* regulates *Ccnd1* expression in the uterus, corroborating previous findings in *Drosophila* and mouse cerebellar granule neuron precursors (Fig. 2A) [38, 39]. Interestingly, this loss of expression appears confined to the uterine stroma, where the proliferation defect was observed in the *Pgr^{tm2}(cre)Lydl⁺ Ihh^{tm1Blan}* mice (Fig. 2B) [11]. The MCM family of proteins is characterized by their binding to chromatin to regulate DNA replication. One of these proteins is MCM5 (also known as CDC46), which has been shown to be critical for replication in both yeast [40] and humans [26]. Here, we show that *Ihh* regulates the expression of *Mcm5* (Fig. 2A). Another member of this family, MCM3, was also reduced upon *Ihh* ablation, and this reduction appears confined to the uterine stroma at this time point (Fig. 2C). Previously, the localization of MCM3 has been shown to be regulated by steroid hormones in the uterus, with E2 plus P4 treatment resulting in strong stromal staining and weak epithelial staining [41]. Many of the cell cycle genes regulated by *Ihh*, such as *Ccnd1*, *Mcm5*, and *Mcm3*, have also been implicated in E2-induced luminal epithelial proliferation at the time of implantation (Supplemental Table S6) [41]. However, even though we observed increased E2 sensitivity in the epithelium of the *Pgr^{tm2}(cre)Lydl⁺ Ihh^{tm1Blan}* uteri, there was no difference in epithelial proliferation when compared to controls. Decreased proliferation was only observed in the endometrial stroma. Thus, the changes in proliferation that were observed were due to the direct action of *Ihh* ablation and not because of the heightened E2 sensitivity of the epithelium.

Previously, we demonstrated that *Ihh* regulated the expression of EGFR in the subepithelial stroma [11]. Here, we show that *Ihh* also regulates the expression of the other members of the ERBB family of receptors using real-time RT-PCR analysis, with *ErbB2* and *ErbB3* being downregulated and *ErbB4* upregulated in the *Pgr^{tm2}(cre)Lydl⁺ Ihh^{tm1Blan}* uterus (Fig. 3). These genes were not identified as being *Ihh* regulated by microarray analysis, but this may be due to the false-negative rate of the analysis. Previous work on the role of EGF signaling in the uterus during embryo implantation examined the EGF ligands *Areg* and *Hbgef*, neither of which was altered in the *Pgr^{tm2}(cre)Lydl⁺ Ihh^{tm1Blan}* uterus. Both *Hbgef^{-/-}* and *Pgr^{tm2}(cre)Lydl⁺ Hbgef^{flf}* mice exhibit embryo implantation defects, suggesting an important role for EGF signaling during early pregnancy, whereas *Areg^{-/-}* mice fail to exhibit a uterine

phenotype, possibly because of compensation by the other EGF ligands [42, 43]. *Adam12*, a known processor of EGF ligands, was downregulated by ablation of *Ihh* (Fig. 2). Recently, ADAM12 has been shown to be a critical regulator of stromal cell decidualization, which is necessary for successful embryo implantation [44]. ADAM12 has been shown to process not only HBEGF but also Delta-like 1, a ligand for the Notch receptors [28, 45]. In addition, ADAM12 has been shown to act in focal adhesion formation through the $\beta 1$ and $\beta 3$ integrins (ITGB1 and ITGB3) and in transforming growth factor- β (TGFB) signaling through interactions with the TGFB type II receptor [46, 47]. All of these pathways have been shown to be active during embryo implantation, and thus *Ihh* through ADAM12 may regulate these pathways during embryo implantation [48–50]. Thus, in the uterus, *Ihh* may be a major mediator of not only EGF but also other signaling pathways. Interestingly, its regulation of EGF signaling occurs not at the level of ligand expression but at the receptor level and also in the processing of the ligands.

Estrogen signaling is critical to uterine function during early pregnancy because it defines the window of receptivity [3]. High or sustained levels of E2 make the uterus refractory toward embryo implantation. In the *Pgr^{tm2(cre)}Lyd1⁺ Ihh^{tm1Blan}* uterus, we observed increased levels of ESR1 at both the mRNA and protein levels with the protein markedly increased in the uterine luminal epithelium, which was accompanied by increased expression of E2-regulated genes (Fig. 4). To identify these E2-regulated genes, our gene list was compared to that from Hewitt et al. [29], in which microarrays were performed on ovariectomized wild-type mice treated with E2. We identified 19 genes induced by E2 in the Hewitt et al. data set that were upregulated upon ablation of *Ihh* (Supplemental Table S5). These results agree with previous findings in the *Pgr^{tm2(cre)}Lyd1⁺ Nr2f2^{tm1Vco}* mice, in which the E2-regulated genes lactoferrin, component C3, and chloride channel-activated 3 were upregulated [14]. Thus, the repressive actions of *Ihh* on the luminal epithelium may occur through *Nr2f2* in the uterine stroma. In addition, the E2 target gene *Muc1* was upregulated in both the *Pgr^{tm2(cre)}Lyd1⁺ Ihh^{tm1Blan}* and *Pgr^{tm2(cre)}Lyd1⁺ Nr2f2^{tm1Vco}* uterus [11, 14]. *Muc1* is a glycoprotein that lines the uterine epithelium and whose loss is necessary for embryo attachment and subsequent implantation to occur [12]. It was previously shown to be E2 regulated, although it failed to appear in the Hewitt et al. data set [12, 29]. Therefore, there may be additional E2-regulated genes that were altered by *Ihh* ablation that were not identified in this analysis. Thus, the *Ihh* signaling axis regulates embryo implantation through a downregulation of E2 signaling.

Furthermore, at 15 mo of age, *Pgr^{tm2(cre)}Lyd1⁺ Ihh^{tm1Blan}* uteri exhibit characteristics of an estrogenized uterus, such as cystic glands and hyalinized stroma (Fig. 5). This phenotype may be attributed to altered ovarian function as serum estradiol and testosterone levels decrease with age because of atresia of the ovarian follicles, and a defect in this decrease could result in increased circulating estradiol levels [51]. However, we detected serum testosterone and estradiol levels that were comparable between *Pgr^{+/+} Ihh^{tm1Blan}* and *Pgr^{tm2(cre)}Lyd1⁺ Ihh^{tm1Blan}* mice, indicating that ovarian function was not affected by *Ihh* ablation. In addition, in our previous analysis of the *Pgr^{tm2(cre)}Lyd1⁺ Ihh^{tm1Blan}* mice, we did not observe an ovarian phenotype, likely because of the nonoverlapping expression patterns of *Ihh* and *Pgr^{tm2(cre)}Lyd1⁺* in the ovary [11, 15, 52]. An indirect hypothalmo-pituitary-ovarian action may also be a potential reason for this phenomenon; however, previously we observed normal serum progesterone levels, and here we demonstrate normal serum testosterone and estradiol

levels, indicating that this axis is intact [11]. Thus, the observed phenotype is likely due to the increased E2 signaling resulting from *Ihh* ablation, suggesting that *Ihh* may also play a role in endometrial dysfunction as aberrant E2 signaling is a hallmark of uterine diseases such as endometriosis and endometrial cancer [53–55]. Recently, *IHH* was identified as a gene significantly downregulated in patients with endometriosis [56]. Thus, the *Ihh* signaling axis may be an important therapeutic target for these diseases as a potential means to target increased E2 signaling.

In conclusion, conditional ablation of *Ihh* in the murine uterus renders female mice infertile [11]. Microarray analysis conducted at the point at which the *Ihh* target genes have their highest expression identified 863 *Ihh*-regulated genes. Of these genes, those that regulate the cell cycle, EGF signaling, and E2 signaling were identified and validated. Thus, *Ihh* affects embryo implantation through its regulation of stromal cell proliferation and inhibition of epithelial E2 signaling, events that are necessary for successful embryo implantation. In addition, the increased E2 signaling observed in the *Pgr^{tm2(cre)}Lyd1⁺ Ihh^{tm1Blan}* uterus and the downregulation of *IHH* observed in endometriosis suggest that Hedgehog signaling may play a role in the development of endometrial diseases, such as endometriosis and endometrial cancer. Further examination of the role of Hedgehog signaling in the uterus may open avenues for new treatments of these diseases.

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