Loss of MIG-6 results in endometrial progesterone resistance via ERBB2

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Abstract

Female subfertility is highly associated with endometriosis. Although the exact etiology of endometriosis-related infertility remains to be determined, endometrial progesterone resistance has recently been suggested as a crucial element in the development of endometrial diseases. Here, we report that MIG-6, a progesterone-induced gene, is downregulated in the endometrium of infertile women with endometriosis and in a non-human primate model of endometriosis. In an endometriosis mouse model with a fluorescent reporter used to identify lesions, an increase of endometriosis development and implantation failure were observed in mice with Mig-6 deficient endometrium compared to controls. MIG-6 is known to inhibit ERBB2, which we found overexpressed in the endometrium from uterine-specific Mig-6 knock-out mice (Pgrcre/+/Mig-6f/f, Mig-6d/d). To investigate the effect of ERBB2 targeting on endometrial progesterone resistance, fertility, and endometriosis, we introduced Erbb2 ablation in Mig-6d/d mice (Mig-6d/dErbb2d/d mice). The additional knockout of Erbb2 rescued all phenotypes seen in Mig-6d/d mice including endometrial progesterone resistance, infertility, and endometriosis lesion development. Transcriptomic analysis showed that genes differentially expressed in Mig-6d/d mice reverted to their normal expression amounts in Mig-6d/dErbb2d/d mice. Together, our results demonstrate that MIG-6-induced ERBB2 overexpression causes endometrial progesterone resistance and a nonreceptive endometrium in endometriosis-related infertility and that ERBB2 targeting reverses these effects.

Introduction

Critical for fertility, the uterine endometrium's epithelial and stromal compartments undergo dynamic hormonally controlled molecular and morphological changes to prepare for embryo implantation and development. Estrogen (E2) stimulates the proliferation of uterine epithelial cells, and progesterone (P4) suppresses E2-induced proliferation. Endometrial P4 resistance implies decreased responsiveness of target tissue to bioavailable P4. Endometrial P4 resistance is seen in women with a nonreceptive endometrium, endometriosis, polycystic ovary syndrome (PCOS), and endometrial cancer. Moreover, P4-induced molecular changes in the eutopic (intrauterine) endometrial tissue of women with endometriosis are either blunted or undetectable.

Endometriosis affects about 10% of all women of reproductive age, and the incidence increases to 50–60% of women with chronic pelvic pain and infertility. While progestin-based therapies are commonly used to treat endometriosis and lead to disease regression in some women, other women with endometriosis and pelvic pain do not respond effectively to progestins. Moreover, many P4-induced molecular changes in the eutopic endometrial tissue of women with endometriosis are either blunted or dysregulated, but an impaired P4 response is seen in the endometrium of women with endometriosis. Despite knowing the effects, the molecular mechanism responsible for endometrial P4 resistance and dysregulation remains unclear. Therefore, understanding the molecular mechanisms of endometrial P4 resistance is critical.
The present study revealed that the amount of mitogen inducible gene 6 (MIG-6) was decreased in endometrium from infertile women with endometriosis. We used uterine-specific Mig-6 knock-out mice to demonstrate that MIG-6 loss results in endometrial progesterone resistance via ERBB2. Our findings provide new insight into the etiology of female infertility and provide a new molecular framework useful for the design of new therapeutic strategies.

Results

**MIG-6 expression is decreased in endometrium from women with endometriosis.**

We previously identified Mig-6 as a P4-regulated gene that mediates the ability of P4 to repress E2 action in the mouse uterus. During the menstrual cycle, P4 amounts rise at the early secretory phase. As measured by RT-qPCR, MIG-6 expression in the human endometrium was significantly higher in the early secretory phase of the menstrual cycle than in the proliferative phase (p < 0.001) (Fig. 1A), suggesting that MIG-6 is a P4-induced gene in the human endometrium as has been demonstrated in the mouse. Because many P4-induced endometrial molecular changes are either blunted or eliminated in women with endometriosis, we examined MIG-6 expression in endometrial biopsies from infertile women with endometriosis. RT-qPCR and immunohistochemistry showed that amounts of MIG-6 mRNA (p < 0.01) and protein (p < 0.001) were significantly lower in the eutopic endometrium of infertile women with endometriosis compared to controls in the early secretory phase (Fig. 1, A-C). To assess how MIG-6 expression is affected by endometriosis progression, we used a baboon model. Intraperitoneal inoculation with autologous menstrual effluent in female non-human primates results in formation of endometriotic lesions highly similar in histomorphology to those seen in women. We found that endometrial MIG-6 protein abundance was significantly reduced in baboons during the progression of endometriosis after experimental disease induction as compared to paired pre-inoculation control samples (Fig. 1D; p < 0.001). These results demonstrate that reduced MIG-6 expression can be caused by the development of endometriotic lesions.

Uncovering pathophysiological mechanisms of endometriosis-related infertility with animal models requires easy identification of lesions to distinguish them from the surrounding normal tissues. With this in mind, we developed a mouse model of endometriosis using mT/mG reporters. In Pgr\textsuperscript{cre/+}Rosa26\textsuperscript{mTmG/+} mice, progesterone receptor (Pgr)-positive uterine cells express mG, while Pgr-negative cells express mT (fig. S1 A and B). Using this model, we surgically induced endometriosis in Pgr\textsuperscript{cre/+}Rosa26\textsuperscript{mTmG/+} mice by inoculating autologous endometrial tissue fragments into the peritoneal cavity after 3 days of E2 treatment (fig. S1C). This method leads to the development of endometriotic lesions similar to those in humans without the need for ovariectomy or unopposed E2 treatment (fig. S1 D-F). To examine the responsiveness of our endometriosis model to E2 and P4, Pgr\textsuperscript{cre/+}Rosa26\textsuperscript{mTmG/+} mice induced with endometriosis were treated with vehicle, E2, or E2 + P4 for 2 weeks. While E2 treatment after endometriosis induction significantly increased the number of endometriotic lesions compared to the vehicle group, the addition of P4 suppressed the E2-induced increase in lesion number (fig. S1 G and H; p
<0.01). Our mouse model thus closely mirrors human endometriosis as an E2-dependent and P4-suppressed disorder. To determine whether MIG-6 expression is dysregulated after endometriosis development in a distinct mammalian system, we examined MIG-6 amount in the eutopic endometrium from Pgr\textsuperscript{cre/+}Rosa26\textsuperscript{mTmG/+} mice with endometriosis. MIG-6 protein expression was significantly reduced in eutopic endometrium from the mice with endometriosis compared to the sham group (Fig. 1E; p < 0.001).

**Mig-6 loss accelerated the development of endometriosis and endometriosis-related infertility.**

Next, we assessed whether endometriosis in mice causes infertility by assessing implantation and decidualization success. One month after endometriosis induction, the number of implantation sites in mice with endometriosis was not changed compared to the sham group. However, 63.6% (7 out of 11) of mice with endometriosis experienced implantation failure 3 months after endometriosis development (fig. S2A). We next examined the impact of endometriosis on decidualization using an artificial decidualization model. One month after endometriosis induction, mice with endometriosis displayed a uterine horn that responded well to artificial decidualization; however, after 3 month of endometriosis development, the mice with endometriosis exhibited a significant defect in decidual response compared to control and sham mice (fig. S2B; p < 0.001). Our result suggests that endometriosis development causes implantation failure and a defect of decidualization, as has been hypothesized in humans.

Having established the link between endometriosis development and MIG-6 attenuation in the eutopic endometrium, we sought to determine if MIG-6 depletion is involved in endometriotic lesion development. In a comparison of MIG-6 expression in paired ectopic and eutopic endometrial biopsies taken from women with endometriosis, MIG-6 amounts were significantly reduced in the ectopic endometrial specimens (p < 0.01) (Fig. 2A and B). To assess the effect of MIG-6 deficiency in endometriosis development, we induced endometriosis in control (Pgr\textsuperscript{cre/+}Rosa26\textsuperscript{mTmG/+}) and Mig-6\textsuperscript{d/d}Rosa26\textsuperscript{mTmG/+} mice and found that uterine MIG-6 attenuation significantly increased incidence (p < 0.01) and weight of endometriotic lesions (p < 0.05) (Fig. 2C and D). To address the role of MIG-6 in endometriosis-related infertility, we surgically induced endometriosis in wild type females using endometrial fragments from donor control (Pgr\textsuperscript{cre/+}Rosa26\textsuperscript{mTmG/+}) and Mig-6\textsuperscript{d/d}Rosa26\textsuperscript{mTmG/+} mice (Fig. 2E). One month after endometriosis induction, the number of implantation sites was significantly reduced in the mice with Mig-6\textsuperscript{d/d}Rosa26\textsuperscript{mTmG/+} ectopic lesions compared to the mice with control ectopic lesions (p < 0.05). Furthermore, implantation sites were entirely absent from mice with Mig-6\textsuperscript{d/d}Rosa26\textsuperscript{mTmG/+} ectopic lesions after 2 months of endometriosis development (Fig. 2F and G). These results demonstrate that MIG-6 attenuation in ectopic lesions increased endometriosis development and accelerated implantation failure compared to controls.

Cessation of epithelial E2-induced proliferation is essential for implantation in all eutherian mammal species studied. In mice, abundant proliferation of epithelial and stromal cells is detectable at day 2.5 of gestation (GD 2.5). However, just before implantation, P4 inhibits epithelial proliferation and
induces differentiation to an embryo receptive state. Establishing uterine receptivity by sequential actions of E2 and P4 on endometrial cells is critical for successful embryo apposition, attachment, implantation, and pregnancy maintenance, and lack of sufficient E2 and P4 action can result in infertility and pregnancy loss in humans and mice.

Mig-6 knockout mice are infertile due to P4 resistance and implantation failure. To determine whether a defect of embryo implantation is caused by an alteration in endometrial cell proliferation, we examined expression of a proliferation marker (Ki67) at pre-implantation (GD 3.5). Epithelial proliferation was significantly increased in the Mig-6 knockout endometrium compared to controls (p < 0.001) (Mig-6+/f; fig. S3 A and B). To identify the molecular explanation for the effect of MIG-6 loss on epithelial proliferation, we examined amounts of several E2 signaling molecules, including epidermal growth factor receptor (EGFR), erb-b2 receptor tyrosine kinase 2 (ERBB2; also known as CD340, proto-oncogene Neu, or HER2) and extracellular-signal-regulated kinase 1/2 (ERK1/2) at GD 3.5 in Mig-6+/f and Mig-6−/− mice. EGFR amounts were unchanged, but ERBB2 and phospho-ERK1/2 (pERK1/2) amounts were selectively increased in Mig-6−/− mice (fig. S3 C and D). These results suggest MIG-6 is a negative regulator of ERBB2/ERK signaling in the pre-implantation endometrium.

Erbb2 overexpression causes infertility seen in Mig-6 mutant mice.

In order to investigate the effect of ERBB2 targeting on nonreceptive endometrium and endometriosis with Mig-6 deficiency, we introduced Erbb2 ablation in Mig-6−/− mice (Mig-6−/−Erbb2−/−; fig. S4). To address the effect of conditional Erbb2 knockout on the infertility phenotype of Mig-6−/− mice, we mated female control, Mig-6−/−, and Mig-6−/−Erbb2−/− (Pgrcre+/fMig-6+/fErbb2+/f) mice with wild type male mice for 6 months to determine their overall fertility. As expected, Mig-6−/− mice were infertile, but surprisingly, Mig-6−/−Erbb2−/− exhibited normal fecundity compared to controls (6.40 ± 0.49 and 7.29 ± 0.29 average pups/litter, respectively; table S1). This is the first report of molecular targeting to correct infertility caused by endometrial P4 resistance.

To further dissect the reversal of Mig-6-related infertility by attenuation of Erbb2, we examined implantation rates. Uterine horns of Mig-6−/− mice had no grossly visible implantation sites at GD 5.5, whereas Mig-6−/−Erbb2−/− mice averaged 7.00 ± 0.41 implantation sites that appeared normally spaced (Fig. 3A). Subsequent histology revealed all embryos in Mig-6−/−Erbb2−/− uteri were positioned as expected alongside the anti-mesometrial luminal epithelium, and the stromal cells had the normal decidual response surrounding the embryo (Fig. 3B). To identify the effect of additional Erbb2 knockout on the aberrantly increased epithelial proliferation of GD 3.5 Mig-6−/− mice, we assessed Ki67 and cyclin D1 expression in Mig-6−/−Erbb2−/− mice. In contrast to Mig-6−/− mice, Mig-6−/−Erbb2−/− endometrial epithelial cells exhibited normal cyclin D1 and Ki67 (Fig. 3C). Since the increase of epithelial proliferation in Mig-6−/− mice is accompanied by increased E2 signaling, we investigated whether excess E2 signaling is abrogated by Erbb2 ablation. The expression of the E2-responsive genes mucin 1 (Muc-1) (p < 0.001), chloride channel calcium activated 3 (Clca3)(p < 0.01), lactoferrin (Ltf)(p < 0.001), and complement
component 3 (C3) (p < 0.05) were significantly increased in Mig-6<sup>d/d</sup> mice but restored to normal amounts in Mig-6<sup>d/d</sup>Erbb2<sup>2/d</sup> mice (Fig. 3D). The same pattern was apparent for MUC1 and LTF protein amounts (Fig. 3E). These results imply that ERBB2 overexpression resulting from Mig-6 attenuation causes female infertility due to a nonreceptive endometrium, and this effect may be reversed by ablation of Erbb2.

**Erbb2 ablation overcomes P4 resistance in Mig-6 mutant mice.**

Mig-6 attenuation causes endometrial P4 resistance demonstrated by P4’s inability to inhibit E2-induced uterine weight gain in Mig-6<sup>d/d</sup> mice<sup>20</sup>. In order to determine if Erbb2 ablation restores endometrial P4 responsiveness in Mig-6<sup>d/d</sup> mice, ovariectomized control, Mig-6<sup>d/d</sup>, and Mig-6<sup>d/d</sup>Erbb2<sup>2/d</sup> mice were treated with vehicle or E2 + P4 for 3 days. While Mig-6<sup>d/d</sup> mice treated with E2 + P4 experienced significant increases in uterine weight (p < 0.05), vascularization, and expression of the E2 target genes Muc1, Clca3, Ltf, and C3 compared to E2 + P4 treated control mice (p < 0.001), Mig-6<sup>d/d</sup>Erbb2<sup>2/d</sup> mice exhibited normal P4 responsiveness and expression of E2 target genes (Fig. 4, A-C). We then examined the effect of Erbb2 ablation in the endometriosis development of Mig-6<sup>d/d</sup> mice and found the number and weight of endometriotic lesions were restored to control amounts by the additional ablation of Erbb2 (Fig. 4D and E; fig. S5).

Uterine Mig-6 ablation causes endometrial hyperplasia by 5 months of age<sup>20</sup>. To investigate the impact of additional Erbb2 knockout on endometrial hyperplasia development due to Mig-6 attenuation, we examined uterine weight and gross histological morphology in control, Mig-6<sup>d/d</sup>, and Mig-6<sup>d/d</sup>Erbb2<sup>2/d</sup> mice at 5 months of age. Uterine weight was significantly decreased in Mig-6<sup>d/d</sup>Erbb2<sup>2/d</sup> mice when compared to Mig-6<sup>d/d</sup> mice (p < 0.001), and histological analysis revealed that Mig-6<sup>d/d</sup>Erbb2<sup>2/d</sup> mice did not develop endometrial hyperplasia (fig. S6). These results demonstrate that all known female reproductive phenotypes caused by knocking out uterine Mig-6 are restored to baseline by also knocking out Erbb2.

To identify the signaling pathways that Mig-6 regulates at pre-implantation, we performed transcriptomic analysis on the uteri from control, Mig-6<sup>d/d</sup>, and Mig-6<sup>d/d</sup>Erbb2<sup>2/d</sup> mice at GD 3.5. We found 1,022 and 771 increased or decreased transcripts, respectively, in the Mig-6<sup>d/d</sup> uterus as compared with controls (Fig. 5A and table S2). Remarkably, 1,722 of the altered genes (96.04%) in Mig-6<sup>d/d</sup> mice reverted to their normal expression amounts in Mig-6<sup>d/d</sup>Erbb2<sup>2/d</sup> mice. Pathway analysis showed that major altered pathways in the Mig-6<sup>d/d</sup> uterus included cell-cycle control and DNA replication. P4 blocks E2-induced DNA synthesis by inhibiting replication licensing including mini-chromosome maintenance (MCM) proteins<sup>33,34</sup> which have a role in both the initiation and elongation phases of eukaryotic DNA replication as part of the MCM complex<sup>35,36</sup>. Fifteen genes associated with cell cycle and DNA replication were significantly changed in the Mig-6<sup>d/d</sup> uterus (table S3). RT-qPCR analysis confirmed that the additional knockout of Erbb2 in Mig-6<sup>d/d</sup> mice restored dysregulated cell-cycle control and DNA-replication-related gene transcripts to normal (Fig. 5B). IHC results showed that at the protein level as well, aberrant
overexpression of MCM2 and MCM6 occurred in Mig-6\(^{d/d}\) mice at the pre-implantation stage but reverted to normal in Mig-6\(^{d/d}\)Erbb2\(^{d/d}\) mice (Fig. 5C). A similar action can be ascribed to P4 and E2 in the human endometrial epithelium, since a loss of MCM proteins occurs in the secretory phase, and P4 dominates this phase of the menstrual cycle\(^{37}\). Additionally, aberrant overexpression of MCM2 and MCM6 may cause abnormal epithelial proliferation and nonreceptive endometrium in infertile women with endometriosis\(^{38}\). Two Kruppel-like transcription factors (KLFs) are implicated in E2 and P4 modulation of uterine proliferation\(^{38}\). Klf4 expression is increased by E2 and promotes DNA replication, whereas Klf15 is increased by P4 and inhibits growth via regulation of Mcm2\(^{38}\). The expression of KLF4 was significantly increased in Mig-6\(^{d/d}\) mice compared to control mice while the expression of KLF15 was decreased in Mig-6\(^{d/d}\) mice, and the amounts reverted to normal in Mig-6\(^{d/d}\)Erbb2\(^{d/d}\) mice (p < 0.001) (Fig. 5, B and D). These results suggest that Erbb2 overexpression due to Mig-6 ablation causes E2-induced epithelial proliferation and P4 resistance by disrupting cell cycle regulation.

**Discussion**

This study reveals the attenuation of MIG-6 in eutopic endometrium from infertile women with endometriosis compared to controls. MIG-6 expression was higher in human endometrium from the early secretory phase than in endometrium from the proliferative phase. Because of the complexity and dynamic nature of implantation, the molecular processes underlying these changes are poorly understood. Improving fertility rates requires unraveling molecular mechanisms of implantation. However, how regulation occurs between P4 and E2 is still not fully understood\(^{39,40}\), which is a critical barrier to better therapies for infertility. Amounts of MIG-6 mRNA and protein were lower in the eutopic endometrium of infertile women with endometriosis compared to controls in the early secretory phase. These results suggest that MIG-6 is a P4-responsive gene in human endometrium as in the mouse\(^{20}\), and MIG-6 loss may result in a non-receptive endometrium in endometriosis-related infertility.

Nonhuman primates are advantageous for studying endometriosis because they are phylogenetically similar to humans\(^{41,42,43}\). Intraperitoneal inoculation with autologous menstrual effluent results in formation of endometriotic lesions similar in histology and morphology to those seen in women\(^{22}\). Paired sequential analysis showed MIG-6 protein amounts were decreased in the eutopic endometrium of baboons during progression of endometriosis as compared to pre-inoculation control. Furthermore, MIG-6 protein expression was reduced in the eutopic endometrium from the mice with endometriosis compared to the sham group. This result demonstrated reduced MIG-6 expression is associated with endometriosis development.

We developed a mouse model of endometriosis based on Pgr\(^{cre/+}\) and mT/mG reporters that produces endometriotic lesions highly similar to those in humans. A mouse model in which excised human endometrial fragments are introduced into the peritoneum of immunocompromised mice is widely used, but is limited by lack of a normal immune system, which is thought to be important in endometriosis pathophysiology\(^{44,45,46}\). In contrast, the mouse model of induced endometriosis is a versatile model that
has been used to study how the immune system, hormones, and environmental factors affect endometriosis. The availability of a large number of transgenic mice in which specific genes can be either eliminated or overexpressed make this induced endometriosis model ideal for studying specific pathways in development and progression of endometriosis and other diseases. However, current mouse models of endometriosis that involve ovariectomy and E2 treatment are impractical for studies of physiological functions that require natural fluctuations in ovarian steroid hormones, such as fertility. On the other hand, our mouse model alleviates the need to apply ovariectomy and E2 treatment to enlarge endometriotic lesions because fluorescence reporter genes allow us to visualize in vivo and in real-time endometriotic lesions like those found in humans. Moreover, similarities between our mouse model and human endometriosis include: 1) development and progression of disease; 2) steroid hormone regulation; 3) fertility defect with implantation failure; and 4) P4 resistance in endometrium with Mig-6 deficiency. Furthermore, the fluorescence reporters enable us to quantitatively examine endometriotic lesions in these mice more accurately and easily than in prior models.

Because Mig-6<sup>d/d</sup> mice have a fertility defect, we applied a syngeneic mouse model to examine the effect of endometriotic lesions with Mig-6 ablation on the eutopic endometrium. Several groups have used syngeneic mouse models of endometriosis, in which the uterus of one mouse is removed, minced and injected intraperitoneally into recipient mice. Syngeneic murine models have several potential advantages over the rodent surgical model: 1) peritoneal seeding of uterine fragments is more similar to retrograde menstruation in women; 2) either the donor or recipient animal can receive therapeutic intervention or be otherwise manipulated prior to induction of disease; and 3) a large number of transgenic mice in which specific genes can be either eliminated or overexpressed are available. These advantages make syngeneic murine models ideal for studying the role of specific pathways in development and progression of endometriosis and other diseases.

P4 is absolutely required for uterine implantation, decidualization, and maintenance of pregnancy. How endometriosis contributes to infertility remains elusive, although P4 resistance is likely involved. P4 resistance is seen in the endometrium of infertile women with endometriosis, and Mig-6<sup>d/d</sup> mice exhibit P4 resistance by the inability of P4 to inhibit E2-induced uterine weight gain. We demonstrate that MIG-6 mediates P4 inhibition of E2-induced cell proliferation by inhibition of ErbB2-ERK signaling. MIG-6 plays an important role in inhibiting epithelial cell proliferation and facilitating implantation. Epithelial cell proliferation and cyclin D1 amounts were higher in the epithelial cells of Mig-6<sup>d/d</sup> mice, whereas both Mig-6<sup>d/d</sup>Erbb2<sup>d/d</sup> and control mice lacked elevated cyclin D1 amounts and epithelial cell proliferation. These results suggest that MIG-6 is a negative regulator of ErbB2 and suppresses E2-induced epithelial cell proliferation at the pre-implantation stage.

We evaluated the potential therapeutic value of ErbB2 as a target for correcting endometrial P4 resistance in infertility. In our transcriptomic analysis in Mig-6<sup>d/d</sup> and Mig-6<sup>d/d</sup>Erbb2<sup>d/d</sup> mice at GD 3.5, altered genes in Mig-6<sup>d/d</sup> mice reverted to their normal expression amounts in Mig-6<sup>d/d</sup>Erbb2<sup>d/d</sup> mice. Pathway analysis using Ingenuity Systems Software showed that major altered pathways in the Mig-6<sup>d/d</sup> uterus included
cell-cycle control and DNA replication. Dr. Pollard’s group showed that P4 blocks E2-induced DNA synthesis by inhibiting replication licensing including mini-chromosome maintenance (MCM) proteins\textsuperscript{33, 34}. The MCM complex has a role in both the initiation and elongation phases of eukaryotic DNA replication\textsuperscript{35, 36, 53}. The overlap of genes associated with cell cycle and DNA replication between the Pollard group’s microarray results and ours is striking. In the uterine epithelium, E2 stimulates expression of MCMs, while P4 decreases transcripts of MCM2 through MCM6\textsuperscript{33, 54}. Our IHC results showed aberrant overexpression of MCM2 and MCM6 in \textit{Mig-6\textsuperscript{d/d}} mice at the pre-implantation stage. A similar action can be ascribed to P4 and E2 in the human endometrial epithelium, since a loss of MCM proteins occurs in the secretory phase, and P4 dominates this phase of the menstrual cycle\textsuperscript{7, 37}. However, aberrant overexpression of MCM2 and MCM6 may cause abnormal epithelial proliferation and nonreceptive endometrium in infertile women with endometriosis\textsuperscript{38}. Our results regarding MIG-6 and ERBB2/ESR1 signaling in regulating uterine function in response to hormonal signals will bring insight into uterine pathophysiology and likely lead to new therapies for endometrial diseases. Deeper inquiry into endometrial epithelial-stromal crosstalk between ErbB2/ERK/ESR1 and PGR/MIG-6 signaling pathways will be of major importance to understanding infertility and endometriosis.

In summary, our findings reveal that attenuation of MIG-6 occurs both in endometriotic lesions and in the endometriosis-effected eutopic endometrium. Evidence from mice indicates that loss of \textit{Mig-6} in endometriotic lesions promotes their development and accelerates endometriosis-related infertility, while loss of \textit{Mig-6} in the eutopic endometrium causes infertility due to defects in implantation and endometrial receptivity. We found that increased epithelial proliferation caused by \textit{Mig-6} loss is caused by E2 through the ERBB2/ERK pathway (Fig. 6). However, targeting \textit{Erbb2} can reverse all apparent female reproductive defects caused by \textit{Mig-6} loss including endometrial hyperplasia, infertility, and endometriosis lesion development. Attenuation of \textit{Mig-6} causes the inability of P4 to properly control the cell cycle and inhibit E2-induced aberrant epithelial proliferation that results from increases in MCMs. However, counteracting the overexpression of \textit{Erbb2} restores normal gene expression patterns, providing a molecular explanation for the rescue of normal reproductive function. These findings not only elucidate a critical pathway for understanding the hormonal control of normal uterine physiology, but they also provide the potential for new treatment strategies for uterine disease.

\section*{Methods}

\subsection*{Study design}

The main objective of this study was to evaluate the role of MIG-6 in endometrial P4 resistance. First, the expression of MIG-6 was assessed in eutopic endometrium of infertile women with endometriosis compared to fertile women. To determine whether endometriosis affects MIG-6 expression, we examined MIG-6 expression in a nonhuman primate and mouse model of endometriosis. Subsequently, we identified ERBB2 as a MIG-6 target and evaluate the impact of \textit{Erbb2} ablation on the infertility and endometrial P4 resistance of \textit{Mig-6\textsuperscript{d/d}} mice. Finally, transcriptomic analysis was applied to dissect the
molecular mechanisms of Mig-6 in the uterus. The control and treatment groups and the number of biological replicates (sample sizes) for each experiment are specified in the figure legends. Animal numbers for each study type were determined by the investigators on the basis of previous experience with the standard disease models that were used or from pilot studies. Animals were randomly allocated to the control and treatment groups and housed together to minimize environmental differences and experimental bias. Analysis of endpoint readouts was carried out in a blinded fashion.

Ethics Statement

The institutional review board of Michigan State University, Greenville Health System, and University of North Carolina approved this study. The Institutional Animal Care and Use Committee at Michigan State University approved all experiments relating to mice. The Institutional Animal Care and Use Committees of both the University of Illinois at Chicago and Michigan State University approved the endometriosis baboon animal model.

Human Endometrium Samples

The human endometrial samples used to examine MIG-6 expression patterns were obtained from Michigan State University’s Center for Women’s Health Research Female Reproductive Tract Biorepository, the University of North Carolina, and the Greenville Hospital System in accordance with the guidelines set by the Institutional Review Boards of Michigan State University (Grand Rapids, MI), the University of North Carolina (Chapel Hill, NC), and Greenville Health System (Greenville, SC), respectively. Written informed consent was obtained from all participants. For experiments examining MIG-6 mRNA expression throughout the menstrual cycle, endometrial samples were analyzed from 22 cycling premenopausal women without endometriosis (n = 6 proliferative, n = 7 early secretory, n = 3 mid secretory, and n = 6 late secretory) and from 20 cycling premenopausal women with endometriosis (n = 2 proliferative, n = 6 early secretory, n = 9 mid secretory, and n = 3 late secretory). Control endometrial tissues were laparoscopically negative for endometriosis and had not been on any hormonal therapies for at least three months prior to surgery. Endometrial menstrual staging was confirmed by an experienced pathologist familiar with female reproduction. To investigate MIG-6 amounts in the endometrium from women, 10 control and 10 eutopic endometrium with endometriosis were used. To compare MIG-6 amounts in the eutopic endometrium and ectopic lesions of women with endometriosis, each of 12 samples were used. All women with endometriosis were infertile. Samples used for immunohistochemistry were fixed in 10% buffered formalin prior to embedding in paraffin wax.

Animals And Tissue Collection

Animals were maintained in a designated animal care facility according to Michigan State University’s Institutional Guidelines for the care and use of laboratory animals. All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University. For all animal studies, animals were randomly distributed among different conditions by the investigator as the animals did not show any size or appearance differences at the onset of the experiments. No animals were excluded, and the investigator was not blinded to group allocation during the experiment. Erbb2 conditional knockout
mice were generated by crossing *Pgr*<sup>cre/+<sup>*Mig-6*/<sup>f/+<sup>*Erbb2*/<sup>f/+<sup>*Mig-6*/<sup>d/d<sup>*Erbb2*/<sup>d/d<sup>. Pregnant uterine samples were obtained by mating control (*Mig-6*/<sup>f/+<sup>, *Mig-6*/<sup>d/d<sup>*Erbb2*/<sup>d/d<sup>, *Mig-6*/<sup>d/d<sup>*Erbb2*/<sup>d/d<sup> with C57BL/6 male mice the morning of a vaginal plug designated as day 0.5 of gestation (GD 0.5). Mice were sacrificed at GD 3.5 and 5.5. For the study of steroid hormone regulation, control, *Mig-6*/<sup>d/d<sup> and *Mig-6*/<sup>d/d<sup>*Erbb2*/<sup>d/d<sup> mice at 6 weeks of age were ovariectomized. Two weeks postsurgery, ovariectomized mice were injected with vehicle (sesame oil; Veh) or estradiol (0.1 µg/mouse; E2) plus progesterone (1 mg/mouse; P4) for 3 days and euthanized at 6 hours after injection. For the fertility studies, adult female control, *Mig-6*/<sup>d/d<sup> and *Mig-6*/<sup>d/d<sup>*Erbb2*/<sup>d/d<sup> mice were placed with wild type C57BL/6 male mice. The mating cages were maintained for 6 months and the number of litters and pups born during that period was recorded. Uterine tissues were then immediately processed at the time of dissection and either fixed with 4% (vol/vol) paraformaldehyde for histology or immunohistochemistry or snap frozen and stored at -80 °C for RNA/protein extraction.

**Induction Of Endometriosis**

For baboon uterine samples, endometriosis was induced by intraperitoneal inoculation of menstrual endometrium on two consecutive menstrual cycles and harvested using laparotomy via endometriectomy from four female baboons as previously described<sup>55</sup>. For mouse uterine samples, 8-weeks-old female mice which have conditional double-fluorescent Cre reporter gene (*Pgr*<sup>cre/+<sup>*Rosa26<sup>mTmG</sup>, *Pgr*<sup>cre/+<sup>*Mig-6*/<sup>f/+<sup>*Rosa26<sup>mTmG</sup>, and *Pgr*<sup>cre/+<sup>*Mig-6*/<sup>f/+<sup>*Erbb2*/<sup>f/+<sup>*Rosa26<sup>mTmG</sup> were injected with 1 µg/ml of E2 per a day at three times and had a surgical procedure to induce endometriosis. Under anesthesia, a midline abdominal incision was made to expose the uterus in female mice, and one of uterine horn was removed. In a Petri dish containing phosphate-buffered saline (PBS; pH 7.5), the uterine horn was opened longitudinally with scissors. The excised uterine horn was cut into small fragments of about 1 mm<sup>3</sup>, and then injected back into the peritoneum of same mouse. The abdominal incision and wound were closed with sutures and skin was closed with surgical wound clips, respectively. After a designated time, the mice were euthanized, and endometriosis-like lesions were removed using a fluorescence microscope and counted.

**Endometriosis-related Infertility Analysis**

Endometriosis were induced in 8 week old control female mouse recipient (fertile) receiving endometrial fragments from donor control (*Pgr*<sup>cre/+<sup>*Rosa26<sup>mTmG</sup> or *Mig-6*/<sup>d/d<sup>*Rosa26<sup>mTmG</sup> endometrium. A sham surgery group was included as a control. After 1, 2, and 3 months of the endometriosis induction, the mice with endometriotic lesions of control or *Mig-6*/<sup>d/d<sup> were mated with wild-type male mouse and then collected at GD 7.5.

**RNA Isolation And Microarray Analysis**

Total RNA was extracted from the uterine tissues using the RNeasy Total RNA Isolation Kit (Qiagen, Valencia, CA). RNA was pooled from the uteri of more than three mice per genotype at GD 3.5 and microarray analysis was performed using GeneChip® Mouse Genome 430 2.0 Arrays (Affymetrix) as
described previously (Gene Expression Omnibus accession code GSE138185). Array data were analyzed using Bioconductor for quantile normalization. We selected aberrantly expressed genes in the uteri of control, Mig-6/d/d and Mig-6/d/d Erbb2/d/d mice at GD 3.5 using a two-sample comparison according to significant fold change greater than 1.5. Aberrantly expressed genes were classified with canonical pathway analyzed by Ingenuity System Software (Ingenuity Systems Inc.).

**Reverse Transcription - Quantitative PCR**

The complementary DNAs (cDNAs) were synthesized with MMLV Reverse Transcriptase (Invitrogen Crop) according to the manufacturer's instructions. RT-qPCR was performed on cDNA to assess the expression of genes of interest with SYBR Green (Bio-Rad) or TaqMan primers (Applied Biosystems). Experimental gene expression data were normalized against the housekeeping gene, 18S ribosomal RNA. Analysis of mRNA expression was first undertaken by the standard curve method, and results were corroborated by cycle threshold values assessing gene expression. Primer sequences used in these studies are shown in table S4.

**Immunohistochemistry Analyses**

Immunohistochemistry and immunofluorescence analyses were performed as previously described. Briefly, dewaxed hydrated paraffin-embedded tissue sections were pre-incubated with 10% normal goat (for anti-MIG-6, Ki67, Cyclin D1, ErB2, pERK1/2, ERK1/2, MUC1, LTF, and KLF4 antibodies) or donkey (for anti-MCM2, MCM6, and KLF15 antibodies) serum in PBS and then incubated with anti-MIG-6 (1:200 dilution; Customized antibody by Dr. Jeong Lab), anti-Ki67 (1:1000 dilution; #ab15580; Abcam), anti-Cyclin D1 (1:1000 dilution; #eo-RB9041-p0; Thermo Fisher Scientific), anti-ErbB2 (1:200 dilution; #2165; Cell Signaling), anti-pERK1/2 (1:500 dilution; #4370; Cell Signaling), anti-ERK1/2 (1:1000 dilution; #4695; Cell Signaling), anti-MUC1 (1:1000 dilution; #ab15481, Abcam), anti-LTF (1:2000 dilution; #07-682, Millipore Corp.), anti-MCM2 (1:20000 dilution; #sc9839, Santa Cruz Biotechnology), anti-MCM6 (1:20000 dilution; #sc9843; Santa Cruz Biotechnology), anti-KLF4 (1:5000 dilution; #sc20691; Santa Cruz Biotechnology), and anti-KLF15 (1:5000 dilution; #ab2647; Abcam) antibodies in PBS supplemented with 10% normal serum overnight at 4 °C. For immunohistochemistry, the sections were incubated with secondary antibody conjugated to horseradish peroxidase (Vector Laboratories) for one hour at room temperature. Immunoreactivity was detected using diaminobenzidine (DAB-Vector Laboratories) and analyzed using microscopy software from NIS Elements, Inc. (Nikon). A semi-quantitative grading system (H-score) was calculated to compare the immunohistochemical staining intensities. The H-score was calculated using the following equation: H-score = ∑ Pi (i), where i = intensity of staining with a value of 1, 2, or 3 (weak, moderate, or strong, respectively) and Pi is the percentage of stained cells for each intensity, varying from 0 to 100%. The overall score ranged from 0 to 300.

**Western Blot Analysis**
Western blot analyses were performed as described previously\textsuperscript{59}. Proteins were extracted using lysis buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2.5 mM EDTA, and 0.125% Nonidet P-40 (vol/vol)) supplemented with both a protease inhibitor cocktail (Roche, Indianapolis, IN) and a phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Protein lysates were electrophoresed via SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Membrane was blocked with Casein (0.5% w/v) in PBS with 0.1%Tween 20 (v/v; Sigma-Aldrich) prior to exposure to anti-ErbB2 (#2165; Cell Signaling, Danvers, MA), anti-EGFR (#2646; Cell Signaling), anti-phospho-ERK1/2 (pERK1/2; #4370; Cell Signaling), anti-ERK1/2 (#4695; Cell Signaling), anti-MIG-6 (Customized antibody by Dr Jeong Lab) or anti-β-actin (#sc1616; Santa Cruz Biotechnology) antibodies diluted to 1:1000. Immunoreactivity was visualized by incubation with a horseradish peroxidase-linked secondary antibody followed by exposure to Electrochemiluminescence reagents (ECL) according to manufacturer's instructions (GE Healthcare Biosciences).

**Statistical Analysis**

No statistical method was used to predetermined sample size for in vivo studies. Based on prior experience, all experiments used 5 mice per group to achieve adequate statistical power. For all animal experiments, block randomization was used to ensure a balance in sample size across groups. The investigators were blinded during the evaluation of results variations in the group. For all animal experiments, over three biological replicates were analyzed for each condition, and results are presented as the mean ± SEM. For data with only two groups, Student's t test was used. For data containing more than two groups, an analysis of variance (ANOVA) test was used, followed by Tukey test for pairwise t-test. $p < 0.05$ was considered statistically significant. All statistical analyses were performed using the Instat package from GraphPad. The original data are provided in table S5.

**Data Availability**

All data are available in the manuscript or the supplementary material. The accession number for microarray generated in this study is GSE138185.

**Declarations**

**Competing interests:**

The authors declare that they have no competing interests.

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Author contributions:

H.-G.Y. and J.-W.J. were responsible for the concept of the study; A.T.F. collected baboon samples; S.L.Y. and B.A.L. collected human samples; J.-Y.Y. and T.H.K. carried out experiments; J.-Y.Y., T.H.K. and J.-H.S. analyzed data; U.M. provided transgenic mice; R.M.M. contributed to write the manuscript. All authors contributed to the final manuscript version.

Ethics declarations

Competing interests: The authors declare that they have no competing interests.

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