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Mig-6 regulates endometrial genes involved in cell cycle and progesterone signaling

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ABSTRACT

Mitogen inducible gene 6 (*Mig-6*) is an important mediator of progesterone (P4) signaling to inhibit estrogen (E2) signaling in the uterus. Ablation of *Mig-6* in the murine uterus leads to the development of endometrial hyperplasia and E2-induced endometrial cancer. To identify the molecular pathways regulated by *Mig-6*, we performed microarray analysis on the uterus of ovariectomized *Mig-6*$^{f/f}$ and *PGR*$^{cre/+}$*Mig-6*$^{f/f}$ (*Mig-6*$^{d/d}$) mice treated with vehicle or P4 for 6 h. The results revealed that 772 transcripts were significantly regulated in the *Mig-6*$^{d/d}$ uterus treated with vehicle as compared with *Mig-6*$^{f/f}$ mice. The pathway analysis showed that *Mig-6* suppressed the expression of gene-related cell cycle regulation in the absence of ovarian steroid hormone. The epithelium of *Mig-6*$^{d/d}$ mice showed a significant increase in the number of proliferative cells compared to *Mig-6*$^{f/f}$ mice. This microarray analysis also revealed that 324 genes are regulated by P4 as well as *Mig-6*. *Cited2*, the developmentally important transcription factor, was identified as being regulated by the P4-*Mig-6* axis. To determine the role of *Cited2* in the uterus, we used the mice with *Cited2* that were conditionally ablated in progesterone receptor-positive cells (*PGR*$^{cre/+}$*Cited2*$^{f/f}$; *Cited2*$^{d/d}$). Ablation of *Cited2* in the uterus resulted in a significant reduction in the ability of the uterus to undergo a hormonally induced decidual reaction. Identification and analysis of these responsive genes will help define the role of P4 as well as *Mig-6* in regulating uterine biology.

1. Introduction

The uterus is an important hormone-responsive reproductive organ in mammals. The ovarian steroid hormones progesterone (P4) and estrogen (E2) are essential mediators of reproductive events associated with the establishment and maintenance of pregnancy [1,2]. E2 stimulates the proliferation of both uterine luminal and glandular epithelium [3]. In contrast, P4 inhibits E2-mediated proliferation of the luminal and glandular epithelium [4,5]. P4 is a critical regulator of reproductive events associated with embryo implantation, decidualization of the endometrial stromal cells and maintenance of pregnancy [2,6]. The physiological effects of P4 are mediated through its cognate receptor, the progesterone receptor (PGR) [7]. The fertility defects exhibited by the progesterone receptor knockout (PRKO) mice unequivocally demonstrated the critical importance of P4 and its receptor in the establishment and maintenance of pregnancy [2,8].

Progestin is a synthetic progesterone. Progestin has been used in the conservative endocrine treatment of early endometrial cancer patients in order to preserve their fertility, as well as in palliative treatment of advanced-stage patients [9,10]. Interruption of P4

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signaling, occurring from the loss of the PGR itself or through the loss of its interacting partners or downstream effectors, leads to a physiological state of P4 resistance [11]. P4 resistance is seen in a wide variety of diseases. P4 resistance is a hallmark of endometriosis [12,13]. P4 resistance is also seen in the endometrium of women with polycystic ovary syndrome (PCOS) [14,15]. Expression of the progesterone receptor (PGR) was known to be positively correlated with a good prognosis and response to progestin treatment [16]. However, more than 30% of patients with progestin treatment did not respond to progestin due to de novo or acquired progestin resistance [9,17,18]. The mechanism of progestin resistance is still unknown. Understanding the precise mechanism of P4 regulation in the endometrium is of critical importance in developing therapeutic approaches to alleviate this women's health crisis.

Mig-6 has been shown to be critical for uterine functioning because conditional ablation of Mig-6 ($PGR^{cre/+}Mig\text{-}6^{f/f}$; $Mig\text{-}6^{d/d}$) in the mouse uterus results in infertility due to a defect of embryo implantation [19,20]. Additionally, $Mig\text{-}6^{d/d}$ mice leads to the development of animals with epithelial hyperplasia, adenoma and adenocarcinomas in organs, such as the uterus, lung, gallbladder and bile duct [19,21–23]. Endometrial tumorigenesis is accelerated by double ablation of *Mig-6* and *Pten* compared to single ablation of *Mig-6* or *Pten* [20]. However, the precise mechanism of *Mig-6* in endometrial cancer remains poorly understood.

Here, we identified *Mig-6* regulated uterine genes using $Mig\text{-}6^{d/d}$ mice in combination with high density DNA microarray analysis. This analysis indicates that *Mig-6* plays an important role in uterine functioning by modulating the regulation of cell cycle related genes and the ability of P4 to regulate specific genes. The results of our investigation provide significant insights into our understanding of the importance of steroid hormone regulation in female reproduction and endometrial cancer.

2. Materials and methods

2.1. Animals and tissue collection

Mig-6 "floxed" ($Mig\text{-}6^{f/f}$) and $PGR^{cre/+}Mig\text{-}6^{f/f}$ ($Mig\text{-}6^{d/d}$) mice [22,24] were maintained in the designated animal care facility according to the Michigan State University institutional guidelines. All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University. Eighteen $Mig\text{-}6^{f/f}$ and 18 $Mig\text{-}6^{d/d}$ mice were ovariectomized at 6 weeks of age. After 2 weeks of rest, vehicle (sesame oil) or P4 (in sesame oil; Sigma–Aldrich, St. Louis, MO; 1 mg/mouse in 100 μl) was administered into ovariectomized mice via s.c. injection. At 6 h following the P4 or vehicle injection, mice were euthanized for tissue collection. $Cited2^{f/f}$ mice [25] were bred with PGR-Cre mice [24] to generate $PGR^{cre/+}$ $Cited2^{f/f}$ ($Cited2^{d/d}$) mice. Uterine tissues were immediately frozen at the time of dissection and stored at −80 °C for RNA extraction or fixed with 4% (v/v) paraformaldehyde for immunohistochemistry.

2.2. 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 3 mice per genotype and treatment. All RNA samples were analyzed with a Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE) before microarray hybridization. Microarray data analysis was performed as previously described [26]. To adjust arrays to a common baseline using invariant set normalization, DNA-Chip Analyzer dChip was used and the perfect match (*PM*) model described by Li and Wong [27] was used to estimate expression. We selected differentially expressed genes within each treatment in the $Mig\text{-}6^{f/f}$ and $Mig\text{-}6^{d/d}$ 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 difference between group means greater than 80. Differentially expressed genes were classified according to canonical pathway analyzed by Ingenuity System Software (Ingenuity Systems Inc., Redwood City, CA).

2.3. Quantitative real-time PCR analysis

Total RNA was extracted from the uterine tissues using the RNeasy total RNA isolation kit (Qiagen, Valencia, CA). The expression levels of mRNA were measured by real-time PCR TaqMan analysis using an Applied Biosystems StepOnePlusTM system (Applied Biosystems, Foster City, CA) and real-time PCR SYBR Green detection system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. mRNA quantities were normalized against the housekeeping gene, 18S RNA. Primer sequences used in these studies are shown in Supplementary Table 1.

2.4. Immunohistochemistry

Immunohistochemistry analysis was performed as previously described [20]. Uterine sections from paraffin-embedded tissue were preincubated with 10% normal serum in phosphate-buffered saline (PBS) and incubated with anti-KI67 (ab15580; Abcam, Cambridge, MA) antibody in 10% normal serum in PBS. On the following day, sections were washed in PBS and incubated with a secondary antibody (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Immunoreactivity was detected using the Vectastain Elite DAB kit (Vector Laboratories).

2.5. Induction of decidualization

The hormonally induced decidual response has been previously described [28]. The ovariectomized $Cited2^{f/f}$ and $Cited2^{d/d}$ female mice were subjected to the following hormonal regimen: 100 ng of E2 per day for three days; two days rest; then three daily injections of 1 mg of P4 plus 6.7 ng of E2. At 6 h following the third P4 and E2 injection, the left uterine horn was mechanically stimulated by scratching the full length of the anti-mesometrial side with a burred needle. The other horn was left unstimulated as a control. Daily injections of P4 (1 mg/mouse) plus E2 (6.7 ng/mouse) were continued for 5 days to maximize the decidual response. The mice were sacrificed on day 5. The uteri were then excised, weighed and fixed in 4% paraformaldehyde for histological analysis.

2.6. Statistical analysis

For data with two groups, Student's t test was used. For data containing more than two groups, one way ANOVA was used, followed by Tukey's post hoc multiple range. All data are presented as means ± SEM. $p < 0.05$ was considered statistically significant. All statistical analyses were performed using the Instat package from GraphPad (San Diego, CA, USA).

3. Results

3.1. Mig-6 suppresses cell cycle progression

Previously, $Mig\text{-}6^{d/d}$ mice were infertile due to an inability of the uterus to undergo embryo implantation [19,20]. To identify the molecular pathways regulated by *Mig-6* in a steroid-hormone independent and dependent manner, we performed high-density

DNA microarray analysis on the uteri of ovariectomized *Mig-6*$^{f/f}$ and *Mig-6*$^{d/d}$ mice treated with vehicle or progesterone (P4) for 6 h. The 772 genes were identified as differentially expressed when comparing the nonhormone-stimulated *Mig-6*$^{f/f}$ to *Mig-6*$^{d/d}$ uterus (Supplementary Table 2). This difference could be accounted for by the impact of *Mig-6* in the uterus. To determine which pathways are regulated by *Mig-6* ablation, we performed pathway analysis using Ingenuity Systems Software. The altered pathways included G1/S checkpoint regulation, G2/M DNA damage checkpoint regulation, cell cycle regulation, ILK signaling, p53 signaling, HIF1α signaling, and Wnt/β-catenin signaling (Supplementary Table 3). Among these, we observed an increase in genes necessary for cell cycle progression in the *Mig-6*$^{d/d}$ uterus compared to *Mig-6*$^{f/f}$. To validate our microarray analysis, we determined the transcript levels on uteri of *Mig-6*$^{f/f}$ and *Mig-6*$^{d/d}$ mice treated with vehicle for 6 h. The mRNA expression level of *Ccnd1*, *Ccnd2*, *E2f3*, and *Rbl1*, cell cycle G1/S checkpoint regulation genes, *Ccnb2*, *Cdk1*, *Chek1*, *Cks1b*, *Cks2*, and *Top2a*, cell cycle G2/M DNA damage checkpoint regulation genes, and *Ccna2* and *Ppp2r2b*, cell-cycle-related genes were significantly increased and *Rprm* was significantly decreased in the *Mig-6*$^{d/d}$ uteri as compared with *Mig-6*$^{f/f}$ (Fig. 1). Immunohistochemical staining of KI67 as a proliferation marker showed that proliferation was significantly increased in the epithelium of *Mig-6*$^{d/d}$ mice compared with *Mig-6*$^{f/f}$ mice (Fig. 2A and B). These results suggest that *Mig-6* is a negative regulator of cell cycle progression and epithelial cell proliferation.

3.2. Identification of P4- and Mig-6-regulated genes

Previously, we have shown that *Mig-6*-ablated mice uteri increased estrogen signaling in the presence of P4, which typically antagonizes ESR activity [19,20]. The overall goal of this investigation was to identify genes that are regulated by the P4-*Mig-6* axis. A total of 324 genes were common in both vehicle-treated vs. P4-treated control *Mig-6*$^{f/f}$ mice and P4-treated *Mig-6*$^{d/d}$ vs. *Mig-6*$^{f/f}$ mice (Fig. 3A). Of the 324 genes, 107 genes were more highly expressed in the P4 treated control uteri and 217 genes decreased. Of the 107 genes, 39 genes were markedly decreased in the P4-treated *Mig-6*$^{d/d}$ mice compare to P4-treated *Mig-6*$^{f/f}$ mice (Fig. 3B). A complete list is presented in Supplementary Table 4. To validate our microarray analysis, we performed qPCR analysis on the uteri of *Mig-6*$^{f/f}$ and *Mig-6*$^{d/d}$ mice treated with vehicle or P4 for 6 h. The transcript levels of FK506 binding protein 5 (*Fkbp5*), interleukin 13 receptor, alpha 2 (*Il13ra2*), Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (*Cited2*), and genes regulated by estrogen in breast cancer protein (*Greb1*) were significantly increased in the uteri of *Mig-6*$^{f/f}$ mice treated with P4 compared to vehicle. However, these inductions were significantly decreased in the *Mig-6*$^{d/d}$ mice treated with P4 compared to *Mig-6*$^{f/f}$ mice (Fig. 3C).

3.3. Cited2 is required for uterine decidualization

Cited2 is a member of the Cited transcription coactivator family that binds the p300/corticosterone-binding protein coactivator and a P4- target gene. To determine the role of *Cited2* in the uterus, we generated the mice with *Cited2* conditionally ablated in progesterone receptor-positive cells (*PGR*$^{cre/+}$*Cited2*$^{f/f}$; *Cited2*$^{d/d}$) [24,25]. P4 signaling is crucial for decidualization of the endometrial stromal cells for successful pregnancy [29]. To determine whether decidualization of the uterus is affected by *Cited2* ablation, we examined the ability of *Cited2*$^{d/d}$ mice to undergo a well-characterized induced decidualization after artificial hormonal induction. Ovariectomized female *Cited2*$^{f/f}$ and *Cited2*$^{d/d}$ mice were provided with E2+P4, and the uterus mechanically stimulated to mimic the presence of an implanting embryo and induce decidualization. First, we checked the transcript levels of *Cited2* in uteri from *Cited2*$^{f/f}$ and *Cited2*$^{d/d}$ mice after artificially induced decidualization by real-time PCR Analysis. *Cited2* mRNA expression was significantly lower in *Cited2*$^{d/d}$ mice compared with *Cited2*$^{f/f}$ mice in the uterine horn with/without stimuli (Fig. 4A). *Cited2*$^{f/f}$ mice showed a decidual uterine horn that responded well to stimuli for artificial induction. However, *Cited2*$^{d/d}$ mice displayed remarkable reduction in the decidual response (Fig. 4B). Weight ratio of stimulated to control horn was significantly decreased in *Cited2*$^{d/d}$ mice compared to *Cited2*$^{f/f}$ control mice (Fig. 4C). Histological analysis confirmed that well-developed decidual cells were detected in the decidual uterine horn of *Cited2*$^{f/f}$ control mice, but differentiated decidual stromal cells were decreased in the decidual uterine horn of *Cited2*$^{d/d}$ mice (Fig. 4D). In addition, the expression of the known decidualization marker, bone morphogenetic protein 2 (*Bmp2*), was reduced in the decidual uterine horn of the *Cited2*$^{d/d}$ mice compared with the decidual uterine horn of *Cited2*$^{f/f}$ control mice (Fig. 4E). Collectively, the uteri of *Cited2*$^{d/d}$ mice were impaired to undergo a hormonally induced decidual reaction. These results

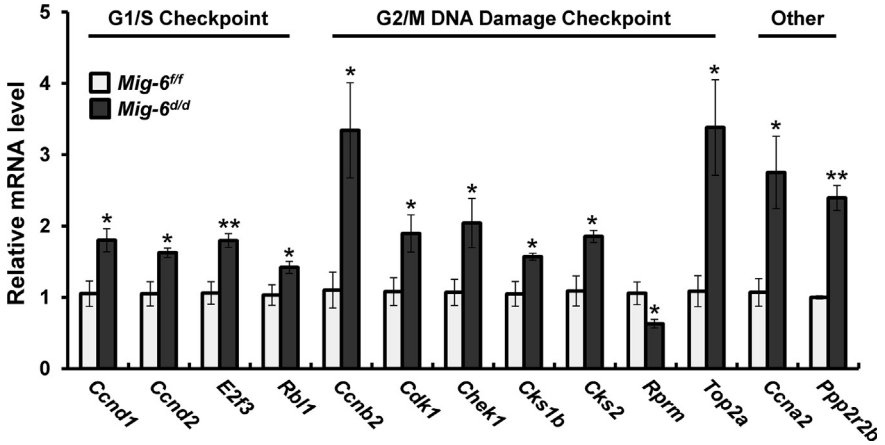

Fig. 1. Regulation of cell cycle genes and epithelial proliferation by *Mig-6*. Validation of *Mig-6* regulated genes in the murine uterus. Real-time RT-PCR analysis were performed on uteri of *Mig-6*$^{f/f}$ and *Mig-6*$^{d/d}$ mice treated with vehicle for 6 h. The results represent the mean ± SEM. *p < 0.05 and **p < 0.01.

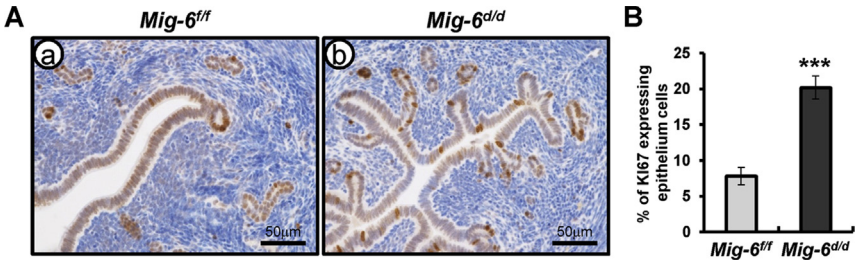

Fig. 2. A) Immunohistochemical analysis of KI67 as a proliferation marker in uteri of *Mig-6*$^{f/f}$ (a) and *Mig-6*$^{d/d}$ (b) mice treated with vehicle for 6 h B) Quantification of KI67 positive cells in epithelium cells. The results represent the mean ± SEM. ***p < 0.001.

suggest that *Cited2* plays an important role for decidualization as a P4 and *Mig-6* target gene.

4. Discussion

The ablation of *Mig-6* increased proliferation of endometrial epithelial cells [19,30]. However, the molecular mechanism of *Mig-6* has not been studied in uterine biology. In this study, we have identified *Mig-6*- and P4-regulated uterine genes using the *Mig-6*$^{d/d}$ mouse and high-density DNA microarray analysis. The design of our microarray analysis was to determine the role of *Mig-6* in the uterus. This analysis identified P4-dependent as well as -independent genes whose expression was altered by *Mig-6* ablation. The choice of conducting the microarray analysis on uteri isolated for 6 h after a single dose of P4 was for the purpose of identifying direct targets of P4 activity. Previously, we showed that *Mig-6* is a P4 signaling mediator that suppresses E2 signaling in the uterus [19,20,31]. However, the majority of genes are altered in ovariectomized *Mig-6*$^{d/d}$ mice treated with vehicle (772 genes). Our microarray analysis showed an increase in genes necessary for cell cycle progression, such as cell cycle G1/S checkpoint regulation genes, cell cycle G2/M DNA damage checkpoint regulation genes, and cell-cycle-related genes in the *Mig-6*$^{d/d}$ uterus compared to *Mig-6*$^{f/f}$. Furthermore, proliferation of luminal epithelium was significantly increased in the uterus of *Mig-6*$^{d/d}$ mice treated with vehicle. These results showed that *Mig-6* has an intrinsic hormone-independent tumor suppressor role in cell cycle progression and epithelial cell proliferation.

This microarray analysis identified 324 genes in which the ablation of *Mig-6* impacted the P4 regulation of genes in ovariectomized mice given a pharmacological dose of P4. We compared and analyzed direct P4-regulated genes and P4 regulated genes in *Mig-6* response to identify the P4-responsive transcriptome that require *Mig-6*. The genes whose regulation by P4 is altered by *Mig-6* ablation reflect metabolic proteins, cell cycle, structural proteins, transcription factors, and transport protein. Investigation of the expression pattern of these genes during natural pregnancy combined with functional analysis of the role of these genes in the establishment and maintenance of pregnancy will determine the significance of the *Mig-6* regulation of these genes during pregnancy. The 39 of 324 genes showed *Mig-6* dependent P4-regulation in the uterus. As validation of our microarray analysis, the transcriptional regulation of several P4 responsive genes such as *Fkbp5*, *Il13ra2*, *Cited2*, and *Greb1* [31,32] are significantly attenuated in the uterus of the *Mig-6*$^{d/d}$ mice compared to *Mig-6*$^{f/f}$. Therefore, the identification of the precise mechanism of P4-PGR regulation in the endometrium is crucial for understanding the causes of impairments in proper uterine functioning.

Cited2 is a member of the Cited (CBP/p300-Interacting Transactivators with glutamic acid (E)/aspartic acid (D)-rich C-terminal domain) transcription coactivator family. CITED2 can bind to DNA binding proteins, such as LHX2, TFAP2, PPARα, PPARγ, and Smads, recruit the histone acetyltransferases CBP/p300, and act as a transcriptional regulator [33–36]. *Cited2* is induced by the P4-PGR signaling and expressed in the uterus during early pregnancy [31]. *Cited2* is identified as a *Mig-6* target gene in our microarray analysis. However, the function of *Cited2* in reproductive biology is elusive. To explore the function of *Cited2* in the uterus, we generated uterine specific *Cited2* ablation mice since targeted deletion of

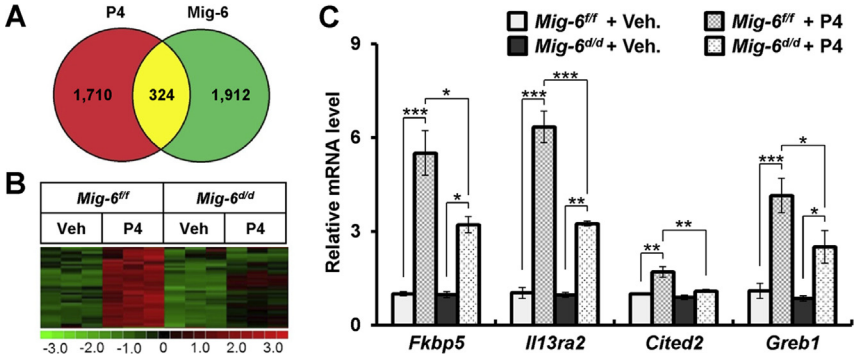

Fig. 3. Validation of *Mig-6* and progesterone regulated genes in the mouse uterus. A) Venn diagrams demonstrating the relationship between genes modulated in the *Mig-6*$^{f/f}$ and *Mig-6*$^{d/d}$ uterus in response to acute treatment with P4. Red circles indicate genes selected by vehicle-treated *Mig-6*$^{f/f}$ vs P4-treated *Mig-6*$^{f/f}$; Green, by P4-treated *Mig-6*$^{f/f}$ vs P4-treated *Mig-6*$^{d/d}$. The numbers, displayed within the intersections of the circles indicate the common genes by two comparisons. B) Clustering analysis of *Mig-6* dependent induced genes by P4 ($p < 0.05$, 1.2-fold change) in uteri. The extent of gene expression changes is represented by a green-red color scale (green: low expression and red: high expression). C) Quantitative real time PCR analysis of transcript levels of *Mig-6* dependent induced genes by P4 in *Mig-6*$^{f/f}$ and *Mig-6*$^{d/d}$ mice treated with vehicle or P4 for 6 h. The results represent the mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.

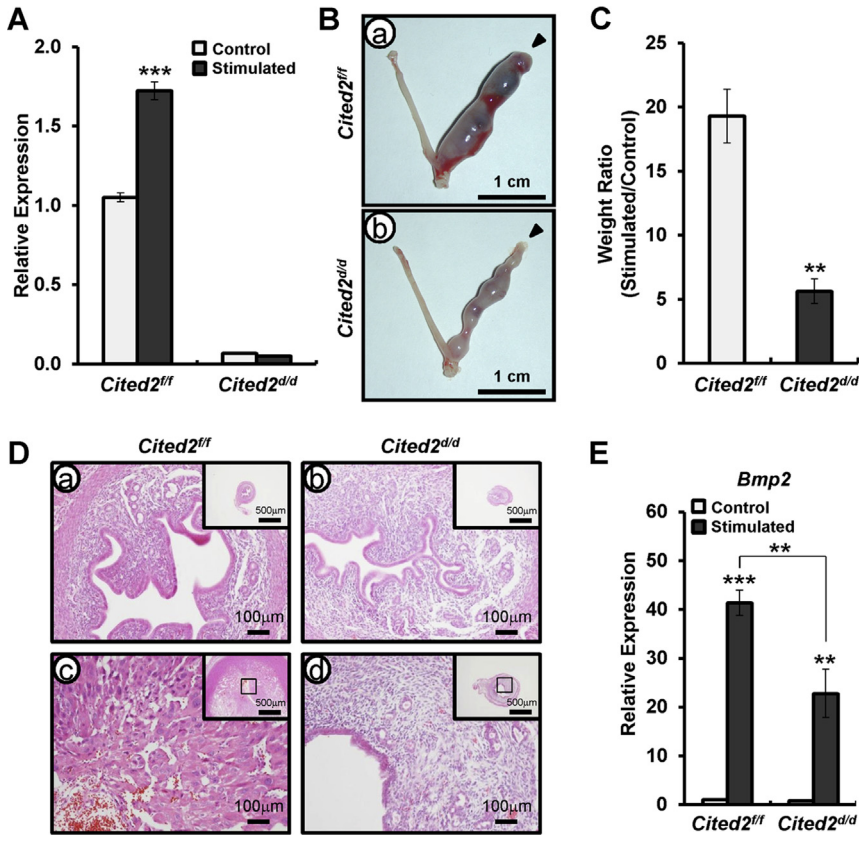

Fig. 4. Decidual defect of *Cited2*$^{d/d}$ mice. A) Quantitative real time PCR analysis of transcript levels of *Cited2* in uteri from *Cited2*$^{f/f}$ and *Cited2*$^{d/d}$ mice after artificially induced decidualization. Ovariectomized mice were primed with E2 plus P4, and one uterine horn was mechanically stimulated to mimic the presence of an implanting embryo and induce decidualization. The other horn was unstimulated and served as a control. The results represent the mean ± SEM. ***p < 0.001. B) Decidualization response of *Cited2*$^{d/d}$ mice. Gross morphology of the uteri of *Cited2*$^{f/f}$ (a) and *Cited2*$^{d/d}$ (b) mice after artificially induced decidualization. C) Ratio between the weight of stimulated and control horn collected from *Cited2*$^{f/f}$ and *Cited2*$^{d/d}$ mice. Results represent means ± SEM of 3 animals per group. **p < 0.01. D) Histology of uteri was investigated by H&E staining. E) Quantitative real time PCR analysis of *Bmp2* as a decidual differentiation marker in uteri of *Cited2*$^{f/f}$ and *Cited2*$^{d/d}$ mice after artificially induced decidualization. The results represent the mean ± SEM. **p < 0.01, and ***p < 0.001.

Cited2 results in embryonic lethality [35]. We show that *Cited2*$^{d/d}$ mice show a decrease in the uterine response to P4 during in the decidual reaction. Our results suggest that *Cited2* plays an important role in P4-dependent decidualization.

In summary, the *Mig-6*-dependently regulated target genes have been identified by microarray analysis. Epithelial cell proliferation of the ovarian steroid hormone independently increased in *Mig-6*$^{d/d}$ mice. The uteri of *Mig-6*$^{d/d}$ mice showed an increase of gene expression including cell cycle G1/S checkpoint regulation genes, cell cycle G2/M DNA damage checkpoint regulation genes, and cell-cycle-related genes. The alteration of P4-responsive genes in *Mig-6*$^{d/d}$ mice suggest a dual *Mig-6* function as a positive regulator as well as negative regulator for P4 functioning. The results of our investigation provide significant insights into our understanding of the importance of *Mig-6* in pregnancy and female reproduction.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.146.

Transparency document

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