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A calcium-dependent phospholipase A2 (cPLA2) expression is regulated by MIG-6 during endometrial tumorigenesis.

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Abstract

The ovarian steroid hormones, estrogen (E2) and progesterone (P4), are essential regulators of uterine biology. The imbalance of these ovarian steroid hormones leads to uterine diseases such as endometrial cancer, endometriosis, and infertility. Mitogen-inducible gene 6 (MIG-6) is an adaptor protein. MIG-6 mediates P4 signaling and acts as a tumor suppressor during endometrial tumorigenesis in both humans and mice. In previous studies, we developed the conditional knockout of *Mig-6* in all uterine compartments (*Pgr*$^{cre/+}$*Mig-6*$^{f/f}$; *Mig-6*^{KO}) and endometrial epithelial cell-specific *Mig-6* knockout (*Sprr2f*$^{cre/+}$*Mig-6*$^{f/f}$; *Mig-6*$^{Ep\text{-}KO}$) mice. Both mouse models developed endometrial hyperplasia and E2-dependent endometrial cancer. P4 treatment significantly decreases aberrant epithelial proliferation and AKT signaling in *Mig-6*$^{Ep\text{-}KO}$ mice but not in *Mig-6*^{KO} mice. In the present study, we identified a calcium-dependent phospholipase A2 (*cPla2*) as one of the genes down-regulated by *Mig-6* in the uterus. We performed immunohistochemistry and Western Blot analysis to investigate the regulation of cPLA2 by MIG-6 as well as determine the expression patterns of cPLA2 in the uterus. While the expression of cPLA2 was stronger at the uterine epithelial cells of *Mig-6*^{KO} and *Mig-6*$^{Ep\text{-}KO}$ mice compared to control mice, P4 suppressed the expression of cPLA2 in *Mig-6*$^{Ep\text{-}KO}$ mice but not in *Mig-6*^{KO} mice. To determine the ovarian steroid hormone regulation of cPLA2, we examined the expression of cPLA2 in ovariectomized control, *Mig-6*^{KO}, *Mig-6*$^{Ep\text{-}KO}$, and PRKO mice treated with P4 or E2. After P4 treatment, cPLA2 expression was remarkably reduced in *Mig-6*$^{Ep\text{-}KO}$ mice but not in *Mig-6*^{KO} mice. However, the expression of cPLA2 was not changed in PRKO mice. Our results identified cPLA2 as a novel target of MIG-6 in the murine uterus and identified its important role during endometrial tumorigenesis.

Keywords

cPLA2; MIG-6; Uterus; Endometrial hyperplasia

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1. Introduction

Endometrial cancer (EC) is the most common gynecological cancer [1]. Most EC cases occur in post-menopausal women, but 20~25% of case can occur in pre-menopausal women [2]. Incidence of EC is expected to rise due to increasing rates of obesity and diabetes which are known risk factors for EC in younger women [3]. A hysterectomy is the standard management procedure of EC that enables tumor removal. However, a hysterectomy is not an option for some EC patients, including those who are extremely obese with related cardiovascular disease, organ failure due to diabetes mellitus, ventilation difficulties, and elderly patients exposed to a high surgical risk [4, 5]. Additionally, at least 20–30% of the young women with early EC might be eligible for the fertility sparing approach [6]. Therefore, there is a tremendous need for nonsurgical approaches to EC, in order to help women looking for an alternative treatment due to high-risk surgical conditions or those looking to preserve their fertility [7, 8].

Endometrioid endometrial cancer (EEC), the most common type of EC (80–85%), is associated with or preceded by abnormal multiplication of endometrial epithelial cells, known as complex atypical hyperplasia (CAH) [9]. EEC's major pathologic phenomenon is the loss of ovarian steroid hormone control [10]. Therapies with progesterone (P4) or similar drugs (called progestins) have been used for reproductive-aged women with early-stage, well-differentiated EEC in cases where surgery is not an option or in recurrent or advanced EEC. However, many studies recommend limiting the use of P4 therapy for EEC due to its low response rates [11, 12].

Steroid hormone regulation is known to be influential in endometrial cancer development. Chronic estrogen (E2) exposure has been found to increase endometrial carcinogenesis through promotion of proliferation and transformation of cells [13]. Alternatively, P4 has been shown to inhibit cell growth and invasion of cancer cells, increasing apoptosis of differentiated cells [14]. Progesterone receptors (PGR) allow P4 to repress endometrial epithelial proliferation [15]. MIG-6 is a P4-responsive gene in the human and mouse endometrium [16, 17]. Mitogen-inducible gene 6 (*MIG-6*) is a 50 kDa adaptor protein and its decreased expression is observed in human endometrial cancer [16]. After P4 treatment, aberrant activation of AKT signaling and epithelial proliferation were significantly decreased in the uteri of P4-responsive ($Mig\text{-}6^{Ep\text{-}KO}$) and control mice. However, our P4-resistant ($Mig\text{-}6^{KO}$) mice did not show any P4 effect in the uterus [18, 19]. Therefore, *Mig-6* mediates P4 action to suppress endometrial epithelial cell proliferation through inhibiting AKT phosphorylation [18].

While there are no current approved targeted therapies available for endometrial cancer, many clinical trials have aimed to target inhibition of cell signaling pathways involved with cell growth and proliferation, including the PI3K/PTEN/AKT pathway [20]. Studies that analyze the PI3K and PTEN pathways suggest the importance of phospholipids in cell polarity and migration [21], leading to the importance of the PLA2 enzymes. The PLA2 superfamily is composed of hydrolytic enzymes that free membrane fatty acids, producing lysophospholipids and arachidonic acid from gylcerophospholipds, which is crucial in the formation of bioactive lipid signaling molecules [22].

cPLA2 is a calcium-dependent phospholipase [23] which has a critical role in regulation of phospholipids on the plasma membrane [24]. Following changes in intracellular calcium, cPLA2 translocate from the cytoplasm of a cell to the endoplasmic reticulum or nuclear envelope, leading to phosphorylation by MAP kinase, where it then mobilizes arachidonic acid [25]. There are six isoforms of the cPLA2 enzyme, with cPLA2α being the most widely studied [24]. Overexpression of the cPLA2α isoform is associated with promotion of cell invasion and metastasis through the PI3K/AKT signaling pathway [26]. Current understanding of steroid hormonal regulation of cPLA2 is limited. Several studies have indicated that E2 alone has no effect on cPLA2 expression [27, 28], while P4 has shown to be a potential inhibitor of cPLA2 expression [29]. PLA2 expression was found to be elevated in endometrial cancerous tissue compared to normal endometrium tissue [30]; however, little is known about its role in endometrial cancer. In this study, we identified that cPLA2 expression is negatively regulated by *Mig-6* in the endometrial hyperplasia, and its expression is independently regulated by PGR.

2. Materials and Methods

2.1 Animals and tissue collection

Mice were cared for and used in the designated animal care facility according to Michigan State University's institutional guidelines. All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University. We used *Sprr2f*$^{cre+}$*Mig-6*$^{f/f}$ (*Mig-6*$^{Ep\text{-}KO}$) and *Pgr*$^{cre/+}$ *Mig-6*$^{f/f}$ (*Mig-6*^{KO}) mouse models [18, 31] to investigate the P4 resistance. For the study of P4 responsiveness, vehicle (beeswax) or P4 (40 mg/pellet) pellets were placed subcutaneously into control (*Mig-6*$^{f/f}$), *Mig-6*^{KO}, and *Mig-6*$^{Ep\text{-}KO}$ mice respectively at 8 weeks of age for 3 months (n=5/treatment/genotype).

For the study of steroid hormone regulation, control, progesterone knock-out (PRKO), *Mig-6*^{KO}, and *Mig-6*$^{Ep\text{-}KO}$ mice at 8 weeks of age were ovariectomized. After this, there was a 2-week period of rest, enabling the mice to heal. The ovariectomized mice were utilized for injection, in which mice received vehicle (sesame oil; Sigma–Aldrich, St. Louis, MO), P4 (10mg/mL; Sigma–Aldrich, St. Louis, MO), or both E2 and P4 (1 μg/mL E2, 10 mg/mL P4). Following the time line (either 6 hours treatment, or 3 days treatment), mice were euthanized, and uterine tissues were immediately fixed with 4% (v/v) paraformaldehyde for immunohistochemistry and stored at −80°C for Western Blot.

2.2 Immunohistochemistry

Immunohistochemistry analyses were performed as previously executed [18]. Concisely, uterine sections were exposed to anti-CPLA2 (CS-5249S; 98 kDa; Cell Signaling, Danvers, MA). The sections were then exposed to anti-rabbit secondary antibody solution (Vector Laboratories, Burlingame, CA) and then incubate in horseradish peroxidase (ThermoFisher Scientific, Waltham, MA). The signal was detected by the Vectastain Elite DAB kit (Vector Laboratories, Burlingame, CA). Normal rabbit immunoglobulin G (IgG) was performed as a negative control to validate cPLA2 antibody. The H-score was calculated as previous reported [18] as a semi-quantitative grading system.

2.3 Western blot Analysis

Isolation of uterine tissue and western blot were performed as described previously [18]. Briefly, total 20μg protein was loaded onto gels and transferred to membrane. The membrane were incubated with 0.5% (w/v) casein (Sigma Aldrich) for 3 hours and then treated used anti-cPLA2 (CS-5249S; Cell Signaling, Danvers, MA). Anti-actin (SC-1615, Santa Cruz Biotechnology, Dallas, TX) were used for loading controls. The band density was determined by relative densitometry using Image J (National Institude of Health, USA), and normalized against the bands obtained for actin.

2.4 Statistical analysis

Statistical analyses were performed using one-way ANOVA analysis, Tukey's post hoc multiple range test using the Instat package from GraphPad (San Diego, CA). $p<0.05$ was considered statistically significant.

3. Results

3.1 cPLA2 is increased in endometrial hyperplasia.

Conditional knockout of *Mig-6* in all uterine compartments ($Pgr^{cre/+}Mig\text{-}6^{f/f}$; $Mig\text{-}6^{KO}$) [16] and endometrial epithelial cell-specific *Mig-6* knockout ($Sprr2f^{cre/+}Mig\text{-}6^{f/f}$; $Mig\text{-}6^{Ep\text{-}KO}$) [19] mice developed CAH and E2-dependent EEC. Previously, we reported the endometrial hyperplastic phenotype with aberrant activation of AKT signaling in *Mig-6* knockout (P4-responsive ($Mig\text{-}6^{Ep\text{-}KO}$) and P4-resistant ($Mig\text{-}6^{KO}$)) mice [18, 32]. cPLA2α is an important member of the cPLA2 family [33] and is associated with promotion of cell invasion and metastasis through the PI3K/AKT signaling pathway [26]. Therefore, we first examined expression of cPLA2 in uteri with endometrial hyperplasia of $Mig\text{-}6^{KO}$ and $Mig\text{-}6^{Ep\text{-}KO}$ mice. Immunohistochemical analysis indicated that levels of cPLA2 were remarkably increased in the endometrial epithelial cells of $Mig\text{-}6^{KO}$ and $Mig\text{-}6^{Ep\text{-}KO}$ mice (mean H-score ± SEM; 208.00 ± 5.83 and 175.00 ± 10.25, respectively) compared to control mice (63.00 ± 24.32) at 5 months of age (Fig. 1). However, the expression of cPLA2 was not detected in the stromal cells. Furthermore, the signal was not detected in IgG staining, supporting the specific staining of cPLA2 (Fig.1). These data suggest that *Mig-6* negatively regulates cPLA2 expressions in endometrial epithelial cells of mouse uterus.

3.2 cPLA2 expression is negatively regulated by P4 in the endometrial hyperplasia with epithelial Mig-6 deficiency.

P4 therapy prevents the development of endometrial cancer associated with unopposed E2 by blocking E2 actions [34]. P4 treatment significantly decreases aberrant epithelial proliferation and AKT signaling in $Mig\text{-}6^{Ep\text{-}KO}$ mice [18], but not in $Mig\text{-}6^{KO}$ mice. In order to assess the effect of P4 treatment on $Mig\text{-}6^{KO}$ and $Mig\text{-}6^{Ep\text{-}KO}$ mice, we placed P4 or vehicle pellets into the control, $Mig\text{-}6^{KO}$, and $Mig\text{-}6^{Ep\text{-}KO}$ mice subcutaneously at 2 months of age. After 3 months of the P4 treatment, control and $Mig\text{-}6^{Ep\text{-}KO}$ mice (mean relative intensity ± SEM; 0.69 ± 0.05 and 1.14 ± 0.09, respectively) showed significantly lower expression of cPLA2 compared to $Mig\text{-}6^{KO}$ mice (3.15 ± 0.49), while vehicle-treated $Mig\text{-}6^{KO}$ mice (2.71 ± 0.20) exhibited no differences compared to vehicle-treated mice (2.99

± 0.27) by Western Blot analysis (Fig. 2A and 2B). In addition, immunohistochemical analysis revealed an increase of cPLA2 expression in *Mig-6*^{KO} (225.00 ± 5.70) and *Mig-6*$^{Ep\text{-}KO}$ (188.00 ± 16.48) mice compared to control (85.00 ± 32.37) but no difference between *Mig-6*^{KO} and *Mig-6*$^{Ep\text{-}KO}$ mice after vehicle treatment. Interestingly, the levels of cPLA2 were remarkably lower in the endometrial epithelial cells of control and *Mig-6*$^{Ep\text{-}KO}$ mice (69.00 ± 7.81 and 49.00 ± 12.59, respectively) compared to *Mig-6*^{KO} mice (189.00 ± 7.31) after P4 treatment. These data suggest that P4 suppresses the expression of cPLA2 in endometrial epithelium of *Mig-6*$^{Ep\text{-}KO}$ mice but not *Mig-6*^{KO} mice.

3.3 The expression of cPLA2 is down-regulated in E2 plus P4 3 days treated P4-responsive not P4-resistant mice.

Mig-6$^{Ep\text{-}KO}$ mice showed that E2 plus P4 treatment led to significant reduction of the uterine weight gain compared to E2 treatment, but there were no differences in *Mig-6*^{KO} mice indicating P4 resistance in *Mig-6*^{KO} mice [31]. Therefore, we next determined whether P4 antagonizes the effect of E2 in cPLA2 expression. Ovariectomized control, *Mig-6*^{KO}, and *Mig-6*$^{Ep\text{-}KO}$ mice were treated with either the vehicle or E2 plus P4 for 3 days. Western Blot analysis showed strong cPLA2 expression in *Mig-6*^{KO} mice (vehicle vs. E2 plus P4; 4.85 ± 0.50 vs. 6.54 ± 0.41) compared to control (vehicle vs. E2 plus P4; 1.00 ± 0.17 vs. 3.08 ± 0.63) after vehicle or E2 plus P4 treatment (Fig. 3A and 3B). The immunohistochemistry showed that vehicle treated *Mig-6*^{KO} and *Mig-6*$^{Ep\text{-}KO}$ samples showed relatively higher expression patterns (172.00 ± 49.86 and 212.00 ± 14.54, respectively), with expression presence in the luminal and glandular epithelium only, compared to control (13.00 ± 11.79). After 3 days of E2 plus P4 treatment, expression within the luminal and glandular epithelium were significantly lower in control and *Mig-6*$^{Ep\text{-}KO}$ mice (46.00 ± 26.57 and 117.00 ± 19.21, respectively) compared to *Mig-6*^{KO} mice (197.00 ± 6.04) as results observed in Western Blot analysis. The results suggest that cPLA2 expression is negatively regulated by P4 in P4-responsive *Mig-6*$^{Ep\text{-}KO}$ mice.

3.4 The expression of cPLA2 is independently regulated by PGR in the uterus

P4 effects are mediated by the progesterone receptor (PGR) [35]. To examine whether cPLA2 expression is regulated by PGR, immunohistochemistry was performed on ovariectomized control, *Mig-6*^{KO}, and progesterone receptor knockout (PRKO) mice treated with either the vehicle or P4 for 6 hours (Fig. 4). The cPLA2 expression was highly detected in the uteri of *Mig-6*^{KO} mice (vehicle vs. P4; 188.00 ± 48.72 and 234.00 ± 22.93, respectively) compared with control (vehicle vs. P4; 14.00 ± 11.66 and 9.00 ± 5.34, respectively) and PRKO (vehicle vs. P4; 15.00 ± 8.37 and 14.00 ± 8.57, respectively) mice. These results suggest that cPLA2 expression is *Mig-6* dependent.

4. Discussion

Mig-6 is an antiproliferative gene allowing it to function as a tumor suppressor in humans [36]. Previously, we reported that endometrial epithelial cells exhibited a hyperplastic phenotype in response to aberrant activation of AKT signaling that is observed in *Mig-6* knockout mice [32]. In order to investigate the regulatory affect *Mig-6* has on cPLA2, we analyzed cPLA2 expression in control (*Mig-6*$^{f/f}$), *Mig-6*^{KO}, and *Mig-6*$^{Ep\text{-}KO}$ mouse models.

We found a significant increase in cPLA2 expression in the epithelial cells of both the *Mig-6*$^{Ep\text{-}KO}$ and *Mig-6*^{KO} mouse models when compared to controls at 5 months. This result shows that *Mig-6* is important in cPLA2 suppression and furthering development of hyperplasia. The observed dysregulation of cPLA2 acts through aberrant promotion of cell invasion and metastasis through PI3K/AKT activation in order to contribute to hyperplasia and ultimately endometrial cancer development [26]. cPLA2 acts as an oncogene through its respective activation and elevation of COX-2, which is known to be associated with EEC [37]. Our mouse models help to then connect the expression of *Mig-6*, cPLA2, and COX-2 to furthered endometrial cancer progression, helping to elucidate a potential pathway at play in endometrial cancer development.

It is well known that both ovarian steroid hormones P4 and E2 are critical in the uterine activity as well as the regulation of epithelial-stromal crosstalk through their respective receptors [38]. An imbalance in steroid hormone regulation can ultimately lead to aberrant endometrial proliferation, furthering hyperplasia development, and endometrial cancer development [34]. P4 therapy is known to prevent further development of endometrial cancer that is associated with chronic E2 expression through its blocking of E2 actions [34]. However, many studies recommend limiting use of P4 therapy for endometrial cancer due to its low response rates [11, 12]. Critical barriers exist in studying endometrial cancer, including a lack of potential biomarkers for P4 response, as well as few preclinical animal models for translational study. In order to attempt to overcome these barriers, we utilized P4-resistant (*Mig-6*^{KO}) and P4-responsive (*Mig-6*$^{Ep\text{-}KO}$) mouse models and examined hormonal regulation of a potential biomarker, cPLA2, for hyperplasia as well as endometrial cancer development. Our results revealed a decrease of cPLA2 expression in response to P4 treatment in *Mig-6*$^{Ep\text{-}KO}$ mice compared to controls. However, there was no difference in cPLA2 expression in *Mig-6*^{KO} mice when compared to controls. The revealed decrease in cPLA2 expression in response to P4 treatment is in accordance with previous results, helping to further confirm the potential role of P4 in inhibition of cPLA2 [29]. The difference in cPLA2 expression exhibited between the two mouse models, *Mig-6*$^{Ep\text{-}KO}$ and *Mig-6*^{KO}, revealing that the effects of P4 are only applicable in *Mig-6*$^{Ep\text{-}KO}$ model. This result suggests the further study for the role cPLA2 plays in hyperplasia development through aberrant PI3K/AKT proliferation. In addition, we found the different cPLA2 expression in random cycling control mice, indicating that further studies are required to understand cycle dependent expression of cPLA2.

To further analyze the role of ovarian steroid hormone regulation on cPLA2 expression, uterine mouse models were ovariectomized, and controlled ovarian steroid hormone treatment was utilized. Our results showed that there was a decrease in cPLA2 expression in both the luminal and glandular epithelium in *Mig-6*$^{Ep\text{-}KO}$ mice, whereas the expression of cPLA2 was not changed in the *Mig-6*^{KO} mouse model. These results indicate that cPLA2 is negatively regulated by P4 in P4-responsive *Mig-6*$^{Ep\text{-}KO}$ mice while cPLA2 is not regulated by P4 in the P4-resistant *Mig-6*^{KO} mice. These results are consistent with current findings of the hormonal regulation of cPLA2, further confirming that the expression of cPLA2 is not E2-dependent, but regulated through progesterone [29].

The PRKO mouse model is incredibly useful in assessing the role of P4 in gene expression profiling and its respective protein expression [39]. We utilized this mouse model in order to observe the expression of cPLA2 in response to ablation of both known PGR isoforms. Through our analysis, we revealed that there was no cPLA2 expression in ovariectomized PRKO or control mice after both vehicle and P4 treatment. These results indicate that PGR does not inhibit the expression of cPLA2 in the mouse uterus. In addition, there was strong epithelial staining in the *Mig-6*^{KO} mouse model with both vehicle and P4 treatment. Therefore, further study for the relationship between cPLA2 and MIG-6 is required to reveal its functions in the uterus.

Overall, these findings show that cPLA2 is a novel target of *Mig-6,* and its regulation is critical to overcome P4 resistance in endometrial hyperplasia. Knockout of *Mig-6* in a complete uterine knockout, as well as a conditional epithelial knockout, leads to an increase in the expression of cPLA2 leading to an increase in cell proliferation as well as all characteristics of cancer progression. P4 treatment helps to lessen the expression of cPLA2; however, this decrease is only exhibited in P4-responsive models. Therefore, understanding the role of cPLA2 will help further translational research in order to overcome P4 resistance in endometrial cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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cPLA2 is a novel target of tumor suppressor gene, Mig-6, in the uterus.

The level of cPLA2 is down-regulated by progesterone in P4 responsive uterus.

cPLA2 expression is critical to overcome P4 resistance in endometrial hyperplasia.

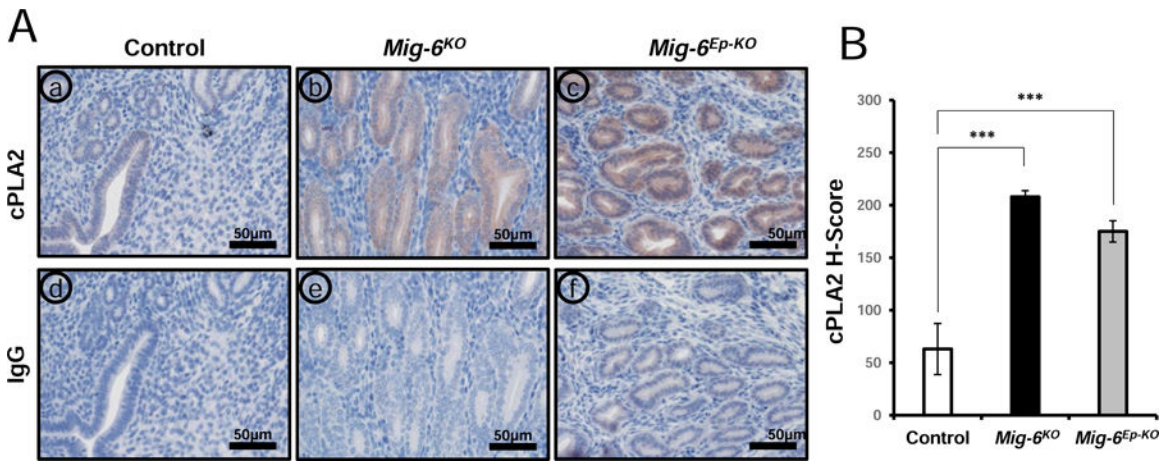

Figure 1. cPLA2 is up-regulated in endometrial hyperplasia in mouse.

(A) Immunohistochemistry analysis revealed that cPLA2 expression is higher in *Mig-6*^{KO} and *Mig-6*$^{Ep\text{-}KO}$ compared to control mice at 5 months of age (a, b, and c). Immunoglobulin G (IgG) were used to validate the specific staining by cPLA2 antibody (d, e, and f). (B) The quantification data of cLPA2 were determined by H-score. The results represent the mean ± SEM. ***, $p < 0.001$.

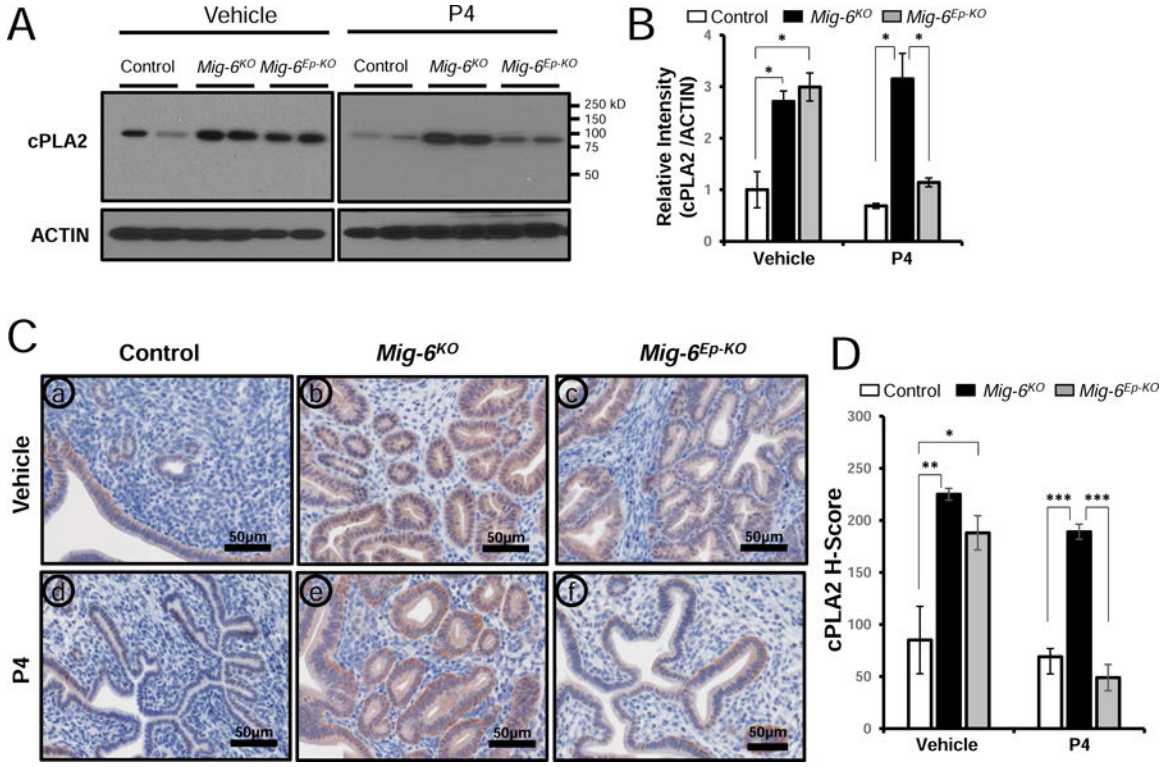

Figure 2. The expression of cPLA2 are down-regulated after P4 treatment in *Mig-6*$^{Ep\text{-}KO}$ mice but not *Mig-6*^{KO} mice.

cLAP2 expression was examined by Western Blot (A) and immunohistochemistry (C) in the uteri of control, *Mig-6*^{KO} and *Mig-6*$^{Ep\text{-}KO}$ mice after vehicle or P4 treatment. The uterine samples were prepared from 8-week old control, *Mig-6*^{KO} and *Mig-6*$^{Ep\text{-}KO}$ mice after vehicle or P4 treatment for 3 months. The quantification data of cPLA2 were determined by relative intensity (B) and H-score (D). The results represent the mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

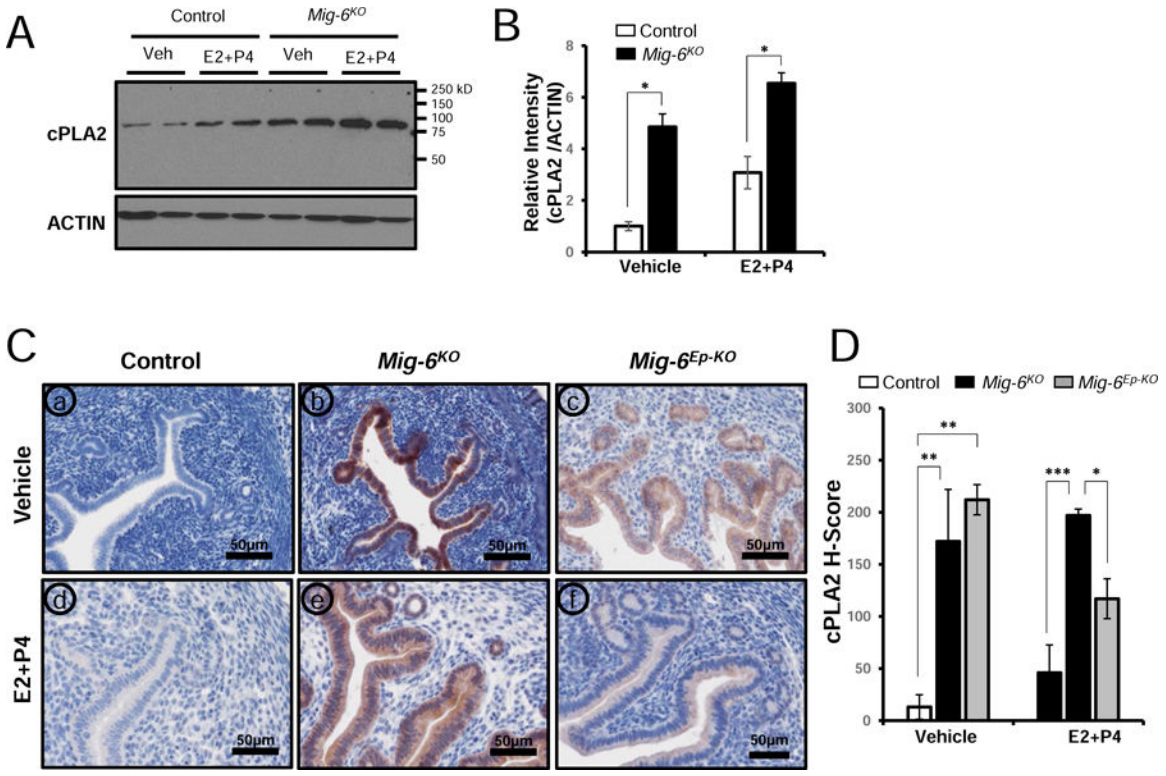

Figure 3. The regulation of cPLA2 expression by E2 plus P4 treatment in the uterus.

The expression of cPLA2 were determined by Western Blot (A) and immunohistochemistry (C) in the uterus from mice treated with vehicle or E2 plus P4 for 3 days. Uterine samples were collected from ovariectomized control, *Mig-6*^{KO} and *Mig-6*$^{Ep\text{-}KO}$ mice after vehicle or E2 plus P4 for 3 days. The quantification data of cPLA2 were determined by relative intensity (B) and H-score (D). The results represent the mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

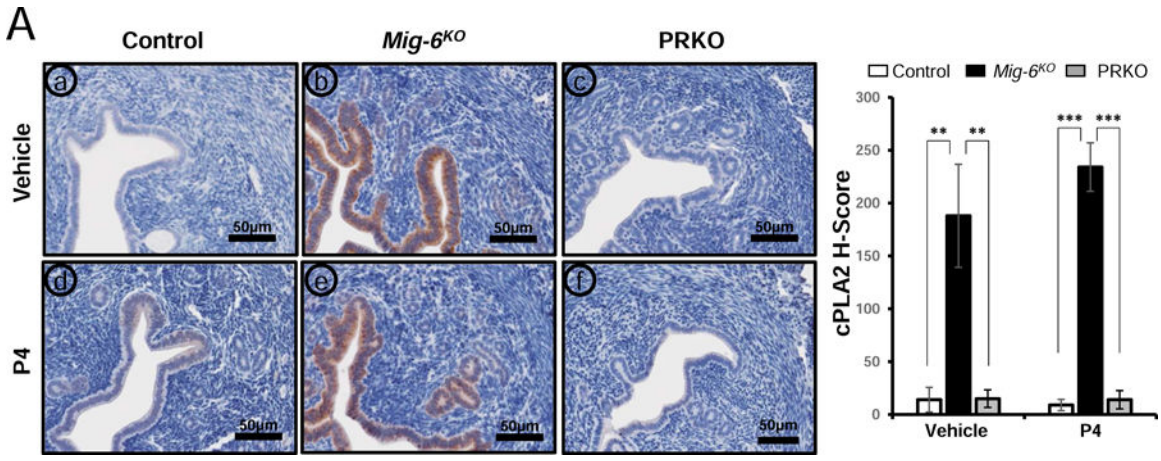

Figure 4. The cPLA2 expression is independent of PGR expression.

Immunohistochemistry analysis showed the cPLA2 expression is higher in uteri of *Mig-6*^{KO} (b and e) mice compared with control (a and d) and PRKO (cand f) mice after vehicle or P4 treatment for 6 hours. The expression of cPLA2 were not detected in control and PRKO mice. The samples were obtained from uteri of ovariectomized control, *Mig-6*^{KO} and *Mig-6*$^{Ep\text{-}KO}$ mice treated with vehicle or P4 for 6 hours. (B) The quantification data of cPLA2 were determined by H-score. The results represent the mean ± SEM. **, $p < 0.01$; ***, $p < 0.001$.