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The synergistic effect of *Mig-6* and *Pten* ablation on endometrial cancer development and progression

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Abstract

Ablation of *Mig-6* in the murine uterus leads to the development of endometrial hyperplasia and estrogen-induced endometrial cancer. An additional endometrial cancer mouse model is generated by ablation of *Pten* (either as heterozygotes or by conditional uterine ablation). To determine the interplay between *Mig-6* and the PTEN/PI3K signaling pathway during endometrial tumorigenesis, we have generated mice with *Mig-6* and *Pten* conditionally ablated in progesterone receptor positive cells ($PR^{cre/+}Mig\text{-}6^{f/f}Pten^{f/f}$; $Mig\text{-}6^{d/d}Pten^{d/d}$). The ablation of both *Mig-6* and *Pten* dramatically accelerated the development of endometrial cancer compared to single ablation of either gene. The epithelium of $Mig\text{-}6^{d/d}Pten^{d/d}$ mice showed a significant decrease in the number of apoptotic cells compared to $Pten^{d/d}$ mice. The expression of the estrogen-induced apoptotic inhibitors *Birc1* was significantly increased in the $Mig\text{-}6^{d/d}Pten^{d/d}$ mice. We identified ERK2 as a MIG-6 interacting protein by co-immunoprecipitation and demonstrated that the level of ERK2 phosphorylation was increased upon *Mig-6* ablation either singly or in combination with *Pten* ablation. These results suggest that *Mig-6* exerts a tumor suppressor function in endometrial cancer by promoting epithelial cell apoptosis through the down-regulation of the estrogen-induced apoptosis inhibitors *Birc1* and the inhibition of ERK2 phosphorylation.

Keywords

Mig-6; *Pten*; Uterus; Endometrial cancer

Introduction

Endometrial cancer is the most frequently diagnosed malignancy of the female genital tract. According to the National Cancer Institute (NCI), endometrial cancer is the most common

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type of gynecological cancer. In the United States, approximately 41,200 cases are diagnosed and about 7,350 women die from the disease each year (Jemal *et al.*, 2006). The majority of endometrial cancers (~90%) are adenocarcinomas, which originate in uterine epithelial cells. All endometrial cancers can be further delineated into two types (Deligdisch and Holinka, 1987; Di Cristofano and Ellenson, 2007). Type I endometrial cancers are estrogen (E2)-dependent and appear mostly in pre- and peri-menopausal women. Frequently, these cancers show mutations in DNA-mismatch repair genes (MLH1, MSH2, MSH6), PTEN, K-ras and β-catenin (Di Cristofano and Ellenson, 2007). In contrast, Type II endometrial cancers are E2-independent and are diagnosed mostly in post-menopausal women, thin and fertile women, or women with normal menstrual cycles.

PTEN (phosphatase and tensin homologue deleted from chromosome 10) is one of the most frequently mutated tumor suppressor genes in human cancers (Steck *et al.*, 1997). PTEN is completely lost or mutated in >50% of primary endometrioid endometrial cancers (Sun *et al.*, 2001) and in at least 20% of endometrial hyperplasias, the precancerous lesions of the endometrium (Levine *et al.*, 1998; Sun *et al.*, 2001). Thus, loss of PTEN is a very early event in the multi-step process leading to endometrioid endometrial cancer. PTEN acts as a negative regulator of phosphoinositide 3-kinases (PI3K) signaling which regulates a number of cellular functions through the activation of Akt (Jiang and Liu, 2008). Previously, loss of *Pten* (either as a heterozygote or by uterine specific ablation) has been shown to induce endometrial cancer in mice highlighting its important role in endometrial cancer development (Daikoku *et al.*, 2008; Vilgelm *et al.*, 2006). This mutation and subsequent Akt activation resulted in the activation of ERα-dependent pathways which play an important role in endometrial cancer tumorigenesis (Vilgelm *et al.*, 2006). Interestingly, the PI3K signaling pathway can also be activated by E2 suggesting a complex interaction exists between these two signaling pathways (Chambliss *et al.*, 2002).

Mig-6 is an immediate early response gene that can be induced by various mitogens and commonly occurring chronic stress stimuli (Makkinje *et al.*, 2000; Saarikoski *et al.*, 2002). It is an adaptor molecule containing a CRIB domain, a src homology 3 (SH3) binding domain, a 14-3-3 binding domain and an EGFR binding domain, but no domain with enzymatic activity (Burbelo *et al.*, 1995; Makkinje *et al.*, 2000). Previously, the interaction between MIG-6 and the 14-3-3 proteins has been demonstrated (Makkinje *et al.*, 2000). Ablation of *Mig-6* in mice leads to the development of animals with epithelial hyperplasia, adenoma, and adenocarcinomas in organs, such as the uterus, lung, gallbladder, and bile duct (Anastasi *et al.*, 2005; Ferby *et al.*, 2006; Jeong *et al.*, 2009; Jin *et al.*, 2007; Zhang *et al.*, 2006). Decreased expression of *Mig-6* is observed in human breast carcinomas which correlate with reduced overall survival of breast cancer patients (Amatschek *et al.*, 2004; Anastasi *et al.*, 2005). These data point to *Mig-6* as a tumor suppressor gene in both mice and humans. Previously, we demonstrated that the absence of *Mig-6* in mice results in the inability of P4 to inhibit E2-induced uterine weight gain and expression of E2-responsive target genes (Jeong *et al.*, 2009). $PR^{cre/+}$ $Mig\text{-}6^{f/f}$ ($Mig\text{-}6^{d/d}$) mice develop hyperplasia and endometrial cancer in a hormone-dependent manner. Additionally, the observation that endometrial carcinomas from women have a significant reduction in *MIG-6* expression provides compelling support for an important growth regulatory role for *Mig-6* in the uterus

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of both humans and mice (Jeong *et al.*, 2009). This demonstrates that *Mig-6* is a critical regulator of the tumorigenesis of endometrial cancer. However, the mechanism of *Mig-6* action in endometrial cancer remains unknown.

In this study, we utilized conditional *Pten* and *Mig-6* ablation in the uteri of mice to demonstrate a synergistic effect of dysregulation of the *Pten* and *Mig-6* signaling pathways during endometrial tumorigenesis. Ablation of both genes dramatically accelerated the development of endometrial cancer compared to single mutation of either gene. Thus, these results demonstrate the importance of *Pten* and *Mig-6* regulation in the tumorigenesis of endometrial cancer by promoting epithelial cell apoptosis.

Results

Generation of mice with Pten and Mig-6 ablation in the murine uterus

The most common genetic mutations in human endometrioid carcinoma are found in the *Pten* gene (Di Cristofano and Ellenson, 2007; Podsypanina *et al.*, 1999). $Pten^{+/-}$ and mice with *Pten* conditionally ablated in the uterus ($PR^{cre/+}Pten^{f/f}$; $Pten^{d/d}$) develop endometrioid endometrial adenocarcinoma (Daikoku *et al.*, 2008; Lian *et al.*, 2006). In order to investigate the effects of the MIG-6 and the PTEN/PI3K/AKT signaling pathways on uterine tumorigenesis, mice with *Pten* floxed ($Pten^{f/f}$) (Lesche *et al.*, 2002) and *Mig-6* floxed ($Mig\text{-}6^{f/f}$) (Jin *et al.*, 2007) were bred to the PR^{Cre} mouse model (Soyal *et al.*, 2005) to generate ablation of *Pten* and *Mig-6* in the uterus. Ablation of *Pten* and *Mig-6* ($Mig\text{-}6^{d/d}Pten^{d/d}$) was assayed by real-time RT-PCR, Western blot and immunohistochemical analysis (n=3). *Mig-6* mRNA expression was detected in the control (WT, $PR^{cre/+}$, $Mig\text{-}6^{f/f}$, and $Pten^{f/f}$) uteri while not in the $Mig\text{-}6^{d/d}$ and $Mig\text{-}6^{d/d}Pten^{d/d}$ uteri (Fig. 1A). There was no effect on *Mig-6* expression by *Pten* ablation. The expression of *Pten* mRNA was detected in the control, but not in the $Pten^{d/d}$ and $Mig\text{-}6^{d/d}Pten^{d/d}$ uteri (Fig. 1B). While there was a slight decrease in *Pten* expression in the $Mig\text{-}6^{d/d}$ uteri, it was not significant. The decrease in *Pten* expression correlated with a decrease in protein expression as observed both by western blot and immunohistochemical analysis (Fig. 1 C and D). Ablation of *Pten* also resulted in increased activation of AKT as expected (Fig. 1C). These results suggest that PR^{cre} efficiently ablated *Pten* and *Mig-6* in the mouse uterus.

Endometrial cancer development in mice with Pten and Mig-6 ablation in PR-expressing cells

Previously, ablation of *Pten* in the uterus was shown to decrease survival due to the development of endometrial cancer (Daikoku *et al.*, 2008). Therefore, we first examined the lifespan of control, $Mig\text{-}6^{d/d}$, $Pten^{d/d}$ and $Mig\text{-}6^{d/d}$ $Pten^{d/d}$ mice. The survival time of $Mig\text{-}6^{d/d}$ $Pten^{d/d}$ mice was significantly shorter compared with control, $Mig\text{-}6^{d/d}$ and $Pten^{d/d}$ mice ($p < 0.0001$; Fig. 2A). To investigate the impact of *Pten* and *Mig-6* ablation on endometrial cancer development and progression, control, $Mig\text{-}6^{d/d}$, $Pten^{d/d}$ and $Mig\text{-}6^{d/d}$ $Pten^{d/d}$ mice were sacrificed at 2 and 4 weeks of age and uterine weight, gross and histological morphology were examined (n=8 per genotype per age). $Pten^{d/d}$ and $Mig\text{-}6^{d/d}$ $Pten^{d/d}$ mice showed a significant increase in uterine weight at 2 weeks of age compared to control and $Mig\text{-}6^{d/d}$ mice (Fig. 2B). The uterine weight of $Mig\text{-}6^{d/d}$ $Pten^{d/d}$ mice was

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significantly increased compared to other mice including *Pten*$^{d/d}$ mice at 4 weeks of age (Fig. 2B and C). Gross morphology at 4 weeks of age showed that the ablation of *Mig-6* and *Pten* dramatically accelerated the development of endometrial cancer compared to single ablation of either gene (Fig. 2C). Histological analysis demonstrated that the uteri of *Pten*$^{d/d}$ and *Mig-6*$^{d/d}$*Pten*$^{d/d}$ mice exhibit a similar endometrial hyperplastic phenotype at 2 weeks of age (Fig. 2D). The *Mig-6*$^{d/d}$*Pten*$^{d/d}$ mice developed endometrial adenocarcinoma at 4 weeks of age characterized by neoplastic endometrial glands invading through the myometrium (Fig. 2F). However, the *Pten*$^{d/d}$ mice still exhibited endometrial hyperplasia at 4 weeks of age (Fig. 2D). Endometrial adenocarcinoma with invasion into the myometrium was observed in the *Pten*$^{d/d}$ mice at 2 months of age (Daikoku *et al.*, 2008). While endometrial hyperplasia and adenocarcinoma were observed, myometrial hyperplasia was not observed in the uteri of *Mig-6*$^{d/d}$*Pten*$^{d/d}$ mice. The *Mig-6*$^{d/d}$*Pten*$^{d/d}$ mice displayed distant metastases into the ovary (Fig. 2G), diaphragmatic skeletal muscle (Fig. 2H), lymph node, colon, and pancreas. These results suggest that *Mig-6* plays an important role as a suppressor of the development of endometrial cancer caused by *Pten* ablation.

Mig-6 exerts a propaoptotic effect as an endometrial cancer tumor suppressor

In order to determine if the endometrial hyperplasia and cancer in the *Pten*$^{d/d}$ and *Mig-6*$^{d/d}$ *Pten*$^{d/d}$ mice is caused by an alteration in cell proliferation and/or apoptosis, we performed immunohistochemical staining for phospho-histone H3, a mitotic marker, and cleaved caspase 3, an apoptotic marker, in mice at 2 weeks of age. Immunohistochemical staining of phosphohistone H3 demonstrated that proliferation was significantly increased in the epithelium of *Pten*$^{d/d}$ and *Mig-6*$^{d/d}$*Pten*$^{d/d}$ compared to control and *Mig-6*$^{d/d}$ mice (Fig. 3 A and B) . However, no significant difference was observed between the *Pten*$^{d/d}$ and *Mig-6*$^{d/d}$*Pten*$^{d/d}$ mice. Immunohistochemical staining of cleaved caspase 3 showed a significant decrease in the number of apoptotic cells in the epithelium of *Mig-6*$^{d/d}$*Pten*$^{d/d}$ mice compared to *Pten*$^{d/d}$ mice (Fig. 3 C and D). There was also no difference in stromal cell proliferation and apoptosis. These results suggest that *Mig-6* acts as a tumor suppressor to induce apoptosis when *Pten* is mutated.

Mig-6 represses Birc1 expression in the murine uterus

The decision as to whether or not a cell undergoes apoptosis is determined by the opposing actions of pro- and anti-apoptotic effectors (Song and Santen, 2003). It is known that E2 can tip this balance toward cell survival in uterine epithelial cells by inducing the expression of baculoviral inhibitors of apoptosis repeat-containing 1 (*Birc1*), a family of anti-apoptotic proteins (Yin *et al.*, 2008). To determine if *Mig-6* promotes uterine epithelial apoptosis by suppressing *Birc1* expression, the expression of *Birc1a, Birc1b, and Birc1e* was determined in the *Mig-6*$^{f/f}$ and *Mig-6*$^{d/d}$ mice treated with E2 for 3 days by real-time RT-PCR (n=3). Interestingly, the expression of *Birc1* genes was significantly increased in the *Mig-6*$^{d/d}$ mice treated with E2 compared to *Mig-6*$^{f/f}$ mice (Fig. 4A). These results suggest that *Mig-6* induces uterine epithelial apoptosis via down-regulation of *Birc1* expression. We also examined the expression of *Birc1a, Birc1b, and Birc1e* in control, *Mig-6*$^{d/d}$, *Pten*$^{d/d}$, and *Mig-6*$^{d/d}$*Pten*$^{d/d}$ mice at 2 weeks of age (n=3) (Fig. 4B). The expression of *Birc1a* and *Birc1b* but not *Birc1e* was significantly increased in *Mig-6*$^{d/d}$*Pten*$^{d/d}$ mice compared to the

other groups. While the expression of *Birc1a, Birc1b, and Birc1e* was slightly increased in *Pten*$^{d/d}$ mice compare to control and *Mig-6*$^{d/d}$ mice, the increase was not significant. These results suggest that *Mig-6* exerts a tumor suppressor function by inducing uterine epithelial apoptosis through the suppression of *Birc1* expression in tumorigenic conditions such as unopposed E2 action or *Pten* ablatxion.

The Interaction of MIG-6 with ERK2 and its regulation of ERK2 phosphorylation

Although these results have established *Mig-6*'s role in steroid hormone regulation and tumorigenesis, the molecular mechanism of *Mig-6* action is not clear. As MIG-6 is an adaptor molecule, we have turned to a biochemical and proteomics approach to identify MIG-6 associating proteins in order to gain insight into its mechanism of action. One of the hallmarks of endometrial cancer is the loss of ovarian steroid hormone (E2 and P4) control over uterine epithelial cell proliferation and apoptosis (Franco *et al.*, 2008; Ito *et al.*, 2007). E2 promotes endometrial cancer by stimulating proliferation and inhibiting apoptosis while P4 antagonizes these actions of E2 in the uterus. Our previous results showed that the absence of *Mig-6* in mice results in the inability of P4 to inhibit E2-induced uterine weight gain and E2-responsive target genes expression (Jeong *et al.*, 2009). These results suggest that *Mig-6* suppresses E2 signaling in the presence of P4. Therefore, we isolated endogenous MIG-6 protein complexes using an anti-MIG-6 antibody (Sigma-Aldrich, St. Louis, MO) from the uteri of *Mig-6*$^{f/f}$ and *Mig-6*$^{d/d}$ mice treated with E2+P4 for 3 days and identified associated proteins using mass spectrometry. Proteins that were identified from lysates prepared by immunoprecipitation in the *Mig-6*$^{f/f}$ mice but not in the *Mig-6*$^{d/d}$ mice were considered true interacting proteins. The use of the *Mig-6*$^{d/d}$ tissue serves as the control for non-specific and cross-reacting proteins. Using this criterion, the identified MIG-6 interacting proteins are listed in Table 1.

14-3-3 proteins are known MIG-6-associating proteins (Zhang and Vande Woude, 2007) that regulate the phosphorylation of proteins involved in PTEN/PI3K/AKT signaling (Kakinuma *et al.*, 2008; Slaets *et al.*, 2008). We also found novel MIG-6 associated molecules such as signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinase 2 (ERK2), and growth factor receptor bound protein 2 (GRB2). ERK2 is a classical MAPK that is activated mainly by growth factors or mitogenic stimuli. It is activated by phosphorylation which results in its translocation into the nucleus where it phosphorylates transcription factors (Eldredge *et al.*, 1994; Fukuda *et al.*, 1997). It has been reported that ERK affects apoptosis by promoting the expression of inhibitor of apoptosis proteins (IAPs) (Erhardt *et al.*, 1999; Tashker *et al.*, 2002; Xia *et al.*, 1995). To validate the interaction between MIG-6 and ERK2 proteins, we performed coimmunoprecipitation experiments using lysates from the uteri of *Mig-6*$^{f/f}$ and *Mig- 6*$^{d/d}$ mice treated with E2+P4 for 3 days. Co-immunoprecipiatation was performed with anti-IgG, anti-MIG-6 and anti-ERK2 antibodies, and analyzed by Western blot analysis using anti-ERK2 antibodies to detect ERK2. ERK2 protein could be detected from immunoprecipitates with the anti-MIG-6 and anti-ERK2 antibodes confirming the interaction between MIG-6 and ERK2 (Fig. 5A). These results indicate that the interaction between MIG-6 and ERK2 may play an important role in the regulation of the phosphorylation of ERK2 and subsequent regulation of apoptosis.

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To determine if the hyperplasia phenotypes observed may be due to altered ERK2 phosphorylation in the *Mig-6*$^{f/f}$ uteri, we examined the expression of ERK2 by Western blot analysis in the *Mig-6*$^{f/f}$ uteri at 5 months of age. The level of phospho-ERK2 but not total ERK2 was increased in *Mig-6*$^{d/d}$ uteri compared to *Mig-6*$^{f/f}$ uteri (Fig. 5B). We also observed an increased level of phospho-ERK2 but not total ERK2 in *Mig-6*$^{d/d}$ and *Mig-6*$^{d/d}$ *Pten*$^{d/d}$ mice compared to control and *Pten*$^{d/d}$ mice (Fig. 5C).

In order to determine if the uteri of *Mig-6*$^{d/d}$ *Pten*$^{d/d}$ exhibited altered ERK2 signaling, control, *Mig-6*$^{d/d}$, *Pten*$^{d/d}$ and *Mig-6*$^{d/d}$ *Pten*$^{d/d}$ mice were sacrificed at 2 weeks of age (n = 6) and the expression of ERK2 target genes, *Fos* (FBJ osteosarcoma oncogene) (Kyriakis and Avruch, 2001), *Junb* (Jun-B oncogene) (Gesty-Palmer *et al.*, 2005), *Ptgs2* (prostaglandin-endoperoxide synthase 2; *Cox2*) (Smith *et al.*, 2000), *Gdf15* (growth differentiation factor 15) (Malathi *et al.*, 2005), *Vegfa* (vascular endothelial growth factor A) (Milanini-Mongiat *et al.*, 2002), *F3* (coagulation factor III) (Gesty-Palmer *et al.*, 2005) and *Serpine1* (serine (or cysteine) peptidase inhibitor, clade E, member 1) (Gesty-Palmer *et al.*, 2005), was examined. The expression of these ERK2 target genes was not altered in *Mig-6*$^{d/d}$ mice compared to control mice. Interestingly, *Mig-6*$^{d/d}$ *Pten*$^{d/d}$ uteri showed significantly increased expression of these ERK2 target genes compared to *Pten*$^{d/d}$ uteri (Fig. 5D). These results suggest that *Mig-6* plays a tumor suppressor function in the context of *Pten* ablation by promoting epithelial cell apoptosis and by inhibiting ERK2 phosphorylation.

Discussion

Endometrial cancer is the most common gynecological cancer and has been shown to be associated with mutations in the tumor suppressor gene *Pten* among others (Di Cristofano and Ellenson, 2007). Loss of *PTEN* is an early event in the multi-step process leading to endometrioid endometrial cancer. *Pten*$^{+/-}$ and mice with *Pten* conditionally ablated in the uterus (*Pten*$^{d/d}$) develop endometrioid endometrial adenocarcinoma (Daikoku *et al.*, 2008; Lian *et al.*, 2006). This mutation and subsequent Akt activation play an important role in the tumorigenesis of endometrial cancer (Vilgelm *et al.*, 2006). The expression of *MIG-6* is decreased in human endometrial cancer patients and *Mig-6*$^{d/d}$ mice develop invasive endometrioid-type endometrial adenocarcinoma with unopposed estrogen treatment (Jeong *et al.*, 2009). Introduction of *Mig-6* ablation into the *Pten*$^{d/d}$ mice accelerated the tumorigenesis of endometrial cancer as compared to *Pten* ablation alone (Fig. 2). The neoplastic endometrial glands in the double mutant mice invaded through the uterine muscle wall and, with age, led to the development of distant metastases. These results suggest that the tumor suppressor function of *Mig-6* is important to prevent the development of endometrial hyperplasia or endometrial cancer in the tumorigenic conditions of unopposed estrogen or *Pten* loss.

It is well known that endometrioid endometrial cancer is an estrogen-dependent disease and progestin hormone therapy has been used to slow the growth of endometrial cancer due to its inhibitory effects on E2 action. The impact of unopposed E2 treatment on the development of endometrial cancer in the context of *Pten* ablation remains unknown. However, ovariectomized *Pten*$^{d/d}$ mice develop endometrial hyperplasia albeit at a slower rate than

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intact *Pten*$^{d/d}$ mice suggesting that the tumorigenesis of the *Pten*$^{d/d}$ mice is partially steroid hormone dependent (unpublished data). Thus, further investigations into the impact of E2 and E2 plus P4 treatment on the development of endometrial cancer in the *Pten*$^{d/d}$ and *Mig-6*$^{d/d}$ *Pten*$^{d/d}$ mice need to be performed in order to further elucidate the effect of steroid hormone signaling on endometrial cancer development.

Most endometrial cancers are characterized by actively proliferating glands, increased Akt signaling and decreased apoptosis (Ejskjaer *et al.*, 2007; Khalifa *et al.*, 1994; Sivridis and Giatromanolaki, 2004). Loss of *Pten* in the uteri of mice and humans results in increased epithelial proliferation (Fig. 3A and 3B), increased phosphorylation of Akt (Fig. 1C), and altered E2 signaling (Daikoku *et al.*, 2008; Lian *et al.*, 2006; Vilgelm *et al.*, 2006) as well as the development of endometrioid endometrial cancer (Daikoku *et al.*, 2008; Kanamori *et al.*, 2001). *Mig-6*$^{d/d}$ mice exhibit altered E2 signaling (Jeong *et al.*, 2009) and a slight but not significant increase in epithelial proliferation (Fig. 3A and 3B) with no effect on Akt signaling or epithelial apoptosis. The double mutant mice also have comparable levels of epithelial proliferation (Fig. 3A and 3B) when compared to the *Pten*$^{d/d}$ mice. However, in contrast to the *Pten*$^{d/d}$ mice, these mice exhibit dramatically decreased epithelial apoptosis (Fig. 3C and 3D). As these mice develop endometrial cancer earlier than the *Pten*$^{d/d}$ mice, these data suggest that the decreased epithelial apoptosis may contribute to the accelerated tumorigenesis.

As E2 signaling is altered by both *Pten* and *Mig-6* ablation, it suggests that E2 signaling may play a role in the enhanced tumorigenesis of the double mutant mice. Estrogen suppresses uterine epithelial apoptosis by inducing BIRC1 expression (Yin *et al.*, 2008). The *Birc1* genes encode a family of antiapoptotic proteins which can physically interact with active caspase-3 and -7 and with active caspase-9 in the presence of ATP (Davoodi *et al.*, 2004; Maier *et al.*, 2002). The expression of *Birc1a, Birc1b,* and *Birc1e* is significantly increased in *Mig-6*$^{d/d}$ mice treated with E2 (Fig. 4). Also, the expression of *Birc1a* and *Birc1b* was significantly increased in the *Mig-6*$^{d/d}$ *Pten*$^{d/d}$ mice compared to controls (Fig. 4). As these genes are well-known apoptosis inhibitors (Endrizzi *et al.*, 2000; Roy *et al.*, 1995), their increased expression may contribute to the decreased apoptosis of the double mutant mice. Thus, *Mig-6* may act as a tumor suppressor in the context of *Pten* ablation by promoting apoptosis through the expression of the *Birc1* family of proteins.

The estrogen receptors (ERs) mediate the effect of E2 under physiological and pathological conditions either by the activation of E2-target gene transcription (Acconcia and Kumar, 2006) or by nongenomic mechanisms which result in the rapid activation of several signal transduction pathways to regulate different cellular processes, such as proliferation, apoptosis, and differentiation. The nongenomic action of E2 has been linked to numerous pathways (EGFR, IGF-IR, c-MET) resulting in the activation of two key signaling cascades, the PTEN/PI3K/AKT and the MAPK pathways (Bhat-Nakshatri *et al.*, 2008; Cheskis *et al.*, 2008; Freeman *et al.*, 2006; Thomas *et al.*, 2008; Vilgelm *et al.*, 2006). One consequence of this nongenomic E2 action is an inhibition of cellular apoptosis which has been observed in various cell types, such as vascular endothelial, smooth and skeletal muscles, and breast cancer cells (Bjornstrom and Sjoberg, 2005; Boland *et al.*, 2008; Song and Santen, 2003; Spyridopoulos *et al.*, 1997). MAPK/ERK-kinases (MEKs) trigger the activation of ERKs by

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phosphorylating a threonine and a tyrosine in their activation loop. Specificity in the signaling between these modules is achieved by protein-protein interactions and scaffolding molecules (Kolch, 2005). Here, we have identified a novel interaction of MIG-6 with ERK2 and demonstrated that ablation of *Mig-6* leads to increased phosphorylation of ERK2 and expression of its target genes (Fig. 5). Abnormal or constitutive phosphorylation of ERK2 leads to tumorigenesis through inappropriate suppression of apoptosis (Evan and Vousden, 2001; Lowe *et al.*, 2004). Thus, these data suggest the *Mig-6* may also exert its tumor suppressor function in the context of *Pten* ablation by regulating the phosphorylation status of ERK2. This increase in phosphorylation may also contribute to the decreased apoptosis observed either by regulating *Birc1* expression or by an unknown mechanism. These results suggest that *Mig-6* may exert its tumor suppressor function in endometrial cancer by inhibiting ERK2 phosphorylation. Further studies need to be conducted to determine the precise mechanism by which these various pathways are integrated by *Mig-6* to regulate epithelial apoptosis during endometrial tumorigenesis which may lead to the development of additional diagnostics or therapeutics for endometrial cancer.

In conclusion, our results demonstrate the synergistic effect of conditional *Pten* and *Mig-6* loss on endometrial cancer development. This accelerated tumorigenesis is likely due to decreased epithelial apoptosis partly through increased expression of the *Birc1* family of apoptotic inhibitors and increased phosphorylation of ERK2. This study has established an endometrial cancer mouse model which replicates common characteristics of the human disease providing a model system to further investigate the genetic and molecular events involved in the transition from normal to hyperplastic/neoplastic endometrium. These and future results will contribute to the understanding of the molecular mechanism of tumorigenesis and to the development of therapeutic approaches for endometrial cancer.

Materials and methods

Animals and Tissue Collection

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. $PR^{Cre/+}$ mice were previously generated (Soyal *et al.*, 2005). The $Pten^{f/f}$ were acquired from Dr. Hong Wu (University of California, Los Angeles, Los Angeles, CA) (Lesche *et al.*, 2002). Mice of various genotypes were sacrificed at 2 and 4 weeks of age. At the time of dissection, uterine tissues were placed in the appropriate fixative or flash frozen and stored at −80°C. Statistical analysis for the survival curve was performed using the logrank test.

Western Blot Analysis

Samples containing 15 μg proteins were applied to SDS-PAGE. The separated proteins were transferred onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Membranes were blocked overnight with 0.5% casein (wt/vol) in PBS with 0.1% Tween 20 (vol/vol) (Sigma–Aldrich, St. Louis, MO) and probed with anti-MIG-6 (Sigma–Aldrich, St. Louis, MO; PE-16), anti-PTEN (Cell Signaling Technology, Inc., Danvers, MA; #9559), anti-ERK2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; SC-1647), anti-phospho-ERK2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; SC-7883), anti-AKT (Santa Cruz

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Biotechnology, Inc., Santa Cruz, CA; SC-55523), or anti-phospho-AKT (Cell Signaling Technology, Inc., Danvers, MA; #9275S) antibodies. Immunoreactivity was visualized by incubation with a horseradish peroxidase-linked secondary antibody and treatment with ECL reagents. To control for loading, the membrane was stripped and probed with anti-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; SC-1615) and developed again.

Immunohistochemistry

Uterine sections from paraffin-embedded tissue were preincubated with 10% normal serum in PBS (pH 7.5) and then incubated with anti-PTEN (Cell Signaling Technology, Inc., Danvers, MA; #9559), anti-phospho-Histone H3 (Upstate Biotechnology, Lake Placid, NY; 06-570) or anti-cleaved caspase 3 (Cell Signaling Technology, Inc., Danvers, MA; #9661L) antibody in 10% normal serum in PBS (pH 7.5). On the following day, sections were washed in PBS and incubated with a secondary antibody (5 µl/ml; Vector Laboratories, Burlingame, CA) for 1 hr. at room temperature. Immunoreacitivity was detected using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA).

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR analysis was conducted on isolated RNA. Expression levels of *Mig-6, Pten, Birc1a, Birc1b,* and *Birc1e* were measured by Real-time RT-PCR TaqMan analysis (Applied Biosystems, Foster City, CA). cDNA was made from 1 µg of total RNA using random hexamers and M-MLV Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA). RTPCR was performed using RT-PCR Universal Master Mix reagent (Applied Biosystems, Foster City, CA). All real-time RT-PCR results were normalized against 18S RNA using ABI rRNA control reagents. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc multiple range test with the Instat package from GraphPad (San Diego, CA, USA).

Immuno-affinity purification

We isolated endogenous MIG-6 protein complexes using an anti-MIG-6 antibody (Sigma-Aldrich, St. Louis, MO) from lysate prepared from uteri of *Mig-6*$^{f/f}$ and *Mig-6*$^{d/d}$ mice treated with E2+P4 for 3 days as previously reported (Jung *et al.*, 2005). The immunoprecipitates was washed 3 times with NETN (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP- 40) and boiled with Laemmli buffer and subjected to SDS-PAGE (4-20% Tris/Glycine NOVEX Gel, Invitrogen, Carlsbad, CA). The Coomassie brilliant blue-stained protein bands were excised and in gel digested with trypsin.

Protein Identification

Nano-HPLC/MS/MS for peptide identification was carried out as described before (Jung *et al.*, 2008). An 50 mm × 75 um, C18 column (BioBasic C18, 5 µm, 300 Å pore diameter, PicoFrit^{TM}, New objective) was used on-line with a LTQ mass spectrometer (Finnigan LTQ^{TM}, ThermoFinnigan, San Jose, CA). The LTQ were operated in the data-dependant mode acquiring fragmentation spectra of the top 20 strongest ions. Obtained MS/MS spectra were analyzed against modified NCBI-ref protein sequence database using BioWorks database search engine (BioWorksBrowser ver 3.2, Thermo Electron, San Jose, CA). All

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peptide identification with stringent BioWorksBrowser filtering criteria - peptide probability > 1 X 10^{-6} and Xcorr score > 2.0 - was manually examined and all peptides have to be identified by consecutive b- or y- ions so that false identifications were eliminated.

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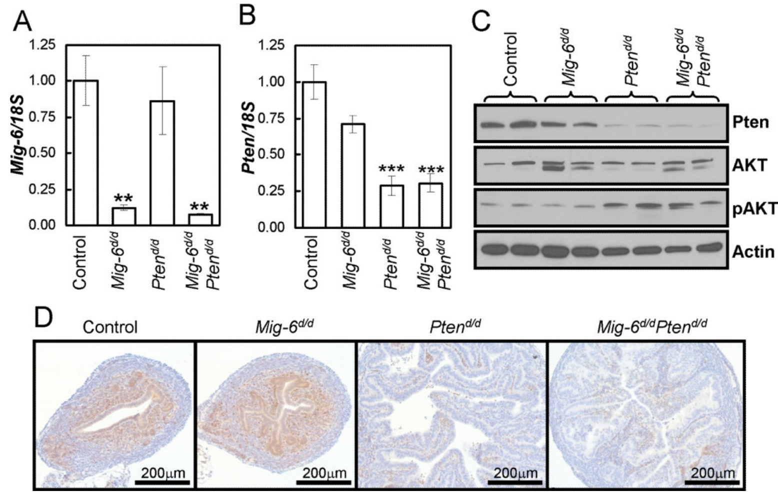

Figure 1.

Analysis of conditionally ablated *Pten* and *Mig-6* in the murine uterus. (A and B) Real-time RT-PCR analysis of *Mig-6* (A) and *Pten* (B) in whole uterine extracts from control, *Mig-6*$^{d/d}$, *Pten*$^{d/d}$ and *Mig-6*$^{d/d}$ *Pten*$^{d/d}$ 2 week old mice. **, $p<0.01$; ***, $p<0.001$ (C and D) Western blot analysis (C) and Immunohistochemical analysis (D) for PTEN in control, *Mig-6*$^{d/d}$, *Pten*$^{d/d}$ and *Mig-6*$^{d/d}$ *Pten*$^{d/d}$ 2 week old mice.

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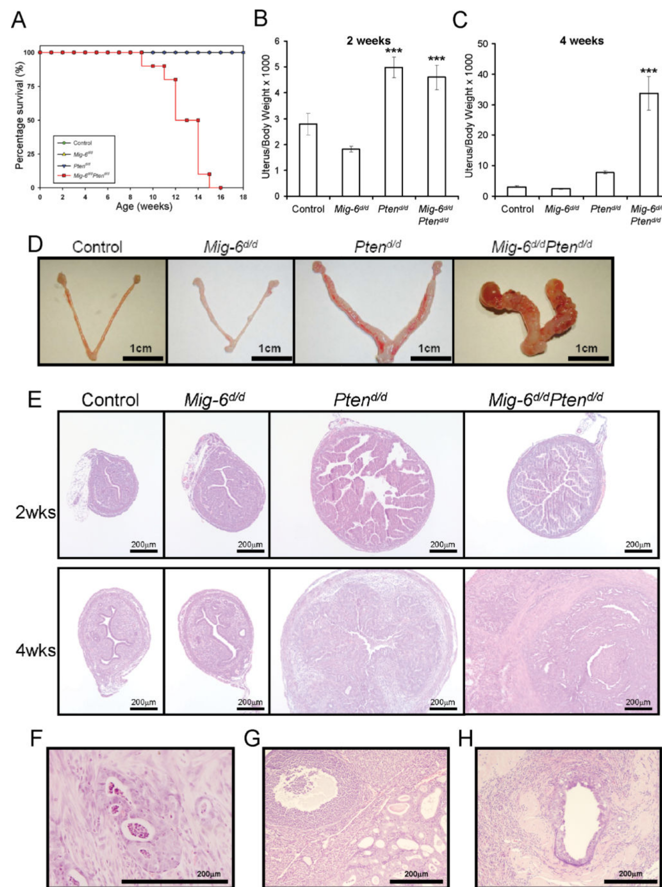

Figure 2.

Development of endometrial cancer in *Mig-6*$^{d/d}$*Pten*$^{d/d}$ mice. (A) Survival curve in control (*PR*$^{cre/+}$, *Mig-6*$^{f/f}$, *Pten*$^{f/f}$, and *Mig-6*$^{f/f}$*Pten*$^{f/f}$), *Mig-6*$^{d/d}$, *Pten*$^{d/d}$, and *Mig-6*$^{d/d}$*Pten*$^{d/d}$ mice. $p < 0.0001$, logrank test. (B and C) The ratio of uterine weight to body weight in control, *Mig-6*$^{d/d}$, *Pten*$^{d/d}$, and *Mig-6*$^{d/d}$*Pten*$^{d/d}$ mice at 2 (B) and 4 (C) weeks of age. ***, $p < 0.001$, one-way ANOVA followed by Tukey's post hoc multiple range test. (D) Gross anatomy of control, *Mig-6*$^{d/d}$, *Pten*$^{d/d}$, and *Mig-6*$^{d/d}$*Pten*$^{d/d}$ mice at 4 weeks of age. (E) Histology of uteri from mice with *Pten* and *Mig-6* ablation. H&E staining of control, *Mig-6*$^{d/d}$, *Pten*$^{d/d}$, and *Mig-6*$^{d/d}$*Pten*$^{d/d}$ mice at 2 and 4 weeks of age. (F) Endometrial cancer that has invaded through the myometrium. (G and H) Endometrial cancer that has metastasized into the ovary (G) and skeletal muscle (H).

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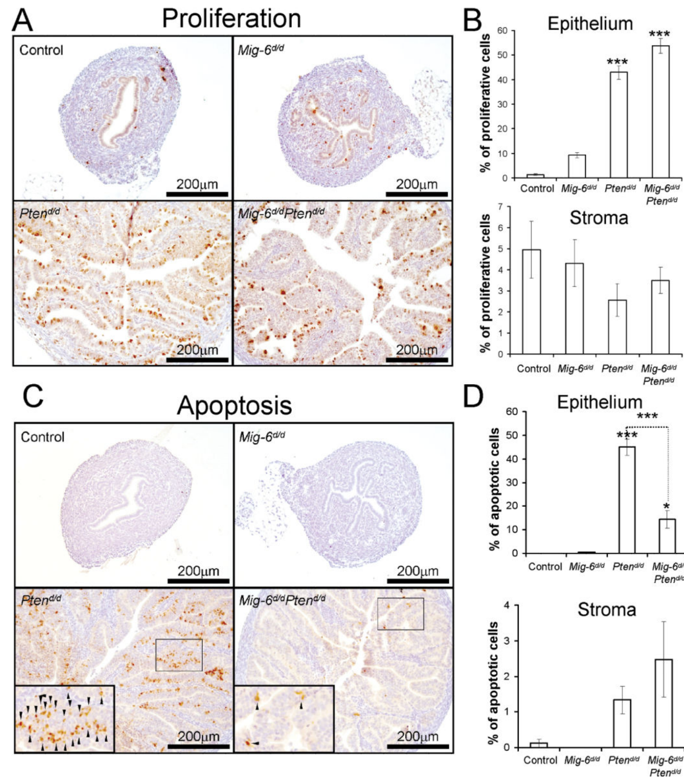

Figure 3.

The regulation of proliferation and apoptosis by *Mig-6*. (A) Immunohistochemical analysis of phospho-histone H3 as a proliferation marker in uteri of control, *Mig-6*$^{d/d}$, *Pten*$^{d/d}$, and *Mig-6*$^{d/d}$ *Pten*$^{d/d}$ mice at 2 weeks of age. (B) Quantification of phospho-histone H3 positive cells in epithelial and stromal cells (C) Immunohistochemical analysis of cleaved caspase 3 as an apoptotic cell marker. Small arrows indicate apoptotic cells. (D) Quantification of cleaved caspase 3 positive cells in epithelial and stromal cells. *, $p<0.05$; ***, $p<0.001$

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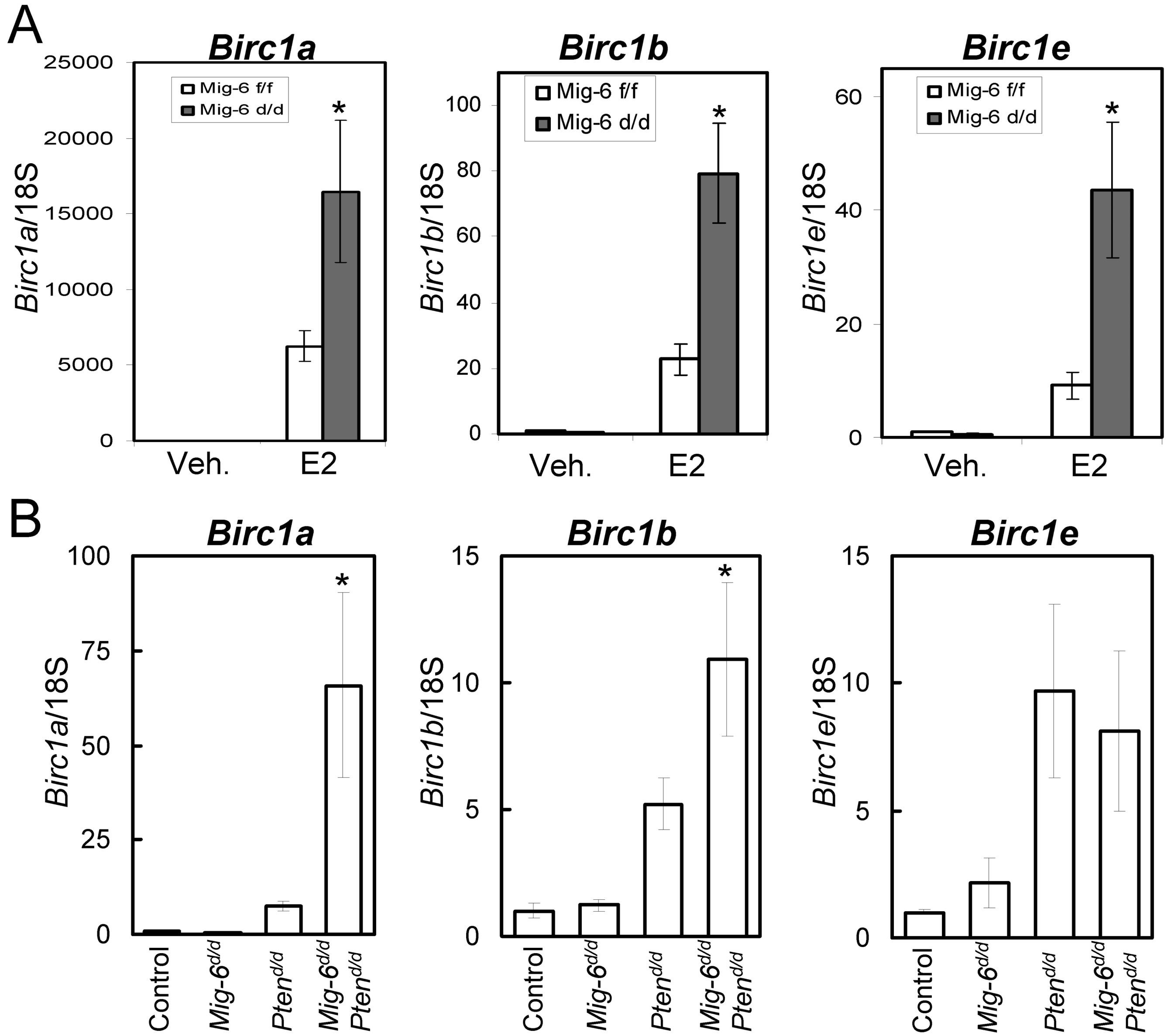

Figure 4.

The regulation of *Birc1* genes in the uteri of *Mig-6* ablation. (A) Real-time RT-PCR analysis of *Birc1a, Birc1b*, and *Birc1e* was performed on uteri of *Mig-6*$^{d/d}$ and *Mig-6*$^{f/f}$ mice treated with E2 for 3 days. (B) Real-time RT-PCR analysis of *Birc1a*, *Birc1b*, and *Birc1e* was performed on uteri of control, *Mig-6*$^{d/d}$, *Pten*$^{d/d}$, and *Mig-6*$^{d/d}$ *Pten*$^{d/d}$ mice at 2 weeks of age. *, $p < 0.05$

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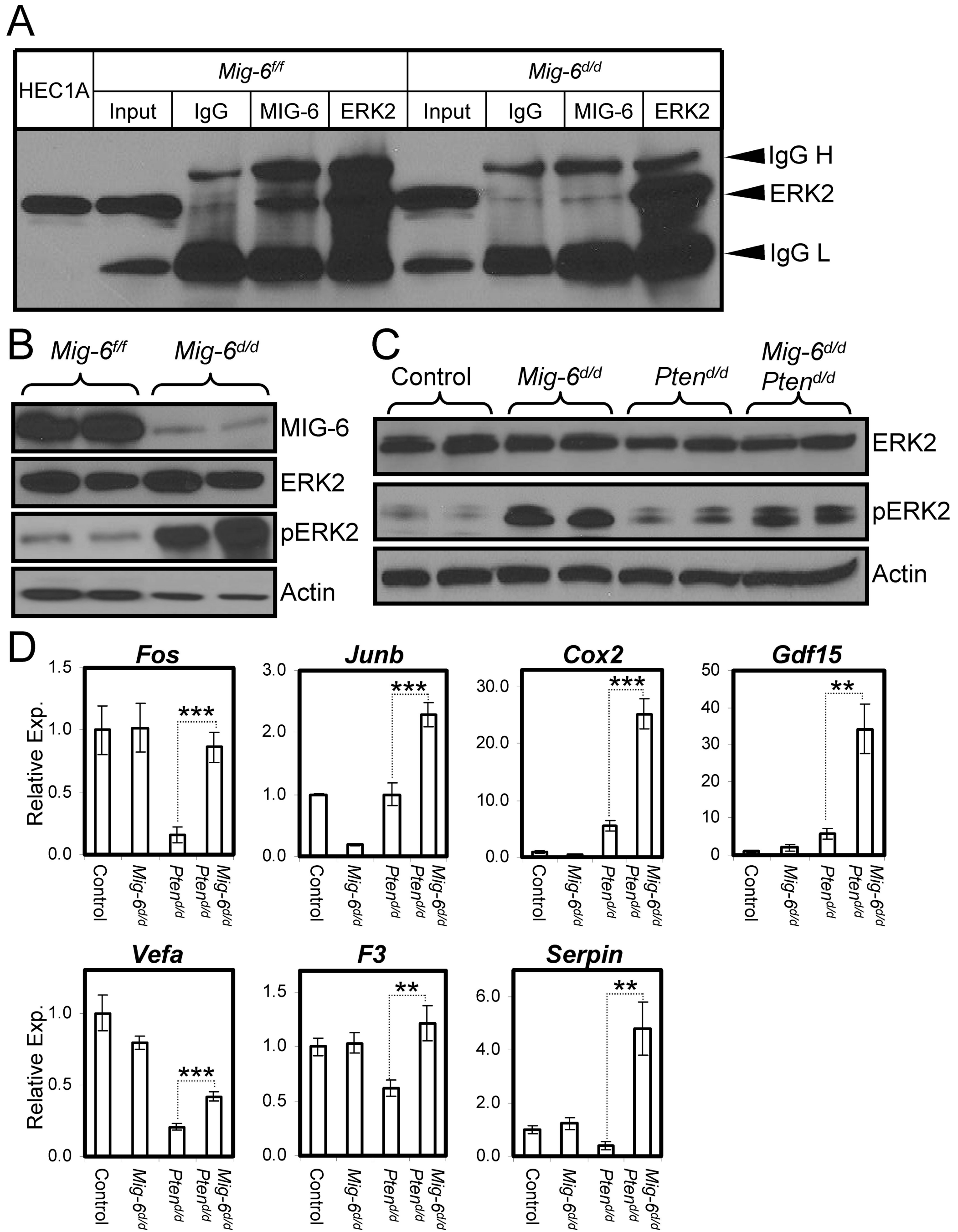

Figure 5.

Interaction between MIG-6 and ERK2 in the murine uterus. (A) Validation of the MIG-6 interaction with ERK2 by co-immunoprecipitation and Western blot analysis. (B) Western blot analysis of ERK2 and phospho-ERK2 in the uteri of 5 month old *Mig-6*$^{f/f}$ and *Mig- 6*$^{d/d}$ mice. (C) Western blot analysis of ERK2 and phospho-ERK2 in the uteri of 2 weeks old control, *Mig-6*$^{d/d}$, *Pten*$^{d/d}$, and *Mig-6*$^{d/d}$ *Pten*$^{d/d}$ mice. (D) The expression of ERK2 target genes. Real-time RT-PCR analysis of *Fos, Junb, Ptgs2, Gdf15, Vegfa, F3*, and *Serpin1* was performed on uteri of control, *Mig-6*$^{d/d}$, *Pten*$^{d/d}$, and *Mig-6*$^{d/d}$ *Pten*$^{d/d}$ mice. **, p<0.01; ***, p<0.001