HHS Public Access

Author manuscript

Reproduction. Author manuscript; available in PMC 2021 May 01.

Published in final edited form as:

Reproduction. 2020 May ; 159(6): 707–717. doi:10.1530/REP-19-0402.

AMPK IS REQUIRED FOR UTERINE RECEPTIVITY AND NORMAL RESPONSES TO STEROID HORMONES

Richard M. Griffiths IV[1], **Cindy A. Pru**[1], **Susanta K. Behura**[2], **Andrea R. Cronrath**[1], **Melissa L. McCallum**[1], **Nicole C. Kelp**[1], **Wipawee Winuthayanon**[1], **Thomas E. Spencer**[2], **James K. Pru**[1,*]

[1]Department of Animal Sciences, School of Molecular Biosciences, and Center for Reproductive Biology, Washington State University, Pullman, WA

[2]Division of Animal Sciences and Department of Obstetrics, Gynecology, and Women's Health, University of Missouri, Columbia, MO

Abstract

We previously demonstrated that 5'-adenosine monophosphate-activated protein kinase (AMPK) is essential for normal reproductive functions in female mice. Conditional ablation of *Prkaa1* and *Prkaa2*, genes that encode the α1 and α2 catalytic domains of AMPK, resulted in early reproductive senescence, faulty artificial decidualization, uterine inflammation and fibrotic postparturient endometrial regeneration. We also noted a delay in the timing of embryo implantation in *Prkaa1/2*$^{d/d}$ female mice, suggesting a role for AMPK in establishing uterine receptivity. As outlined in new studies here, conditional uterine ablation of *Prkaa1/2* led to an increase in ESR1 in the uteri of *Prkaa1/2*$^{d/d}$ mice resulting in prolonged epithelial cell proliferation and retention of E_2-induced gene expression (*e.g., Msx1, Muc1, Ltf*) through the implantation window. Within the stromal compartment, stromal cell proliferation was reduced by five-fold in *Prkaa1/2*$^{d/d}$ mice, and this was accompanied by a significant decrease in cell cycle regulatory genes and aberrant expression of decidualization marker genes such as *Hand2, Bmp2, Fst, Inhbb*. This phenotype is consistent with our prior study demonstrating a failure of the *Prkaa1/2*$^{d/d}$ uterus to undergo decidualization. Despite these uterine defects, ovarian function seemed to be normal following ablation of *Prkaa1/2* from peri-ovulatory follicles in that ovulation, luteinization and serum progesterone levels were not different on day 5 of pregnancy or pseudopregnancy between *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ mice. These cumulative findings demonstrate that AMPK activity plays a prominent role in mediating several steroid hormone-dependent events such as epithelial cell proliferation, uterine receptivity and decidualization as pregnancy is established.

[*]**Correspondence**: Dr. James K. Pru, jpru@wsu.edu.

AUTHOR CONTRIBUTIONS

RMG, CAP, SKB, ARC and JKP conceived the project, designed the experiments, and analyzed data. RMG, CAP, SKB, MLM, NCK, WW, and TES performed gene and protein expression studies. RMG, CAP and JKP wrote the manuscript and integrated comments from other authors.

Competing Financial Interests: The authors declare no competing or financial interests.

Keywords

AMPK; decidualization; endometrium; estrogen; fertility; implantation; pregnancy; progesterone; uterus

INTRODUCTION

Uterine receptivity is established by the sequential actions of estrogen (E_2) and progesterone (P_4) that act through their nuclear receptors to change the expression of critical genes. Several of these genes have been shown through conditional mutagenesis in mice to be fundamentally required for the establishment of early pregnancy, some of which include leukemia inhibitory factor (*Lif*), follistatin (*Fst*), bone morphogenetic protein 2 (*Bmp2*), heart- and neural crest derivatives-expressed protein 2 (*Hand2*), Msh homeobox 1 (*Msx1*), and Indian hedgehog (*Ihh*) (Stewart et al. 1992, Simon *et al.* 2009, Daikoku *et al.* 2011, Li *et al.* 2011, Li *et al.* 2013, Bhurke *et al.* 2016, Cheng *et al.* 2017, Fullerton *et al.* 2017). The proliferative and differentiative actions of sex steroid hormones are not direct. Rather, the hormones up-regulate expression of paracrine factors that, in turn, coordinate proliferative events induced by E_2 and differentiation events directed by P_4. While many of the paracrine ligands and their cognate receptors that mediate female sex steroid hormone actions in the uterus have been characterized, much less is known about the signal transduction pathways linked to these receptors. It is known that the MAPK, AKT and STAT3 signaling pathways play prominent roles in uterine receptivity and decidualization (Cheng *et al.* 2001, Yoshino *et al.* 2003, Singh *et al.* 2011, Yin *et al.* 2012, Salleh & Giribabu 2014, Makker *et al.* 2018, Yoo *et al.* 2018).

Adenosine monophosphate-activated protein kinase (AMPK) is an essential component of the signal transduction pathway that serves as a cellular sensor of energy status (Viollet *et al.* 2009). AMPK is a highly conserved heterotrimeric protein complex consisting of a catalytic α subunit and regulatory β and γ subunits. The *Prkaa1* and *Prkaa2* genes encode the α1 and α2 AMPK catalytic domains, respectively. In response to elevated cellular AMP, AMPK initiates catabolic processes while simultaneously inactivating energy depleting anabolic pathways (Hardie *et al.* 2006, Horman *et al.* 2012, Hardie & Lin 2017). AMPK plays a vital role in placental development (Kaufman & Brown 2016). Stable shRNA knockdown of *Prkaa1* and *Prkaa2* (*Prkaa1/2*) in trophoblast labyrinth cells results in changed morphology, growth rate and nutrient transport (Carey *et al.* 2014). More specifically, *Prkaa1/2* knockdown reduce trophoblast glycolysis, mitochondrial respiration and ATP coupling efficiency (Waker *et al.* 2017). In contrast, excessive AMPK activity alters fatty acid metabolism and impairs trophoblast invasiveness in preeclampsia (Yang *et al.* 2018). These collective findings indicate that AMPK activity must be tightly regulated for normal placental development. AMPK may also play an anti-inflammatory role in fetal membranes, as its expression decreases at term. Furthermore, pharmacological activation of AMPK with phenformin or 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) reduced infection-induced expression of pro-inflammatory cytokines in extraembryonic membranes of pregnancies with preterm premature rupture of the membranes (PPROM) (Lim *et al.* 2015). It is interesting to note that obese women have reduced placental expression and

activity of AMPK, which correlates with poor placental and pregnancy outcomes (Martino *et al.* 2016). Reduced placental expression of the active form of AMPK inversely correlates with birth weight (Jansson *et al.* 2013). Reduced placental AMPK expression/activity also generates a lipotoxic placental environment and an associated decrease in angiogenesis and increased expression of markers of inflammation and oxidative stress (Saben *et al.* 2014). The correlation between obesity and reduced placental AMPK expression/activity is also present in other animals. AMPK activity is reduced in cotyledonary tissue in obesogenic ewes (Zhu *et al.* 2009). Administration of AICAR reduced hypertension and angiogenic imbalance in a rat model of preeclampsia where increased AMPK activity reestablished placental antioxidant activities (Banek *et al.* 2013).

The known functions of AMPK in the female reproductive system are limited. Based on pharmacological studies, AMPK activation decreases steroidogenesis in rat and bovine granulosa cells (Tosca *et al.* 2005, Tosca *et al.* 2006, Tosca *et al.* 2007a, Tosca *et al.* 2010). A role for AMPK in oocyte maturation is species-dependent where AMPK catalytic activity results in oocyte activation in mice, but inhibition in pigs and cattle (Mayes *et al.* 2007, Tosca *et al.* 2007b, Santiquet *et al.* 2014, Bertoldo *et al.* 2015). Within the uterus, P53 was suggested to modulate uterine AMPK activity, which in turn controlled the timing of parturition in a murine model of preterm birth (Deng *et al.* 2016). Polymorphisms in the *PRKAA1* gene are thought to enhance adaptation to high altitudes during pregnancy in Andean women by influencing uterine artery diameter, fetal growth, and birth weight outcomes (Bigham *et al.* 2014). Steroid hormones were shown to regulate expression of facilitative glucose transporters in murine uterine epithelial tissue in an AMPK-dependent fashion (Kim & Moley 2009). Beyond these limited findings, relatively little is known about the role of AMPK in female reproduction, particularly on the uterine side of the maternal:fetal interface during pregnancy. We recently established that conditional mutagenesis of *Prkaa1* and *Prkaa2* in the uterus results in subfertility that rapidly progresses to complete infertility and significantly reduced fecundity (McCallum *et al.* 2018). Additional phenotypes included uterine inflammation and abnormal postparturient deposition of fibrotic tissue in the endometrium, indicative of endometritis. Here, we evaluated AMPK as a mediator of steroid hormone responses and uterine receptivity.

MATERIALS AND METHODS

Animals

All animal experiments were reviewed and approved by the Washington State University Institutional Animal Care and Use Committee. *Prkaa1/2*$^{d/d}$ mice were developed by crossing *Prkaa1/2*$^{fl/fl}$ mice with *Pgr-cre* mice to conditionally ablate genes encoding the α1 and α2 catalytic domains of AMPK within the female reproductive tract (McCallum *et al.* 2018, Soyal *et al.* 2005). The exact genetic background of these mice is not known. However, based on the number of crosses we have made in our own lab, the mice are on a greater than 95% C57BL/6 background. The sub/infertility and faulty artificial decidualization observed in *Prkaa1/2*$^{d/d}$ female mice (McCallum *et al.* 2018) prompted us to further evaluate the conditional ablation of *Prkaa1/2* during the time of uterine receptivity and early decidualization. We were also interested in comparing uterine steroid hormone responses in

Prkaa1/2$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ female mice. For the early pregnancy studies, female mice were placed with males of proven breeding capacity and were considered day of pregnancy (DOP) 0.5 upon observation of a vaginal plug. Female reproductive tracts were collected for histological analyses on DOP4, 5 and 8. On DOP5, blood was collected from *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ female mice for serum isolation and the mice were then immediately euthanized. Serum samples were submitted to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core for P_4 hormone assays.

For evaluating uterine steroid hormone responses around the time of embryo implantation, 6–10 week old *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ female mice were bred by vasectomized male mice of proven breeding capacity to induce pseudopregnancy. On day of pseudopregnancy (DOPP) 4, a 0.5 inch 30 gauge needle was inserted entirely into the uterine lumen and a scratch was given when the needle was drawn back as 25 μl of sesame oil was injected into the uterine lumen to initiate decidualization. Uteri were collected 24 h later for mRNA isolation and histological evaluation. Ovaries were also collected and processed for paraffin embedding. This model relies on endogenous steroids to prime the uterus for decidualization. In a second pseudopregnancy model, we ovariectomized *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ female mice and allowed them to recover for two weeks. The mice were then given a steroid hormone regimen consistent with early pregnancy and artificially decidualized on the equivalent of DOPP4 (Zhang *et al.* 2012, McCallum *et al.* 2018). Uteri from *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ female mice were collected 24 h later and processed for mRNA isolation and paraffin embedding.

RNA Isolation and Quantitative Polymerase Chain Reaction (qPCR)

Uterine expression of genes associated with receptivity and decidualization was determined by qPCR using primer sets identified in Table 1. For these studies, RNA was isolated from uteri obtained from *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ mice using Tri-Reagent (Sigma-Aldrich; St. Louis, MO) following the manufacturer's instruction. Isolated RNA was DNase treated (Roche Applied Science, Indianapolis, IN) and quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific; Waltham, MA) prior to cDNA synthesis. Messenger RNA was then reverse transcribed using BioRad iScript (BioRad; Hercules, CA). Quantitative PCR was completed using BioRad's C1000 CFX96 Real-Time Thermal Cycler System with an annealing temperature of 56–58°C (1 min) for 40 cycles. Data were analyzed with CFX Manager Software (BioRad; Hercules, CA). *Rpl13a* was used as an internal control to normalize expression data. A no reverse transcriptase negative control was included to confirm the absence of genomic DNA.

Immunohistochemistry

All tissues were fixed in 4% paraformaldehyde and stored in 70% ethanol until paraffin embedding. Tissues were processed in an ethanol gradient and xylenes, embedded in paraffin, and sectioned at 5 μm. Tissue sections were heated at 65°C for 60 minutes then deparaffinized in xylenes, rehydrated in a series of decreasing ethanol washes and placed in PBS. Slides were stained with hematoxylin and eosin (Scytek Laboratories Inc., Logan, UT) as per manufacturer's instructions, or they were used for immunohistochemistry (IHC). For IHC, tissue sections underwent quenching (10 minutes in 3% hydrogen peroxide) and

antigen retrieval (brought to a boil in 0.1M sodium citrate followed by incubation in the heated solution for 20 minutes and then cooling to room temperature). Sections were then blocked (0.1% bovine serum albumin, 0.1% normal goat serum or normal donkey serum, and 1% Triton-X100 in phosphate-buffered saline) for 1 h and then incubated overnight at 4°C in blocking solution containing primary antibody shown in Table 2. Slides were washed in phosphate-buffered saline (3×10 minutes) and incubated with secondary antibody diluted at 1:500 in blocking solution for 30 minutes at room temperature. Sections were washed as before and then incubated with horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA). Washes were again performed and the sections were exposed to 3,3'-diaminobenzidine (BD Biosciences, San Diego, CA) followed by a five-minute inactivation in PBS. Sections were then counterstained with methyl green or hematoxylin, dehydrated and mounted.

Data Analysis

Data are presented as a mean ± SEM for n=3–8 independent experiments in which one mouse represented one experimental replicate. The exact number of experimental replicates are indicated in each figure legend. Animals were randomly assigned to each group based on genotype. Differences between treatment groups were analyzed using the Student's *t*-test. A p-value ≤0.05 was considered significant. All data were analyzed using GraphPad Prism 5.0 (San Diego, California).

RESULTS

We previously demonstrated that AMPK is required for fertility in the female and that uterine expression of E_2 (ESR1) and P_4 (PGR) receptors did not differ between *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ mice following E_2 synchronization (McCallum *et al.* 2018). As E_2 and P_4 ensure that the uterus is receptive to the implanting embryo as pregnancy is established, we first evaluated the expression of *Esr1* and *Pgr* on DOPP5 by qPCR. A significant increase in *Esr1* mRNA levels were observed in the *Prkaa1/2*$^{d/d}$ uterus compared to the *Prkaa1/2*$^{fl/fl}$ uterus, whereas *Pgr* mRNA levels did not differ (Fig. 1A and 1B). This difference in expression at the mRNA level was also evident at the protein level based on IHC analysis, particularly in the epithelial compartment (Fig. 1C).

In evaluating our prior breeding trial data (McCallum *et al.* 2018), we noted that the time to first weaning for nulliparous *Prkaa1/2*$^{d/d}$ female mice was about twice that of female *Prkaa1/2*$^{fl/fl}$ mice (Fig. 2A). Many of the pups born to *Prkaa1/2*$^{d/d}$ female mice appeared smaller at birth than pups from *Prkaa1/2*$^{fl/fl}$ female mice and this translated to a smaller mean pup mass at the time of weaning (Fig. 2B). Evaluation of ovaries from *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ mice on DOP5 and DOPP5 revealed no histological differences (Fig. 2C). Furthermore, serum P_4 levels were not different on DOP5 (Fig. 2D). Despite no difference in ovarian histology or luteal function, we observed faulty embryo implantation and embryonic growth in *Prkaa1/2*$^{d/d}$ dams on DOP4, DOP5 and DOP8 (Fig. 3). Embryos within uteri from *Prkaa1/2*$^{d/d}$ mice were commonly not implanting (DOP4) or were smaller in size or degenerate (DOP4, DOP5 and DOP8) compared with embryos in uteri from *Prkaa1/2*$^{fl/fl}$ mice. Interestingly, while the initiation of stromal cell decidualization was visible in

Prkaa1/2$^{fl/fl}$ mice on DOP4 and DOP5, the decidualization response was commonly blunted or absent during these times in *Prkaa1/2*$^{d/d}$ mice. Furthermore, the zippering of the luminal epithelium on DOP5 was often incomplete in *Prkaa1/2*$^{d/d}$ mice.

At the time of embryo apposition and implantation, endometrial epithelial cells should be non-proliferative and the underlying stromal cells should be proliferating in response to luteal-derived P_4. In this next experiment, we generated pseudopregnant *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ mice by placing intact females with vasectomized male mice to evaluate endometrial cell proliferation. While uteri from *Prkaa1/2*$^{fl/fl}$ mice displayed normal endometrial cell proliferation, epithelia of *Prkaa1/2*$^{d/d}$ mice remained proliferative and the stromal cell compartment showed a significant decrease in proliferation on DOPP5 based on expression of the mitosis marker phospho-histone H3 (Fig. 4). This was accompanied by a significant decrease in the expression of the cell cycle regulatory genes *Cyclin A2* (*Ccna2*), *Cyclin B1* (*Ccnb1*), *Cyclin B2* (*Ccnb2*), *Cyclin E1* (*Ccne1*), *Cyclin E2* (*Ccne2*), *Cyclin F* (*Ccnf*), and *Cyclin Dependent Kinase 1* (*Cdk1*) (Fig. 5A) and the decidualization marker genes Follistatin (*Fst*), *Inhibin βB* (*Inhbb*), *Bone morphogenetic protein 2* (*Bmp2*), *heart-and neural crest derivatives-expressed protein 2* (*Hand2*), *S100 calcium-binding protein A9* (*S100a9*), and *serine protease 28* (*Prss28*) (Fig. 5B) in *Prkaa1/2*$^{d/d}$ mice. Interestingly, *wingless-type MMTV family member 4* (*Wnt4*), which is also essential for decidualization, was not differentially regulated. Three common E_2-induced genes were up-regulated in uteri of *Prkaa1/2*$^{d/d}$ mice and these include *Msh homeobox 1* (*Msx1*), *Mucin 1* (*Muc1*), and *Lactotransferrin* (*Ltf*, P=0.07) (Fig. 5C). Several other genes such as *Tachykinin 2* (*Tac2*), *E2F transcription factor 8* (*E2f8*), *Glutathione peroxidase 3* (*Gpx3*), *Arachidonate 15-lipoxygenase* (*Alox15*) and *Interleukin 1 beta* (*IL1b*) were also differentially expressed (Fig. 5D). The *Hand2* gene is fundamentally required for uterine receptivity and decidualization in mice (Li *et al.* 2011). Evaluation of HAND2 protein expression by IHC revealed a significant decrease in stromal cell expression at a time when decidualization should be just underway (Fig. 6). These cumulative findings are consistent with the failure of uteri from *Prkaa1/2*$^{d/d}$ mice to undergo artificial decidualization (McCallum *et al.* 2018) and for faulty embryo implantation and luminal closure in these mice, and these defective uterine functions may stem from elevated or unopposed E_2-regulated signaling.

To provide additional evidence that uteri from *Prkaa1/2*$^{d/d}$ mice fail to properly respond to steroid hormones and establish a receptive uterus, we ovariectomized *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ mice, provided an exogenous steroid hormone regimen consistent with early pregnancy (McCallum *et al.* 2018), and then evaluated cell proliferation and gene expression on DOPP5 as before. Unlike uteri from *Prkaa1/2*$^{fl/fl}$ mice, uteri from *Prkaa1/2*$^{d/d}$ mice retained mitotic epithelial cells and tended (p=0.07) to weigh more than uteri from *Prkaa1/2*$^{fl/fl}$ mice (Figure 7). Likewise, we observe the same differential expression for *Fst*, *Inhbb*, *Muc1*, *Alox15*, and *Tac2* as we did in intact mice that were exposed to endogenous steroid hormones.

DISCUSSION

We previously established an essential role for the AMPK signal transduction pathway in uterine physiology (McCallum *et al.* 2018). Ablation of genes encoding the α1 and α2

catalytic subunits of AMPK from the female reproductive tract resulted in subfertility that rapidly progressed to complete infertility. Associated features of this broad phenotype included faulty decidualization, parturition-dependent fibrosis of the stromal compartment (*i.e.*, endometritis), and chronic inflammation. It was noted from breeding trial data that the time to first weaning of pups born to *Prkaa1/2*$^{d/d}$ dams was twice as long as that observed for *Prkaa1/2*$^{fl/fl}$ dams despite no difference in estrous cyclicity. Pups born to *Prkaa1/2*$^{d/d}$ dams were also smaller at the time of weaning, suggesting additional functions for AMPK during pregnancy beyond those previously described (McCallum *et al.* 2018). As such, the objective of the present study was to evaluate the role of AMPK in establishing the uterine receptive state during early pregnancy. In addition to evaluating early pregnancy, we used two models to appraise AMPK functions around the time of uterine receptivity. The first involved the use of intact *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ mice that were bred to vasectomized male mice to induce a state of pseudopregnancy. This model relies on endogenous steroid hormones to develop the receptive endometrium. The second model used *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ mice that were first ovariectomized and then given a series of daily steroid hormone injections that mimicked early pregnancy. Even though estrous cyclicity and steroidogenesis were not different between intact *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ mice (McCallum *et al.* 2018, Fig. 2), and a histological evaluation of ovaries on DOP5 and DOPP5 collectively indicated that *Prkaa1/2* are dispensable for normal ovarian functions (McCallum *et al.* 2018), Fig. 2), this second model was implemented to directly evaluate the effects of steroid hormone actions on the uterus under controlled conditions.

Uterine receptivity is established in response to the synchronous secretion of ovarian-derived E_2 and P_4. These two steroid hormones work in series with E_2 first priming the endometrium to undergo epithelial cell proliferation. This is followed by P_4, which attenuates epithelial cell proliferation causing them to take on a differentiated secretory function and stimulating stromal cell proliferation (Das & Martin 1973, Wang & Dey 2006). In addition to appropriately timed ligand availability, uterine expression of the classical receptors for E_2 and P_4 are tightly regulated. Dysregulation of these receptors compromises fertility in the female. Whereas ESR1 expression normally decreases during the window of implantation (Lessey *et al.* 2006), an increase in ESR1 mRNA and protein was observed in uteri from *Prkaa1/2*$^{d/d}$ mice during the window of implantation. Elevated ESR1 expression in the endometrium of women during the window of implantation is implicated in some cases of unexplained infertility (Lessey *et al.* 2006, Dorostghoal *et al.* 2018).

Because of the increased expression of ESR1 during receptivity, we evaluated implantation and early embryo development on DOP4, 5 and 8. Murine implantation occurs between DOP4 and DOP5. Absorption of uterine fluid and closure, or zippering, of the luminal epithelium promote proper antimesometrial eccentric implantation in mice (Zhang *et al.* 2013). We previously noted upon gross observation that implantation sites from 4–6 month old nulliparous *Prkaa1/2*$^{d/d}$ mice appear normal. However, upon histological evaluation of implantation sites at the time of embryo implantation (*i.e.,* DOP 4 and 5; Figure 3) in 6–10 week old *Prkaa1/2*$^{d/d}$ mice, we did observe clear signs of an unzippered luminal epithelium, lack of formation of an embryonic chamber, and stunted embryonic growth in some sites, all of which are clear signs of dyssynchrony between the mother and implanting embryo. While exhibiting the same gestation length, the delay in implantation likely explains the decrease in

pup mass observed in offspring born to *Prkaa1/2*$^{d/d}$ mice. Use of the *Pgr-cre* mouse results in peri-ovulatory ablation of floxed genes in ovarian follicles and subsequent corpora lutea given that *Pgr* is normally expressed for a 12 h window on either side of ovulation in mice. Despite peri-ovulatory follicular ablation of *Prkaa1* and *Prkaa2*, ovulation, luteinization and P_4 steroidogenesis were normal in *Prkaa1/2*$^{d/d}$ mice. In support of our previous findings (McCallum *et al.* 2018), these data suggest that AMPK is dispensable for late follicular and luteal functions in the ovary and that early pregnancy issues observed in *Prkaa1/2*$^{d/d}$ mice most likely derive from disrupted uterine functions.

To characterize the faulty uterine receptivity phenotype in more detail, we next evaluated steroid hormone-induced proliferation in the epithelial and stromal compartments, as well as expression of markers of stromal decidualization and inflammation in both models of pseudopregnancy. Coupled with elevated expression of ESR1 in uteri obtained from *Prkaa1/2*$^{d/d}$ mice on DOPP5 was the prolonged retention of luminal epithelial cell mitosis and elevated expression of the E_2 marker genes *Msx1*, *Muc1*, and *Ltf*. While *Msx1* expression normally increases in epithelial tissue on DOP4 in the receptive mouse uterus, its expression is almost undetectable following implantation or the equivalent time during pseudopregnancy (Daikoku *et al.* 2011). *Msx1* is fundamentally required for establishing the receptive state in mice through its modulation of epithelial junctional activity in luminal tissue (Sun *et al.* 2016). *Msx1* expression was more recently shown to be widely disrupted in infertile women where reduced *Msx1* expression associated with loss of epithelial cell polarity and a receptive uterine state. (Bolnick *et al.* 2016).

Within the stromal compartment, a 5-fold decrease in stromal cell proliferation was also observed in DOPP5 *Prkaa1/2*$^{d/d}$ uteri, a phenotype that was accompanied by down-regulation of several cell cycle regulatory genes. This likely contributed to faulty artificial decidualization (McCallum *et al.* 2018) and the diminished size of implantation sites during early pregnancy (Fig. 3). We also noted decreased expression of decidualization markers, a number of which are discussed below, have been shown through mutagenesis studies in mice and knockdown studies in human endometrial stromal cells to be essential for decidualization. *Bone morphogenetic protein 2* (*Bmp2*), *follistatin* (*Fst*), and *heart- and neural crest derivatives-expressed* protein 2 (*Hand2*) are among the most well-studied (Lee *et al.* 2007, Li *et al.* 2013, Bhurke *et al.* 2016, Fullerton *et al.* 2017). The *Prkaa1/2*$^{d/d}$ uterine phenotype is strikingly similar to the *Fst* conditional mutant phenotype in which uterine ablation of *Fst* resulted in subfertility caused by a failure to achieve uterine receptivity, retention of features of E_2 signaling such as luminal epithelial cell proliferation at a time when P_4 signaling should dominate, impaired embryo implantation, and faulty decidualization (Fullerton *et al.* 2017). Another interesting feature of the *Fst*$^{d/d}$ phenotype is the increased expression of inhibin βB. When dimerized, inhibin βB forms activin, which in turn antagonizes BMP signaling. Importantly, BMP signaling, particularly that initiated by BMP2 and BMP7 (Li *et al.* 2013, Lee *et al.* 2007, Monsivais *et al.* 2017), is an essential component of the differentiation program that establishes the receptive state and initiates decidualization in the uterus. The *Prkaa1/2*$^{d/d}$ uterus similarly shows a pattern of decreased *Fst* and increased *Inhbb* during receptivity (Fig. 5b). We suspect that AMPK signaling is essential for reducing *Inhbb* expression, which then ensures robust BMP signaling that is necessary for initiating the deciduogenic response. The decreased expression of *Hand2*

mRNA was validated at the protein level by immunohistochemistry. This protein is a mediator of the antiproliferative action of P_4 (Li *et al.* 2011). The significant decrease in HAND2 expression in the *Prkaa1/2*$^{d/d}$ uterus likely contributes to aberrantly retained epithelial cell expression during receptivity. Of note, the expression of *Wnt4,* another essential gene required for decidualization, was not different in uterine tissues isolated from *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ mice. This indicates that AMPK, either directly or indirectly, is necessary for mediating steroid hormone regulated expression of a select cadre of genes essential for implantation and decidualization, but not others.

In summary, this study demonstrates the functional requirement of AMPK signaling in establishing the uterine receptive state for embryo implantation. The actions of AMPK are commonly associated with sensing and regulating available cellular energy. While we have not ruled out the possibility that pregnancy issues observed in *Prkaa1/2*$^{d/d}$ mice stem specifically from faulty energy sensing, we do show aberrant expression of E_2 responsive genes, as well as genes that coordinates stromal decidualization, strongly support the idea that AMPK is functionally linked to normal steroid hormone signaling in the uterus. What now remains to be determined is: 1) identifying the factor(s) that increase AMPK signaling prior to transitioning to the receptive state; and 2) determining the molecular mechanism by which AMPK differentially regulates E_2- and P_4-dependent genes. With regard to identifying factors that increase AMPK signaling, AMPK activity was recently shown to increase in human stromal cell decidualization assays in an epidermal growth factor receptor-dependent fashion (Large *et al.* 2014.) As EGFR and several EGF-like ligands (*e.g.*, HB-EGF, amphiregulin) have been shown to be important for implantation and decidualization (Large *et al.* 2014, Das *et al.* 1994, Das *et al.* 1995, Paria *et al.* 2001, Leach *et al.* 1999), this would place AMPK near the top of the molecular pyramid that controls establishment of the receptive state should AMPK be confirmed as one of the phosphorylation cascades downstream of the EGFR.

ACKNOWLEGMENTS

We thank The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core, supported by the Eunice Kennedy Shriver NICHD/NIH P50 grant (HD28934), for performing the serum P_4 hormone assays. We are grateful for funding support from the National Institutes of Health (HD086402 to JKP and HD097087 to WW).

Funding: This study was funded in part by NIH R21HD086402 (JKP) and HD097087 (WW) and USDA National Institute of Food and Agriculture, Functional Genomics in Animal Improvement, Food Safety and Human Health Hatch Project 1014918.

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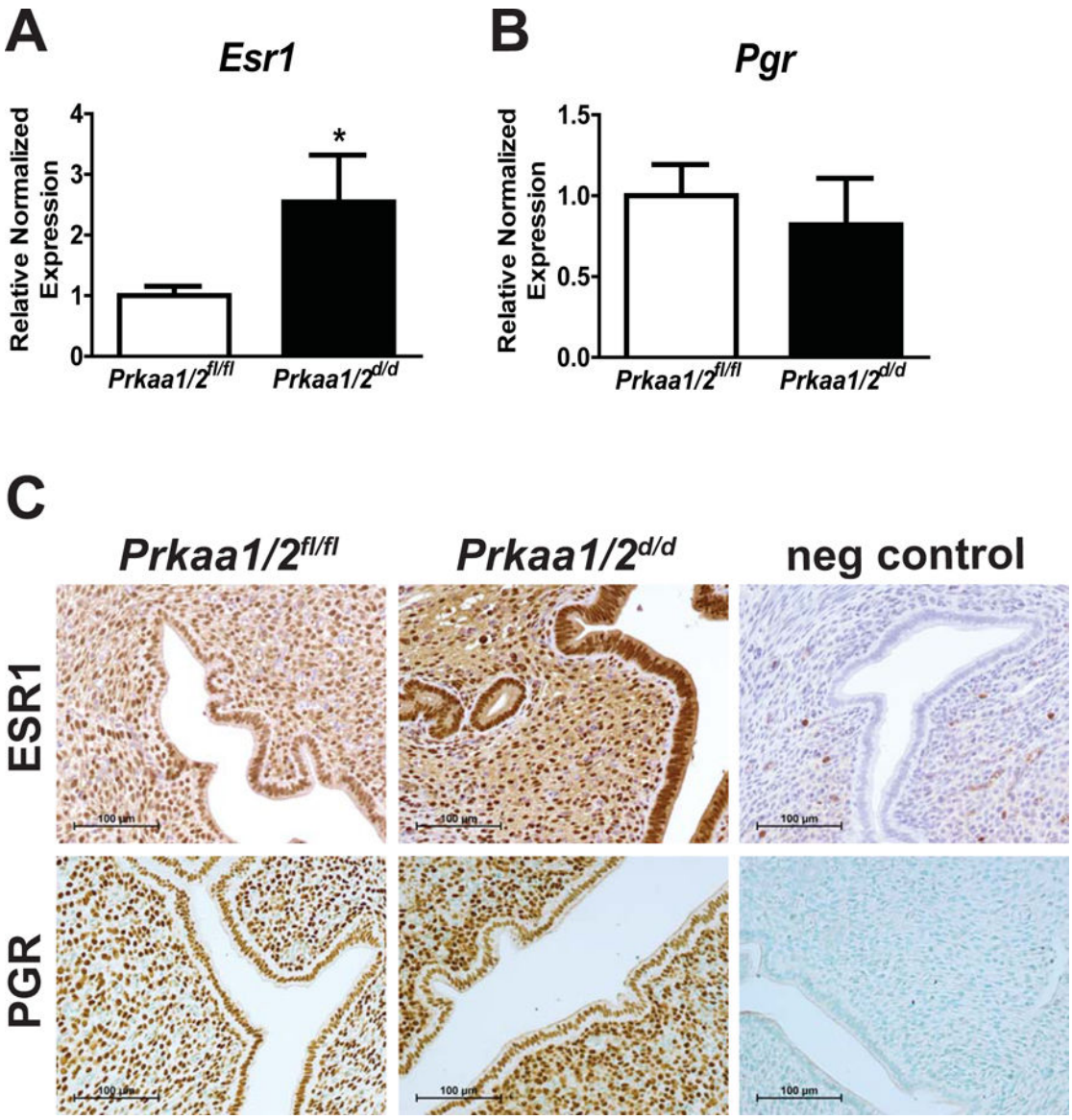

Figure 1. Disrupted ESR1, but not PGR expression in the DOPP5 *Prkaa1/2*$^{d/d}$ uterus.

Shown are uterine RT-qPCR (**A**, **B**) and immunohistochemical (**C**) expression data for ESR1 and PGR on day of pseudopregnancy (DOPP) 5 in *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ mice. Values are expressed as mean ± SEM, *P≤0.05, n=4.

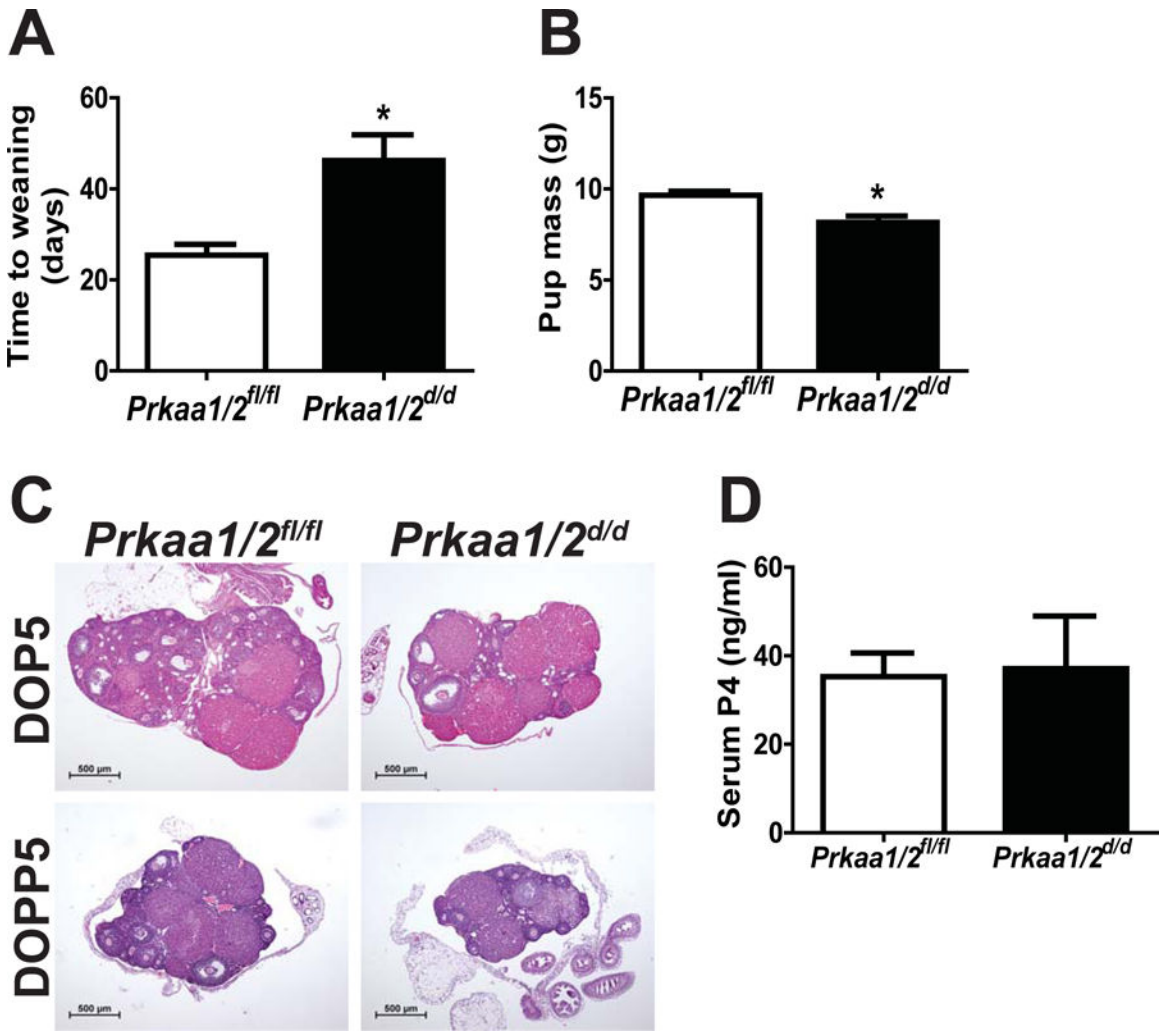

Figure 2. PRKAA1 and PRKAA2 deficiency disrupts time to first weaning with no impact on ovarian structure and function.

(**A**) Overall time to weaning of pups is dramatically increased in primiparous *Prkaa1/2*$^{d/d}$ females (p=0.014). (**B**) Pups born to *Prkaa1/2*$^{d/d}$ dams weighed significantly less at the time of weaning than pups born out of *Prkaa1/2*$^{fl/fl}$ females. (**C**) A histological analysis demonstrated no difference in ovarian architecture or corpora lutea formation between *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ mice during early pregnancy (DOP5) or pseudopregnancy (DOPP5) (n=4–6). (**D**) Serum P_4 measured on DOP5 was not different between *Prkaa1/2*$^{fl/fl}$ (n=6) and *Prkaa1/2*$^{d/d}$ (n=4) animals demonstrating that post-ovulatory steroidogenesis is not dependent on AMPK activity. Values are expressed as mean ± SEM, *P≤0.05.

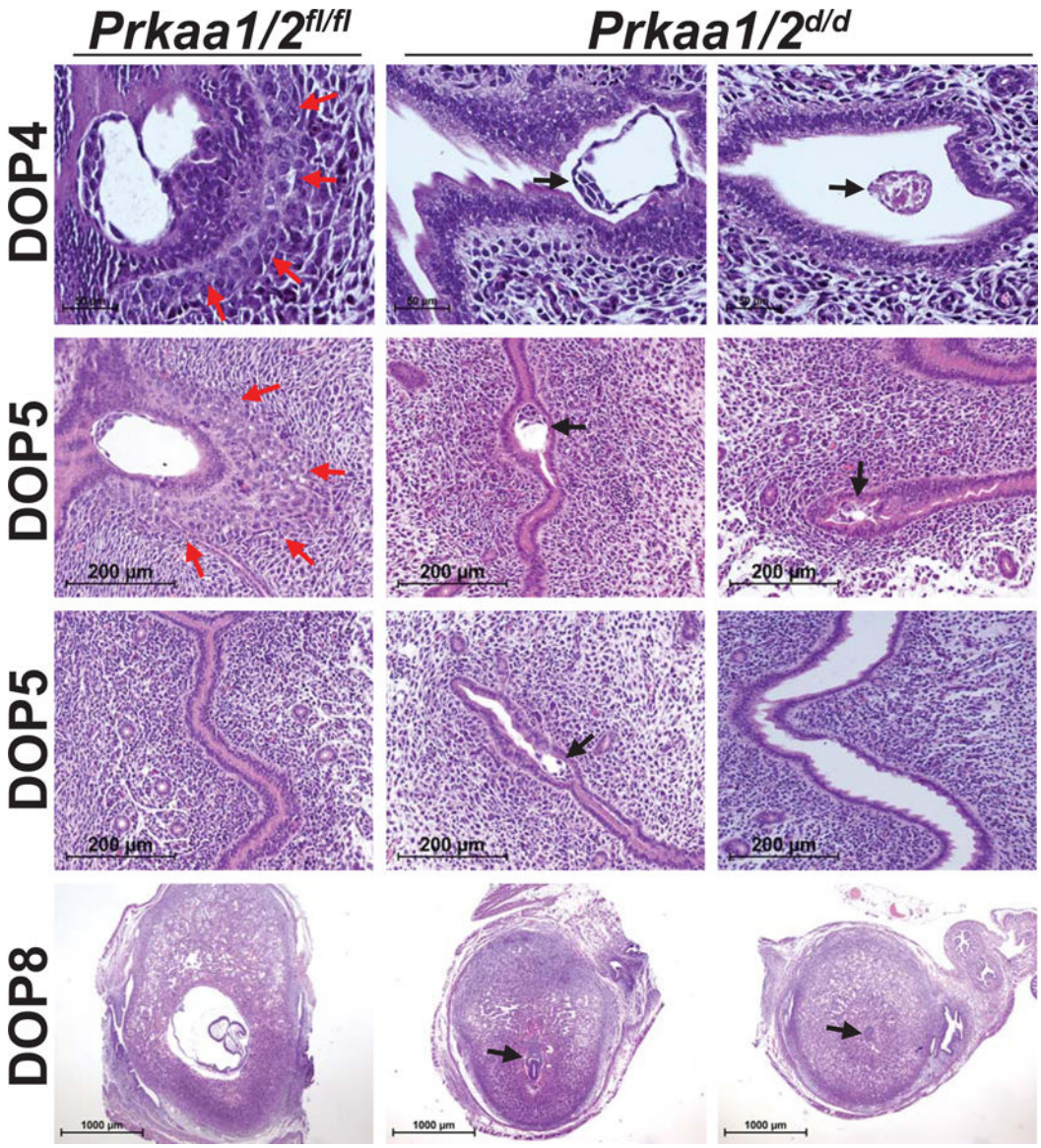

Figure 3. Embryo implantation is delayed or disrupted in *Prkaa1/2*$^{d/d}$ mice.

Embryos from *Prkaa1/2*$^{fl/fl}$ females implanted normally and the stroma decidualized as expected on DOP4, DOP5 and DOP8, (red arrows). Embryos from *Prkaa1/2*$^{d/d}$ females commonly failed to implant properly, were smaller in size or did not survive the implantation process (black arrows). The luminal epithelium of gravid *Prkaa1/2*$^{d/d}$ female mice failed to zipper shut on DOP5. While DOP8 embryos had greatly expanded in *Prkaa1/2*$^{fl/fl}$ mice with a clear presence of an amniotic cavity, DOP8 embryos from *Prkaa1/2*$^{d/d}$ mice were often small and stunted in development with reduced decidual expansion. n=4–8

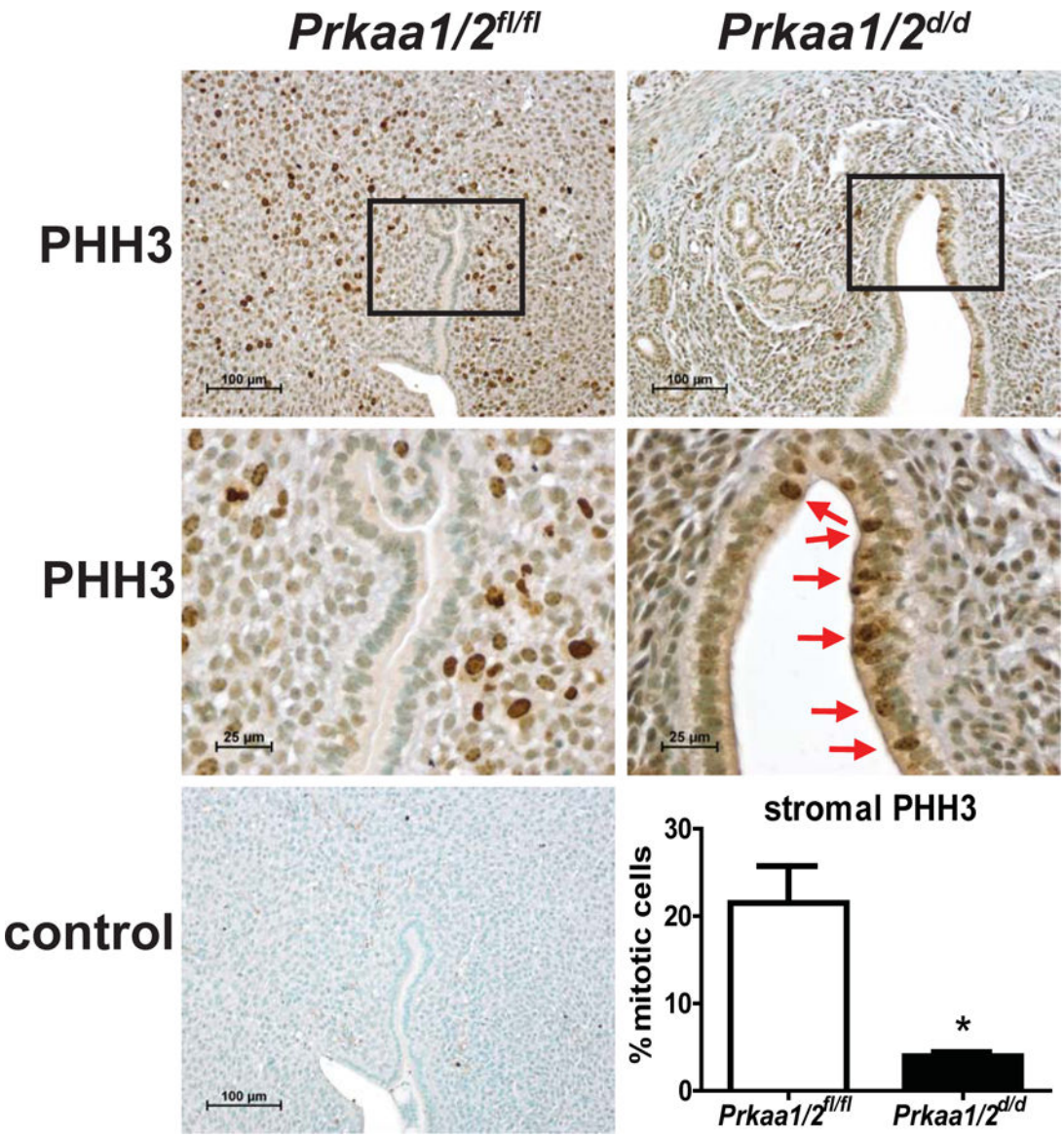

Figure 4. Aberrant retention of luminal epithelial proliferation and decreased stromal proliferation in the DOPP5 *Prkaa1/2*$^{d/d}$ uterus.

Immunohistochemical staining for the mitosis marker phosphohistone-H3 (PHH3) shows retention of luminal epithelial proliferation in the DOPP5 *Prkaa1/2*$^{d/d}$ uterus with significantly diminished proliferation in the stromal compartment compared to the *Prkaa1/2*$^{fl/fl}$ uterus (*P=0.02). Values are expressed as mean ± SEM, n=3–4.

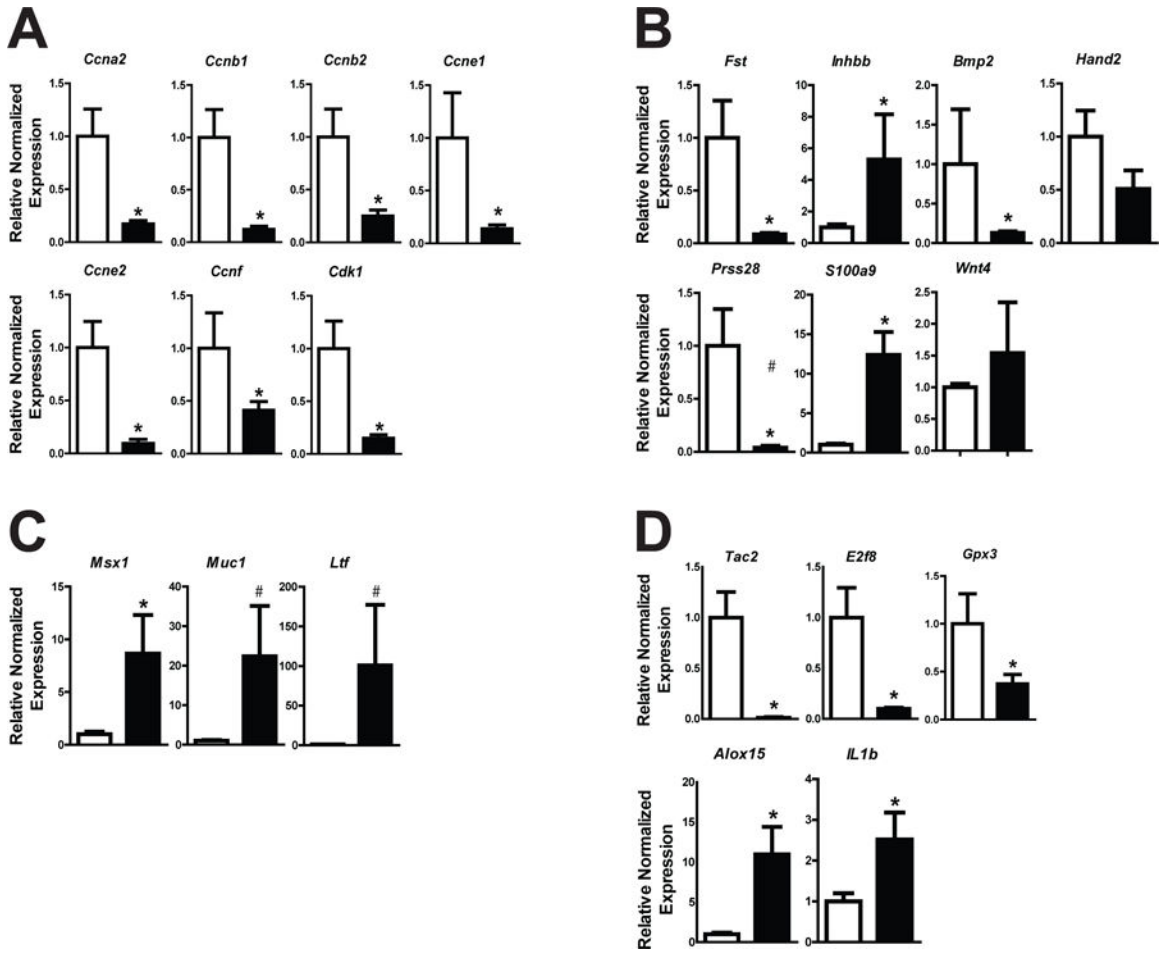

Figure 5. PRKAA1 and PRKAA2 deficiency results in differential expression of genes associated with the cell cycle, decidualization, estrogen-signaling, and inflammation on DOPP5.

Using an intact pseudopregnancy model, RT-qPCR was used to evaluate expression of cell cycle regulatory genes *Ccna2, Ccnb1, Ccnb2, Ccne1, Ccne2, Ccnf, and Cdk1* (**A**) and genes important for decidualization (**B**; *Fst, Inhbb, Bmp2, Hand2, S100a9, Prss28, Wnt4*) in uteri obtained from *Prkaa1/2*$^{fl/fl}$ (white bars) and *Prkaa1/2*$^{d/d}$ (black bars) mice. E_2 responsive genes (**C**; *Msx1, Muc1* and *Ltf*), as well as genes commonly associated with inflammation (**D**; *Tac2, E2f8, Gpx3, Alox15* and *IL1b*) were also differentially regulated between groups. Values are expressed as mean ± SEM, *P≤0.05; $^{\#}$P=0.07, n=3.

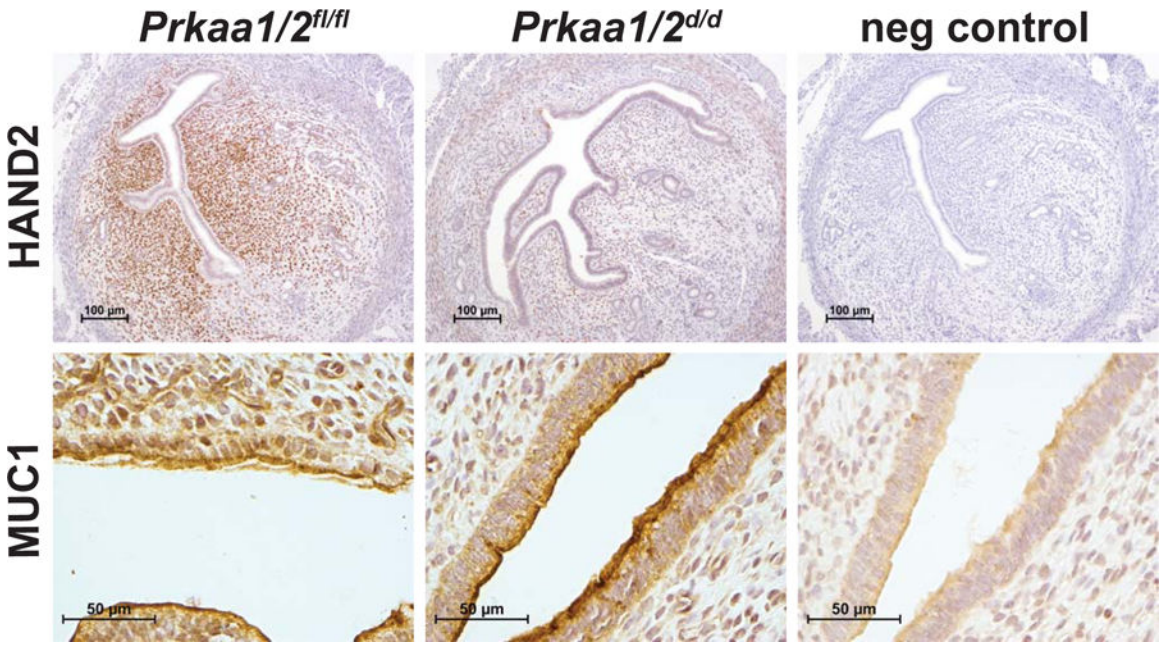

Figure 6. HAND2 expression is decreased in the DOPP5 *Prkaa1/2*$^{d/d}$ uterus.

HAND2 and MUC1 were evaluated in intact *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ mice on DOPP5 using immunohistochemistry. *Prkaa1/2*$^{fl/fl}$ animals show strong expression of HAND2 in the stroma whereas *Prkaa1/2*$^{d/d}$ animals show a dramatic decrease in stromal staining. No difference in MUC1 expression was observed between the two groups. n=3

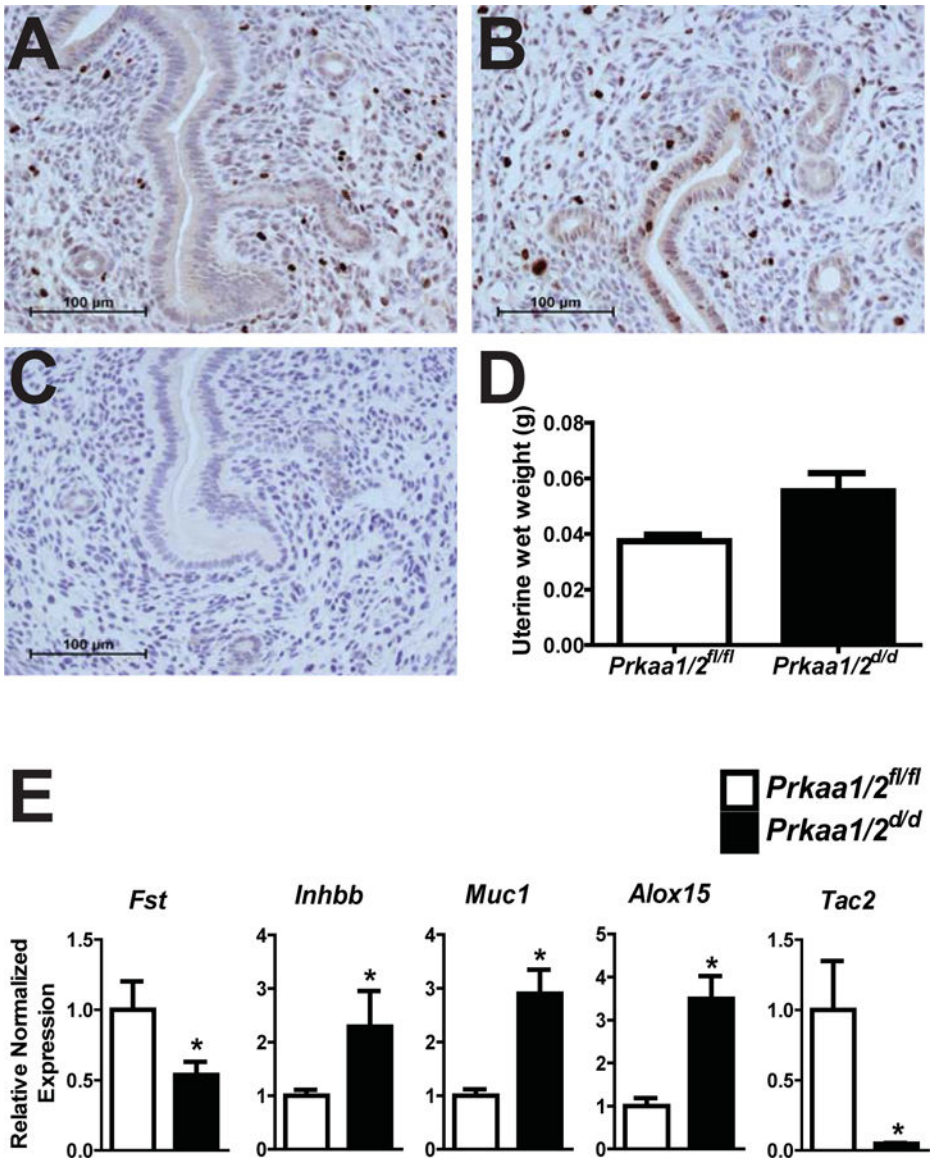

Figure 7. Aberrant steroid hormone signaling in uteri from ovariectomized *Prkaa1/2*$^{d/d}$ mice treated with exogenous steroid hormones mimicking early pregnancy.

(**A**) Following ovariectomy and provision of a steroid hormone regimen consistent with early pregnancy (McCallum *et al.* 2018), immunohistochemical analysis of the mitosis marker phosphohistone-H3 shows some proliferation in the stromal compartment with no proliferation in the luminal epithelium in *Prkaa1/2*$^{fl/fl}$ uteri. (**B**) In contrast, *Prkaa1/2*$^{d/d}$ uteri show retention of proliferation in the luminal epithelium with some proliferation in the stroma. (**C**) No primary antibody negative control. (**D**) *Prkaa1/2*$^{d/d}$ uteri tended (P=0.07) to weight more than *Prkaa1/2*$^{fl/fl}$ uteri. (**E**) RT-qPCR analysis of *Fst, Inhbb, Muc1, Alox15* and *Tac2* in uteri obtained from *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ mice. Values are expressed as mean ± SEM, *P≤0.05, n=3.

Table 1.

Primers used for genotyping and qPCR

Gene	Forward Primer	Reverse Primer(s)
qPCR		
Alox15	5’-AGAGTGGCCACACCAAGATG-3’	5’-GTAGACCGCTTCAGCACCAT-3’
Bmp2	5’-ACGAGAAAAGCGTCAAGCCA-3’	5’-CCAGTCATTCCACCCCACAT-3’
Ccna2	5’-TAAGCCTTGTCTTGTGGACCT-3’	5’-TGTCTCTGGTGGGTTGAGAAG-3’
Ccnb1	5’-ACAACGGTGAATGGACACCA-3’	5’-TCATGTGCTTTGTGAGGCCA-3’
Ccnb2	5’-TGCCTGTCTCAGAAGGTGCT-3’	5’-AGCGATGAACTTGGTACGGTT-3’
Ccne1	5’-GTTACAGATGGCGCTTGCTC-3’	5’-AGCCAGGACACAATGGTCAG-3’
Ccne2	5’-CCAGTAACAGTCATCTCCTGGT-3’	5’-AGTCGATGGCTAGAATGCACA-3’
Ccnf	5’-TTCCACGATGATGCACCCAA-3’	5’-GTCAGCATAGCTCAGCACCT-3’
Cdk1	5’-ACGGCGACTCAGAGATTGAC-3’	5’-ACTTCTGGCCACACTTCGTT-3’
E2f8	5’-TAACGACATCTGCCTGGACG-3’	5’-CCATGTGCAGGCTCTCTAGG-3’
Esr1	5’-TGTTTGCTCCTAACTTGCTCCT-3’	5’-TCATGCGGAACCGACTTGAC-3’
Follistatin	5’-GTGACAATGCCACATACGCC-3’	5’-ACTTCAAGAAGCACGCCAGA-3’
Gpx3	5’-CCGGGGACAAGAGAAGTCTAAG-3’	5’-GATGGTGAGGGCTCCATACTC-3’
Hand2	5’-TCAAGGCGGAGATCAAGAAGAC-3’	5’-TTCTTGTCGTTGCTGCTCACT-3’
Il1b	5’-TGCCACCTTTTGACAGTGATGA-3’	5’-ATCAGGACAGCCCAGGTCAA-3’
Inhbb	5’-TGTGGCACGAGAGACTCCTA-3’	5’-CGCATCCGTTTGCTGGTATC-3’
Ltf	5’-CCTGCTTGCTAACCAGACCA-3’	5’-ACACAGGGCACAGAGATTGG-3’
Msx1	5’-CTAGATCGGACCCCGTGGAT-3’	5’-TGGTCTTGTGCTTGCGTAGG-3’
Muc1	5’-TACCACACTCACGGACGCTA-3’	5’-ACTGCCATTACCTGCCGAAA-3’
Pgr	5’-AGCATGTCGTCTGAGAAAGTGT-3’	5’-AACACCGTCAAGGGTTCTCAT-3’
Prss28	5’-CAACCTTCTCCAACGTGTGC-3’	5’-TTGTGCTCGTCGGATGACTT-3’
Rpl13a	5’-TTGCTTACCTGGGGCGTCT-3’	5’-CCTTTTCCTTCCGTTTCTCCTC-3’
S100a9	5’-ACCACCATCATCGACACCTTC-3’	5’-AAAGGTTGCCAACTGTGCTTC-3’
Tac2	5’-TAGCGTGGGACCAAAGGAGA-3’	5’-GGCTGTTCCTCTTGCCCATA-3’
Genotyping		
Pgr-Cre	5’-ATGTTTAGCTGGCCCAAATG-3’	5’-TATACCGATCTCCCTGGACG-3’
		5’-CCCAAAGAGACACCAGGAAG-3’
Prkaa1	5’-TATTGCTGCCATTAGGCTAC-3’	5’-GACCTGACAGAATAGGATATGCCCAACCTC-3’
Prkaa2	5’-GCTTAGCACGTTACCCTGGATGG-3’	5’-GTTATCAGCCCAACTAATTACAC-3’

Table 2.

Antibodies used for IHC

Target protein	Manufacturer, Catalog No.	Species, Clonal Status	Dilution
ESR1	Thermo Fisher Scientific, MA5–13191	Mouse, Monoclonal	1:200
HAND2	Santa Cruz Biotechnology, SC-9409	Goat, Polyclonal	1:100
PGR	Thermo Scientific, RM-9102	Rabbit, Monoclonal	1:250
PHH3	Millipore, 06–570	Rabbit, Polyclonal	1:500
MUC1	Abcam, 15481	Rabbit, Polyclonal	1:100