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Citation of this paper:
De Oliveira, Vanessa; Schaefer, Jennifer; Abu-Rafea, Basim; Vilos, George A.; Vilos, Angelos G.; Bhattacharya, Moshmi; Radovick, Sally; and Babwah, Andy V., "Uterine aquaporin expression is dynamically regulated by estradiol and progesterone and ovarian stimulation disrupts embryo implantation without affecting luminal closure" (2020). *Paediatrics Publications*. 597.
https://ir.lib.uwo.ca/paedpub/597

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This article is available at Scholarship@Western: https://ir.lib.uwo.ca/paedpub/597

Molecular Human Reproduction, Vol.26, No.3, pp. 154–166, 2020
Advance Access Publication on January 16, 2020 doi:10.1093/molehr/gaaa007

molecular human reproduction

ORIGINAL RESEARCH

Uterine aquaporin expression is dynamically regulated by estradiol and progesterone and ovarian stimulation disrupts embryo implantation without affecting luminal closure

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Submitted on September 26, 2019; resubmitted on December 18, 2019; editorial decision on January 10, 2020

ABSTRACT: The study investigated the effect of normal and supraphysiological (resulting from gonadotropin-dependent ovarian stimulation) levels of estradiol (E2) and progesterone (P4) on mouse uterine aquaporin gene/protein (*Aqp*/AQP) expression on Day 1 (D1) and D4 of pregnancy. The study also examined the effect of ovarian stimulation on uterine luminal closure and uterine receptivity on D4 of pregnancy and embryo implantation on D5 and D7 of pregnancy. These analyses revealed that the expression of *Aqp3*, *Aqp4*, *Aqp5* and *Aqp8* is induced by E2 while the expression of *Aqp1* and *Aqp11* is induced by P4. Additionally, P4 inhibits E2 induction of *Aqp3* and *Aqp4* expression while E2 inhibits *Aqp1* and *Aqp11* expression. *Aqp9*, however, is constitutively expressed. Ovarian stimulation disrupts *Aqp3*, *Aqp5* and *Aqp8* expression on D4 and AQP1, AQP3 and AQP5 spatial expression on both D1 and D4, strikingly so in the myometrium. Interestingly, while ovarian stimulation has no overt effect on luminal closure and uterine receptivity, it reduces implantation events, likely through a disruption in myometrial activity and embryo development. The wider implication of this study is that ovarian stimulation, which results in supraphysiological levels of E2 and P4 and changes (depending on the degree of stimulation) in the E2:P4 ratio, triggers abnormal expression of uterine AQP during pregnancy, and this is associated with implantation failure. These findings lead us to recognize that abnormal expression would also occur under any pathological state (such as endometriosis) that is associated with changes in the normal E2:P4 ratio. Thus, infertility among these patients might in part be linked to abnormal uterine AQP expression.

Key words: Water homeostasis / fluid homeostasis / AQP / aquaporin / ovarian stimulation / superovulation / embryo implantation / uterus

Introduction

Embryo implantation remains the least understood rate-limiting step in pregnancy (Koot *et al*., 2012; Su and Fazleabas, 2015), and studies suggest that uterine luminal (or endometrial cavity) fluid volume is an important regulator of implantation. In humans, excessive luminal fluid can have a negative impact on pregnancy. For example, in women with hydrosalpinx (blocked and fluid filled fallopian tubes), the implantation rates following IVF and embryo transfer (ET) is low (Zeyneloglu *et al*., 1998; Camus *et al*., 1999) and it is thought to be due to the leakage of hydrosalpinx fluid into the uterine cavity (Chien *et al*., 2002; Strandell and Lindhard, 2002; Hinckley and Milki, 2003) since treatments that prevent the leakage improve pregnancy rates (Strandell *et al*., 2001; Kontoravdis *et al*., 2006). It is also reported that luminal fluid accumulation following controlled ovarian stimulation, although not a common complication, can be detrimental to embryo implantation (Sharara

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and Prough, 1999; Chien *et al*., 2002; Akman *et al*., 2005; He *et al*., 2010; Liu *et al*., 2016). The idea is that too much fluid reduces contact between the embryo and luminal epithelium and for that reason during ET, the embryo is buffered in a very small volume of fluid (usually 20–60 µl).

Water movement in and out the endometrial cavity (lumen) across the uterine epithelium is controlled by many molecules, and among the key players are the estradiol (E2)-induced cystic fibrosis transmembrane conductance regulator (CFTR) that mediates secretion and the progesterone (P4)-induced epithelial sodium channel (ENaC) that mediates absorption (reviewed in Chan *et al*., 2009). Aquaporins (AQPs) are also important regulators of water homeostasis, but the roles of uterine AQPs in regulating uterine receptivity and embryo implantation are poorly understood. AQPs are selective bidirectional transporters that move water, gases and some neutral solutes along a concentration gradient, and compared with simple diffusion across the lipid bilayer, AQPs exhibit a greater capacity for water movement (Agre *et al*., 2002). To date, 13 mammalian AQPs (AQP0–12) have been identified (Li and Wang, 2017), but in the mouse, *Aqp10* is a pseudogene (Morinaga *et al*., 2002) and thus only 12 *Aqp* genes encode functional proteins.

While it is clear that many of the mouse uterine AQP genes and proteins are regulated by E2 and/or P4, the unambiguous determination as to which specific AQPs are E2- and/or P4-regulated requires further investigation (Jablonski *et al*., 2003; Richard *et al*., 2003; Zhang *et al*., 2015). In a recent study, it was considered that abnormally high levels of E2 and P4 resulting from ovarian stimulation (an important step in IVF protocols) could alter uterine AQP expression with the potential of disrupting uterine fluid homeostasis and embryo implantation. To explore this, E2 was administered to pregnant mice at a supraphysiological dose on D4 and this triggered a dramatic rise in *Aqp5* and *Aqp8* expression that was coupled to uterine fluid accumulation and reduced embryo implantation, suggesting a role for AQP5 and AQP8 in mediating the E2-induced secretion of water into the lumen (Zhang *et al*., 2015).

To develop an unambiguous understanding of the regulation of mouse uterine AQP expression by E2 and P4 under normal and ovarian-stimulated conditions in the preimplantation period, we conducted a multi-faceted analysis of expression. This analysis included the recently discovered *Aqp11* and *Aqp12* (Li and Wang, 2017). We reasoned that by analyzing the expression of a given AQP using multiple approaches, ambiguous or conflicting data could be accurately reconciled thereby proving a stronger and clearer understanding of uterine AQP expression. Analyses were conducted on uteri isolated from Day 1 (D1), D4 and D7 pregnant non-ovarian-stimulated mice, from D4 pregnant non-ovarian-stimulated RU486-treated mice and from ovariectomized (OVX) mice that received E2, P4 or E2 + P4. Studies were also conducted on D1, D4 and D7 pregnant ovarian-stimulated mice to determine the effect of supraphysiological levels of E2 and P4 on AQP gene and protein expression, luminal volume, uterine receptivity and embryo implantation. Finally, to determine whether the preimplantation embryo modulated uterine AQP expression, expression was compared between D4 pregnant and pseudopregnant mice. In these studies, D1, D4 and D7 of pregnancy were selected for analysis because on D1, gene expression is largely regulated by pre-ovulatory E2 while on D4, following corpora lutea formation, regulation by P4 is also prevalent (Wang and Dey, 2006). Additionally, D4 represents the day that uterine receptivity is acquired and on that afternoon embryo implantation is initiated (Wang and Dey, 2006). D7 was studied as it shows the presence of implantation and resorption sites.

Taken together, our studies clearly revealed that *Aqp1*, *Aqp3*, *Aqp4*, *Aqp5*, *Aqp8*, *Aqp9* and *Aqp11* are expressed in the D1 and D4 pregnant mouse uterus while the expression of *Aqp2*, *Aqp6*, *Aqp7* and *Aqp12* is weakly or barely detected. Our investigations also revealed that uterine AQP genes and proteins are dynamically regulated by E2 and P4 where E2 induces the expression of *Aqp3*, *Aqp4*, *Aqp5* and *Aqp8* while P4 induces the expression of *Aqp1* and *Aqp11*. Our study also showed that ovarian stimulation significantly disrupted normal expression patterns of AQP1, *Aqp3*/AQP3, *Aqp5*/AQP5 and *Aqp8* on D4 and while this had no overt effect on luminal closure and uterine receptivity it disrupted embryo spacing along the uterine horns and reduced implantation rates.

Materials and Methods

Animal studies

Animal studies were approved by Rutgers University according to guidelines established by the Institutional Animal Care and Use Committee. The mice were maintained under a 12-h light/dark cycle and provided with standard rodent chow and water *ad libitum*. WT C57 Bl/6 mice were used in all studies.

Unstimulated pregnancy

Females (8–10 weeks old) in proestrus were mated either to vasectomized males (to establish a pseudopregnancy) or to stud males (to establish a true pregnancy). The day of mating is defined as Day 0. Only females that showed a copulatory plug (evidence of successful mating) on the morning of D1 were studied further. Mice were euthanized between 8:00 and 9:00 a.m. on D1 or D4 of pregnancy and uteri collected and stored in RNAlater (Life Technologies Inc., Burlington, ON, Canada). In a second cohort of unstimulated mice, pregnancy was allowed to progress to D7 and uteri were isolated and analyzed for implantation swellings and resorption sites by visual inspection.

Ovarian-stimulated pregnancy

Females (8 week old) were administered 7.5 IU pregnant mare serum gonadotropin (PMSG; Folligon; Intervet) i.p. followed 48 h later by 7.5 IU hCG (Chorulon; Intervet) i.p. Immediately after the hCG injection, mice were mated to vasectomized or stud males. The day of mating is defined as Day 0. Only females that showed a copulatory plug (evidence of successful mating) on the morning of D1 were studied further. Mice were euthanized between 8:00 and 9:00 a.m. on D1 or D4 of pregnancy and uteri collected and stored in RNAlater (Life Technologies Inc., Burlington, ON, Canada). In a second cohort of ovarian-stimulated mice, pregnancy was allowed to progress to D7 and uteri were isolated and analyzed for implantation swellings and resorption sites by visual inspection.

Zygote transfer study

Three-week old ovarian-stimulated WT C57 Bl/6 females were mated to WT C57 Bl/6 stud males, and on the morning of D1 of preg-

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nancy, females were euthanized and zygotes were collected from the oviducts with M2 medium (Sigma, St. Louis, MO, USA). Zygotes were then transferred into the left and right oviducts of WT D1 ovarian-stimulated ($n = 3$) and non-stimulated ($n = 3$) 8-week old pseudopregnant WT C57 Bl/6 females. In each case, 15 zygotes were transferred into each oviduct. On the morning of D5 of pregnancy, uterine horns were collected and flushed to recover unimplanted blastocysts and other embryos. Recovered embryos were then quantified and characterized by light microscopy. Data were analyzed by Student's *t* test, where $P < 0.05$ was considered statistically significant.

E2, P4 or E2 + P4 regulation of gene expression

Eight-week-old ovariectomized mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and 2 weeks after ovariectomy mice were administered by s.c. injection either peanut oil (100 µl), E2 (100 ng in a final volume of 100 µL), P4 (1 mg in a final volume of 100 µL) or E2 (100 ng) + P4 (1 mg) (in a final volume of 200 µl) (Jeong *et al*., 2010). Twenty-four and 48 h after hormone administration, mice were euthanized and uteri were collected and stored in RNAlater (Life Technologies Inc., Burlington, ON, Canada). E2 and P4 were purchased from Tocris Bioscience (Minneapolis, MN).

RU486 administration

Ovarian-stimulated mice following hCG injection were mated to vasectomized adult males (D0 of pseudopregnancy). On the following morning (D1) females exhibiting a copulatory plug were set aside, and at 9:00 a.m. on D3 each mouse was administered either 200 µl oil s.c. or 200 µl RU486 (400 µg) s.c. (de Oliveira *et al*., 2019). On the morning of D4, mice were euthanized and uteri were collected and stored in RNAlater (Life Technologies Inc., Burlington, ON, Canada). RU486 was purchased from Sigma (St. Louis, MO, USA). The RU486 protocol is modified after Haraguchi *et al*. (2014).

H&E staining

Mouse uteri were fixed in 4% paraformaldehyde (PFA) overnight, transferred to 70% ethanol, paraffin embedded and sectioned at 5 µm. Uterine sections were deparaffinized, rehydrated and stained with H&E using standard techniques. Sections were then dehydrated in ethanol, cleared in xylene, overlaid with Permount mounting medium (Fisher Scientific, Ottawa, ON, Canada) and sealed with a coverslip.

Immunohistochemistry

Mouse uteri were fixed in 4% (w/v) PFA overnight, washed 3× in PBS and then transferred to 70% ethanol, paraffin embedded and sectioned at 5 µm. Uterine sections were deparaffinized, rehydrated and blocked with normal blocking serum (horse) and then incubated with the following primary antibodies: (i) rabbit monoclonal anti-AQP1 (1:150 dilution, catalogue # AB168387, (ii) rabbit polyclonal anti-AQP3 (1:100 dilution, catalogue # AB125219, (iii) rabbit polyclonal anti-AQP5 (1:125 dilution, catalogue # AB78486. Primary antibodies were purchased from Abcam, Cambridge, MA, USA. Antigen-bound primary antibodies were detected with the ImmunoCruz rabbit ABC Staining System (Santa Cruz Biotechnology, Inc. Dallas, TX, USA). Stained sections were overlaid with Permount mounting medium (Fisher Scientific, Ottawa, ON, Canada) and sealed with a coverslip. For a given AQP, all samples were processed in parallel and treated with 3,3′-diaminobenzidine for an identical period of time. This permitted a comparison of protein levels under different experimental conditions. Experimental conditions were carefully maintained between independent assays and analyses were conducted three independent times. A no primary antibody control was conducted to determine the nonspecific binding of the secondary antibody. The same secondary antibody was used in all immunohistochemistry (IHC studies.

Image acquisition

For both H&E- and 3,3′-diaminobenzidine-stained tissue sections, slides were scanned using the Aperio ScanScope XT. Sections were then viewed and measured using the ImageScope software.

qPCR analysis of gene expression

Gene expression studies were conducted using PrimePCR Assays (Bio-Rad Laboratories): *Aqp1* (Unique Assay ID: qMmuCID0020860), *Aqp2* (Unique Assay ID: qMmuCID0006873), *Aqp3* (Unique Assay ID: qMmuCID0024368), *Aqp4* (Unique Assay ID: qMmuCID0006859), *Aqp5* (Unique Assay ID: qMmuCID0006880), *Aqp6* (Unique Assay ID: qMmuCED0003333), *Aqp7* (Unique Assay ID: qMmuCID0025269), *Aqp8* (Unique Assay ID: qMmuCID0007218), *Aqp9* (Unique Assay ID: qMmuCID0022285), *Aqp11* (Unique Assay ID: qMmuCED0048241), *Aqp12* (Unique Assay ID: qMmuCID0007984), *Ltf* (Unique Assay ID: qMmuCIP0034255), *Hand2* (Unique Assay ID: qMmuCID0009541), *Rpl13a* (Unique Assay ID: qMmuCED0040629). Based on full wet-lab validation, all assays met the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al*., 2009). Assays were validated in terms of specificity, efficiency and linear dynamic range. At least 10 qPCR reactions were conducted for each assay where cDNA was prepared from reference RNA, no template control, gDNA and seven points from a 10-fold dilution series of synthetic templates (from 20 million down to 20 copies). All assays used in this study exhibited efficiencies between 90 and 110%. Since the PrimePCR Assays (Bio-Rad Laboratories) exhibit similar efficiencies (90–110%), gene expression was calculated as relative expression to the housekeeping gene (*Rpl13a*), using the $2^{-\Delta\Delta Ct}$ method.

Gene expression was determined on total RNA prepared from the uterine horns of mice. In the case of 'true pregnancies', D4 pregnant horns were flushed free of embryos immediately after isolation. Uteri were collected in RNAlater (Life Technologies Inc., Burlington, ON, Canada), homogenized and RNA was isolated using the Qiagen RNeasy mini kit according to manufacturer's instructions (Qiagen, Missassauga, ON, Canada). cDNA was synthesized using iScript™ Reverse Transcription Supermix from Bio-Rad. Briefly, 1 µg of total RNA was reverse transcribed and diluted 2-fold in water. Five microliters of diluted cDNA was used in each qPCR reaction. qPCR was conducted using the iTaq Universal SYBR Green Supermix from Bio-Rad.

Statistics

The differences between groups were determined using unpaired Student's *t* test or one-way ANOVA followed by post hoc Bonferroni test (GraphPad Prism Software, Inc, La Jolla, CA). All values are expressed as mean ± SEM and a value of $P < 0.05$ was considered statistically significant.

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Results

The uterus from the D1 and D4 pseudopregnant, non-ovarian-stimulated mouse expresses several *Aqp* genes as well as *Rpl13a*

qPCR analysis revealed that in the whole uterus on D1 and D4 of pseudopregnancy, the expression of several *Aqp* genes as well as *Rpl13a* was readily detected. In this study, *Rpl13a* was selected as the reference gene because it is highly expressed in the mouse uterus and expression is not influenced by E2 and P4 (Schroder *et al.*, 2009). In the current study, it was determined that the cycle-threshold (Ct) value for *Rpl13a* on D1 and D4 did not vary more than ±0.5 units among independent samples within a given day or between both sample days. Given the almost invariable expression of *Rpl13a* among independent samples coupled to the similar efficiencies (90–110%) exhibited by all qPCR primers used in this study (PrimePCR Assays, Bio-Rad Laboratories), unprocessed Ct values of gene expression were initially analyzed to obtain an overview of the expression of the genes under investigation. Based on these values, only genes that exhibited a Ct value of 30 or less on either D1 or D4 of pseudopregnancy in the uterus of the non-ovarian-stimulated mouse were selected for further analysis. The selected genes were *Aqp1*, *Aqp3*, *Aqp4*, *Aqp5*, *Aqp8*, *Aqp9* and *Aqp11*. As for *Aqp6* and *Aqp7*, on both D1 and D4 of pseudopregnancy, Ct values were consistently greater than 30 while values for *Aqp2* and *Aqp12* were greater than 35. Accordingly, these genes were excluded from this study.

Uterine *Aqp1* is induced by P4 but repressed by E2

Uterine *Aqp1* expression was assessed in D1 and D4 pseudopregnant females. These females had not undergone ovarian stimulation

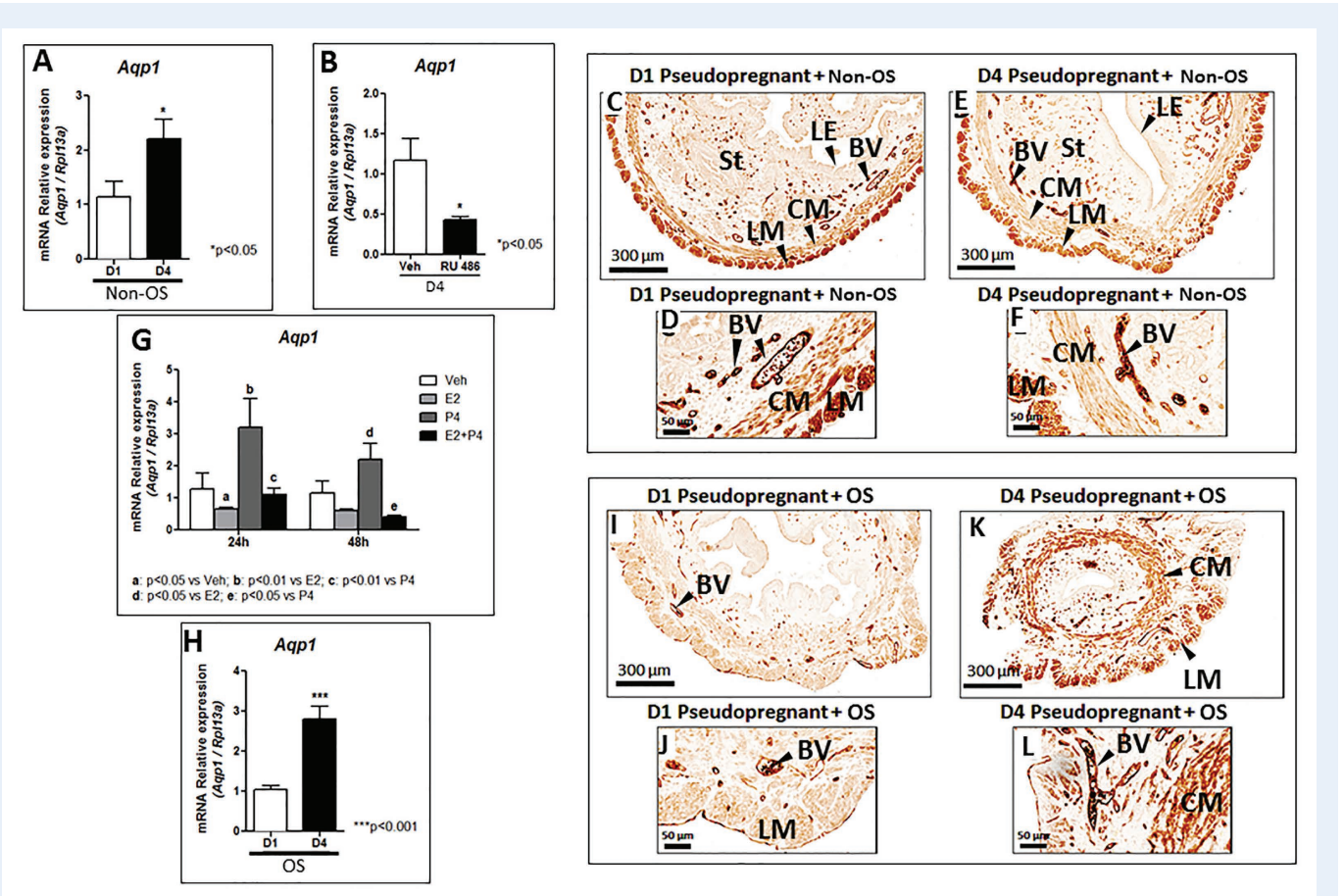

Figure 1 Uterine *Aqp1* is induced by progesterone (P4) but repressed by estradiol (E2) in the ovariectomized mouse model. Uterine *Aqp1* expression was measured in the non-ovarian-stimulated, pseudopregnant mouse (**A**) by qPCR on Day 1 (D1) and D4 of pregnancy, (**B**) by qPCR on D4 in the oil- and RU486-treated mouse and (**C**–**F**) by immunohistochemistry (IHC) on D1 and D4. Expression was also measured by qPCR in the (**G**) OVX mouse + oil, E2, P4 or E2 + P4 following 24 and 48 h of treatment, (**H**) in the ovarian-stimulated, pseudopregnant mouse on D1 and D4 of pregnancy and finally (**I**–**L**) expression was also measured by IHC on D1 and D4 in the ovarian-stimulated, pseudopregnant mouse. For each experimental group, uteri from 10–15 mice were analyzed by qPCR while uteri from three mice were analyzed by IHC. BV: blood vessel; CM: circular muscle of the myometrium; LE: luminal epithelium; LM: longitudinal muscle of the myometrium; St: stroma; non-OS: non-ovarian-stimulated; OS: ovarian-stimulated; vs, versus.

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Table I Summary of *Aqp* gene expression changes during early pregnancy and in response to E2 or P4 administration.

Gene	Non-OS D4 vs. D1	Effect of E2 or P4 administered to the OVX mouse	Effect of E2 on P4- induced expression or P4 on E2-induced expression in the OVX mouse	OS D4 vs. D1
Aqp1	increased	Induced by P4	E2 blocks P4-induced expression	increased
Aqp3	decreased	Induced by E2	P4 transiently represses E2-induced expression	increased
Aqp4	decreased	Induced by E2	P4 represses E2-induced expression	decreased
Aqp5	decreased	Induced by E2	P4 has no effect on E2-induced expression	increased
Aqp8	decreased	Induced by E2	P4 has no effect on E2-induced expression	increased
Aqp9	unchanged	No effect by E2 or P4	E2 and P4 have no effect on expression	unchanged
Aqp11	increased	Induced by P4	E2 blocks P4-induced expression	increased

(see Materials and Methods) and are referred to as 'Non-OS' in the figures. The results showed that there was a small but significant increase in expression on the morning of D4 raising the possibility that *Aqp1* is a P4-induced gene (Fig. 1A, Table I). To test this further, females were administered RU486 (a P4 receptor modulator that blocks the action of P4 by binding to the P4 receptor) (Bagchi *et al.*, 2005) on D3 of pseudopregnancy and on the morning of D4, *Aqp1* expression was determined. As seen, relative to oil-treated mice, RU486 downregulated *Aqp1* expression strengthening the finding that its expression is induced by P4 (Fig. 1B). At the protein level on both D1 and D4 in the non-ovarian-stimulated mouse, AQP1 was strongly expressed in the longitudinal muscle of the myometrium with weaker but discernible expression in the circular muscle (Fig. 1C–F, Supplementary Fig. S1). AQP1 was also highly expressed in endothelial cells lining blood vessels as well as in the blood cells within these vessels (Fig. 1C–F, Supplementary Fig. S1). Overall, both the level and spatial distribution of AQP1 protein on D1 and D4 appeared similar suggesting the small but significant increase in mRNA expression observed on D4 (Fig. 1A) did not dramatically alter AQP1 expression (Fig. 1C–F, Supplementary Fig. S1).

To further understand the regulation of uterine *Aqp1* expression by P4 and E2, *Aqp1* expression was assessed in females that received either vehicle (oil), E2, P4 or E2 + P4 for 24 or 48 h, 2 weeks after ovariectomy (OVX). The results confirmed that *Aqp1* expression is indeed induced by P4, but interestingly the P4 induction is blocked by E2 (Fig. 1G, Table I). This repressive effect was powerful as the E2 + P4 treatment was the same as the E2-treatment only (Fig. 1G, Table I).

Following ovarian stimulation in both animals and humans, E2 and P4 rise to supraphysiological levels and the E2:P4 ratio is disrupted; this can alter gene expression profiles observed in the non-stimulated female during the pre- and peri-implantation period. Such changes can have adverse effects on the acquisition of uterine receptivity and embryo implantation (Gidley-Baird *et al.*, 1986; Simon *et al.*, 1995; Valbuena *et al.*, 2001; Zhang *et al.*, 2015). To determine whether ovarian stimulation alters *Aqp1* expression on the morning of D4 of pregnancy (the day of uterine receptivity), expression was assessed in D1 and D4 pseudopregnant, ovarian-stimulated females (referred to as 'OS' in the figures). The results revealed that following stimulation, the D1 to D4 *Aqp1* expression profile closely resembles that seen in the non-stimulated females indicating that stimulation had no effect on gene expression (Fig. 1H vs. 1A, Table I). Surprisingly, at the protein level, stimulation triggered striking changes. Here it was observed that while AQP1 was still strongly expressed on endothelial and blood cells on both D1 and D4 (Fig. 1I–L compared to 1C–F), expression in the myometrium was completely lacking on D1 (Fig. 1I and J compared to 1J and D 1C and D) while on D4 it was again expressed on the myometrial longitudinal muscle but greatly intensified on the circular muscle (Fig. 1K and L compared to 1E and F).

Uterine *Aqp3* is induced by E2 while P4 blocks the E2 stimulatory effect

Uterine *Aqp3* expression was assessed in D1 and D4 pseudopregnant, non-ovarian-stimulated females, and the results showed that expression was significantly reduced on the morning of D4 suggesting that *Aqp3* is an E2-induced gene (Fig. 2A, Table I). At the protein level on D1 in the non-stimulated mouse, AQP3 was localized to the luminal and glandular epithelia as well as the myometrial longitudinal and circular muscles (Fig. 2B and C). On D4, AQP3 expression was only weakly detected in the myometrium and absent in the epithelium (Fig. 2D and E).

In the OVX model, it was confirmed that *Aqp3* expression was induced by E2 and while P4 alone had no effect on expression, 24 h following treatment it significantly reduced the E2-induced expression (Fig. 2F, Table I). In pseudopregnant, stimulated females, when compared to the pseudopregnant, non-stimulated females, expression was greatly increased on D4 compared to D1 (Fig. 2G compared to 2A, Table I) suggesting that following ovarian stimulation, the supraphysiological levels of E2 triggered an induction of *Aqp3* expression not seen in non-stimulated females.

At the protein level, ovarian stimulation also affected AQP3 expression compared to that observed in the non-stimulated mouse. Following stimulation, on D1, AQP3 was localized to the luminal epithelium in a strong punctate pattern (Fig. 2H and I compared to 2B and C) while on D4 it was strongly expressed in the myometrial circular muscle and endometrial stromal cells (Fig. 2J–L compared to 2D and E).

Uterine *Aqp4* is induced by E2 while P4 blocks the E2 stimulatory effect

Uterine *Aqp4* expression was assessed in D1 and D4 pseudopregnant, non-ovarian-stimulated females, and the results showed that expression was significantly reduced on the morning of D4 suggesting that

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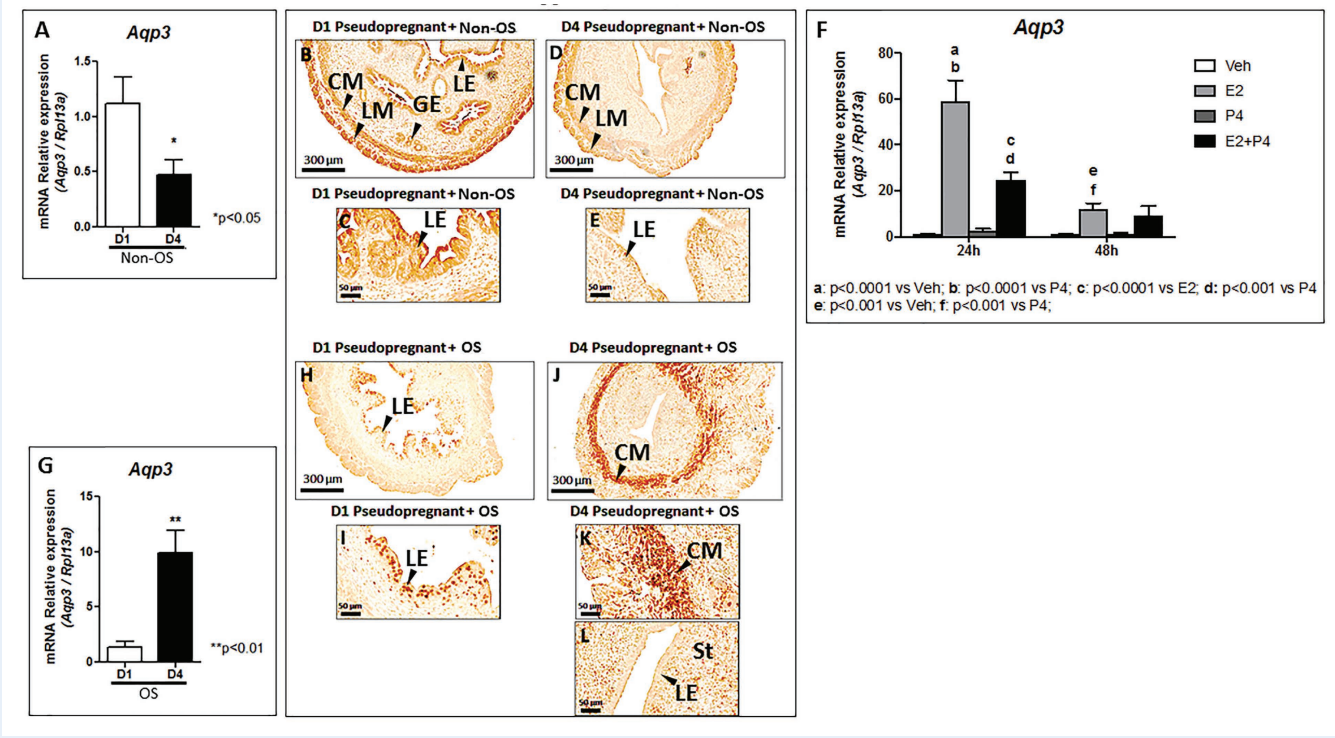

Figure 2 Uterine *Aqp3* is induced by estradiol (E2). Uterine *Aqp3* expression was measured in the non-ovarian-stimulated, pseudopregnant mouse (**A**) by qPCR on Day 1 (D1) and D4 of pregnancy, and (**B**–**E**) by immunohistochemistry (IHC) on D1 and D4. Expression was also measured by qPCR in the (**F**) ovariectomized (OVX) mouse + oil, E2, progesterone (P4) or E2 + P4 following 24 and 48 h of treatment, (**G**) in the ovarian-stimulated, pseudopregnant mouse on D1 and D4 of pregnancy and finally (**H**–**L**) expression was also measured by IHC on D1 and D4 in the ovarian-stimulated, pseudopregnant mouse. For each experimental group, uteri from 10 to 15 mice were analyzed by qPCR while uteri from three mice were analyzed by IHC. CM: circular muscle of the myometrium; GE: glandular epithelium; LE: luminal epithelium; LM: longitudinal muscle of the myometrium; St: stroma. Non-OS: non-ovarian-stimulated; OS: ovarian-stimulated; vs, versus.

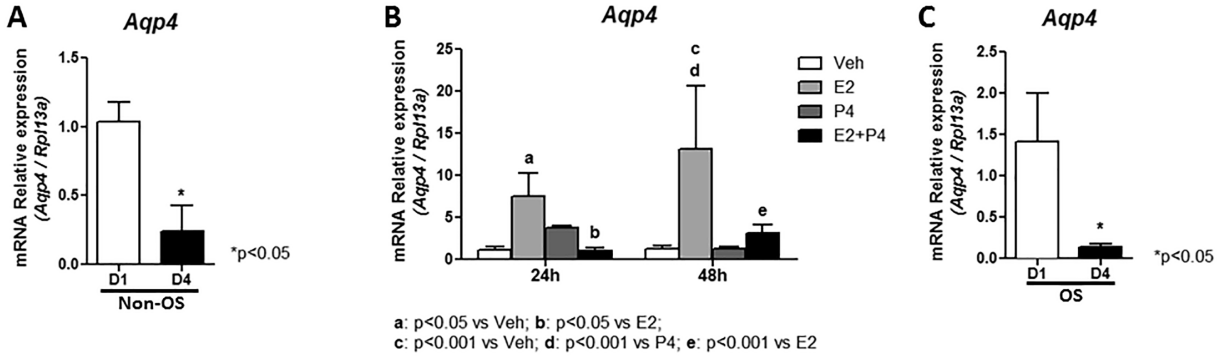

Figure 3 Uterine *Aqp4* is induced by estradiol (E2) while progesterone (P4) blocks the E2 stimulatory effect. Uterine *Aqp4* expression was measured by qPCR in the (**A**) non-ovarian stimulated, pseudopregnant mouse on day 1 (D1) and D4 of pregnancy, (**B**) ovariectomized (OVX) mouse + oil, E2, P4 or E2 + P4 following 24 and 48 h of treatment and (**C**) in the ovarian-stimulated, pseudopregnant mouse on D1 and D4 of pregnancy. For each experimental group, uteri from 10 to 15 mice were analyzed by qPCR. Non-OS: non-ovarian-stimulated; OS: ovarian-stimulated; vs, versus.

Aqp4 is an E2-induced gene (Fig. 3A, Table I). In the OVX model, it was confirmed that *Aqp4* expression was induced by E2, and while P4 alone had no effect on expression, when co-administered with E2, P4 blunted the induction by E2 (Fig. 3B, Table I). In stimulated females, like non-stimulated females, expression was significantly reduced on the morning of D4 (Fig. 3C compared to 3A, Table I).

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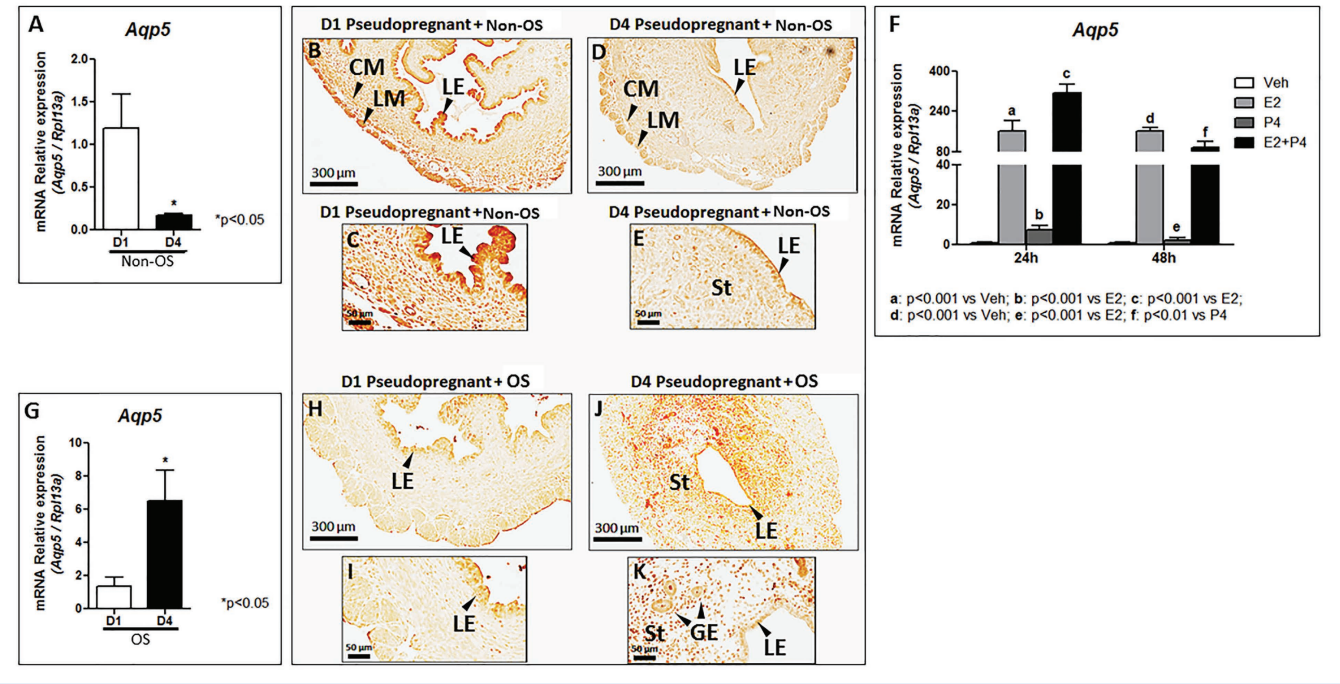

Figure 4 Uterine *Aqp5* is induced by estradiol (E2). Uterine *Aqp5* expression was measured in the non-ovarian-stimulated, pseudopregnant mouse (**A**) by qPCR on Day 1 (D1) and D4 of pregnancy and (**B**–**E**) by immunohistochemistry (IHC on D1 and D4. Expression was also measured by qPCR in the (**F**) ovariectomized (OVX) mouse + oil, E2, P4 or E2 + P4 following 24 and 48 h of treatment, (**G**) in the ovarian-stimulated, pseudopregnant mouse on D1 and D4 of pregnancy and finally (**H**–**L**) expression was also measured by IHC on D1 and D4 in the ovarian-stimulated, pseudopregnant mouse. For each experimental group, uteri from 10 to 15 mice were analyzed by qPCR while uteri from three mice were analyzed by IHC. CM: circular muscle of the myometrium; GE: glandular epithelium; LE: luminal epithelium; LM: longitudinal muscle of the myometrium; St: stroma. Non-OS: non-ovarian-stimulated; OS: ovarian-stimulated; vs, versus.

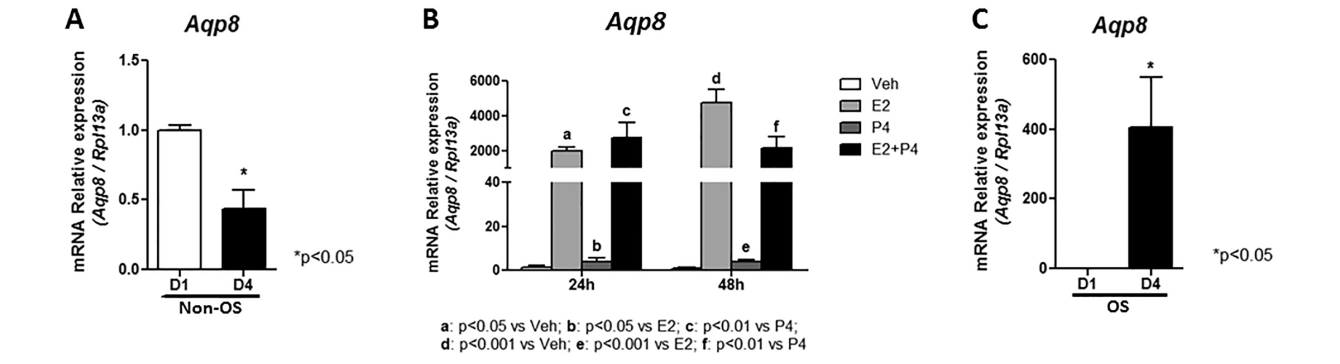

Figure 5 Uterine *Aqp8* is induced by estradiol (E2). Uterine *Aqp8* expression was measured by qPCR in the (**A**) non-ovarian stimulated, pseudopregnant mouse on Day 1 (D1) and D4 of pregnancy, (**B**) ovariectomized (OVX) mouse + oil, E2, progesterone (P4) or E2 + P4 following 24 and 48 h of treatment and (**C**) in the ovarian-stimulated, pseudopregnant mouse on D1 and D4 of pregnancy. For each experimental group, uteri from 10 to 15 mice were analyzed by qPCR. Non-OS: non-ovarian-stimulated; OS: ovarian-stimulated; vs, versus.

Uterine Aqp5 is induced by E2

Uterine *Aqp5* expression was assessed in D1 and D4 pseudopregnant, non-ovarian-stimulated females and the results revealed that expression was significantly reduced on the morning of D4 suggesting that *Aqp5* is an E2-induced gene (Fig. 4A, Table I). At the protein level on D1 in the non-stimulated mouse, AQP5 was observed in the luminal epithelium as well as in the longitudinal muscle of the myometrium (Fig. 4B and C). On D4, however, AQP5 expression was almost completely absent in all uterine compartments (Fig. 4D and E).

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In the OVX model, it was confirmed that *Aqp5* expression was robustly induced by E2 while P4 had no effect (Fig. 4F, Table I). In pseudopregnant, stimulated females, mRNA expression was greatly increased on D4 compared to D1 suggesting that following stimulation, the supraphysiological levels of E2 triggered an induction of *Aqp5* expression not seen in non-stimulated females (Fig. 4G compared to 4A, Table I). At the protein level, stimulation triggered changes in AQP5 expression compared to that observed in the non-stimulated mouse. On D1, AQP5 was expressed on the luminal epithelium and in the longitudinal muscle of the myometrium (Fig. 4H and I compared to 4B and C) while on D4, it was expressed strongly throughout the stroma and weakly in the myometrium (Fig. 4J and K compared to Fig. 4D and E).

Uterine *Aqp8* is induced by E2

Uterine *Aqp8* expression was assessed in D1 and D4 pseudopregnant, non-ovarian-stimulated females, and the results revealed that expression was significantly reduced on the morning of D4 suggesting that *Aqp8* is an E2-induced gene (Fig. 5A, Table I). In the OVX model, it was confirmed that *Aqp8* expression is very strongly induced by E2 while P4 had no effect (Fig. 5B, Table I). In pseudopregnant, stimulated females, expression was greatly increased on D4 compared to D1 suggesting that following stimulation, the supraphysiological levels of E2 triggered an induction of *Aqp8* expression not seen in non-stimulated females (Fig. 5C compared to 5A, Table I).

Uterine *Aqp9* is unresponsive to E2 and P4

Uterine *Aqp9* expression was assessed in D1 and D4 pseudopregnant, non-ovarian-stimulated females, and the results revealed that expression was not significantly different between D1 and D4 suggesting that *Aqp9* is unresponsive to E2 or P4 (Fig. 6A, Table I). In the OVX model, it was confirmed that *Aqp9* expression was unaffected by E2, P4 or E2 + P4 (Fig. 6B, Table I). The observation that *Aqp9* was unresponsive to E2 or P4 was again observed in pseudopregnant, stimulated females where expression was not significantly different between D1 and D4 (Fig. 6C, Table I).

Uterine *Aqp11* is induced by P4 but repressed by E2

Uterine *Aqp11* expression was assessed in D1 and D4 pseudopregnant, non-ovarian-stimulated females, and the results revealed that expression was significantly increased on the morning of D4 suggesting that *Aqp11* is a P4-induced gene (Fig. 7A, Table I). In the OVX model, it was confirmed that *Aqp11* expression was clearly induced by P4 but interestingly it was strongly repressed by E2 (Fig. 7B, Table I). The repressive effect of E2 on expression was powerful as the E2 + P4 treatment was the same as the E2-treatment only (Fig. 7B, Table I). In pseudopregnant, stimulated females, the results clearly revealed that stimulation does not alter the *Aqp11* expression profile from D1 to D4, similar to non-stimulated females (Fig. 7C compared to 7A, Table I).

The preimplantation embryo does not exert any effect on the expression of uterine Aqp genes on D4 of pregnancy

It has been reported that for some genes, uterine expression is modulated by the preimplantation embryo (Das *et al.*, 1994; Passaro *et al.*, 2018; Passaro *et al.*, 2019). In the studies described thus far, expression of the *Aqp* genes was assessed in the pseudopregnant mouse. Therefore, to determine whether their expression is influenced by unimplanted embryos in the uterine horn on the morning of D4, gene expression was compared between the pseudopregnant, ovarian-stimulated female (mated to a vasectomized male) and pregnant, stimulated female (mated to a stud male) following mating. The results revealed that for each gene under investigation, the presence of unimplanted embryos had no significant effect on total gene expression in the uterus (Fig. 8). In a preliminary investigation, it was also observed that D4 uterine *Aqp* gene expression was similar between the non-stimulated pseudopregnant and pregnant female, further strengthening the finding that uterine *Aqp* gene expression is not modulated by the unimplanted embryo.

Ovarian stimulation disrupts embryo spacing and implantation despite having no overt effect on luminal closure and uterine receptivity

Administration of 50 ng of E2 to mice on the morning of D4 of pregnancy was found to be enough to increase luminal fluid retention (by

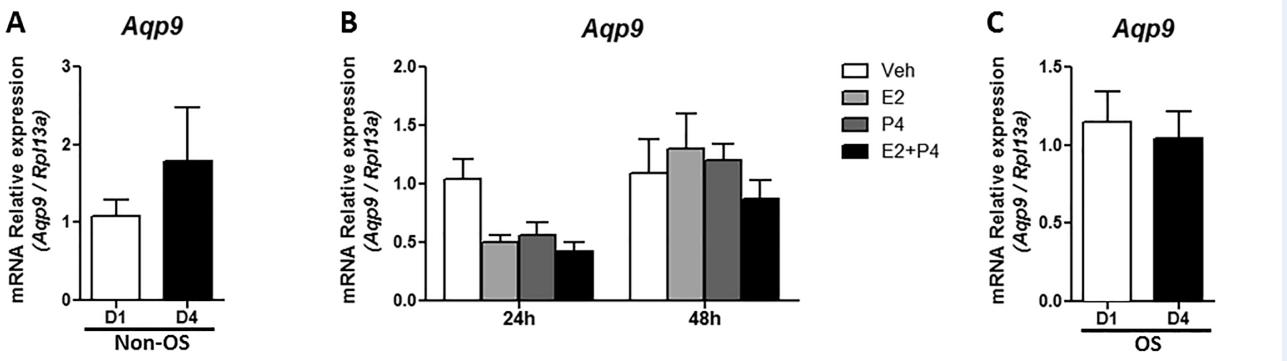

Figure 6 Uterine *Aqp9* is unresponsive to estradiol (E2) and progesterone (P4). Uterine *Aqp9* expression was measured by qPCR in the (**A**) non-ovarian stimulated, pseudopregnant mouse on Day 1 (D1) and D4 of pregnancy, (**B**) ovariectomized (OVX) mouse + oil, E2, P4 or E2 + P4 following 24 and 48 h of treatment and (**C**) in the ovarian-stimulated, pseudopregnant mouse on D1 and D4 of pregnancy. For each experimental group, uteri from 10 to 15 mice were analyzed by qPCR. Non-OS: non-ovarian-stimulated; OS: ovarian-stimulated; vs, versus.

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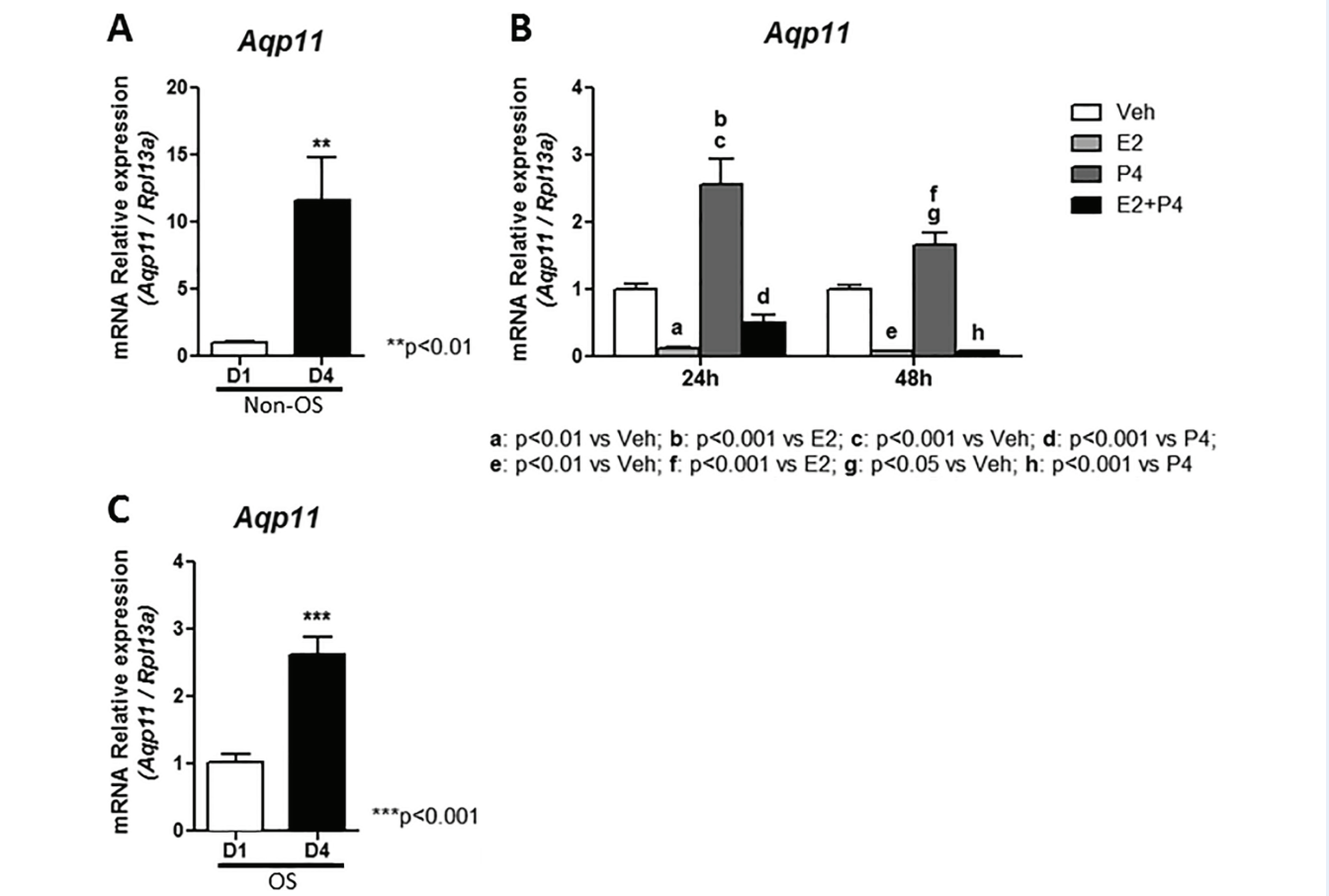

Figure 7 Uterine *Aqp11* is induced by progesterone (P4) but repressed by estradiol (E2). Uterine *Aqp11* expression was measured by qPCR in the (**A**) non-ovarian stimulated, pseudopregnant mouse on Day 1 (D1) and D4 of pregnancy, (**B**) ovariectomized (OVX) mouse + oil, E2, P4 or E2 + P4 following 24 and 48 h of treatment, (**C**) in the ovarian-stimulated, pseudopregnant mouse on D1 and D4 of pregnancy and (**D**) in the non-ovarian-stimulated and ovarian-stimulated mouse on D4 of pregnancy. For each experimental group, uteri from 10 to 15 mice were analyzed by qPCR. Non-OS: non-ovarian-stimulated; OS: ovarian-stimulated; vs, versus.

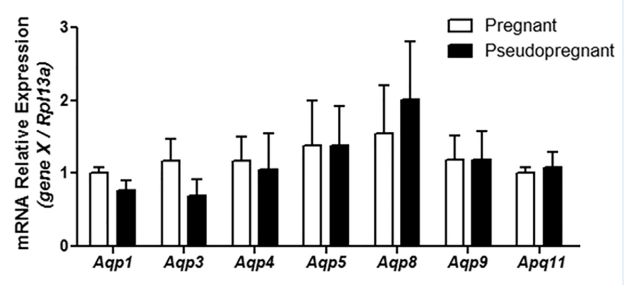

Figure 8 The preimplantation embryo does not exert any effect on the expression of uterine *Aqp* genes on day 4 (D4) of pregnancy. Uterine *Aqp1, 3, 4, 5, 8, 9* and *11* expression was measured by qPCR in the ovarian-stimulated pregnant and pseudopregnant mouse on D4 of pregnancy ($n = 10–15$ mice).

about 4 µl) on the afternoon of D4 and disrupt embryo implantation that begins around that time (Zhang *et al.*, 2015). This study was done to understand the effects of ovarian stimulation employed in human IVF protocols. However, in IVF protocols supraphysiological levels of E2 on the day of ET arise in response to gonadotropin administration given several days prior to ET. Accordingly, to more closely recapitulate the human IVF protocol, mice were administered superovulating levels of PMSG (the equine placental analog of pituitary FSH) and hCG (the human placental analog of pituitary LH) 6 and 4 days, respectively, prior to the start of embryo implantation (see Materials and Methods).

On the morning of D1 and D4 of pregnancy, uteri from ovarian-stimulated and non-stimulated mice were collected and analyzed by histology for luminal size. The results clearly showed that on D1, the lumen of both stimulated and non-stimulated mice was in a wide-open state (Fig. 9A and B) while on D4 it had undergone closure resulting in a similar luminal size in both groups of mice (Fig. 9C and D). The D1 and D4 uteri were also examined by qPCR for the expression of E2-induced *Ltf* and P4-induced *Hand2*, genes that are critical for the acquisition of uterine receptivity on D4. The results clearly revealed that stimulation had no significant effect on gene expression, and as expected there was a downregulation of *Ltf* and an upregulation of *Hand2* on D4 in both treatment groups (Fig. 9E–H). Finally, in another group of mice, pregnancy was allowed to progress to D7, and on the morning of D7

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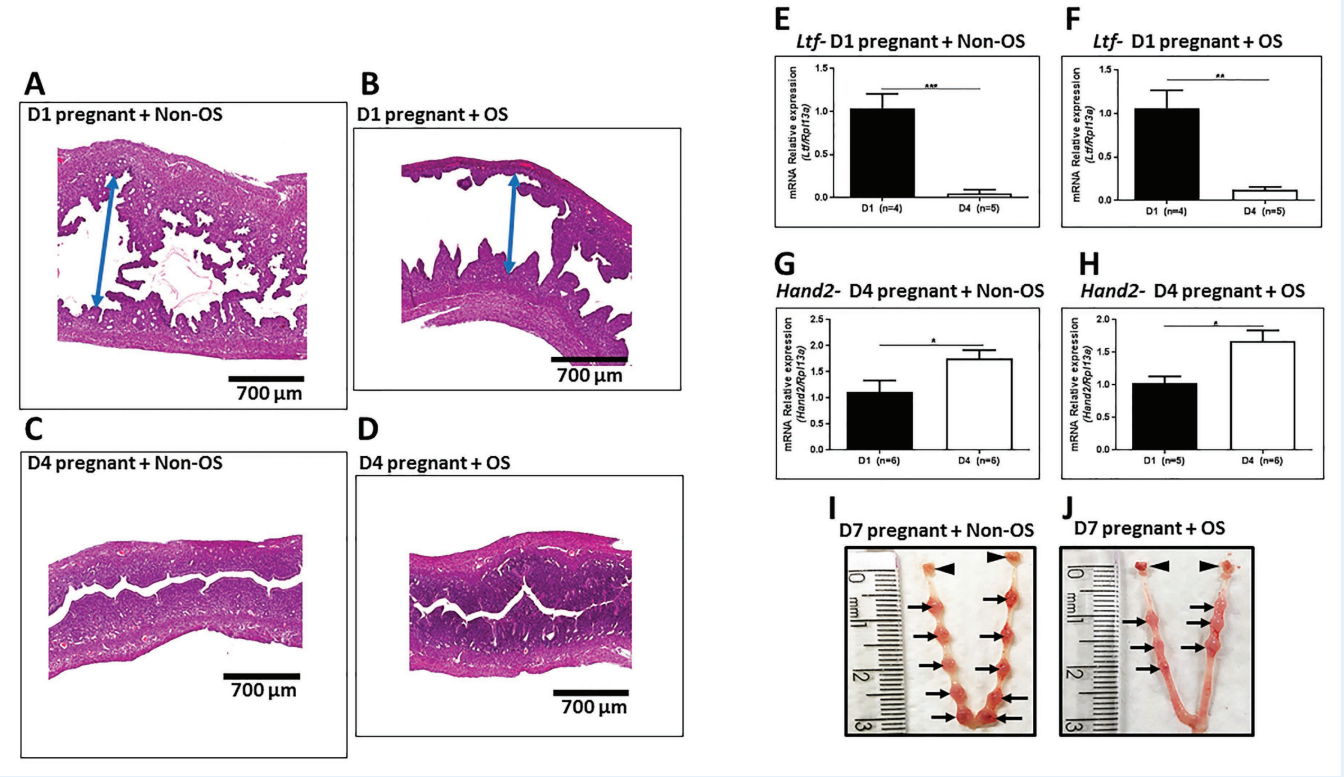

Figure 9 Ovarian stimulation disrupts embryo implantation despite having no overt effect on luminal closure and uterine receptivity. Luminal state (open or closed) was assessed in hematoxylin and eosin (H&E)-stained uterine longitudinal sections from non-ovarian-stimulated and ovarian-stimulated mice on Day 1 (D1) and D4 of pregnancy (**A**–**D**). *Ltf* and *Hand2* expression was quantified by qPCR in uteri from non-ovarian-stimulated and ovarian-stimulated mice on D1 and D4 of pregnancy (**E**–**H**) and embryo implantation sites in uteri from non-ovarian-stimulated and ovarian-stimulated mice were visualized on D7 of pregnancy (**I** and **J**). Arrowheads point to the ovaries; arrows point to implantation sites.

uteri were collected and implantation sites were quantified. The results showed that there was a reduction in the number of implantation sites in the stimulated females versus the non-stimulated females (5.0 ± 0.6 versus 8.0 ± 1.4; $P < 0.05$) (Fig. 9I and J). Absorption sites were absent in both groups, but the average implantation swelling in the stimulated group was about 30% smaller. Finally, it was observed that implantation sites along the uterine horn were not distributed equidistantly strongly implicating a defect in myometrial activity (Fig. 9I and J).

Ovarian stimulation results in the survival of a larger cohort of eggs where a stimulated 3-week-old female readily ovulates 40 or more eggs. This number declines rapidly with age, and we have reported that stimulated 8–10-week-old females (C57 Bl/6 and Sv/129 inbred strains) ovulate about 15–20 eggs (Calder *et al.*, 2014; de Oliveira *et al.*, 2019). In contrast, 8–10-week-old C57 Bl/6 and Sv/129 non-stimulated females ovulate about 10 eggs. In the studies described above, comparisons were made between stimulated and non-stimulated 8-week-old C57 Bl/6 pregnant females raising the possibility (though counterintuitive) that the reduction in the number of implantation sites in the stimulated females was the result of a higher ovulation rate. To address this possibility, 15 zygotes were transferred into each oviduct (total of 30 zygotes per mouse) of stimulated ($n = 3$) and non-stimulated ($n = 3$) 8-week old C57 Bl/6 females on D1 of pseudopregnancy. Implantation rates were then indirectly assessed by flushing uterine horns on the morning of D5 and quantifying the number of flushed embryos. The results revealed that the flushing from non-stimulated females contained significantly fewer embryos compared to stimulated females (12.2 ± 1.7 versus 19.1 ± 2.2 embryos, $P < 0.05$). Additionally, characterization of the flushed embryos revealed that in non-stimulated females compared to stimulated females, there were more blastocysts (62 versus 44%, $P < 0.05$) and fewer morulae (11 versus 21%, $P < 0.05$).

Discussion

Taking a multi-faceted approach to analyzing AQP/*Aqp* expression, this study clearly revealed that *Aqp3*, *Aqp4*, *Aqp5* and *Aqp8* exhibit greater expression on D1 compared to D4 and are induced by E2; in contrast, *Aqp1* and *Aqp11* show greater expression on D4 compared to D1 and are induced by P4. This study also revealed that ovarian stimulation, a major component of IVF protocols, has dramatic effects on AQP1, *Aqp3*/AQP3, *Aqp5*/AQP5 and *Aqp8* expression, and this is associated with reduced implantation rates in the absence of any overt effects on luminal closure and uterine receptivity. Our study also showed that total uterine expression of a given *Aqp* is largely under the influence of E2 and/or P4 and independent of the preimplantation embryo.

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It was reported that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a compound that exhibits antiestrogenic properties, reduced *Aqp3* expression in the adult mouse uterus (Burns et al., 2013). Supporting the likelihood that *Aqp3* is regulated by E2, an estrogen response element (ERE) was identified in the human *AQP3* (Huang et al., 2015). More recently, a P4 response element (PRE) was also identified in the human *AQP3* and it was found that P4 upregulates *AQP3* expression (Cui et al., 2018). In our studies, P4 is not observed to induce *Aqp3* expression, but instead represses E2-induced expression. Thus, it is possible that on regulatory domains in the mouse *Aqp3* gene, P4 forms part of an inhibitory complex while in human *AQP3* it is part of a stimulatory complex. This exciting possibility awaits further investigation.

An examination of *Aqp4* expression revealed it was highly expressed on D1 but weakly expressed on D4 (Richard et al., 2003) suggesting that *Aqp4* is induced by E2, but until our current study, there was no direct evidence in support of this. It was reported that *Aqp5* is P4-regulated (Lindsay and Murphy, 2006) and *Aqp8* is constitutively expressed (Jablonski et al., 2003); however, a recent study suggest that *Aqp5* and *Aqp8* are E2-induced genes (Zhang et al., 2015). Our data fully support the finding that *Aqp5* and *Aqp8* are E2-induced genes and further reveal that their expression is not regulated by P4. As a result, the expression of *Aqp5* and *Aqp8* is very responsive to the increased E2 levels on D4 in ovarian-stimulated mice without an effect by P4.

In the mouse, it was reported that *Aqp9* was barely expressed on the morning of D4 in the pregnant uterus (Richard et al., 2003) while in the rat it was reported that AQP9 protein was expressed in the glandular epithelium at the apical and secretory surface at the time of implantation (Lindsay and Murphy, 2007). We found that mouse uterine *Aqp9* is constitutively expressed and unresponsive to E2 and P4. AQP9, like AQP3, AQP7 and AQP10, belongs to the group of aquaglyceroporins, where in addition to transporting water they also transport glycerol. Glycerol is an important energy substrate and provides energy to the pregnant uterus. However, in the preimplantation period of pregnancy when energy demands are still relatively low and constant it could explain why *Aqp9* is constitutively expressed.

AQP11 and AQP12, referred to as the 'superaquaporins' or the 'unorthodox aquaporins', were the last of the AQPs to be identified, and their functions are still poorly understood (Li and Wang, 2017). Whether AQP11 has reproductive functions awaits further determination in part because the AQP11 null mouse dies before weaning due to advanced renal failure (Morishita et al., 2005). However, we recently reported that *Aqp11* is expressed in the pregnant uterus and that expression is increased on D4 in response to P4 (de Oliveira et al., 2019). Here we additionally report that among all the *Aqp* genes expressed in the uterus in the preimplantation period, *Aqp11* is highly expressed. It is therefore likely that *Aqp11* plays a role in the acquisition of uterine receptivity and embryo implantation. In this study, we provide additional evidence that *Aqp11* expression is induced by P4 and demonstrated for the first time that it is strongly inhibited by E2.

Despite major advances in assisted reproductive technologies, it is estimated that about half of all human embryos fail to implant following IVF/ET (Margalioth et al., 2006; Boomsma et al., 2009; Koot et al., 2016). Although infrequent, IVF/ET is associated with the accumulation of luminal fluid which can be detrimental to embryo implantation (Sharara and Prough, 1999; Chien et al., 2002; Akman et al., 2005; He et al., 2010; Liu et al., 2016). During IVF/ET, ovarian stimulation triggers an increased number of follicles and corpora lutea resulting in supraphysiological levels of E2 and P4 as well as an altered E2:P4 ratio. As shown in mice, abnormally high levels of E2 on D4 of pregnancy results in the aberrant expression of uterine *Aqp5* and *Aqp8* that is associated with fluid accumulation in the luminal cavity and the disruption in embryo implantation (Zhang et al., 2015). Our study shows that in addition to *Aqp5* and *Aqp8*, ovarian stimulation also triggers the increased and abnormal expression of *Aqp3* on D4. However, while this was associated with reduced implantation rates, luminal closure was unaffected leading us to reconsider the role of luminal fluid retention in disrupting implantation. Additionally, molecular markers of uterine receptivity indicated the uterus was in a receptive state, so implantation failure was due to other factors. Based on our findings that D7 implantation swellings along the uterine horn were about 30% smaller in ovarian-stimulated versus non-stimulated females, we suggest that implantation failure following stimulation is the result of delayed and abnormal embryo development. This is further strengthened by the finding that even when ovarian-stimulated and non-stimulated females are experimentally manipulated to contain an equal number of zygotes at the start of pregnancy, a larger number of these zygotes develop into unimplanted blastocysts in the stimulated females on the morning of D5 of pregnancy. Furthermore, relative to non-stimulated females, on D5 of pregnancy stimulated females contain fewer blastocysts and more morulae suggesting there is delayed embryo development. These observations are fully supported by a previous report which clearly describes how ovarian stimulation delays embryonic and fetal development in the mouse (Van der Auwera and D'Hooghe, 2001).

In the mouse, myometrial contractions act to correctly orient and evenly space the implanting embryos along the uterine horn and improperly oriented embryos do not implant (Yoshinaga, 2013; de Oliveira et al., 2019). Our study showed that following ovarian stimulation, equidistant embryo spacing was disrupted and this was associated with the aberrant expression of myometrial AQP1, AQP3 and AQP5. This finding suggests that abnormal expression of AQP1, AQP3 and AQP5 likely disrupted normal myometrial activity and this would not only affect embryo spacing, it would also reduce implantation events by failing to properly orient embryos in the implantation crypt.

Taken together, our data clearly reveal that uterine *Aqp*/AQP expression in the mouse is dynamically regulated by E2 and P4 in the preimplantation period and ovarian stimulation triggers aberrant expression of AQP1, *Aqp3*/AQP3, *Aqp5*/AQP5 and *Aqp8* on the day of uterine receptivity, disrupting embryo implantation without overtly affecting luminal closure and uterine receptivity. Instead, the data strongly suggest that implantation failure is the result of delayed embryo development and disrupted myometrial activity.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

Acknowledgements

A special note of gratitude to our colleagues (Dr Marianne Polunas, Ramiro Corrales and Pedro Louro) at Rutgers Research Pathology Services for their excellent technical support in our histology studies.

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We would like to thank the Robert Wood Johnson Foundation for their support of the Child Health Institute of New Jersey; the views expressed here do not necessarily reflect the views of the Foundation.

Authors' roles

A.V.B. developed the research study and prepared the manuscript; V.d.O., J.S. and A.V.B. performed the research; M.B. assisted with the statistical analysis, and all authors analyzed the data and critically reviewed the manuscript.

Funding

This work was supported by funds provided to A.V.B. (from the Department of Pediatrics, Robert Wood Johnson Medical School, Rutgers, The State University of New Jersey, New Brunswick, NJ, United States) and funds awarded to S.R. (from the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, R01-HD-068777).

Conflict of interest

The authors declare that there are no competing financial, personal or professional competing interests.

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