Implantation failure in mice with a disruption in Phospholipase C beta 1 gene: lack of embryonic attachment, aberrant steroid hormone signalling and defective endocannabinoid metabolism

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ABSTRACT: Phospholipase C beta 1 (PLCβ1) is a downstream effector of G-protein-coupled receptor signalling and holds central roles in reproductive physiology. Mice with a disruption in the Plcβ1 gene are infertile with pleiotropic reproductive defects, the major reproductive block in females being implantation failure. Here, PLCβ1 was demonstrated at the luminal and glandular epithelia throughout the pre- and peri-implantation period, with transient stromal expression during 0.5–1.5 days post coitum (dpc). Examination of implantation sites at 4.5 dpc showed that in females lacking functional PLCβ1 (knock-out (KO) females), embryos failed to establish proper contact with the uterine epithelium. Proliferating luminal epithelial cells were evident in KO implantation sites, indicating failure to establish a receptive uterus. Real-time PCR demonstrated that KO implantation sites had aberrant ovarian steroid signalling, with high levels of estrogen receptor α, lactoferrin and amphiregulin mRNA, while immunohistochemistry revealed very low levels of estrogen receptor α protein, possibly due to rapid receptor turnover. KO implantation sites expressed markedly less fatty acid amide hydrolase and monoacylglycerol lipase, indicating that endocannabinoid metabolism was also affected. Collectively, our results show that PLCβ1 is essential for uterine preparation for implantation, and that defective PLCβ1-mediated signalling during implantation is associated with aberrant ovarian steroid signalling and endocannabinoid metabolism.

Key words: GPCR / implantation / PLCβ1 / signalling / uterus

Introduction

Implantation failure is one of the major impediments in human reproduction and this is more prominent when assisted reproduction techniques are used (reviewed in Diedrich et al., 2007). Knock-out (KO) mouse models with implantation defects are important for unravelling and examining pathways that lead to successful implantation, the latter being directly relevant for the further development of diagnostic implantation markers in humans. Successful implantation of the conceptus into the uterus is controlled by diverse signalling pathways that regulate uterine receptivity, embryo development and competence, and embryo–uterine communication. Female mice with a homozygous null mutation in the Phospholipase C β1 (Plcβ1) gene (Plcβ1−/−, hereafter referred as KOs), have altered reproductive behaviour, ovulation, pre-implantation embryo development and exhibit an implantation block (Böhm et al., 2002; Ballester et al., 2004; Filis et al., 2009). The Plcβ1 gene encodes the PLCβ1 protein, an immediate downstream effector of G-protein-coupled receptors (GPCRs) that associate with the Gqα family of G-proteins (Hubbard and Hepler, 2005). In mice, Plcβ1 is alternatively spliced to produce the two isoforms PLCβ1α and PLCβ1β; these differ only in their C-terminal tails.

GPCRs have central roles in mediating reproductive physiology; many reproductive hormones, notably GnRH, FSH, LH and oxytocin, signal through GPCRs and disruption of GPCR signalling can have adverse effects on fertility. GPCR-Gqα signalling is mediated by four different PLCβ isozymes, PLCβ1-4, each the product of a different gene. Among the four PLCβ isoforms, PLCβ1 appears to hold an essential role in mediating GPCR signalling in reproductive physiology, as mice with disruption in the Plcβ2, Plcβ3 and Plcβ4 genes all are fertile (Jiang et al., 1997; Kim et al., 1997; Xie et al., 1999; Böhm et al., 2002;...
Investigation of the implantation failure in Plcβ1 KO mice should, therefore, be a powerful way of determining key signalling pathways for successful implantation. In mice, fertilized ova reach the uterus by 3.5 days post coitum (dpc) at which time they are developing towards the blastocyst stage. Implantation is initiated at ~4.0 dpc (midnight) in mice with attachment of hatched blastocysts to the uterine epithelium. Uterine preparation and implantation are under the Control of estrogens and progesterogens, both acting through their cognate receptors. During 0.5–1.5 dpc, preparation of the uterus for implantation is primarily influenced by increasing levels of estrogen secreted by pre-ovulatory follicles, whereas from 2.5 dpc onwards the uterus is primarily under the Control of progesterone secreted from the newly formed corpora lutea (reviewed in Wang and Dey, 2007). In rodents, implantation is then initiated by a surge of estrogen on the morning of the 4th dpc (keeping with the dating system used here for the time of pregnancy), which initiates a transient period of uterine receptivity to implantation, referred to as the implantation window (Huet-Hudson and Dey, 1987). Plcβ1 is rapidly activated in response to progesterone in swine granulosa cells (Lieberherr et al., 1999) and to estrogen in rat osteoblasts (Le Melay et al., 1997), implying that similar pathways may operate in the uterus.

The endocannabinoids anandamide and 2-arachidonoyl glycerol (2-AG) hold central roles in reproduction and Control early embryo development and implantation (as reviewed in Fride, 2008). Given that Plcβ1 is involved in the biosynthesis and release of endocannabinoids in the brain (Hashimoto et al., 2005; Jung et al., 2005), it would not be surprising if it were also involved in uterine endocannabinoid metabolism. Another phospholipase, phospholipase A2 is also involved in the production of endocannabinoids as well as prostanoids (Murakami et al., 2003) and its loss is associated with delayed implantation in mice (Song et al., 2002). High levels of endocannabinoids can have adverse effects in pre-implantation embryo development (Paria et al., 1995, 1998; Yang et al., 1996), while low levels of endocannabinoids are found at the implantation sites (Schmid et al., 1997). The enzymes fatty acid amide hydrolase (FAAH) and monoaoylglycerol lipase (MAGL) are up-regulated at the implantation sites and assist implantation by degrading anandamide and 2-AG, respectively (Wang et al., 2007).

Here, Plcβ1 KO female mice are used to characterize the specific roles of Plcβ1 in uterine physiology and signalling during the pre- and peri-implantation period. This work shows that Plcβ1 is expressed in the pre- and peri-implantation period and that its loss affects ovarian steroid responsiveness, uterine preparation for embryo implantation and endocannabinoid metabolism.

Materials and Methods

Animals

Mice were housed in an environmentally Controlled room on 14-h light and 10-h dark photoperiod. Animals were provided food and water ad libitum, and kept in accordance with UK legal requirements. Transgenic mice had an insertion of a neomycin cassette in the pleckstrin homology domain of the 5′ region of Plcβ1 (Kim et al., 1997), with the colony on a mixed 129Sv x C57BL/6J background. Mice were fed high-protein Teklad 2019 diet (Harlan UK Ltd, Bicester, UK) to provide additional nutrition for the KO offspring (these mice can have stunted general development when provided with standard rodent food). Heterozygous (Plcβ1+/−; HET) pairs were bred to provide wild type (Plcβ1+/+; WT), HET and KO offspring. Offspring were earmarked for identification upon weaning and DNA prepared from ear punch material for subsequent genotyping. For all experiments, female experimental mice were 6–11 weeks of age. Since HET females appear to have no reproductive phenotype, WT's and HETs were both used as ‘Controls’, age matched to the experimental KO females, siblings used where possible. WT stud males were proven fertile Control males from the same colony. Females were identified as in diestrous or estrus when vaginal smears showed primarily leucocytes or cornified cells, respectively. Smears to establish cyclicity were carried out at the same time each day over a 5-day period.

Genotyping of transgenic mice

Genotyping was carried out from PCRs on DNA extracted from ear punches, with all genotyping later confirmed with DNA extracted from tail tips at culling. Sense primer 5′-GTTAAGTCCTCACGCAAACC and antisense primer 5′-ACCTTGGAACCTTGGCTG were used to amplify a 180-bp WT allele band, and sense primer 5′-GTTAAGT CCTACGACAAACC and antisense primer 5′-CTGACTAAGGG AGGAGTAGAG were used to amplify a 290-bp KO allele band (as in Fils et al., 2009).

Ovulation induction and matings

Animals were injected i.p. with 10 IU pregnant mare serum gonadotrophin, followed by 5 IU human chorionic gonadotrophin (hCG) 48 h later (both from Intervet, Milton Keynes, UK), with both hormone preparations administered after 1600 h. Immediately after hCG administration, animals were housed with a stud Control male for a single night. The following day at 1200 h was regarded as 0.5 dpc.

Dye injection and dissection of implantation sites

Between 12:00 h and 14:00 h on 4.5 dpc, females received an ip injection of 0.1 ml of 54.7 mg/ml sodium pentobarbitone (Ceva Sante Animale, Libourne, France). Under anaesthesia, 1 ml of 0.5% Chicago Sky Blue 6B (Sigma-Aldrich, Dorset, UK) was perfused through the heart: Chicago Sky Blue stains areas of increased vascular permeability, thus visualizing implantation sites from 4.5 dpc onward (Nagy et al., 2003). Implantation sites (blue stained uterine pieces) and interimplantation sites (unstained uterine pieces between two blue-stained bands) were dissected out, and either fixed (see below) or frozen on dry-ice and stored at −70°C. KO uteri have fewer and less pronounced implantation sites, presumably the result of only few of their embryos triggering a local increase in vascular permeability (Fils et al., 2009). Histological examination of less pronounced, presumed implantation sites from KO uteri confirmed that implantation is initiated in those sites, as that they contain embryos in apposition to the uterine epithelium (also demonstrated in Fig. 1C–iv); although the implantation process does not undergo successful completion in these sites, they are here termed as ‘implantation sites’.

Histology and immunohistochemistry

Uterine pieces were fixed overnight in 4% phosphate-buffered saline-dissolved paraformaldehyde, washed 3 × with 50% ethanol and stored there until processing in paraffin blocks. Blocks were sectioned at 5 μm and sections stained with haematoxylin and eosin or probed immunohistochemically. Antibodies and fluorescent dyes used, with dilutions, are detailed in Table I. Immunohistochemical expression analysis of uterine Plcβ1 during 0.5–4.5 dpc was performed in three Control uteri from each stage of pregnancy in parallel, to allow for comparison between
Figure 1 Knock-out (KO) uteri of cycling animals have grossly normal morphology but implantation sites at 4.5 dpc show lack of attachment and diminished luminal closure. (Ai) Control and (Aii) KO uteri in diestrous, both showing luminal closure; (Bi) Control and (Bii) KO uteri in diestrous, both showing expanded lumen; (Ci) Control and (Cii–iv) KO implantation sites at 4.5 dpc. Photomicrographs show embryo attachment in Control but not in KO luminal epithelia. le, luminal epithelium; str, stroma; bl, blastocyst. Scale bars: A, B, 900 μm in low power and 150 μm in magnified images; C, 600 μm in low power and 30 μm in magnified images.

| Table I Primary antibodies, secondary antibodies and fluorescent dyes used. |
|--------------------------------------------------|------------|------------------|
| **Primary antibodies**                          | Dilution  | Catalogue #      | Company                      |
| Rabbit anti-phospho S10 H3 histone              | 1:500     | ab5176           | Abcam, Cambridge, UK         |
| Rabbit anti-PLCβ1 R-223                         | 1:100     | sc-205           | Santa Cruz Biotechnology, Santa Cruz, USA |
| Rabbit anti-FAAH                                | 1:100     | 101 600          | Cayman, Tallinn, Estonia     |
| Rabbit anti-MAGL                                | 1:100     | ab24701,         | Abcam Cambridge, UK          |
| Mouse anti-ERα                                  | 1:00      | NCL-ER-6F11      | Novocastra, Milton Keynes, UK|
| **Secondary antibodies**                        |           |                  |                               |
| Biotinylated goat anti-rabbit                   | 1:200     | E0432            | Dako, Cambridge, UK          |
| Biotinylated goat anti-mouse                    | 1:200     | E0433            | Dako, Cambridge, UK          |
| **Fluorescent dyes**                            |           |                  |                               |
| Streptavidin-conjugate Alexa Fluor 568          | 1:200     | S11226           | Invitrogen, Paisley, UK      |
| Hoechst 33342                                   | 1:10 000  | 62 249           | Thermo Scientific, Erembodegem, Belgium |
different stages. A total of six 4.5 dpc implantation sites (from three paired Control and KO uterus) were used for other immunohistochemical protein expression analyses.

**RNA extraction, cDNA synthesis and real-time PCR**

RNA was extracted from snap-frozen uterine pieces using the RNeasy mini kit (#74104; Qiagen, Crawley, UK), coupled with RNase-free DNase set (#79254; Qiagen) treatment to eliminate traces of genomic DNA. cDNA was synthesized using the Quantitect Reverse Transcription kit (#205313; Qiagen). All kits were used according to the manufacturer’s guidelines. Real-time PCR was performed using Green Dye Master Mix (#s2000; Rovalab, Teltow, Germany). Forward and reverse primer sequences are detailed in Table II. All mRNA levels were quantified relative to the housekeeping gene Gapdh. Analyses were carried out on three implantation and three interimplantation sites per animal, from a total of three pairs of Control and KO animals. For each pair of animals, mRNA amounts for a given gene were averaged, to derive the mean implantation and mean interimplantation values for each animal; values were then normalized against Control interimplantation sites, which were given a nominal value of 1.

**Statistics**

For analysis of proliferating cells, statistical significance of differences between means was compared with the Student’s two-tailed paired t-test. For real-time PCR data, the statistical significance of differences between means was compared with a one-sample two-tailed paired t-test.

**Results**

**Lack of embryo attachment in implantation sites of KO females**

In contrast to the well-established implantation sites found in pregnant mice at 4.5 dpc, embryos can be easily flushed out of KO uteri at that stage, indicating a problem with implantation (Filis et al., 2009). To further investigate the uterine physiology of KO females, uterus from non-pregnant and pregnant females were examined histologically. Non-pregnant KO uteri from diestrous and estrous animals appeared comparable with uteri from Control animals, with no gross developmental abnormalities, indicating that KO endometrium can undergo cyclic remodelling changes in response to estrogens and progestogens (Fig. 1 A and B). In contrast, differences between KO and Control uteri were evident during early pregnancy. KO implantation sites showed a lack of embryo attachment, abnormal embryo appearance and diminished luminal closure compared with Controls (Fig. 1 Ci–iv, Table III). Successfully attached blastocysts were defined as those whose trophoectoderm was in close proximity to the uterine epithelium (no greater than 3 μm away) for at least 50% of their periphery. This lack of attachment and abnormal blastocyst appearance complements previously reported observations (Filis et al., 2009). In all KO implantation sites examined, only a single blastocyst was found attached to the uterine epithelium (Table III); in another case, three non-attaching, conjoined blastocysts were observed in a KO implantation site that also exhibited complete absence of luminal closure (Fig. 1 Ci, Table III).

Levels of proliferation in implantation sites of KO uteri were characterized by probing for the presence of the proliferative marker phospho-S10 histone H3 (pH3). Proliferating cells were counted within a defined 1.43 mm² field of view around the blastocyst (Fig. 2A) with pH3-immunoreactivity evident in the stromal cells of implantation sites in both KOs and Controls. There was no significant difference in the overall number of proliferating cells between KOs and Controls (Fig. 2B; P = 0.22). Notably though, proliferating epithelial cells were observed in KO but not Control implantation sites (Fig. 2C and D). This latter observation is striking, as epithelial cell proliferation normally ceases by ~3 dpc after which point differentiation and preparation for embryo reception takes place (Martin et al., 1973; Lundkvist and Nilsson, 1982; Huet-Hudson et al., 1989). Failure of KO uteri to cease epithelial cell proliferation by 4.5 dpc thus points to defective uterine preparation for implantation.

**Expression of PLCβ1 in WT uteri during the pre- and peri-implantation period**

Expression of PLCβ1a in the uterus was probed during the pre- and peri-implantation period (antibodies to specifically detect PLCβ1b were not available). PLCβ1a is consistently expressed in the luminal and glandular uterine epithelia during 0.5–4.5 dpc as well as in the embryonic trophotroctderm at 4.5 dpc (Fig. 3 A–E). Consistent with its role(s) as a GPCR signal-mediating protein, subcellular localization

**Table II Sequences of forward and reverse primers for real-time PCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Plcβ1a</td>
<td>ATGAGAAGCCCAAGCTGCA</td>
<td>CCCCCTTTCATGGGCTTCTTGTA</td>
</tr>
<tr>
<td>Plcβ1a</td>
<td>GAGAAGCCCAAGGGGAAA</td>
<td>CCCCCTTTCATGGGCTTCTTGTA</td>
</tr>
<tr>
<td>Plcβ2</td>
<td>CATGACCAAGGTCACACAG</td>
<td>CCCCCTTTCATGGGCTTCTTGTA</td>
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<tr>
<td>Plcβ3</td>
<td>AGAAGCAGAGAGACCAGGA</td>
<td>GCCAGAGCCAGGATACCATT</td>
</tr>
<tr>
<td>Plcβ4</td>
<td>GCTACACGAGGATCCCATCC</td>
<td>CACCGAGCCAGGATACCATT</td>
</tr>
<tr>
<td>Pgr</td>
<td>CTTGATGATCTTGTGAAACAG</td>
<td>CACCGAGCCAGGATACCATT</td>
</tr>
<tr>
<td>Esr1</td>
<td>TGAAGGCGCCCATAGCGGAA</td>
<td>CACCGAGCCAGGATACCATT</td>
</tr>
<tr>
<td>Areg</td>
<td>FGACTCAGACCGAGGATGACA</td>
<td>GCGGCTGTTTTCTTCCGCTCAT</td>
</tr>
<tr>
<td>Ltf</td>
<td>CAGCCCCCTTCGAAAAACAGT</td>
<td>GCCAGAGCCAGGATACCATT</td>
</tr>
<tr>
<td>Gapdh</td>
<td>GGGTGTGAACCACAGGAAT</td>
<td>GCCAGAGCCAGGATACCATT</td>
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appears to be restricted to the cytoplasm and/or associated with the cell membrane. Immunohistochemical reactions were carried out at the same time to allow for semi-quantitative comparison of PLCβ1α expression. As can be seen in Fig. 3, expression was particularly high at 1.5 dpc and lower around implantation sites at 4.5 dpc. During 0.5 and 1.5 dpc, foci of PLCβ1α expression were also noted at the stroma, but this disappeared from 2.5 dpc onwards. Notably, in the 4.5 dpc implantation site, PLCβ1α is absent both from the apical surface of uterine luminal epithelia and in the trophectoderm. At the same time, levels of Plcβ1a mRNA remained unchanged between interimplantation and implantation sites, whereas Plcβ1b mRNA levels were decreased by ~30% at the implantation sites (Fig. 4A; P = 0.0372). Collectively, the expression pattern of PLCβ1 indicates potential involvement in epithelial–epithelial and epithelial–stromal communication during endometrial preparation, as well as during the implantation process itself.

**Levels of Plcβ1-4 in implantation sites**

Since loss of PLCβ1 should disrupt Gαq-GPCR-mediated signalling, levels of Plcβ2, Plcβ3 and Plcβ4 were examined to determine whether KOs compensate for the loss of PLCβ1 by up-regulating uterine expression of the other PLCβ isozymes. No significant changes were noted in the expression of Plcβ2 and Plcβ3 in Control and KO uteri (Fig. 4B and C), but an effect was seen for Plcβ4, which was significantly down-regulated at the implantation sites of Control but not KO uteri (Fig. 4D; P = 0.001). Since PLCB1 is usually absent from implantation sites at this stage, the maintained expression of Plcβ4 at KO implantation sites is more likely to be the consequence of aberrant implantation process in KOs, rather than compensation for the loss of Plcβ1. Levels of Plcβ2-4 remained comparable among the interimplantation sites of Control and KO uteri, again indicating that the KO uteri do not respond to the loss of PLCβ1 signalling by modifying the mRNA expression of the other Plcβs.

**Ovarian steroid receptors and ovarian steroid responsiveness in KO uteri**

To investigate estrogen and progesterone responsiveness in KO implantation sites, expression of progesterone and estrogen receptor

<table>
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<th>Table III Summary of 4.5 dpc implantation site defects in KO uteri.</th>
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<tr>
<td><strong>Implantation site defects</strong></td>
</tr>
<tr>
<td>Abnormal blastocyst(s) appearance</td>
</tr>
<tr>
<td>Diminished or absent luminal closure</td>
</tr>
<tr>
<td>Attached blastocyst</td>
</tr>
<tr>
<td>Conjoined blastocysts</td>
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**Figure 2** KO implantation sites contain proliferating epithelial cells at 4.5 dpc. (A) Quantification scheme for analysis of proliferative cells; (B) Density of proliferating cells around the blastocyst in Control and KO implantation sites (n = 3 animals per group, with one implantation site section per animal. Histograms show mean ± SEM (C and D) immunohistochemical localization of pH3 mitotic marker in Control and KO implantation sites, respectively. Dotted squares show magnified areas around the blastocyst. Green arrowheads indicate examples of proliferating luminal epithelial cells in KO sections. le, luminal epithelium; str, stroma. Scale bars: 300 μm.
mRNA (Pgr and Esr1, respectively) was examined at 4.5 dpc (Fig. 5). KO uteri had Pgr levels comparable with Controls, and no changes were noted between implantation and interimplantation sites (Fig. 5Ai). Esr1 levels dropped significantly at implantation sites of Controls (P = 0.026) but KO implantation sites fail to show this down-regulation (Fig. 5Aii). The responsiveness of KO uteri to ovarian steroids was examined further by probing for expression of Areg and Ltf, which are positively regulated direct targets of progesterone and estrogen, respectively. Consistent with the roles of progesterone in establishing and maintaining pregnancy, Areg expression tended to be higher in Control implantation sites but this effect was not significant, owning to the large variation among samples; in contrast, KO uteri had uniformly high levels of Areg throughout (Fig. 5Aiii). Ltf expression was significantly lower in the implantation sites of Controls (P = 0.033), mimicking the down-regulation of expression of Esr1 (Fig. 5Aii and iv). The high and extremely variable levels of Ltf in KO uteri (Fig. 5Aiv) suggested overactive estrogen signalling in KO uteri. To investigate this further, Control and KO implantation sites were probed with an anti-ERα antibody. In contrast to the high mRNA levels, KO implantation sites expressed very low levels of ERα protein, possibly due to rapid turnover (Fig. 5B). Taken altogether, these data indicate that KO uteri have abnormal progesterone and estrogen responsiveness at the time of implantation that may, at least in part, account for the implantation failure.

Expression of genes associated with endocannabinoid metabolism in KO uteri

To determine whether aberrant levels of endocannabinoids in KO reproductive tracts might account for implantation and embryo development defects, expression of FAAH and MAGL, key enzymes that degrade endocannabinoids, was probed using immunohistochemistry in Control and KO implantation sites (since endocannabinoids themselves are of low molecular weight and prone to rapid degradation). For both enzymes, at 4.5 dpc expression was strong in Control and weak in KO tissue, both in blastocysts and in surrounding epithelial and stromal cells of implantation sites (Fig. 6). Low levels of both

**Figure 3** Immunohistochemical localization of phospholipase C (PLC)β1 expression in the uterus during early pregnancy. PLCβ1 is expressed in the luminal and glandular epithelia during 0.5–4.5 dpc and in the stroma during 0.5–1.5 dpc, with overall expression transiently higher at 1.5 dpc. White arrowheads show examples of stromal staining foci. le, luminal epithelium; str, stroma; ge, glandular epithelium; bl, blastocyst. Scale bars: 150 μm.
FAAH and MAGL indicate that KO uteri are likely to have abnormally high levels of endocannabinoids; this may contribute not only to the abnormal blastocyst appearance but also to the failure of KO uteri to initiate implantation.

**Discussion**

This study investigates the implantation failure of Plcβ1 KO female mice, examining the roles of PLCβ1 signalling during implantation. It
has previously been reported that the main reproductive block of Plcβ1 KO female mice is implantation failure (Filis et al., 2009). To further characterize this, KO implantation sites were analysed histologically and molecularly. The implantation failure of Plcβ1 KO females manifests as lack of attachment of the blastocysts to the uterine epithelium and is accompanied by abnormal embryo morphology and diminished luminal closure. The lack of attachment and the abnormal blastocyst morphology reported here complement previous observations from uterine flushing and ex vivo blastocyst examinations (Filis et al., 2009).

Successful implantation is dependent on up-regulation of progesterone signalling, with high levels of Areg (a positively regulated direct target of progesterone signalling) expressed in implantation sites at 4.5 dpc (Das et al., 1995). This up-regulated progesterone signalling leads to closure of the uterine lumen by inducing luminal fluid absorption (Salleh et al., 2005), and to decidualization, occurring several hours after embryo attachment. Mice deficient for the progesterone-inducible genes Leukemia inhibitory factor and Fk506 binding protein 4 also show defective luminal closure (Fouladi-Nashta et al., 2005; Tran-guch et al., 2005), while loss of the progesterone receptor abolishes decidualization when uteri are challenged with a decidualogenic stimulus (Lydon et al., 1995). There were no Pgr mRNA level changes between implantation and interimplantation sites in either Control or KO uteri; as Pgr expression shifts from luminal epithelia to decidua during implantation (Tan et al., 1999), it is likely that net Pgr levels between implantation and interimplantation sites remain similar, as our results here indicate. However, consistent with the requirement for progesterone signalling for successful implantation, Control animals here expressed high, if variable levels of Areg only at implantation sites. In contrast, KO uteri, with defective luminal closure had high, if variable levels of Areg throughout the uterus.

Proliferation of luminal epithelial cells ceases at ~3 dpc, after which they differentiate (Martin et al., 1973; Lundkvist and Nilsson, 1982; Huet-Hudson et al., 1989). Cessation of epithelial cell proliferation is a well-established progesterone-mediated effect (Huet-Hudson et al., 1989; Ohta et al., 1993), with the inability of uterine epithelial cells to cease proliferation past 3 dpc also recently reported in mice with a conditional disruption of Pgr exclusively in uterine epithelia (Franco et al., 2012). Here, proliferating epithelial cells were evident in KO implantation sites at 4.5 dpc, again indicative of aberrant progesterone signalling in these mice.

Estrogen signalling is involved in preimplantation uterine preparation and is responsible for initiating the uterine implantation window in rodents (Paria et al., 1993; Ma et al., 2003). Estrogen signalling has been shown to inhibit attachment and implantation (Ma et al., 2003). Low ERα protein levels have been observed in ectopic pregnancies, suggesting that this might be a frequent theme in implantation-related aberrancies (Horne et al., 2009).

Uterine ablation of the homeobox genes Msx1/Msx2 results in implantation failure, with such mice closely mimicking the phenotype of

**Figure 6** Expression of endocannabinoid-degrading enzymes FAAH and MAGL is down-regulated in KO implantation sites. (A) FAAH and (B) MAGL immunolocalization in Control and KO uteri at 4.5 dpc. In both cases, KO implantation sites show markedly lower expression levels of the enzymes than do Control implantation sites. le, luminal epithelium; str, stroma; bl, blastocyst. Scale bars: 150 μm.
**Plcβ1** KO females, exhibiting lack of embryonic attachment, diminished luminal closure and lack of decidualization (Nallasamy et al., 2012). Interestingly, these mice fail to cease uterine epithelial proliferation at 3.5 dpc and are characterized by abnormally high Ltf levels during the peri-implantation period (Nallasamy et al., 2012). The close similarities between these mice and the KO mice here could indicate an involvement of PLCβ1 in the Msx1/Msx2 pathway.

Blastocysts developing in the KO reproductive tract can have abnormal development, as demonstrated here and in Filis et al. (2009). As high levels of endocannabinoids can adversely affect the pre- and peri-implantation development of embryos (Fride, 2008), it was hypothesized that aberrant endocannabinoid levels in KO uteri might contribute to the observed phenotype. FAAH and MAGL are responsible for the degradation of the endocannabinoids anandamide and 2-AG, respectively, with cyclo-oxygenase (COX2) also able to degrade 2-AG to some extent, and all three enzymes are expressed at higher levels at implantation sites (Wang et al., 2007). Here, FAAH and MAGL are expressed at low levels in KO implantation sites, as is also the case for COX-2 (Filis et al., 2009), together indicating that levels of endocannabinoids are likely to be higher in the KO reproductive tract, impeding implantation (Fig. 7). These high levels of endocannabinoids may, at least in part, also be responsible for the abnormal blastocyst appearance. FAAH expression is generally positively regulated by progesterone, and during the opening of the implantation window FAAH is expressed by the combined action of progesterone and estrogen (Maccarrone et al., 2000; Xiao et al., 2002), while blastocyst-derived signals can also up-regulate FAAH at the implantation sites (Maccarrone et al., 2004). Faah-deficient mice are subfertile, with delayed implantation and reduced Chicago Sky Blue staining (Wang et al., 2006), the latter being reminiscent of the

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**Figure 7** Proposed roles of PLCβ1 in degradation of endocannabinoids at implantation sites. (A) FAAH expression at the implantation sites can be stimulated by (1) the blastocyst directly (Maccarrone et al., 2004), (2) ovarian steroid hormones (Xiao et al., 2002), or through (3) up-regulation by PLCβ1, which can also act (4) via the estrogen pathway (as demonstrated here). PLCβ1 is involved in both the up-regulation of (5) MAGL (as suggested here) and (6) COX2 (Filis et al., 2009). FAAH then degrades anandamide, while both MAGL and COX2 degrade 2-AG (Wang et al., 2007). (B) In the KO uterus, failure of endocannabinoid degradation contributes to the implantation block in these mice, demonstrating a key role for PLCβ1 in the degradation of endocannabinoids during implantation.
poor staining of implantation sites in Plcβ1 KO uteri (as reported in Filis et al., 2009), where implantation is not delayed but aborted. On the other hand, the roles of MAGL in implantation are unknown and to the best of our knowledge this is the first demonstration of MAGL dysregulation in the context of implantation.

At the time of implantation, Plcβ1 KO uteri have a range of abnormalities associated with defective preparation, and the pre- and peri-implantation expression of Plcβ1 supports this notion. While both Plcβ1a and Plcβ1b can potentially localize to the nucleus where they participate in GPCR-independent nuclear signalling via the hydrolysis of nuclear phosphoinositides (Follo et al., 2010), no nuclear staining was evident for Plcβ1a in the uterus (no Plcβ1b-specific antibody or pan-Plcβ1 antibody was available to investigate expression immunohistochemically). The exclusively cytoplasmic localization of Plcβ1a supports its role as a GPCR signal mediator in the uterus. Interestingly, stromal Plcβ1 expression is evident at 0.5–1.5 dpc, during which time epithelial Plcβ1 expression appears to be upregulated. At this time, the uterus is primarily under the control of estrogen and Plcβ1 may be mediating uterine estrogenic responses, as has been shown for estrogen-mediated Plcβ1 activation in rat osteoblasts and possibly rat endometrium (Le Mellay et al., 1997; Wang et al., 2008; Konigame et al., 2011). At the time of implantation, Plcβ1 is lost from the apical surface of luminal epithelia (as well as the apical surface of trophoderm cells), indicating the possibility that GPCR signalling might need to be damped during embryo–maternal dialogue. Consistent with this idea, mRNA levels of Plcβ1b and those of Plcβ4 are significantly downregulated in the implantation sites of Controls, one possible way to quench GPCR signalling during implantation. Oxytocin and its GPCR-Gqα oxytocin receptor (OTR) have been negatively associated with implantation success in both humans and rodents (Pierzynski et al., 2007; Moraloglu et al., 2010; Chou et al., 2011) and Plcβ1 and Plcβ4 down-regulation, as well as displacement of Plcβ1 from the apical surface of the cell, may be required physiologically to prevent oxytocin signalling.

Current knowledge of GPCR-Gqα signalling in the uterus around the time of implantation is limited, with only few studies focusing on the importance of GPCR-Gqα signalling during the early stages of pregnancy establishment. Notable examples ofGPCRs that signal through the Gqα-PLCβ pathway and whose signalling has been implicated in embryo implantation in humans and rodent models are the lysophosphatidic acid receptor 3, calcitonin receptor, platelet-activating factor receptor, growth hormone secretagogue receptor, histamine H1 receptor, prokineticin receptor 1 and OTR (Hore and Mehrotra, 1988; Ding et al., 1994; Nishi et al., 1995; O’Neil, 1995; Zhu et al., 1998; Kawamura et al., 2003; Ye et al., 2005; Evans et al., 2009; Jensen et al., 2010). Disruption of LPA3, and H1R signalling have also been shown to negatively affect implantation rates, yet to the best of the authors’ knowledge not a single GPCR-Gqα receptor appears to be an absolute requirement of implantation (Johnson and Dey, 1980; Ye et al., 2005). Similarly, from the four Plcβ subtypes, only Plcβ1 affects fertility (Jiang et al., 1997; Kim et al., 1997; Xie et al., 1999; Böhm et al., 2002; Ballester et al., 2004). The results presented here show that Plcβ1 disruption affects uterine steroid responsiveness, luminal epithelial differentiation and endocannabinoid metabolism, suggesting that Plcβ1 acts as a hub for uterine GPCR signalling.

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Authors’ roles

P.F. carried out initial experimental design, experimental work, performed data analysis and drafted the manuscript. P.C.K. helped with experimental design and data interpretation, and participated in manuscript preparation. N.S. helped with experimental design and data interpretation, reviewed and edited the manuscript, and supervised the study.

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Conflict of interest

None declared.

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