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ORIGINAL RESEARCH

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 ($Plc\beta1^{-/-}$, hereafter referred as KOs), have altered reproductive behaviour, ovulation, pre-implanation 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 $G_q\alpha$ family of G-proteins (Hubbard and Hepler, 2005). In mice, *Plcβ1* is alternatively spliced to produce the two isoforms PLCβ1a and PLCβ1b; 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-$G_q\alpha$ signalling is mediated by four different PLCβ isozymes, PLCβ1-4, each the product of a different gene. Among the four PLCβ isozymes, 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;

Ballester *et al.*, 2004). 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 progestogens, 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 Mellay *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 (Hashimotodani *et al.*, 2005; Jung *et al.*, 2005), it would not be surprising if it were also involved in uterine endocannabinoid metabolism. Another phospholipase, phospholipase A2α is also involved in the production of endocannabinoids as well as prostaglandins (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-implantaton 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 monoacylglycerol lipase (MAGL) are up-regulated at the implantation sites and assist implantation by degradating anandamide and 2-AG, respectively (Wang *et al.*, 2007).

Here, *Plcl*β1 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 × 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 ($Plc\beta1^{+/-}$; HET) pairs were bred to provide wild type ($Plc\beta1^{+/+}$; 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, WTs 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′-GTTAAGTCCTCAGGCAAACACC and antisense primer 5′-ACCTTGGGAGCTTTGGCGTG were used to amplify a 180-bp WT allele band, and sense primer 5′-GTTAAGT CCTCAGGCAAACACC and antisense primer 5′-CTGACTAGGGG AGGAGTAGAAG were used to amplify a 290-bp KO allele band (as in Filis *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 (Filis *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. 1Cii–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 1. 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

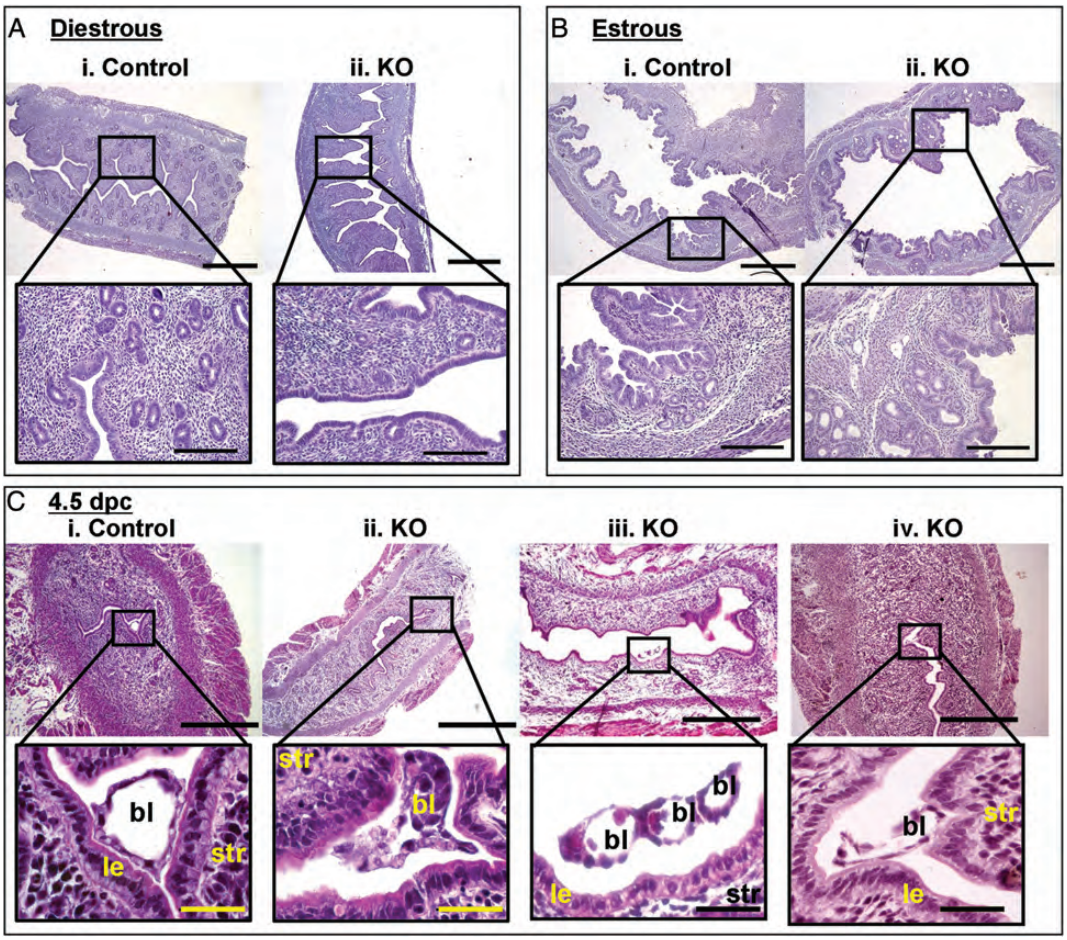

Figure 1 Knock-out (KO) uteri of cycling animals have grossly normal morphology but implantation sites at 4.5 dpc show lack of attachment and diminished luminal closure. (**Ai**) Control and (**Aii**) KO uteri in diestrous, both showing luminal closure; (**Bi**) Control and (**Bii**) KO uteri in diestrous, 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 1 Primary antibodies, secondary antibodies and fluorescent dyes used.

	Dilution	Catalogue #	Company
Primary antibodies			
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 uteri) 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, uteri 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. 1A 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. 1Ci–iv, Table III). Successfully attached blastocysts were defined as those whose trophectoderm 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 attaching 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. 1Ciii, 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 mm^2 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 trophectoderm at 4.5 dpc (Fig. 3A–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.

Gene	Forward	Reverse
Plcβ1 isoform a	ATGAGAAGCCCAAGCTGCA	CCCTTTCATGGCTTCCTGTA
Plcβ1 isoform b	GAGAAGCCCAAGGGGGAA	CCCTTTCATGGCTTCCTGTA
Plcβ2	CATGACCAAGGTCACCACAG	CCCTTCATCTTGGCCTCATA
Plcβ3	AGAAGCAGAGAGACCCGAGA	TCCAGAGGCAGGATACCATT
Plcβ4	GCTACCACGAACATCCATCC	CGGCTCATTTGTCGCTTATT
Pgr	CTTGCATGATCTTGTGAAACAGC	GGAAATTCCACAGCCAGTGTCC
Esr1	TGAAAGGCGGCATACGGAAAG	CACCCATTTCATTTCGGCCTTC
Areg	FGACTCACAGCGAGGATGACA	GGCTTGGCAATGATTCAACT
Ltf	CAGCCCCTTCAGAAAACAGT	AAACTCCTTCTCCAGCTCCA
Gapdh	GGGTGTGAACCACGAGAAAT	CCTTCCACAATGCCAA AGTT

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 PLCβ1a 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 PLCβ1a expression were also noted at the stroma, but this disappeared from 2.5 dpc onwards. Notably, in the 4.5 dpc implantation site, PLCβ1a 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.

Table III Summary of 4.5 dpc implantation site defects in KO uteri.

Implantation site defects	Control	KO
Abnormal blastocyst(s) appearance	0/6	6/6
Diminished or absent luminal closure	0/6	6/6
Attached blastocyst	6/6	1/6
Conjoined blastocysts	0/6	1/6

Levels of *Plcβ1-4* in implantation sites

Since loss of PLCβ1 should disrupt $G_{\alpha}q$-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 *Plcβ*s.

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

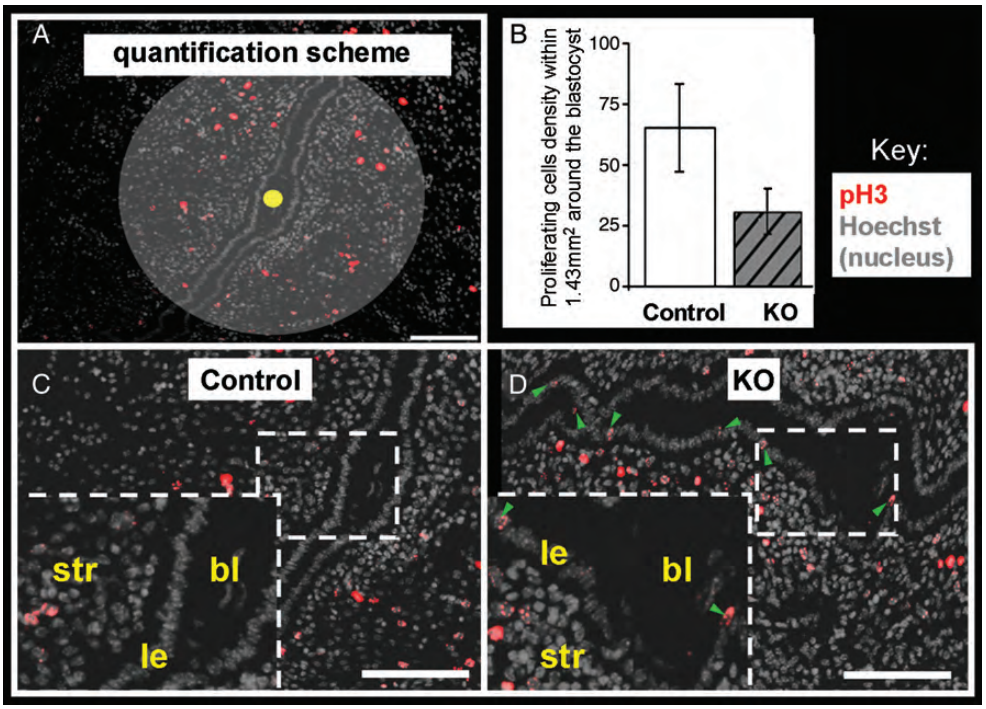

Figure 2 KO implantation sites contain proliferating epithelial cells at 4.5 dpc. (**A**) Quantification scheme for analysis of proliferative cells; (**B**) Density of proliferating cells around the blastocyst in Control and KO implantation sites ($n = 3$ animals per group, with one implantation site 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.

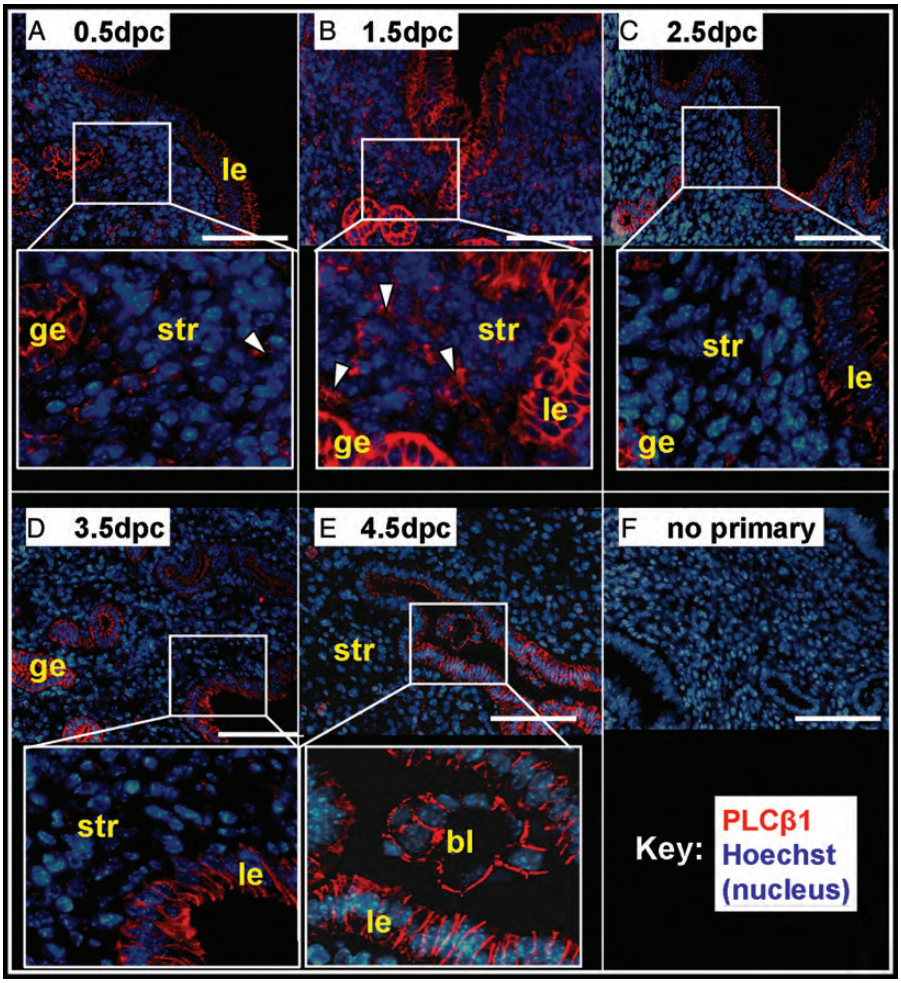

Figure 3 Immunohistochemical localization of phospholipase C (PLC)β1 expression in the uterus during early pregnancy. PLCβ1 is expressed in the luminal and glandular epithelia during 0.5–4.5 dpc and in the stroma during 0.5–1.5 dpc, with overall expression transiently higher at 1.5 dpc. White arrowheads show examples of stromal staining foci. le, luminal epithelium; str, stroma; ge, glandular epithelium; bl, blastocyst. Scale bars: 150 μm.

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

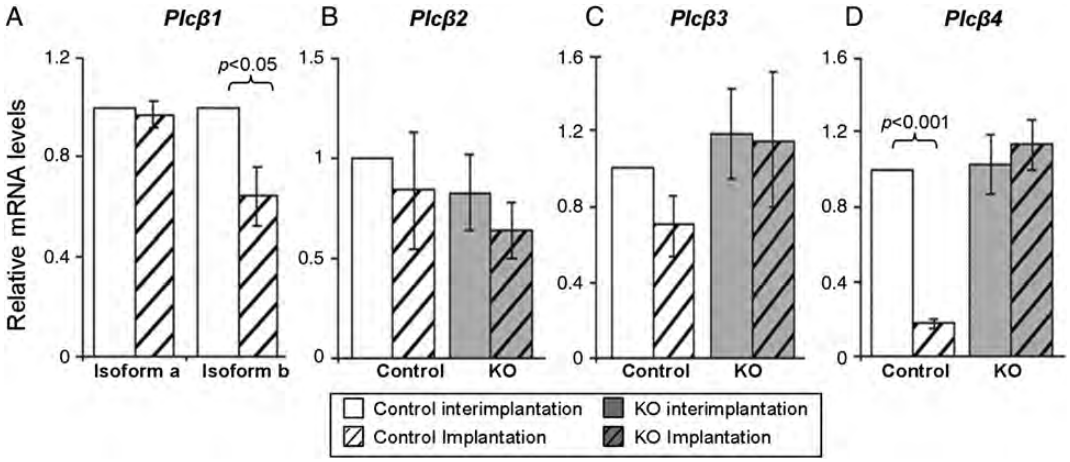

Figure 4 mRNA levels of *Plcβ1-4* in implantation and interimplantation sites of Control and KO animals at 4.5 dpc. (**A**) Levels of *Plcβ1a* and *Plcβ1b* in interimplantation and implantation sites of Control animals. $n = 4$ animals per group, with 3 sites analysed per animal; (**B** and **D**) Levels of *Plcβ2-4* in interimplantation and implantation sites of Control and KO mice. $n = 3$ animals per group, with three sites analysed per animal. Histograms show mean ± SEM relative to Control interimplantation site.

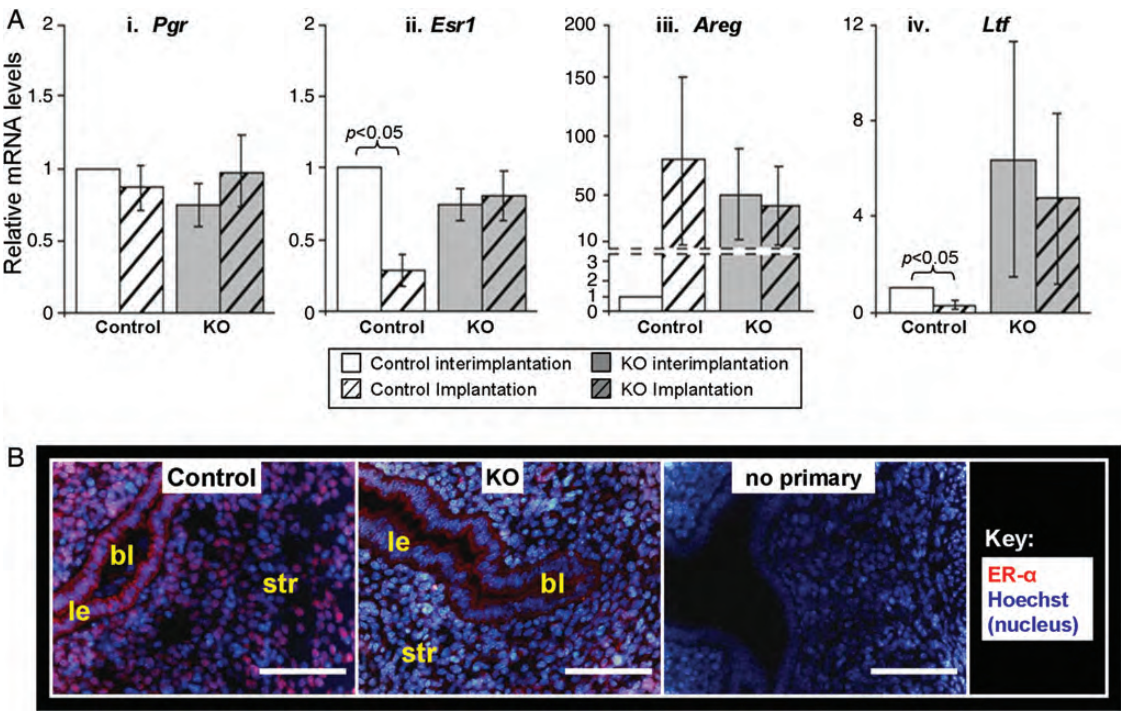

Figure 5 Steroid signalling is aberrant in KO implantation sites. (**Ai–iv**) mRNA levels of **(i)** *Prg*, **(ii)** *Esr1*, **(iii)** *Areg* and **(iv)** *Ltf* in interimplantation and implantation sites of Controls and KOs; expression of *Esr1*, *Areg*, and *Ltf* are all dysregulated in KOs. $n = 4$ animals per group, with three sites analysed per animal. Histograms show mean ± SEM relative to Control interimplantation site. (**B**) Localization of ER-α around implantation sites. KO tissue exhibits markedly lower levels of nuclear ER-α staining. le, luminal epithelium; str, stroma; 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

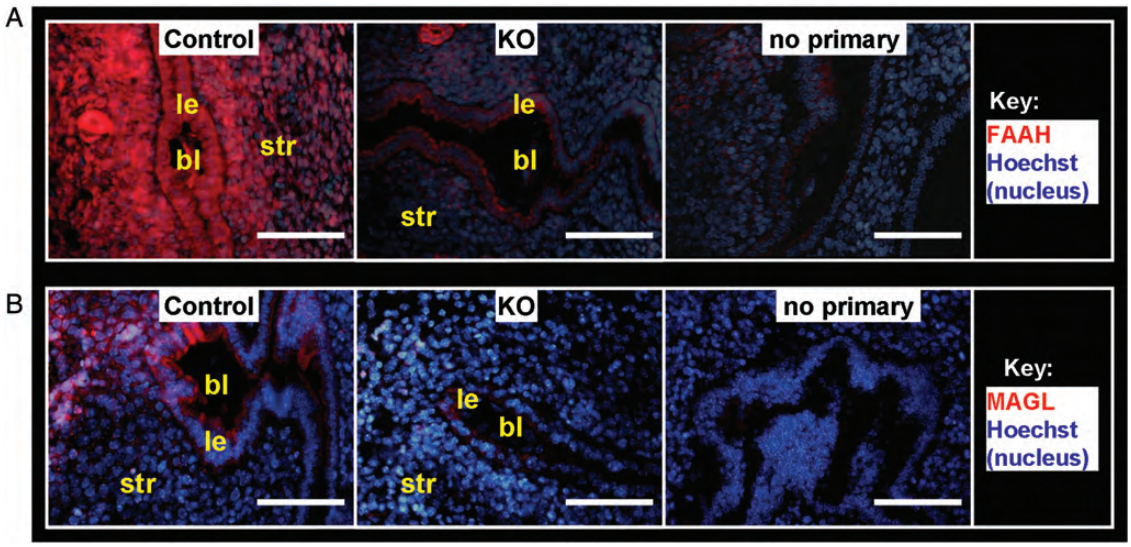

Figure 6 Expression of endocannabinoid-degrading enzymes FAAH and MAGL is down-regulated in KO implantation sites. (**A**) FAAH and (**B**) MAGL immunolocalization in Control and KO uteri at 4.5 dpc. In both cases, KO implantation sites show markedly lower expression levels of the enzymes than do Control implantation sites. le, luminal epithelium; str, stroma; bl, blastocyst. Scale bars: 150 μm.

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 pdc (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; Tranguch *et al.*, 2005), while loss of the progesterone receptor abolishes decidualization when uteri are challenged with a deciduogenic 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). *Esr1* is expressed at the uterine glands and subepithelial stroma during 4.5 dpc (Tan *et al.*, 1999) and its down-regulation in Control implantation sites (Fig. 5) is in agreement with the disappearance of uterine glands from decidua (Stewart and Peel, 1978). However, KO implantation sites failed to down-regulate *Esr1* indicating failure to initiate decidualization. Estrogen signalling is further disrupted with KO uteri expressing low levels of ERα protein and high levels of *Ltf* (a positively regulated direct target of estrogen). Together, these results are most likely to indicate that the implantation sites of KOs have highly up-regulated estrogen signalling, with low protein levels alongside high mRNA levels likely to be due to rapid ERα protein turnover: ERα can degrade in response to agonists and such rapid ERα degradation occurs in ERα-negative human breast cancer cells (Wijayaratne and McDonnell, 2001; Marsaud *et al.*, 2003; Chu *et al.*, 2007; Valley *et al.*, 2008). High levels of *Ltf* suggest overactive estrogen signalling and in agreement with the implantation failure of KOs, increased 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

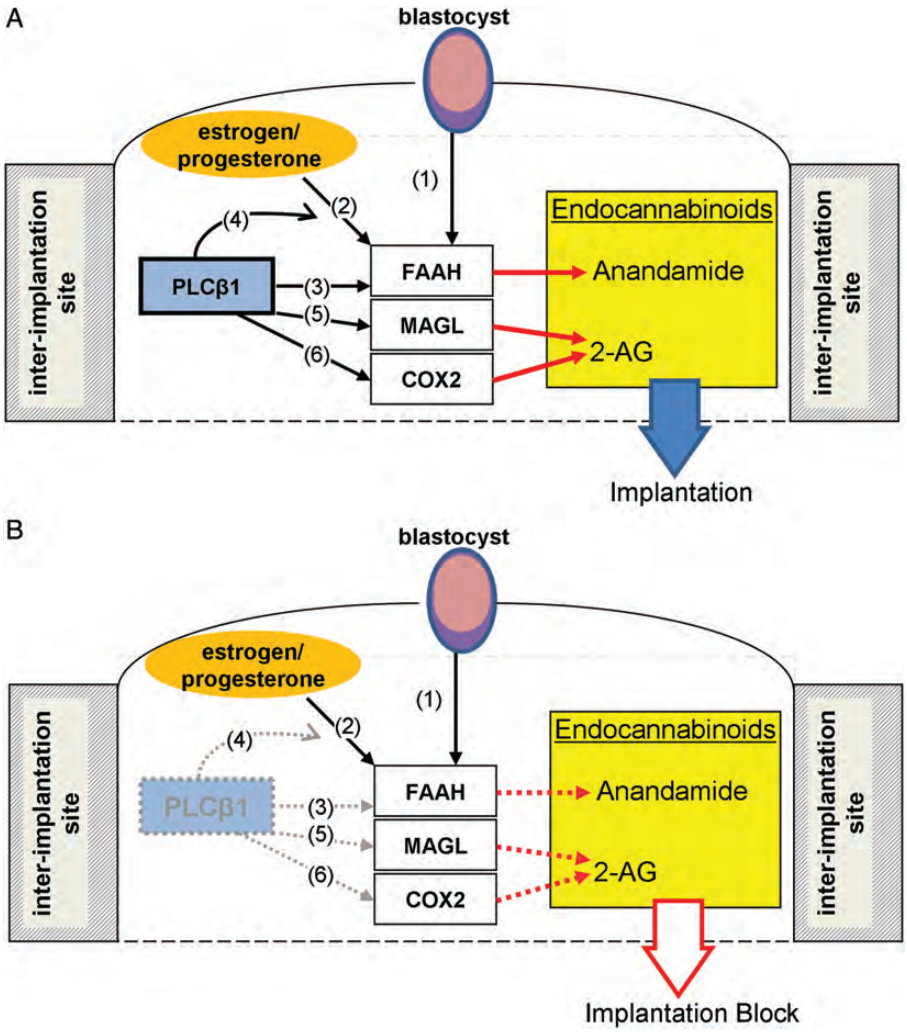

Figure 7 Proposed roles of PLCβ1 in degradation of endocannabinoids at implantation sites. (**A**) FAAH expression at the implantation sites can be stimulated by (1) the blastocyst directly (Maccarrone *et al.*, 2004), (2) ovarian steroid hormones (Xiao *et al.*, 2002), or through (3) up-regulation by 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.

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

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 participates 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 up-regulated. 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 trophectoderm 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 down-regulated in the implantation sites of Controls, one possible way to quench GPCR signalling during implantation. Oxytocin and its GPCR-$G_q\alpha$ 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-$G_q\alpha$ signalling in the uterus around the time of implantation is limited, with only few studies focusing on the importance of GPCR-$G_q\alpha$ signalling during the early stages of pregnancy establishment. Notable examples of GPCRs that signal through the $G_q\alpha$-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-$G_q\alpha$ 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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