Paf receptor expression in the marsupial embryo and endometrium during embryonic diapause

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Abstract

The control of reactivation from embryonic diapause in the tammar wallaby (*Macropus eugenii*) involves sequential activation of the corpus luteum, secretion of progesterone that stimulates endometrial secretion and subsequent changes in the uterine environment that activate the embryo. However, the precise signals between the endometrium and the blastocyst are currently unknown. In eutherians, both the phospholipid Paf and its receptor, platelet-activating factor receptor (PTAFR), are present in the embryo and the endometrium. In the tammar, endometrial Paf release *in vitro* increases around the time of the early progesterone pulse that occurs around the time of reactivation, but whether Paf can reactivate the blastocyst is unknown. We cloned and characterised the expression of PTAFR in the tammar embryo and endometrium at entry into embryonic diapause, during its maintenance and after reactivation. Tammar PTAFR sequence and protein were highly conserved with mammalian orthologues. In the endometrium, PTAFR was expressed at a constant level in the glandular epithelium across all stages and in the luminal epithelium during both diapause and reactivation. Thus, the presence of the receptor appears not to be a limiting factor for Paf actions in the endometrium. However, the low levels of PTAFR in the embryo during diapause, together with its up-regulation and subsequent internalisation at reactivation, supports earlier results suggesting that endometrial Paf could be involved in reactivation of the tammar blastocyst from embryonic diapause.

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Introduction

Embryonic diapause in mammals is a period of developmental arrest, in which the blastocyst is maintained in a dormant state for an extended period of time. Over 100 species of mammals undergo embryonic diapause (Renfree & Calaby 1981, Renfree & Shaw 2000), including the mouse and around 30 species of marsupials. In marsupials, the control of diapause is best understood in the tammar wallaby (*Macropus eugenii*) in which the blastocyst remains quiescent for 11 months. The marsupial blastocyst differs from that of eutherian mammals in that there is no inner cell mass at any stage. The blastocyst in diapause consists of a unilaminar layer of about 80 cells and there is no sign of an embryonic disc until around day 9 after removal of the pouch young (RPY; Renfree 1994, Frankenberg *et al.* 2013). In the tammar, the external and physiological mechanisms controlling entry into and reactivation from diapause have been well established (Tyndale-Biscoe & Renfree 1987). Lactational or seasonal factors inhibit the development of the corpus luteum, keeping the secretion of progesterone low and the endometrium unstimulated

(Tyndale-Biscoe *et al.* 1974, Renfree *et al.* 1979). Removal of this inhibition of the corpus luteum leads to a pulse of progesterone between days 4 and 7, but usually on day 5, after RPY to reactivate the diapausing blastocyst (Hinds & Tyndale-Biscoe 1982, Hinds *et al.* 1983, Shaw & Renfree 1984). This pulse of progesterone induces a secretory endometrium, which in turn reactivates the blastocyst (Renfree 1972, 1994, Renfree & Tyndale-Biscoe 1973). Subsequently, the first signs of reactivation are observed in the blastocyst, including a resumption of mitosis by day 4 RPY and a change in metabolism by day 5 RPY, with the first measurable expansion of the blastocyst occurring at day 8 RPY (Fig. 1; Thorner *et al.* 1981, Shaw & Renfree 1986, Tyndale-Biscoe & Renfree 1987, Shaw 1996, Spindler *et al.* 1998, 1999). However, how these proximal signals in the uterine environment are translated by the embryo to reactivate the diapausing blastocyst is unknown. One potential regulator is Paf (1-*o*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine).

Paf is a potent ether phospholipid that is involved in numerous aspects of pregnancy (Tiemann 2008). The binding of Paf to the platelet-activating factor receptor

Figure 1 Summary of the timing of events during reactivation from diapause of the embryo and endometrium in the tammar wallaby after removal of the pouch young (RPY). The RPY-induced prolactin inhibition on day 0 initiates the events that reactivate the blastocyst from embryonic diapause. Thus, the timings of all subsequent reactivation stages are determined relative to this day of RPY, including the progesterone pulse, coinciding with an oestradiol-17 β pulse, which reactivates the endometrium. Also around this time, the first signs of reactivation are observed in the blastocyst and the first increase in uterine Paf secretion occurs (Kojima *et al.* 1993) followed by the first measurable expansion of the blastocyst. Adapted from Shaw G 1996 *The uterine environment in early pregnancy in the tammar wallaby. Reproduction, Fertility, and Development* 8 811–818.

(PTAFR), a member of the G protein-coupled receptor family, accounts for many of the reported actions of Paf (Ishii *et al.* 2002). Paf is produced and released by embryos during preimplantation development of all eutherian species studied to date: mouse (O'Neill 1985, Ryan *et al.* 1989, Ammit & O'Neill 1991, Roudebush *et al.* 2002), rabbit (Minhas *et al.* 1993), sheep (Battye *et al.* 1991), hamster (Velasquez *et al.* 1995) and human (Collier *et al.* 1990, Ammit & O'Neill 1991, Nakatsuka *et al.* 1992). No embryos of marsupial species have yet been examined, although Paf appears to be produced by the endometrium of the tammar during the period of reactivation (Kojima *et al.* 1993). Similarly, PTAFR mRNA transcripts are expressed in the human blastocyst (Sharkey *et al.* 1995, Roudebush *et al.* 2003) and across all preimplantation stages in both porcine and mouse embryos (Roudebush *et al.* 1997, 2002, Stojanov & O'Neill 1999, Lee *et al.* 2004).

Paf is beneficial for early embryonic development in a number of ways. In the mouse, the addition of Paf can stimulate embryo metabolism (Ryan *et al.* 1989, 1990a), increase total cell number (Ryan *et al.* 1990b, Roudebush *et al.* 1996) and promote overall embryo development and viability (Nishi *et al.* 1995, Stoddart *et al.* 1996, O'Neill 1997, 1998). Furthermore, Paf has the critical function of generating a pro-survival anti-apoptotic transcriptome within the embryo (Jin & O'Neill 2011) and has the net effect of maintaining the tumour suppressor protein p53 in a latent state (Jin *et al.* 2009). However, exposure to high levels of Paf provides either no additional benefit or can even have harmful effects (Ryan *et al.* 1990b).

In the tammar, Paf is present in the culture medium after incubation of endometrial explants during embryonic diapause and at all reactivation stages examined (Kojima *et al.* 1993). Although Paf production is highly variable, its levels appear to increase at reactivation, around four days after RPY. This is coincident with both the first observed pulse of progesterone and the first signs of reactivation and increased metabolism in the tammar blastocyst (Hinds & Tyndale-Biscoe 1982, Gordon *et al.* 1988, Kojima *et al.* 1993, Spindler *et al.* 1998, 1999). However, it is unclear whether Paf is actively secreted by the endometrium or released as a result of collection techniques. Regardless, the release of Paf around the time of the progesterone pulse is consistent with reports on the rat and sheep, which indicate that endometrial Paf release is dependent on progesterone and/or oestradiol that, in the sheep, may occur in a pulsatile manner (Chami *et al.* 1999, 2004, Li *et al.* 1999). As Paf is an important regulator of embryo development in a wide range of eutherian species, and in tammars Paf secretion by the endometrium is increased at the time of reactivation from diapause, it is possible that the increased metabolic and mitotic rates observed in the tammar blastocyst at reactivation from diapause could be attributable to an increase in intrauterine Paf.

Although Paf has beneficial effects on the preimplantation embryo and the endometrium secretes Paf, surprisingly there is no direct, definitive evidence that Paf released from the endometrium acts directly on the embryo in any species, which implies that only autocrine Paf release affects embryo development (O'Neill 2005). In the tammar, it is not known whether Paf is produced and released by the embryo, nor do we know the distribution of PTAFR in either the endometrium or the embryo. In this study, we aimed to characterise the expression of PTAFR in the tammar endometrium and embryo at entry into diapause, during diapause and reactivation from diapause to determine whether endometrial Paf could function in reactivation of the blastocyst from embryonic diapause.

Materials and methods

Animals

Tammar wallabies (*M. eugenii*) of Kangaroo Island origin were kept in open grassy yards in our breeding colony. Their diet was supplemented with fresh fruit, vegetables and lucerne cubes, and water was supplied *ad libitum*. Care and treatment of animals conformed to the National Health and Medical Research Council Australian (2004) guidelines. All animal handling and experimentation was approved by the University of Melbourne Animal Experimentation Ethics Committees.

Tissues

Tammar uterine samples and embryos were obtained as previously described (Renfree & Tyndale-Biscoe 1978).

Other tissues were collected from the pouch young (days 0–350 *post-partum*) for use as positive controls as listed below. When the day of birth was unknown, the ages of pouch young were extrapolated from growth curves based on head length measurements (Poole *et al.* 1991). Before and during embryonic diapause, the stage of pregnancy was based on the *post-partum* age of the pouch young. The tammar has a *post-partum* oestrus and the sucking stimulus of the pouch young acts to inhibit luteal development via prolactin secreted from the pituitary (reviewed in Tyndale-Biscoe & Renfree (1987)). This luteal inhibition prevents the progesterone pulse from occurring and results in the endometrium and subsequently the embryo entering into quiescence (Tyndale-Biscoe 1978, Shaw & Renfree 1986, Renfree & Shaw 2000). In this study, adult females with a pouch young older than day 8 *post-partum* were presumed to be carrying a diapausing blastocyst (Tyndale-Biscoe & Renfree 1987). Before reactivation from diapause can occur, the pouch young-induced luteal inhibition must be removed for 3 consecutive days (Gordon *et al.* 1988, Hinds 1989). Therefore, after diapause the reactivation stages of pregnancy were determined relative to the day of RPY (day 0; Fig. 1).

All tissues were collected under RNase-free conditions. Tissues were either snap frozen in liquid nitrogen for RNA and/or protein extraction or fixed overnight in 4% (w/v) paraformaldehyde, washed twice in 1× PBS and stored in 70% (v/v) ethanol before paraffin embedding and sectioning.

Cloning of tammar PTAFR partial cDNA sequences

Total RNA was isolated from pouch young tissues with TRI Reagent (Ambion, Austin, TX, USA) and DNase-treated using DNA-free (Ambion) according to the manufacturer's instructions. The quality and quantity of the RNA were verified by both electrophoresis and optical density reading using a NanoDrop ND-1000 spectrophotometer (BioLab, Thermo Fisher Scientific, Waltham, MA, USA). Five micrograms total RNA were reverse transcribed using SuperScript III Kit (Invitrogen) with oligo(dT) priming according to the manufacturer's instructions. Cross species primers designed to conserved regions were initially used to amplify partial sequences of PTAFR from day 80 *post-partum* liver. The PCR products were cloned using the pGEM-T-Easy vector (Promega) and sequenced at the Sequencing and Genotyping Facility (Department of Pathology, University of Melbourne, Australia). The resulting sequence was used to design tammar-specific primers for the expression study (see below). The specificity of all transcripts obtained was confirmed by homology with nucleotide sequences using the National Centre for Biotechnology Information (NCBI) BLASTN 2.2.26 program (www.ncbi.nlm.nih.gov/BLAST/). Once the tammar genome had been released, the cloned partial sequence obtained was combined with the corresponding partial sequence available on Ensembl (www.ensembl.org, Ensembl release 67, May 2012). The remainder of the sequence was then obtained by sequence searches of the tammar Whole Genome Shotgun (WGS) database available at NCBI and contigs were aligned using CAP3 (Huang & Madan 1999). The predicted protein sequence

for the full-length tammar PTAFR was obtained using the Translate tool from the Swiss Institute of Bioinformatics (SIB) ExPASy Bioinformatics Resources Portal (Artimo *et al.* 2012). The tammar sequence was then compared against nucleotide and protein sequences from human, mouse, opossum, Tasmanian devil and platypus sequences retrieved from either GenBank (www.ncbi.nlm.nih.gov/) or Ensembl (relevant accession numbers are listed in Table 1). Nucleotide and protein identities were determined using FASTA (version 36.3.5c, March 2012; Pearson & Lipman 1988) and the protein alignment was determined using T-Coffee (Notredame *et al.* 2000, Tommaso *et al.* 2011). The cloned tammar PTAFR nucleotide sequence has been submitted to GenBank (GenBank ID: JX524197).

Endometrial qPCR expression

In order to determine the variation in the expression for PTAFR in the endometrium across the stages of embryonic diapause, total RNA was isolated from the endometrium as described earlier. Oligo(dT)-primed first-strand cDNA synthesis was reverse transcribed from 3 µg of total RNA in a 20 µl reaction using TaqMan (Roche Molecular Systems, Inc. and Applied Biosystems) following the manufacturer's instructions and diluted 1:5 with nuclease-free water. QPCR was performed in a 20 µl reaction volume using 5 µl cDNA, 10 µl QuantiTect SYBR Green PCR Kit (Qiagen) and 0.8 µm of each primer. The relative expression levels of PTAFR transcripts were compared with the reference gene ACTB using the following tammar-specific primers: PTAFR (forward, 5'-CCTTGTTGAGTCAGCCTCTT-3'; reverse, 5'-CG-TGTCCATCAGTACATCA-3') and ACTB (forward, 5'-TTGCT-GACAGGATGCAGAAG-3'; reverse 5'-AAAGCCATGCCAAT-CTCATC-3').

Reactions were run in triplicate on an Opticon 2 (MJ Research, Waltham, MA, USA) using the following conditions: 50 °C, 10 min; 95 °C, 15 min; 50 cycles of 95 °C, 30 s; 53 °C, 30 s; 72 °C, 40 s and a plate read for 1 s; 72 °C for 1 min and a dissociation curve from 55 to 95 °C, reading for 1 s every 0.5 °C and a final extension of 72 °C for 5 min. A minimum of five

Table 1 Homologies for tammar PTAFR nucleotide and protein sequence compared with other vertebrates.

Common names	Species names	Accession numbers	Nucleotide identities (%) ^a	Protein identities (%) ^b
Human	<i>Homo sapiens</i>	NM_001164723.2	75	75 (89)
Mouse	<i>Mus musculus</i>	NM_001081211.1	69	71 (89)
Grey short tailed opossum	<i>Monodelphis domestica</i>	XM_001372834.1	83	81 (93)
Tasmanian devil	<i>Sarcophilus harrissi</i>	ENSSHA-T00000-003177	85	85 (94)
Platypus	<i>Ornithorhynchus anatinus</i>	XM_001508993.2	71	71 (86)

^aOnly the coding sequence for each gene was compared with the tammar sequence. ^bValues in brackets are the percentage amino acid similarity with tammar PTAFR.

replicates were run per stage for a total of ten stages covering entry into diapause, diapause and reactivation from diapause. The negative control reactions contained nuclease-free water instead of template. Plates were excluded if more than one of the negative control triplicates was contaminated. Individual samples of a triplicate were also excluded if they had irregular melting curves or if the coefficient of variation (CV) was >0.05 for the triplicate and the remaining two samples had a CV of <0.05 . If more than one of the triplicates was irregular, the sample was repeated or excluded.

The expression levels of *PTAFR* transcripts were compared with the expression levels of *ACTB* using the $\Delta\Delta C_t$ method to correct the C_t values (Livak & Schmittgen 2001). The subsequent relative quantity values obtained for the replicates of each stage were then averaged and analysed as described below.

Embryo RT-PCR expression

In order to check the expression pattern of *PTAFR* in the embryo across the stages of embryonic diapause, total RNA was isolated from embryos using the RNeasy Plus Micro Kit (Qiagen) with adjustments as described in a study by Lefèvre & Murphy (2009). cDNA was reverse transcribed from total RNA using the SMARTer PCR cDNA synthesis kit (Clontech) and then amplified using Ex Taq polymerase (Takara, Shiga, Japan) for 27 cycles. RT-PCR was performed in a 25 μ l reaction with GoTaq Green Master Mix (Promega) with 0.3 μ M of each forward and reverse primer using the same *PTAFR* primers as for qPCR but using *GAPDH* as the reference gene, because in the tammar we have found *GAPDH* to have a more consistent expression in the embryo than *ACTB*. Primer sequences were *GAPDH* (forward, 5'-CCTACTCCAATGTATCTGTTGT-3'; reverse, 5'-GGTGGAACTCCTTTTGGACTGG-3'). RT-PCR conditions were 95 °C, 2 min; 40 cycles of 95 °C, 30 s; 53 °C, 60 s; 72 °C, 1 min, then 72 °C for 5 min. The negative control reaction contained nuclease-free water instead of template and a day 3 *post-partum* gravid endometrial sample served as a positive control.

Endometrial protein expression

Protein expression and localisation of PTAFR in the endometrium was carried out using immunohistochemistry across a range of 16 gravid uterine stages covering entry into diapause (seven stages, days 0–8 *post-partum*), early and late diapause (five stages, days 9–250 *post-partum*) and reactivation from diapause (four stages, days 3–6 RPY) with three replicates per stage. Each immunohistochemistry run included one uterine tissue at each stage with day 175 *post-partum* lung used as a positive control (initially used to confirm antibody specificity) to ensure consistency in each run. Tissue sections (6 μ m) were prepared as described earlier, deparaffinised and rehydrated. Sections were treated with 5% (v/v) hydrogen peroxide in distilled water for 15 min to block endogenous peroxidases and then washed in Tris-buffered saline with Tween-20 (TBST) containing 100 mM Tris-HCl, pH 7.5, 300 mM NaCl and 0.5% (v/v) Tween-20. The sections were blocked with 10% (v/v) goat serum with 0.1% (w/v) BSA (Sigma-Aldrich) in TBST (diluent) for 1 h, and then incubated overnight at 4 °C with primary

antibody. PTAFR was detected with a rabbit polyclonal antibody (160602, Cayman Chemical Company, Ann Arbor, MI, USA) at a concentration of 2 μ g/ml. The next day, sections were washed in TBST, incubated for 1 h in polyclonal goat anti-rabbit-biotinylated secondary antibody (E0432, Dako, Glostrup, Denmark) at a concentration of 1.44 μ g/ml and washed again in TBST. The signal was then amplified for 30 min with streptavidin/HRP conjugated (P0397, Dako) before a final wash in TBST. The signal was visualised with 3,3'-diaminobenzidine (Dako) and sections were counterstained with haematoxylin. Two negative controls were also included on every slide in every run, diluent only and rabbit IgG fraction (normal) (X0903, Dako) at the same concentration as the primary antibody.

Embryo immunofluorescence

Embryo whole-mount immunofluorescence was performed to detect PTAFR across the stages of entry into diapause (days 2–4 *post-partum*), early and late diapause (days 11–250 *post-partum*) and reactivation from diapause (day 4 RPY). Three replicates from each stage of diapause were tested along with three negative IgG control embryos (day 6 *post-partum*, day 189 *post-partum* or day 3 RPY), where each immunofluorescence run consisted of one embryo from each stage and one negative IgG control embryo. Immunofluorescence used the same primary antibody and IgG listed earlier for the immunohistochemistry. Embryo shell coats were first removed to prevent autofluorescence and then embryos were washed in wash buffer (0.05% (v/v) Tween-20 and 2% (w/v) BSA in 1 $\times$ PBS) before being permeabilised in 0.5% Triton X-100 (v/v) in 1 $\times$ PBS for 30 min. Embryos were washed as mentioned earlier and then blocked in 30% (v/v) donkey serum (D9663, Sigma-Aldrich) for 2–3 h. After blocking, embryos were incubated overnight for at least 16 h with either primary antibody at 1 μ g/ml or rabbit non-immune IgG fraction (normal) (X0903, Dako) (at the same concentration as primary antibody). The next day, embryos were washed as above and then incubated with AF488 donkey anti-rabbit secondary IgG antibody (A21206, Invitrogen) at 10 μ g/ml for 1 h in the dark at room temperature; embryos were kept in the dark from this point. Embryos were then washed as above and incubated with 100 ng/ml DAPI dilactate (D3571, Invitrogen) in PBS for 5 min, washed again and mounted in DABCO antifade (1,4-diazabicyclo[2.2.2]octane).

Confocal microscopy

Embryos were visualised using the Zeiss LSM 510 Meta Confocal Microscope (Carl Zeiss, Jena, Germany) mounted on a Zeiss Axioplan (Imager.Z1/I AXIO) upright microscope and images were captured using the attached Zen 2009 LE program. Embryos were visualised with Argon (Green Alexafluor 488 antibody) and u.v. (DAPI). Pinhole diameter was set as close as possible to 1.5 Airy units on the weaker Argon channel, and the pinhole diameter was then kept constant in the u.v. settings. Visualisation using maximal gain and offset was able to be optimised separately for each channel. Each run contained an IgG control embryo and visualisation settings were initially optimised to eliminate background using

this control embryo to reduce the Argon settings until staining was minimal. These settings were then used to visualise the other embryos in the run. Embryo images were captured using a z-stack of both channels throughout the whole embryo with optical slices taken approximately every 5 μm .

Western blot

Protein for the western blots was extracted from snap frozen tissue using radioimmunoprecipitation buffer with a mix of protease inhibitors (Protease Inhibitor Cocktail Set V, Calbiochem, #535141; Merck, Darmstadt, Germany). Protein concentrations were measured with a spectrophotometer (NanoDrop ND-1000, BioLab), diluted in 4 $\times$ Laemmli sample buffer with 5% (v/v) β -mercaptoethanol (Laemmli 1970) and heated for 5 min at 100 $^{\circ}\text{C}$. PTAFR (48 kDa) was run across 25 μg protein samples of day 156 *post-partum* lung, day 170 *post-partum* liver, day 138 *post-partum* kidney and adult day 5 RPY gravid endometrium. Protein samples were run on a 10% SDS-PAGE separating gel and blotted to a PVDF Hybond-P membrane (Amersham, GE Healthcare, Buckinghamshire, UK). The membrane was blocked with 5% (w/v) skim milk in TBST (containing 200 mM Tris-HCl, pH 7.5, 300 mM NaCl and 0.1% (v/v) Tween-20) for 16 h at 4 $^{\circ}\text{C}$ and then incubated with 2 $\mu\text{g}/\text{ml}$ primary antibody for 1 h. The membrane was then washed three times in TBST for 10 min each, incubated with HRP-conjugated secondary antibody goat anti-rabbit (sc-2004, Santa Cruz Biotechnology, Inc.) at 0.04 $\mu\text{g}/\text{ml}$ for 30 min and washed again as mentioned earlier. Protein bands were detected using chemiluminescence, where the membrane was incubated with ECL western blotting detection reagents (RPN2106, GE Healthcare) for 1 min and exposed to Hyperfilm (Amersham, GE Healthcare) for 10 min.

Statistical analysis

Statistical analyses for qPCR were conducted using R, version 2.11.1 (R Development Core Team 2010). For all analyses, a significance level of $P < 0.05$ was used and data are presented as mean $\pm$ S.E.M. A Shapiro-Wilks test was first performed to check the assumption that the data had a normal distribution. As the distribution of the relative expression values was skewed, the data were log transformed for analysis and the back-transformed means were reported. Log transformed data were analysed by one-way ANOVA, with multiple comparisons of means compared using Tukey contrasts (only if ANOVA was significant). As there was no significant variation with age, samples were further grouped as 'entry into diapause', 'during diapause' and 'reactivation from diapause' and one-way ANOVA was performed as described earlier.

Results

Cloning and sequence analysis of the tammar PTAFR gene

An 801-bp partial PTAFR cDNA sequence was obtained with cross-species primers which, when combined with the partial PTAFR sequence available on Ensembl

(ENSMEUT00000006792), partially overlapped with and extended the sequence to 904 bp. This sequence was then used to search the tammar wallaby WGS database (available at NCBI) to identify the remainder of the 3' end of the tammar PTAFR sequence. The contigs obtained (ti, 1484702988; ti, 1709030349; ti, 1646286453 and ti, 1648958551) overlapped with and extended the above sequence by 131 bp. The whole coding region of the PTAFR gene is contained within only one exon. The full tammar coding sequence was 1035 bp in length and the translated sequence predicted a protein of 344 amino acids. There was moderately high conservation of tammar PTAFR with all mammalian vertebrates examined with nucleotide sequence identities of 69% or greater (Table 1). The protein sequence identities were also highly conserved between tammar PTAFR and other mammals, and showed a very high amino acid similarity (Table 1). Although the predicted tammar protein sequence contained a number of sequence variations compared with the eutherians, the majority of these were conserved in both opossum (*Monodelphis domestica*) and Tasmanian devil (*Sarcophilus harrissi*) (Fig. 2).

Consistent with the structure of a G protein-coupled receptor, PTAFR consists of an extracellular N-terminal domain, an intracellular C-terminal domain and seven transmembrane domains intersected by an additional three extracellular and three cytoplasmic domains (Prescott *et al.* 2000, Ishii *et al.* 2002). In the tammar, the transmembrane domains had the highest conservation with eutherians and these were also highly conserved among all marsupial species. Based on mutation studies, transmembrane domains 5, 6 and 7 are predicted to be involved in Paf binding and, in particular, three histidine residues are thought to constitute the three-dimensional Paf-binding pocket and were completely conserved in the tammar (Ishii *et al.* 1997). Other regions important for PTAFR function that were conserved in the tammar include the third intracellular loop, which is required for intracellular signalling, and the C-terminal domain, which is phosphorylated before desensitisation of the receptor (Prescott *et al.* 2000).

mRNA expression profiles for PTAFR in the endometrium and embryo during embryonic diapause

Endometrial PTAFR mRNA was detected by RT-PCR before, during and after embryonic diapause (Fig. 3A). The expression of PTAFR in endometrium during these stages did not vary quantitatively when assessed by qPCR expression (Fig. 3B; $P > 0.05$) and there was no significant difference in PTAFR expression in the endometrium between entry into diapause, diapause and reactivation from diapause (Fig. 3B; $P > 0.05$). PTAFR was also detected in the embryo at all stages examined (Fig. 3C).

Figure 2 Alignment of the predicted tammar PTAFR sequence with PTAFR from other species. Wallaby PTAFR was highly conserved with known mammalian vertebrates. In particular, the seven transmembrane domains (blue boxes) showed the highest conservation across species and this included conservation of the three histidine residues that are predicted to form the Paf-binding pocket (arrows). Shown here also are the third intracellular loop (underlined in yellow) and the C-terminal intracellular tail (underlined in red). Black backgrounds indicate identical residues of the consensus sequence, grey backgrounds indicate similar residues and white background indicates either no similarity to the consensus sequence or no consensus sequence could be obtained.

Figure 3 mRNA expression profiles of *PTAFR* in the embryo and the endometrium across the stages of entry into diapause, diapause and reactivation from diapause. (A) mRNA expression profile for *PTAFR* in the endometrium across the stages of entry into diapause, diapause and reactivation from diapause, showing expression at all stages. *ACTB* was used as the reference gene; (B) *PTAFR* qPCR endometrium expression profile relative to *ACTB* showing consistent expression across all stages with no significant difference in expression at any stage. Data are mean $\pm$ S.E.M. with $n=5$ samples per individual pregnancy stage except for diapause with $n=7$ samples; (C) *PTAFR* mRNA was expressed across all stages in the embryo from entry into diapause, diapause and reactivation from diapause. *GAPDH* was used as the reference gene. Note that the embryo RT-PCR image is formed from different parts of the same gel for each gene. +ve, positive control (day 3 *post-partum* gravid adult endometrium); -ve, no template negative control; d, day; pp, *post-partum*; RPY, removal of pouch young.

PTAFR protein expression in the uterus during embryonic diapause

The protein expression and localisation of *PTAFR* in the uterus was examined by immunohistochemistry during entry into diapause, diapause and reactivation from diapause. At day 0 of pregnancy (which is also one day *post-partum* of the previous pregnancy), *PTAFR* staining was reduced in the glands (Fig. 4A) but at all other stages examined, including during entry into diapause, *PTAFR* was present predominantly on the membranes of the glandular epithelium (Fig. 4B, C and D). After reactivation, on day 4 RPY, there was a distinct apical staining in the basalis layer of the endometrium that was not present in the functionalis layer (Fig. 4D, inset shows basalis layer). Cytoplasmic staining in the luminal epithelium was first detected during embryonic diapause (Fig. 4C) and remained throughout reactivation (Fig. 4D). In the

stroma, *PTAFR* staining was rare and detected only in some blood vessels; there was also occasional background cytoplasmic staining that was not consistent with stage. Furthermore, the presence of *PTAFR* staining in the pouch young lung (Fig. 4E) and its absence in the IgG control (Fig. 4F) indicated that the expression observed in the uterus was not an artefact of the immunohistochemistry. In addition, a western blot confirmed that the antibody was specific to *PTAFR* and detected a protein of the expected size (Fig. 4G).

Localisation of *PTAFR* protein expression in the embryo during embryonic diapause

PTAFR protein expression and localisation was also examined in the embryo across the stages of entry into diapause, during diapause and reactivation from diapause. *PTAFR* protein was expressed strongly in the apical regions of the cells in the cleavage stage embryo before diapause (Fig. 5A), but there was a very

Figure 4 *PTAFR* protein is present in endometrial glands across all stages of embryonic diapause. *PTAFR* protein was detected in the endometrium across the stages of entry into embryonic diapause (A and B), during diapause (C) and at reactivation from diapause (D). Inset shows high power of selected glands. Also shown is the positive control tissue, day 175 *post-partum* lung (E) and the IgG control for the same tissue (F). A western blot run for *PTAFR* (48 kDa), across a range of tissues determined the specificity of the antibody (G). lu, lumen; m, myometrium; b, basalis layer; f, functionalis layer; lun, lung; liv, liver; kid, kidney; ge, gravid endometrium; Lad, ladder. Scale bar (A, B, C and D)=200 μ m at 20 $\times$ magnification, (E and F)=100 μ m at 40 $\times$ magnification, all insets are at 100 $\times$ magnification.

Figure 5 Representative sections showing PTAFR protein expression in the embryo across the stages of embryonic diapause. (A, B and C) Before diapause, a four-cell embryo showing strong PTAFR staining in the region of the cell membrane; (D, E and F) before diapause, an eight-cell embryo showing overall PTAFR staining and localised cytoplasmic expression; (G, H and I) during diapause, showing very faint PTAFR expression; (J, K and L) reactivation from diapause, showing both membrane and cytoplasmic PTAFR expression; (M, N and O) a view of the same reactivated embryo but through a different plane, showing the distinct localised cytoplasmic expression and (P, Q and R) the IgG negative control embryo. Embryos were visualised using a z-stack, photos shown are representative 5 µm thick optical sections through the middle of the embryo, except for (M, N and O) which is a section near the top of the embryo. Green indicates PTAFR, blue indicates DAPI nuclear stain and the merge shows both channels. Scale bar = 50 µm.

low (almost undetectable) expression in the blastocyst during embryonic diapause (Fig. 5G). At reactivation from diapause, there was strong staining in the region of the plasma membrane of several cells of the unilaminar blastocyst (Fig. 5J). There was also a localised cytoplasmic expression in the perinuclear region at both entry into diapause and reactivation (Fig. 5D, J and M).

Discussion

Successful reactivation from embryonic diapause in the tammar requires a progesterone pulse which induces active secretion from the endometrium (Hinds & Tyndale-Biscoe 1982), but the factors involved in the molecular cross-talk between the endometrium and blastocyst are unknown. The results of this study suggest that endometrial Paf may be one of the important factors for the reactivation of the dormant tammar blastocyst.

The tammar *PTAFR* gene was highly conserved with that of *PTAFR* in eutherian species. Furthermore, the seven transmembrane domains were all highly conserved between tammar and all species examined, and this included conservation of the putative Paf-binding pocket (Ishii *et al.* 1997, Prescott *et al.* 2000). These domains are characteristic of a member of the G protein-coupled receptor superfamily and, together with conservation of the regions involved in translation of PTAFR signalling and desensitisation (Ishii & Shimizu 2000, Prescott *et al.* 2000), suggest that the functional role of PTAFR has been conserved in the tammar.

There was no change in *PTAFR* expression in the tammar endometrium throughout any stage of diapause. Although in mice there are two *Ptafr* transcripts arising from two distinct promoters, the first exon of each is spliced to a common second exon so that both transcripts encode the same protein (Ishii & Shimizu 2000). It was beyond the scope of this study to distinguish between the two transcripts and, as the *PTAFR* region examined contains the whole coding sequence, it is possible that dimorphic expression patterns exist for the two transcripts. However, in eutherians there is a correlation between *PTAFR* mRNA and PTAFR protein expression (Ahmed *et al.* 1998, Chami *et al.* 1999, 2004, Tiemann *et al.* 2005), thus the location of the protein is the critical factor in determining a biological effect.

PTAFR protein staining in the tammar endometrium was present in the glandular epithelium across all stages from day 2 *post-partum*, including throughout diapause and reactivation. Unexpectedly, PTAFR was first detected in the luminal epithelium during embryonic diapause and was subsequently intensely expressed at all reactivation stages examined. Although Paf is detected in the tammar endometrium at low levels during diapause (Kojima *et al.* 1993), the quiescent state of the endometrium at this time suggests that the effects of Paf are likely to be minimal.

Furthermore, the responsiveness of the tammar endometrium to Paf may require the progesterone pulse at reactivation. In the sheep, Paf can act as a pulse generator of uterine prostaglandins, but only in a steroid-primed uterus (Chami *et al.* 1999). Similarly, in humans, the response of the endometrium to Paf appears to depend on the steroid hormone profile and not on the cellular localisation of PTAFR (Ahmed *et al.* 1998).

Consistent with this, at reactivation in the tammar endometrium there did appear to be a difference in PTAFR staining intensity between the basalis and functionalis layers of the endometrium. The low intensity staining within the glands of the functionalis layer suggested that the receptor is activated by the hormonally regulated Paf release at this time and is subsequently internalised and degraded, as is seen in other cell types (see below), but this remains to be established. Regardless, in the tammar, constant expression of PTAFR in the glandular epithelium at all stages and its presence in the luminal epithelium during both diapause and reactivation suggests that the expression of the receptor is not the limiting factor in mediating the response of the endometrium to Paf.

PTAFR mRNA was present in the tammar early embryo as in other eutherian species. This is the first report of PTAFR expression in a marsupial embryo. PTAFR was expressed throughout entry into diapause, diapause and reactivation, but qPCR analysis is required to determine a quantitative difference in expression between samples. In the mouse, *Ptafr* is first expressed after activation of the zygotic genome (Stojanov & O'Neill 1999). Although it is not known when the tammar embryonic genome is activated, it would be expected to be active by the blastocyst stage. Thus these results indicate that PTAFR is presumably expressed from both the maternal and embryonic genomes.

In the embryo, PTAFR protein was strongly expressed at entry into diapause both in the region of the plasma membranes at the four-cell stage and with a distinct, localised cytoplasmic staining at the eight-cell stage. However, despite the presence of PTAFR mRNA, there was very low PTAFR protein detected during embryonic diapause. Hence, regulation of PTAFR expression in the tammar blastocyst appears to be at the protein level. At reactivation from diapause, in addition to the strong cellular membrane staining, there was again localised cytoplasmic staining suggesting that PTAFR was being internalised. Furthermore, this expression pattern did not appear to be localised to a particular area of the blastocyst. The tammar blastocyst has no inner cell mass and there are currently no methods to determine where the pluriblast cells of the embryo will form (Frankenberg *et al.* 2013). No cytoplasmic expression of this type has been reported in the embryos of eutherians, despite evidence that desensitisation occurs in response to Paf in the two-cell mouse embryo (Emerson *et al.* 2000) and that PTAFR can be internalised in other cell types. In Chinese hamster ovary cells, treatment with Paf results in receptor phosphorylation and sequestration into the cytoplasm (Ishii *et al.* 1998). Similarly, in a human cell line, Paf-stimulated PTAFR degradation is dependent on internalisation (Dupré *et al.* 2003). Therefore, it would appear that Paf is present both before diapause and at reactivation and is responsible for the internalisation of PTAFR observed at these stages.

Furthermore, the release of endometrial Paf appears to up-regulate PTAFR expression in the blastocyst at reactivation. However, the progesterone pulse at reactivation stimulates an overall increase in uterine secretions and the activity of these factors can significantly affect the timing of blastocyst reactivation (Shaw & Renfree 1986, Spindler *et al.* 1998). Therefore, there are numerous other uterine factors present, whose downstream effects could result in an increase in blastocyst PTAFR levels. Regardless, these results support the suggestion that at reactivation, the progesterone pulse from the corpus luteum results in an increase in endometrial Paf which binds to, and is involved in, the reactivation of the tammar blastocyst from embryonic diapause.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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