

# Embryo-endometrial interactions during early development after embryonic diapause in the marsupial tammar wallaby

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**ABSTRACT** The marsupial tammar wallaby has the longest period of embryonic diapause of any mammal. Reproduction in the tammar is seasonal, regulated by photoperiod and also lactation. Reactivation is triggered by falling daylength after the austral summer solstice in December. Young are born late January and commence a 9-10-month lactation. Females mate immediately after birth. The resulting conceptus develops over 6-7 days to form a unilaminar blastocyst of 80-100 cells and enters lactationally, and later seasonally, controlled diapause. The proximate endocrine signal for reactivation is an increase in progesterone which alters uterine secretions. Since the diapausing blastocyst is surrounded by the zona and 2 other acellular coats, the mucoid layer and shell coat, the uterine signals that maintain or terminate diapause must involve soluble factors in the secretions rather than any direct cellular interaction between uterus and embryo. Our studies suggest involvement of a number of cytokines in the regulation of diapause in tammars. The endometrium secretes platelet activating factor (PAF) and leukaemia inhibitory factor, which increase after reactivation. Receptors for PAF are low on the blastocyst during diapause but are upregulated at reactivation. Conversely, there is endometrial expression of the muscle segment homeobox gene *MSX2* throughout diapause, but it is rapidly downregulated at reactivation. These patterns are consistent with those observed in diapausing mice and mink after reactivation, despite the very different patterns of endocrine control of diapause in these 3 divergent species. These common patterns suggest a similar underlying mechanism for diapause, perhaps common to all mammals, but which is activated in only a few.

**KEY WORDS:** *embryonic diapause, marsupial, MSX, PAF, LIF*

## Introduction

The ability to arrest embryonic development during the reproductive cycle is widespread amongst mammals (Lopes *et al.*, 2004, Murphy, 2012, Renfree and Calaby, 1981, Renfree and Shaw, 2000). Embryonic diapause is a transient state in which embryos at the blastocyst stage are arrested in growth and metabolic activity in synchrony with uterine quiescence. There are a number of forms of this developmental arrest, but all lengthen the period of gestation. This allows selection for traits that regulate the timing of diapause so that birth of the neonate(s), or weaning in the case of marsupials, coincides with the most favourable time for survival (Renfree and Calaby, 1981, Renfree and Shaw, 2000). However, the mechanisms used to control diapause are variable amongst mammals.

Many marsupial species undergo embryonic diapause. Blastocysts may either cease cell division, as in most macropodid

marsupials, so that their size and cell numbers remain constant (Renfree and Shaw, 2000), or undergo an early period of very slow growth with cell division and expansion, as in the nectarivorous honey possum (Renfree, 1981) and the feathertail glider (Ward and Renfree, 1988). The tammar wallaby, *Macropus eugenii*, is the best studied diapausing marsupial (Renfree and Shaw, 2000). However, whilst the lactational (from the sucking stimulus), photoperiodic (via melatonin) and endocrine (via progesterone) control of diapause is now well understood in the tammar (Tyndale-Biscoe and Renfree, 1987), the specific endometrial-embryo interactions that occur at and after reactivation and their molecular control are only just beginning to be understood. This review will focus on those interactions.

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*Abbreviations used in this paper:* LIF, leukaemia inhibitory factor; MSX, muscle segment homeobox; PAF, platelet activating factor.

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Marsupials are unusual in that they have two completely separate uteri (not uterine horns), and in the macropodids (the kangaroos and wallabies), ovulation occurs from alternate ovaries, so that in any one pregnancy there is one gravid uterus and one non-gravid one. This affords the opportunity to have an “inbuilt” control for any changes that occur during the short pregnancy before they deliver their highly altricial young. In addition, since the oestrous cycle is shorter than the pregnancy (by around a day or two), the changes that occur during a normal oestrous cycle are continuing in the non-gravid uterus during the whole of the pregnancy (Tyndale-Biscoe and Renfree, 1987).

### Entry into and maintenance of diapause

In diapausing mammals, the entry into diapause is controlled by signals including the suckling stimulus, the change in daylength or the availability of nutrition (see Flint *et al.*, 1981). In the tammar wallaby *Macropus eugenii*, a small macropodid marsupial, the young are born towards the end of the pro-oestrous phase. In late gestation the maturing fetus produces increasing amounts of cortisol that may provide the fetal signal for parturition (Ingram *et al.*, 1999, Shaw *et al.*, 1996). At term, progesterone falls rapidly concomitantly with short pulses of prostaglandin F<sub>2α</sub> and mesotocin (Parry *et al.*, 1996, Renfree *et al.*, 1994) that trigger uterine contractions and parturition (Shaw and Renfree, 2001, Shaw and Renfree, 2006). Mating occurs about 1 h post-partum (Rudd, 1994), and ovulation and fertilisation occur about 40 hrs post-partum (Renfree, 1994, Renfree and Lewis, 1996). Transport down the oviduct takes under 24 h, and during this passage the zona-enclosed zygote is further enclosed in a mucin layer derived from oviducal secretions, and a final layer, the shell coat laid down at the utero-tubal junction (Renfree and Lewis, 1996). Cleavage commences once the zygote reaches the uterus (Fig. 1). As with all marsupials, there is no morula stage, no compaction and no inner cell mass in the tammar embryo (McLaren, 1982, Renfree and Lewis, 1996). The formation of the unilaminar blastocyst is complete by about day 7-8 post-coitum (Tyndale-Biscoe, 1979). At this stage, if the female is suckling a young, or is in the non-breeding season, the embryo will enter diapause, remaining dormant until reactivated by the termination of lactation before the winter solstice or the end of the non-breeding season in December at the summer solstice. The tammar diapausing blastocyst is remarkably uniform, consisting of a single layer of approximately 80-100 cells (Fig. 1) (Renfree and Tyndale-Biscoe, 1973, Smith, 1981). If there is no suckling young in the pouch, pregnancy proceeds uninterrupted, and by day 19 of the 26.5 day gestation, embryogenesis is completed, organogenesis is initiated, the shell coat breaks down and the embryo attaches to the endometrium (Denker and Tyndale-Biscoe, 1986, Renfree, 1973b, Renfree, 1994, Renfree and Tyndale-Biscoe, 1973).

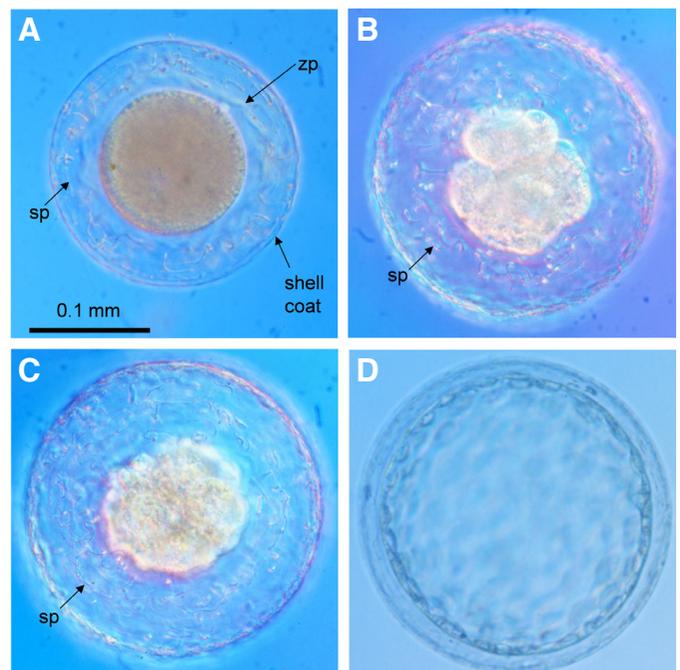
The endocrinology underlying lactational and seasonal diapause is well understood, but the precise details of the molecular changes that occur in the uterus and blastocyst are not known (Renfree, 2000). Few studies have investigated the influence of the ovarian hormones on uterine secretions from the time of ovulation to the formation of the early blastocyst. At oestrus, there is a surge of oestrogen peaking 1-2 days after birth that precedes the ovulation (Shaw and Renfree, 1984). Oestrogen then remains low for the period of embryonic diapause in the tammar. After ovulation, the corpus luteum forms slowly. If there is a newborn young in the

pouch the pituitary releases prolactin as a result of the sucking stimulus. Both the blastocyst and the corpus luteum then remain quiescent. Thus diapause is maintained by elevated prolactin. Luteal cells have a high concentration of prolactin receptors (Sernia and Tyndale-Biscoe, 1979). Prolactin suppresses activity of the corpus luteum and so prevents secretion of progesterone (Hearn, 1973, Hinds, 1989, Hinds and Tyndale-Biscoe, 1982, Sernia and Tyndale-Biscoe, 1979). Thus prolactin is luteostatic in the tammar, in contrast to the luteotrophic actions of prolactin in other mammalian species, and the absence of a hormonal signal (progesterone) from the quiescent corpus luteum maintains diapause. Interestingly, hypophysectomy will terminate pregnancy because it removes the source of prolactin that maintains the delay (Hearn, 1974, Hearn, 1975), clearly demonstrating the CL can continue its development and function without further stimulation by pituitary gonadotrophins.

The tammar blastocyst remains in diapause under a regimen of low progesterone until either the sucking stimulus is removed or lost, or until the longest day (in the Southern Hemisphere December 21-22<sup>nd</sup>). Diapause therefore normally extends from the post-partum oestrus at the end of January immediately after birth, and is maintained for 11 months.

### Reactivation from diapause

Reactivation from diapause occurs at three levels: the corpus luteum of the ovary, the secretory activity of the endometrium and in the blastocyst itself.



**Fig. 1. Representative cleavage stage embryos and blastocyst of the tammar wallaby.** (A) Single cell fertilised egg: note the supernumerary sperm (sp) trapped in the mucin layer (arrows) and the zona pellucida (zp) surrounding the oocyte. (B) 8 cell embryo. (C) 16+ cell embryo. All have a thick mucin layer with numerous sperm trapped in it outside the zona. (D) The 80-100 cell blastocyst. Note the lack of any inner cell mass. The blastocyst is unilaminar, and the mucin layer is attenuated. The outer layer is the shell coat. Scale bar, 0.1 mm.

### The ovary

If the sucking pouch young is removed (RPY=removal of pouch young) prolactin levels decline and both the CL and the blastocyst reactivate. On removal of the sucking stimulus, the luteostatic action of prolactin diminishes, but the sucking stimulus and hence prolactin needs to be absent for at least 3 days (Gordon *et al.*, 1988, Hinds *et al.*, 1992) for the blastocyst to be irrevocably committed to reactivation. Similarly, hypophysectomy (Hearn, 1974, Hearn, 1975) or administration of a dopamine agonist, such as bromocriptine (Tyndale-Biscoe and Hinds, 1984) or cabergoline (Hearn *et al.*, 1998), also suppress prolactin secretion and remove the inhibition on the corpus luteum. Thus the production of progesterone from the corpus luteum is essential for the reactivation after diapause, although the ovaries can be dispensed with from day 8 after RPY (Renfree and Tyndale-Biscoe, 1973, Tyndale-Biscoe, 1970, Young and Renfree, 1979). Indeed, the diapausing tammar embryo can survive many months after ovariectomy or luteectomy (Tyndale-Biscoe and Hearn, 1981). After RPY, there is a pulse of progesterone that occurs on day 4, 5 6 or 7 after RPY and this coincides exactly with a short pulse of oestrogen (Flint and Renfree, 1982, Hinds and Tyndale-Biscoe, 1982, Shaw and Renfree, 1986, Tyndale-Biscoe *et al.*, 1983). However progesterone alone is all that is needed to induce resumption of active pregnancy to term (Renfree and Tyndale-Biscoe, 1973). Nevertheless, and unlike in mice, progesterone is not needed to maintain the tammar uterus during diapause, and oestradiol alone will not reactivate the blastocyst. In fact, treatment of lactating females with oestradiol either has no effect, or initiates expansion followed by collapse of blastocysts (Fletcher *et al.*, 1988).

### The uterus

Maintenance of the viable blastocyst for 11 months and inhibition of further growth during diapause in the tammar is completely dependent on the uterine environment. During diapause the uterus is relatively inactive with low secretion levels and there are no obvious changes (Renfree, 1973a, Shaw, 1996, Tyndale-Biscoe and Renfree, 1987).

During pregnancy, the uteri of all marsupial species so far described becomes highly secretory (the potoroo, Shaw and Rose, 1979; brushtail possum, Arnold and Shorey, 1985; Shorey and Hughes, 1973; the brown antechinus, Cruz and Selwood, 1993; the stripe-faced dunnart, Cruz and Selwood, 1997, Selwood and Woolley, 1991; and the Virginia opossum, Fleming and Harder, 1981, Padykula and Taylor, 1976). However, the uterine response during pregnancy in the period after embryonic diapause is most extensively described in the tammar (Renfree, 1972, Renfree, 1973a, Renfree and Tyndale-Biscoe, 1973, Shaw, 1996). Distinct changes are detectable in the uterus depending on the phase of the cycle and response to changing levels of steroid hormones in the circulation. During the follicular or proliferative phase of the cycle, when follicles grow and mature in the ovary, there is marked hyperplasia of the uterine epithelium and glands (Clark and Poole, 1967). During the luteal phase of the cycle, after oestrus and ovulation, the uterus becomes secretory with marked hypertrophy of uterine cells as a result of stimulation by progesterone (Clark and Poole, 1967, Shorey and Hughes, 1973, Tyndale-Biscoe and Renfree, 1987, Walker and Hughes, 1981). Ultrastructurally, there is an increase in endometrial activity during early pregnancy reflected by the distribution and abundance of various cellular organelles, together with a marked

increase in dilated rough endoplasmic reticulum, Golgi complexes and secretory vesicles in the uterine glands, suggestive of active synthesis and secretion, in mid pregnancy (Freyer *et al.*, 2002). There is structural remodelling that increases sub-epithelial gland density in the endometrial stroma due to both hyperplasia and hypertrophy of glandular cells. The transient rise in progesterone from the corpus luteum induces further uterine secretion from the endometrium, since endometrial weight increases significantly by day 6 after RPY and by day 8 there is a significant increase in the diameter of the blastocyst (Renfree and Tyndale-Biscoe, 1973). The weight of the gravid endometrium is significantly greater than that of the non-gravid side in the same animal, and the composition of uterine secretions also differs between the two sides (Renfree, 1972, Renfree, 1973a, Renfree and Tyndale-Biscoe, 1973). This difference is initially due to the vascular arrangement of the reproductive tract and presence of a single corpus luteum which ensures there is always a higher content of protein in the gravid compared to the non-gravid uterus (Renfree, 1972), and a greater rate of protein synthesis in the gravid uterus (Renfree, 1972, Shaw and Renfree, 1986) because progesterone is preferentially delivered to the gravid uterus (Towers *et al.*, 1986). However, after day 15, differences between the two uteri emerge that are due to the presence of the developing embryo and appear to be the result of stimulation by the placenta or placental hormones demonstrating that there is maternal recognition of pregnancy (Menzies *et al.*, 2011, Renfree, 1972, Renfree, 2000). Thus only the gravid uterus maintains secretory activity as pregnancy progresses (Renfree, 1972, Renfree, 2000, Renfree and Tyndale-Biscoe, 1973, Tyndale-Biscoe and Renfree, 1987, Wallace, 1981).

### The blastocyst

The tammar blastocyst is completely enclosed by three acellular embryonic coats from the time it enters the uterus. The zona pellucida is surrounded by a thick mucin layer (somewhat akin to that of the rabbit) and outside this a shell coat is laid down by the cells around the utero-tubal junction (reviewed in Shaw *et al.*, 1996). The shell coat remains for two-thirds of the active gestation, or 19 days of the 26.5 day gestational period, so early development of the tammar embryo *in utero* is completely dependent on the secretions of the endometrial glands containing small molecules capable of penetrating these tertiary coats (Renfree and Shaw, 2000, Renfree and Tyndale-Biscoe, 1973, Shaw, 1996).

The tammar blastocyst is a relatively passive partner in early development. The cleavage stages proceed relatively autonomously in the tammar and cleavage can continue in culture (Renfree and Lewis, 1996) (Fig. 1), but to date diapause has never been broken *in vitro*, even using media conditioned by incubating reactivated endometrium or in co-culture with endometrium (MB Renfree, unpublished data). This may be due partially to the long time taken for reactivation of the corpus luteum and the resumption of progesterone synthesis. During diapause, the blastocyst remains viable and has a low metabolic rate. Glucose uptake during diapause suggests that this rate of metabolism provides sufficient energy for the maintenance and homeostasis of the embryo during the long period of arrest (Spindler, 1997, Spindler *et al.*, 1995, Spindler *et al.*, 1998). The first sign of metabolic change is at day 4 RPY as are the first mitoses are seen in luteal cells and in the blastocyst itself. Thus the blastocyst remains essentially quiescent for the first three days after the trigger for reactivation.

### Growth and transcription factors

Progesterone and oestradiol induce the production and release of some cytokines and growth factors from the uterus that can have both autocrine and paracrine actions to regulate the pre-implantation embryo and prepare the endometrium for implantation (Sharkey, 1998). These may have an important role to stimulate subsequent resumption of embryonic growth after embryonic diapause (Renfree and Shaw, 2000). Of the known growth factors, leukaemia inhibitory factor (LIF), insulin-like growth factor (IGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), platelet activating factor (PAF) and transforming growth factor- $\beta$  (TGF- $\beta$ ) are present in the uterus and all have been shown to influence the development and growth of the pre-implantation embryo in eutherian mammals. Some of these have now been examined in the tammar, namely PAF and PAFR, MSX and LIF. A brief description of each of these follows.

#### PAF and PAF-receptor

In eutherians, both the phospholipid PAF and its receptor, platelet-activating factor receptor (PAFR) are present in the embryo and the endometrium. PAF may be important for implantation and maternal recognition of pregnancy (O'Neill, 1991) and enhances mitoses in preimplantation embryos in mice (Roberts et al., 1993). Paf generates a pro-survival anti-apoptotic transcriptome within the embryo (Jin and O'Neill, 2011) and maintains the tumour suppressor protein P53 in a latent state (Jin et al., 2009). In the tammar, PAF is present in the culture media after incubation of endometrial explants during embryonic diapause and at all reactivation stages examined (Kojima et al., 1993). Although PAF production was highly variable, its levels appear to increase at reactivation, around four days after removal of the pouch young (RPY) when progesterone secretion from the corpus luteum resumes (Kojima et al., 1993).

The gene for PAFR, *PTAFR*, is expressed in the tammar endometrium throughout the period of entry into diapause, diapause as well as after reactivation and so does not appear to vary greatly (Fenelon et al., 2014). PAFR protein is also expressed strongly in the apical regions in the plasma membrane of tammar cleavage stage embryos before diapause and has a distinct localised cytoplasmic staining at the eight-cell stage. However, while PAFR protein is only expressed in low levels in the embryo during diapause, on reactivation it is up-regulated and internalised with localised cytoplasmic expression in the perinuclear region, suggesting that endometrial PAF is involved in its reactivation (Fenelon et al., 2014) (Fig. 2). However, although *PTAFR* mRNA is expressed before, throughout and after diapause, PAFR protein is very low during diapause and so regulation appears to be at the protein level. Further characterisation of the role of this phospholipid in onset and reactivation from diapause in the tammar is awaited with interest.

#### Muscle segment homeobox gene, MSX

*Muscle segment homeobox (MSX)* genes are important for ensuring a receptive uterus and are members of an ancient, highly conserved homeobox gene family (Daikoku et al., 2011). Ablation of both *Msx1* and *Msx2* in mice uteri prevents implantation by inducing a non-receptive uterus (Nallasamy et al., 2012). In the mouse without diapause, expression is transient at early day 4. In contrast, during diapause, *Msx1* is highly expressed, and in mice with conditional uterine inactivation of *Msx1* and *Msx2* there is reduced blastocyst recovery and survival (Cha et al., 2013). These

mice also fail to achieve true delay, suggesting that the entry into diapause is under maternal control. Oestrogen and progesterone do not directly induce *Msx1* but in mice, the effects of *Msx* genes in diapause are mediated through *Wnt5a*, a known transcriptional target of uterine *Msx* (Cha et al., 2013, Nallasamy et al., 2012). Interestingly, *Msx1* expression persists in *Lif*<sup>-/-</sup> mice but LIF injection induces implantation and downregulation of *Msx1* expression (Cha et al., 2013; also see below). Loss of *Msx1/MSX2* expression correlates with altered uterine luminal/epithelial cell polarity and affects E-cadherin/ $\beta$ -catenin complex formation through the control of *Wnt5a* expression (Daikoku et al., 2011).

The role of *MSX* genes is highly conserved in mammals and it acts as a molecular mediator in the uterus during embryonic diapause. In the mink, *Mustela vison*, and tammar wallaby there is a similar expression pattern of *MSX1* and *MSX2* to that of mice. It persists during diapause and is rapidly downregulated upon blastocyst reactivation and implantation/attachment. However, whilst in mice and mink the predominant gene is *MSX1*, in the tammar it is *MSX2*. *MSX1* is undetectable in the tammar uterus throughout diapause. It is also virtually undetectable on the day before birth and day of birth, but a few days later *MSX2* is highly expressed by d4 post-partum and remains high during diapause. After reactivation it decreases progressively up to day 5 RPY (Cha et al., 2013). It is interesting that this basic mechanism controlling embryonic diapause has been conserved between mammals of different orders that have been separated for around 160 million years (Luo et al., 2011)(Fig. 2).

#### Leukaemia inhibitory factor, LIF

Of the growth factors in the cytokine family, several studies have implicated leukaemia inhibitory factor (LIF) as a potential regulator of early development of the eutherian embryo. Mouse embryos depend on LIF for implantation (Dey et al., 2004, Stewart et al., 1992). LIF binds to a heterodimer of LIF receptor and IL6ST (a.k.a. gp130), and their mRNAs are all expressed in the blastocyst (Nichols et al., 1996). Mouse embryos that are *IL6ST*<sup>-/-</sup> survive unimplanted in the uterus after ovariectomy but cannot resume development subsequently once restored to an oestrogen-rich environment specifically because of the loss of an epiblast component (Nichols et al., 2001). As noted above, there is an interaction between *Msx1* and *Lif*. Implantation failure in *Lif* mutant mice could be due to sustained expression of *Msx1* in the luminal epithelium, but *Hoxa10* is correctly expressed in the uterus lacking *Lif* (Daikoku et al., 2004). There appears to be a positive-negative feedback loop between *Msx1* and *Lif* in regulating each other's activity (Daikoku et al., 2011).

In the mink (Song et al., 1998), Western spotted skunk (Hirzel et al., 1999) and the laboratory mouse (Bhatt et al., 1991) LIF is expressed in the uterus either at very low or undetectable levels during diapause and is dramatically increased with the resumption of development and implantation. This suggests that LIF may have a role in terminating diapause and in subsequent embryonic cell proliferation and differentiation. However, it is not known whether LIF has any effects on diapause in the tammar. There may be a relationship between the persistence of IL6ST/LIFR expression and the duration of diapause (Nichols et al., 2001). Certainly there is some evidence for this in the tammar. We have been measuring the levels of *LIF* mRNA in the endometrium before, during and after blastocyst reactivation. Oestradiol is released from the Graafian

follicle and increases to a peak about 12 hours post-partum (Shaw and Renfree, 1986), and at this time LIF is high. LIF then decreases in the endometrium as the oestradiol levels fall and the conceptus enters diapause. This is similar to that observed in the brushtail possum *Trichosurus vulpecula* in which *LIF* mRNA transcript is expressed at low levels in the uterus containing an embryo at the single cell or 8-16 cell stage (Cui and Selwood, 2000). *LIF* expression is low during the 11 month diapause in the tamarin, and increases dramatically after the blastocyst has reactivated (MB Renfree, H Clark, AJ Pask and G Shaw, unpublished observations) (Fig. 2). However, it is not yet known if this is the initial signal for reactivation or simply accompanies the reactivation of the blastocyst and subsequent embryonic growth.

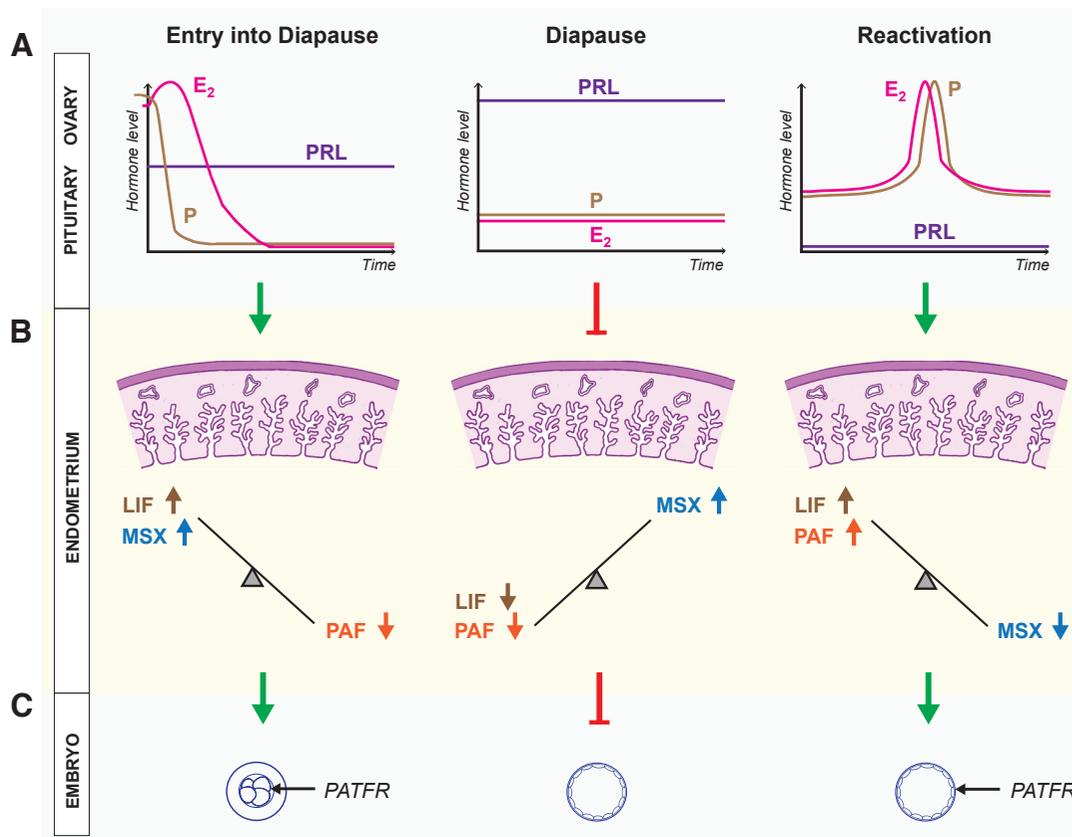
### Other regulatory factors

There are many other growth factors and cytokines produced by the uterine endometrium that may have a role in the regulation of diapause. Mitotic arrest occurs at the G0 or G1 phase of the cell cycle, possibly mediated by p21 and there is now evidence that there are a number of other factors that are involved in the onset and regulation of diapause (Lopes *et al.*, 2004). PCNA1 induces DNA synthesis and is regulated by p21 (Fotedar *et al.*, 2004). FOXO also induces p21 expression and mitotic arrest so may also play a role in the maintenance of diapause (Lopes *et al.*, 2004). Estrogen is known to stimulate expression of the EGF family, and EGF is a potent mitogen expressed in the uterus during implantation in the mouse (Dey *et al.*, 2004). However, surprisingly expression of p53, a cell cycle inhibitor, does not differ between dormant and active mouse blastocysts (Hamatani *et al.*, 2004). Vascular endothelial growth factor (VEGF) induces endothelial

proliferation and vascularisation in the uterus and is upregulated in the mink at reactivation (Lopes *et al.*, 2003, Lopes *et al.*, 2006). In the tamarin, *VEGF* mRNA expression of placental origin certainly increases from mid-pregnancy as does *IGF2* (Ager *et al.*, 2008) but levels during diapause and early reactivation are as yet unknown. Polyamines are another interesting group of molecules that indirectly regulate cell cycling and protein synthesis, and there is accumulating evidence that they may play a role in diapause. In the mink, genes that inhibit polyamine degradation are upregulated (Lefevre *et al.*, 2011a, Lefevre *et al.*, 2011b, Fenelon *et al.*, this volume) and the polyamine putrescine and its synthetic enzyme ornithine decarboxylase are expressed at the termination of diapause in the mink (Murphy, 2012). Further study of these and other numerous growth factors and cytokines will provide new evidence for the molecular control of embryonic diapause.

### Conclusions

New understanding of cytokines and growth factors have opened up some surprising new avenues for understanding the role of the uterus in controlling embryonic diapause. The similarity of the patterns of changes in these cytokines in diapause and reactivation, in tamarins, mice and mink suggests that, despite the very different endocrine controls of diapause, the endocrine pathways converge on common regulatory pathways in the uterus that regulate early embryo development and diapause. Marsupials diverged from eutherian mammals about 160 million years ago, so these pathways are ancient and conserved, and they probably apply to all mammals, but are activated in only a few. It is possible that all mammals once had diapause (reviewed in



**Fig. 2. Regulators of embryonic diapause in the tamarin wallaby.** (A) The signals from the pituitary (PRL: prolactin) and ovary (P: progesterone; E<sub>2</sub>: estradiol 17 $\beta$ ) determine the secretory activity of the endometrium. (B) Endometrial cytokines, transcription factors and growth factors in the uterine secretions are maintained at differing levels to induce entry into diapause, maintain the quiescent phase of diapause and in reactivation shown by the see-saw relationships between them. LIF: leukaemia inhibitory factor protein; MSX: muscle segment homeobox protein; PAF: Platelet activating factor protein. (C) The inhibitory or stimulatory effects act ultimately on the cleavage stage (left) or blastocyst stage (middle, right) embryos. PATFR: Platelet activation factor receptor gene. Data from references cited in text and unpublished results.

Fenelon *et al.*, this issue). Where there is a selective advantage for a delay in gestation, any mutations or epigenetic changes that activate these common underlying mechanisms will be positively selected. Conversely where diapause has evolved, there will be selective pressures to lose this trait if it becomes unnecessary through environmental change. Thus the diversity of endocrine controls for diapause may arise because there are potentially many ways to hormonally regulate the ovarian hormone output or alter the uterine responsiveness to ovarian steroids and consequently modulate these basic pathways to control diapause. However, the uterine signals appear to be remarkably conserved.

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