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PRIMER

The enigma of embryonic diapause

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ABSTRACT

Embryonic diapause – a period of embryonic suspension at the blastocyst stage – is a fascinating phenomenon that occurs in over 130 species of mammals, ranging from bears and badgers to mice and marsupials. It might even occur in humans. During diapause, there is minimal cell division and greatly reduced metabolism, and development is put on hold. Yet there are no ill effects for the pregnancy when it eventually continues. Multiple factors can induce diapause, including seasonal supplies of food, temperature, photoperiod and lactation. The successful reactivation and continuation of pregnancy then requires a viable embryo, a receptive uterus and effective molecular communication between the two. But how do the blastocysts survive and remain viable during this period of time, which can be up to a year in some cases? And what are the signals that bring it out of suspended animation? Here, we provide an overview of the process of diapause and address these questions, focussing on recent molecular data.

KEY WORDS: Embryo, Diapause, Uterus, Endometrial secretions, Blastocyst, Growth factors

Introduction

Embryonic diapause – a period in early development during which an embryo remains suspended at the blastocyst stage – was first observed in the roe deer, *Capreolus capreolus*, when it was noticed that although the rut occurred in August (in the Northern hemisphere) there was no embryo visible in the uterus until January (Ziegler, 1843). Originally, this observation was attributed to a 'silent heat' but Ziegler (1843) and Bischoff (1854) discovered that mating was followed by a period of quiescence, during which there was an almost complete cessation of embryo growth (Short and Hay, 1966). This led to the concept that in some mammalian species there is a delay of implantation, distinct from the delay in fertilisation after sperm storage, and the process was termed 'delayed implantation' or, more accurately, 'embryonic diapause' (Box 1). Interestingly, the roe deer remains the only ungulate (hoofed mammal) in which embryonic diapause has been confirmed, although there is some suggestion that Pere David's deer (*Elaphurus davidianus*) also exhibits a period of diapause (Brinklow and Louden, 1993).

One of the main functions of diapause is to control when birth takes place, independent of the time of mating and the length of pregnancy. Thus, while reproduction and development generally occur when environmental conditions are favourable for a species, unfavourable conditions, especially when they recur periodically, may be avoided by either migration of the species or by the intervention of a dormant stage in the animal's life cycle. In line with this, it has been shown that photoperiod, nutrition, temperature and rainfall can all affect the patterns of diapause so that, for many mammals, reproduction is synchronised to one of these environmental events.

Diapause occurs at the blastocyst stage, which is the end of a phase of relative autonomy in embryonic development. Further development beyond this stage requires closer contact with the uterus and increased uterine secretory activity that, in turn, depends on the level of ovarian steroids. During diapause, blastocysts either remain totally quiescent or expand at a very slow rate (Enders, 1966; Renfree and Calaby, 1981; Renfree and Shaw, 2000; Fenelon et al., 2014a), with some species retaining their zona pellucida – the protective glycoprotein layer that surrounds the blastocyst (Box 1). The precise molecular mechanisms that halt blastocyst development are not well understood, nor are the signals that reactivate development, in any mammal (Shaw and Renfree, 1986; Renfree and Shaw, 2000, 2014; Murphy, 2012; Fenelon et al., 2014a). Nonetheless, recent studies of embryo-uterine interactions and of stem cell states are beginning to provide some clues into these events. In this Primer, we discuss this progress, highlighting the complex interactions that put an embryo to sleep, keep it safe and wake it up again.

Lactational versus seasonal control of diapause

Since the first report in roe deer, embryonic diapause has been identified in a diverse range of over 130 mammalian species – everything from an anteater to a gerbil to a polar bear (Table 1, Fig. 1). It has even been suggested to occur in primates, including in women, although there is no direct evidence for this (Tarín and Cano, 1999; Ptak et al., 2012, 2013). As a reproductive strategy, embryonic diapause can be of benefit in one of two ways: either by allowing the female to produce the maximum number of offspring in a given season or by synchronizing parturition with environmental conditions favourable to offspring survival. Accordingly, embryonic diapause within mammals can be induced via one of two mechanisms: lactational diapause, where arrest is induced selectively; and seasonal diapause, where arrest of the embryo is induced during every gestation (Lopes et al., 2004).

Lactational embryonic diapause is a common reproductive phenomenon best understood in the mouse, *Mus musculus*. In the mouse, if mating occurs at postpartum oestrus, implantation is delayed by the presence of suckling young. This results in an increase in circulating prolactin levels, which prevent the oestrogen surge at day (d) 3.5 of pregnancy and cause the blastocysts to enter into diapause (Mantalenakis and Ketchel, 1966; Psychoyos, 1973). Embryonic diapause in rodents can last from 1 day to several weeks, with the length of delay depending on the number of sucking young (Weichert, 1940, 1942; Mantalenakis and Ketchel, 1966; Pritchett-Corning et al., 2013). Interestingly, the sucking inhibition can be overridden with injections of the dopamine agonist bromocriptine, which depresses sucking-induced prolactin release and allows blastocyst reactivation (Flint and Renfree, 1981). Embryonic diapause can also be experimentally induced in the mouse by ovariectomy on d3.5, prior to the oestrogen surge, and maintained

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Box 1. The definition of diapause

Diapause is a cessation or slowing of growth of the early embryo. In some mammals, this can occur at the blastocyst stage of development and is known as 'embryonic diapause'. It also occurs in some non-mammalian species (see Box 3). In many, but not all, mammals implantation follows immediately after the resumption of development, reflecting the original term 'delayed implantation', but at the Second Symposium on Embryonic Diapause (Flint et al., 1981) it was decided that 'embryonic diapause' is the better term to describe the state, particularly for those species whose blastocysts do not implant immediately, such as marsupials. It should also be noted that delayed fertilisation can also occur, for example in those species in which sperm are stored in the female reproductive tract and fertilisation occurs sometime after copulation, before a new conceptus is formed (Wimsatt, 1975). Finally, 'delayed development' refers to those species that implant but have a period of quiescence before the embryo begins to differentiate, as occurs in some bats (see Box 4).

Within embryonic diapause, five different conditions of the blastocyst can be recognised (from Renfree and Calaby, 1981):

1. No growth: occurs in unilaminar blastocysts with a zona pellucida (e.g. kangaroos)
2. No growth: occurs in unilaminar blastocysts without a zona pellucida (e.g. mice and rats)
3. Some slow growth: occurs in unilaminar blastocysts with a zona pellucida (e.g. mustelids, bears, seals)
4. Some slow growth: occurs in bilaminar blastocysts without a zona pellucida (e.g. roe deer)
5. Implanted but undifferentiated (e.g. some bats; see Box 4)

by daily progesterone injections, or it can be induced by oestrogen receptor inhibitor injections on d2.5 and d3.5 (Yoshinaga and Adams, 1966; Paria et al., 1993b). Reactivation from diapause then occurs after removal of the young, resulting in a surge of oestrogen (Fig. 2) (Psychoyos, 1973). Similarly, diapause can be experimentally terminated by a single injection of oestradiol (Yoshinaga and Adams, 1966; McLaren, 1968; Psychoyos, 1973).

Seasonal diapause is especially predominant among carnivores, including all extant Ursidae, and numerous examples in the orders Mephitidae, Mustelidae, Otariidae and Phocidae (Table 1). In the majority of seasonally diapausing mammals examined to date, embryonic diapause is controlled by seasonal changes in photoperiod. However, the seasonal trigger for reactivation varies greatly and appears to depend on the requirements of each species, independent of their location in either the Northern or Southern hemisphere (Fig. 3). Seasonal diapause has been extensively studied in the American mink, *Neovison vison*, in which the control of diapause is mediated by seasonal changes in photoperiod at the vernal equinox (Murphy, 2012). The mink mates around late February to late March, after which time the blastocyst enters into diapause due to high nocturnal melatonin levels, which cause low prolactin levels. Regardless of the date of mating, reactivation from diapause is triggered by the increasing photoperiod following the vernal equinox (21-22 March, northern hemisphere), which results in an increase in circulating prolactin and a subsequent increase in ovarian progesterone synthesis (Fig. 2) (Murphy and James, 1974; Murphy et al., 1981). However, only prolactin, and not progesterone or oestradiol, can experimentally terminate diapause in the mink (Papke et al., 1980; Murphy et al., 1981; Stoufflet et al., 1989).

In contrast to the examples above, diapause in the tammar wallaby, *Macropus eugenii*, can be either lactationally or seasonally induced depending on the time of year (Figs 2 and 3) (Tyndale-Biscoe and Renfree, 1987). Regardless of the mechanism, both versions are maintained by high levels of prolactin, which inhibit the corpus luteum and result in low progesterone levels (Hinds, 1989; Hinds and Tyndale-Biscoe, 2013). Tammar wallabies give birth to a single, altricial young in late January (in the southern hemisphere), which completes its development in the pouch and emerges around late September. Within a few hours of giving birth, mating occurs at postpartum oestrus, and if a pouch young is present the blastocyst will enter into lactational diapause, maintained by high prolactin levels from the sucking pouch young. If the pouch young is lost during the breeding season (January-May), this prolactin inhibition is removed and the subsequent progesterone pulse reactivates the diapause blastocyst (Fig. 2) (Hinds and Tyndale-Biscoe, 1982, 2013; Shaw and Renfree, 1984). If, however, the pouch young is lost after this time, the embryo will remain in seasonal, photoperiod

Table 1. Mammalian families known to exhibit pre-implantation embryonic diapause

Type	Family	Common name*	Known diapause species	Total species
Eutherian	Cervidae	Deer	1	55
	Mephitidae	Skunks	3	12
	Otariidae	Eared seals	9	16
	Phocidae	True seals	14	19
	Ursidae	Bears	8	8
	Mustelidae	Mink, marten, badgers, polecats, weasel, otters, sable, wolverine	21	64
	Miniopteridae	Long-winged bats	2	23
	Pteropodidae	Megabats	1	187
	Dasypodidae	Armadillos	2	20
	Talpidae	Moles	1	42
	Myrmecophagidae	Anteaters	1	3
	Soricidae	Shrews	3	387
	Cricetidae	New world mice and rats	13	698
	Muridae	Old world mice and rats	14	727
	Chinchillidae	Viscacha	1	7
Marsupialia	Acrobatidae	Feathertail glider and feathertail possum	2	2
	Burramyidae	Pygmy possums	3	5
	Macropodidae	Kangaroos and wallabies	24	67
	Potoroidae	Potoroos and bettongs	7	12
	Tarsipedidae	Honey possum	1	1

*In the interests of space, only the species with diapause are included in the list of common names. Data are compiled from references listed in: Renfree and Calaby (1981); Mead (1993); Renfree and Shaw (2000); Fenelon et al. (2014a); IUCN (2016).

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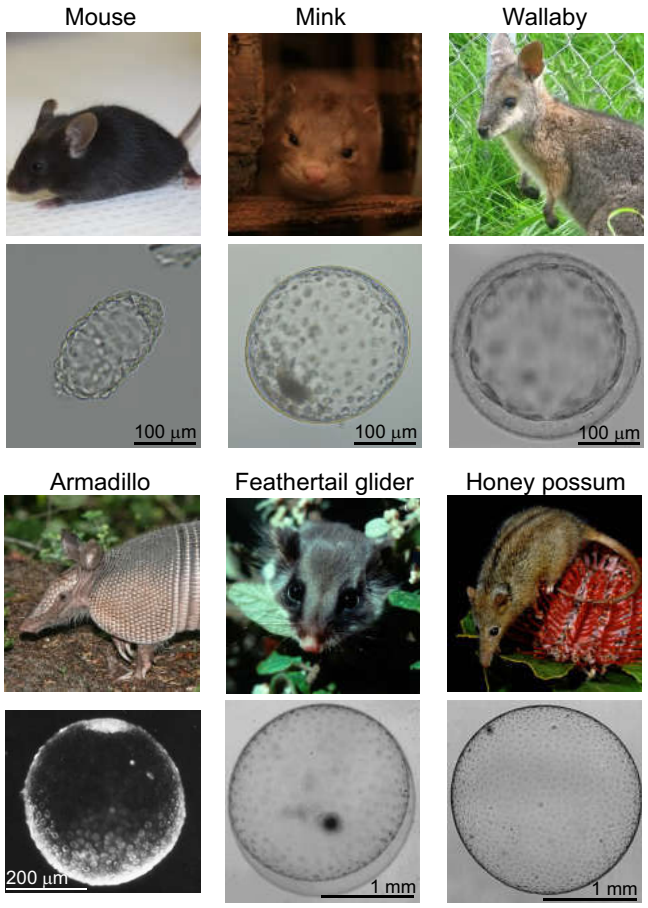

Fig. 1. Mammals and their diapause blastocysts. Blastocysts in diapause of the mouse (*Mus musculus*), mink (*Neovison vison*), tammar wallaby (*Macropus eugenii*), nine-banded armadillo (*Dasypus novemcinctus*), feathertail glider (*Acrobates pygmaeus*) and honey possum (*Tarsipes rostratus*) are shown. Note that the size of the mammals bears no relationship to the size of their blastocysts. Indeed, the smallest two mammals illustrated here, the honey possum and the feathertail glider, have the largest blastocysts (at ~2 mm and ~2000 cells), while the next largest blastocyst is that of the armadillo (~260-400 µm).

induced-diapause until after the summer solstice (21-22 December, southern hemisphere) when, in contrast to the mink, the increase in nocturnal melatonin secretion results in a decrease in prolactin and reactivates the blastocyst (Tyndale-Biscoe et al., 1986; Hinds, 1989). Experimentally, reactivation can be induced by exogenous progesterone alone during lactational quiescence, and by melatonin during the seasonal quiescence period. Thus, the tammar embryo can remain in diapause for 11 months (Fig. 3). This system increases the chances that one offspring will be produced each season and will only emerge from the pouch at an optimal time to ensure its survival in the spring, when food is abundant and lactation is most energy demanding.

In summary, the hormonal control of diapause has been established for many species, with the proximal signals for reactivation depending on the relative levels of prolactin, progesterone and/or oestrogen. An exception to this is the roe deer where, although it appears that the control of diapause depends on the winter solstice and a change in photoperiod, there is no evidence for prolactin-, progesterone- or oestrogen-induced reactivation of the blastocyst (Aitken, 1974; Lambert et al., 2001; Hoffmann et al., 1978). The levels of all three of these hormones remain constant throughout diapause and reactivation, until blastocyst elongation when there is a significant increase in oestrogen (Lambert et al., 2001). Despite this, the *in vivo* administration of oestradiol does not affect blastocyst development, elongation or uterine secretions (Aitken, 1981). Thus, the proximal signal for reactivation might be either a uterine-secreted or a blastocyst-secreted protein, rather than a circulating hormone (Lambert et al., 2001).

Molecular control of diapause

The molecular control of diapause is best understood in three unrelated species: the mouse, mink and tammar wallaby (Fig. 4). Tammar and mink blastocysts are surrounded by multiple acellular layers and do not implant until a number of days after reactivation, suggesting that the factors that control diapause must reach the embryo via uterine secretions (Renfree, 1973; Shaw and Renfree, 1986; Renfree and Shaw, 2000; Murphy, 2012; Fenelon et al., 2014a). Such uterine secretions, which are complex but as yet not fully defined, can provide direct communication between the endometrium and the blastocyst. There is a decrease in uterine gland activity before the onset of diapause in the tammar wallaby (Laird et al., 2016), consistent with a decrease in total protein concentration and a decrease in the volume of secretions in this species during diapause (Renfree, 1972, 1973). The secretion of some stage-specific proteins also decreases as mouse blastocysts enter into diapause and increases upon reactivation (Weitlauf, 1994), as also occurs in the tammar (Renfree, 1973) and roe deer (Aitken, 1974). When these early studies were performed, it was not possible to identify the exact proteins involved in this potential embryo-uterine crosstalk. However, more recent studies have revealed that multiple small proteins, including nutrients, proteases, hormones, cytokines, growth factors and transcription factors, have the potential to regulate embryonic development and hence entry into and exit from diapause. Amino acids in the uterine fluids have also been shown to affect embryo development (Gardner and Lane, 1993; Winkle et al., 2006), although the specific responses to individual amino acids are unknown. Below, we discuss how some of these key factors have been implicated in embryonic diapause and how, at the molecular level, they might act on the embryo to induce and reverse its arrest.

Growth factors and cytokines in the uterine environment

The uterine endometrium of mammals secretes cytokines and growth factors that influence the development of the pre-implantation embryo (reviewed by Cha et al., 2012). It is likely that some of these also control the arrested growth that occurs in diapause. These factors include epidermal growth factors and receptors [e.g. EGF, HB-EGF (or HBEGF) and ERBB4], the phospholipid PAF (formerly known as platelet-activating factor), vascular endothelial growth factor (VEGF) and leukaemia inhibitory factor (LIF). Many of these factors are also present in the blastocyst, and their expression is now known to coincide with blastocyst reactivation after diapause. These will be discussed in more detail below.

Many members of the epidermal growth factor (EGF) family, for example, are expressed in the endometrium and embryo of the mouse during both the peri-implantation period and at reactivation from diapause (reviewed by Hardy and Spanos, 2002; Dey et al., 2004). Of note, the first sign of reactivation from diapause is the detection of endometrial HB-EGF adjacent to the blastocyst, just a few hours before implantation commences (Das et al., 1994). Endometrial HB-EGF is known to be involved in binding to its receptor ERBB4 on the blastocyst to coordinate implantation in both mice and humans (Paria et al., 1993a; Yoo et al., 1997; Leach et al.,

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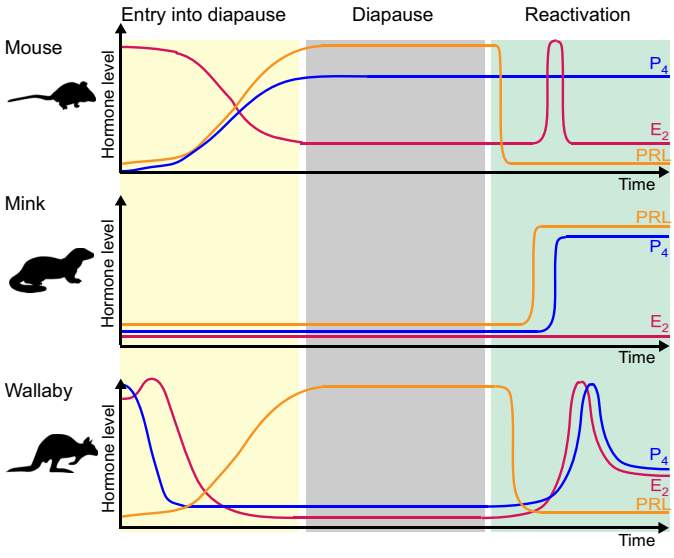

Fig. 2. Hormonal changes during diapause. Hormone profiles of the mouse, mink and tammar wallaby at entry into diapause, during diapause, and after reactivation from embryonic diapause. Although all three species depend to different degrees on the same three hormones (with the exception of oestradiol in the mink), the levels of each vary greatly between species and result in three very distinct species profiles. Blue lines represent progesterone (P_4), red lines represent oestradiol (E_2), and orange lines represent prolactin (PRL).

1999; Paria et al., 1999; Wang et al., 2000; Chobotova et al., 2002), although whether it plays a role in reactivation from diapause is unclear. ERBB4 is also present in the mouse endometrium at reactivation, while HB-EGF is the only EGF family ligand significantly upregulated in the mouse blastocyst at reactivation (Lim et al., 1998). More recently, HB-EGF and ERBB4 have been detected in the uterus and blastocyst of both the tammar and mink specifically at reactivation from diapause, distinct from implantation (Fenelon et al., 2017). In addition, it has been shown that the soluble form of epidermal growth factor receptor (EGFR), which can also bind to HB-EGF, and another mitogen, hepatoma-derived growth factor (HDGF), are present in the tammar uterine fluid from d3 until at least d11 after removal of pouch young (RPY, equivalent to days of reactivation), when these secreted proteins constitute 21% of the uterine fluid proteome (Martin et al., 2016). Together, these findings suggest that reciprocal EGF family signalling between the endometrium and embryo is likely to play a central role in reactivation from diapause. It should also be noted that a high degree of functional redundancy exists within the EGF family and that some of the other EGF ligands that are present throughout reactivation in the luminal epithelium may also be involved in signalling to coordinate reactivation (Huet et al., 1989; Tamada et al., 1991; Riese and Stern, 1998; Cai et al., 2003; Brown et al., 2004).

An additional factor that appears to influence blastocyst reactivation is PAF, a phospholipid that signals via its receptor platelet-activating factor receptor (PTAFR). Both PAF and PTAFR are present in the endometrium and embryos of mice, rabbits, hamsters, humans and marsupials (O'Neill, 1985, 2005; Ammit and O'Neill, 1991; Jin and O'Neill, 2011; Kojima et al., 1993). The release of endometrial PAF is under the control of progesterone and oestradiol (Chami et al., 1999; Li et al., 1999). It stimulates embryo metabolism, enhances cell proliferation and increases overall embryo viability (Emerson et al., 2000; O'Neill, 2005). PAF is detected in the medium of tammar endometrial samples cultured for 24 h *in vitro* (Kojima et al., 1993) and levels appear to increase in the culture medium around the time that the first mitoses are detected in the blastocyst at day 4 after reactivation (Spindler et al., 1996). Endometrial PAF also upregulates the expression of PTAFR in the blastocyst, leading to its internalisation and cytoplasmic localisation in the perinuclear region of blastocyst cells at reactivation (Fenelon et al., 2014b). The ability of embryos to release PAF appears to be an indication of their viability (Ryan et al., 1989).

Another factor that is essential for embryo implantation and may play a role in diapause is the cytokine VEGF, which is a heparin-binding homodimeric glycoprotein and an endothelial cell-specific mitogen. Local expression of VEGF mediates maternal-embryo interactions and facilitates blastocyst implantation (Hannan et al., 2011). In mice, uterine flushings containing VEGF isoforms appear to increase blastocyst cell number (Binder et al., 2014), suggesting that VEGF might increase proliferation. VEGF is also upregulated in the mink endometrium at the time of reactivation from diapause and induces endothelial proliferation and vascularisation in the uterus (Lopes et al., 2003; Lopes et al., 2006).

LIF is a member of the interleukin 6 (IL6) family of pro-inflammatory cytokines that also has multiple roles in regulating blastocyst implantation and is involved in diapause and blastocyst viability in mice. After its secretion into the uterine lumen, LIF binds to a heterodimeric LIF transmembrane receptor complex consisting of LIF receptor (LIFR) and gp130 (IL6ST) (Rosario et al., 2014), leading to activation of the JAK-STAT3 pathway as well as the ERK pathway. Implantation in mice requires LIF produced in endometrial glandular epithelium on day 4 of pregnancy, just before implantation (Stewart et al., 1992). LIF binds to the LIFR-gp130 heterodimer expressed in the blastocyst (Nichols et al., 1996, 2001). LIF is induced in the uterine glands by the actions of oestrogen and TP53 (p53), a tumour suppressor (Rosario and Stewart, 2016). In the absence of LIF, mouse blastocysts enter diapause, whereas blastocysts lacking gp130 do not survive (Hondo and Stewart, 2004; Rosario and Stewart, 2016). LIF in the endometrium is almost undetectable during diapause but increases during reactivation in various species, including mouse, mink, the Western spotted skunk, *Spilogale gracilis* (formerly *Spilogale putorius*), and the wallaby (Bhatt et al., 1991; Stewart et al., 1992; Song et al., 1998; Hirzel et al., 1999; Passavant et al., 2000; Hearn, 2005). In the skunk, the expression of uterine LIFR (LIFRβ) increases when blastocysts resume development, and this is apparently under the stimulatory control of prolactin (Passavant et al., 2000). Since LIF expression in mice is under oestrogenic control it can be used in place of an oestrogen injection to induce reactivation (Chen et al., 2000). LIF affects gene expression in the uterine endometrium by downregulating a suite of genes in the first hour after treatment, and upregulating a different set, including members of the Sox, Kfl, Hes, Hey and Hox families of transcription factors (Rosario et al., 2014). LIF also regulates muscle segment homeobox (MSX) genes (see below). LIF thus appears to play multiple roles in the uterus, inducing a dynamic and complex network of changes that is essential for reproduction (Rosario et al., 2014; Rosario and Stewart, 2016). It is likely that these changes are also important in the control of embryonic diapause. However, LIF also maintains pluripotency in the epiblast of the blastocyst and in stem cells (discussed below).

Other signalling and transcription factors in the uterine environment

A number of other uterine factors known to regulate implantation are likely to be involved in the molecular control of diapause. These include insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth

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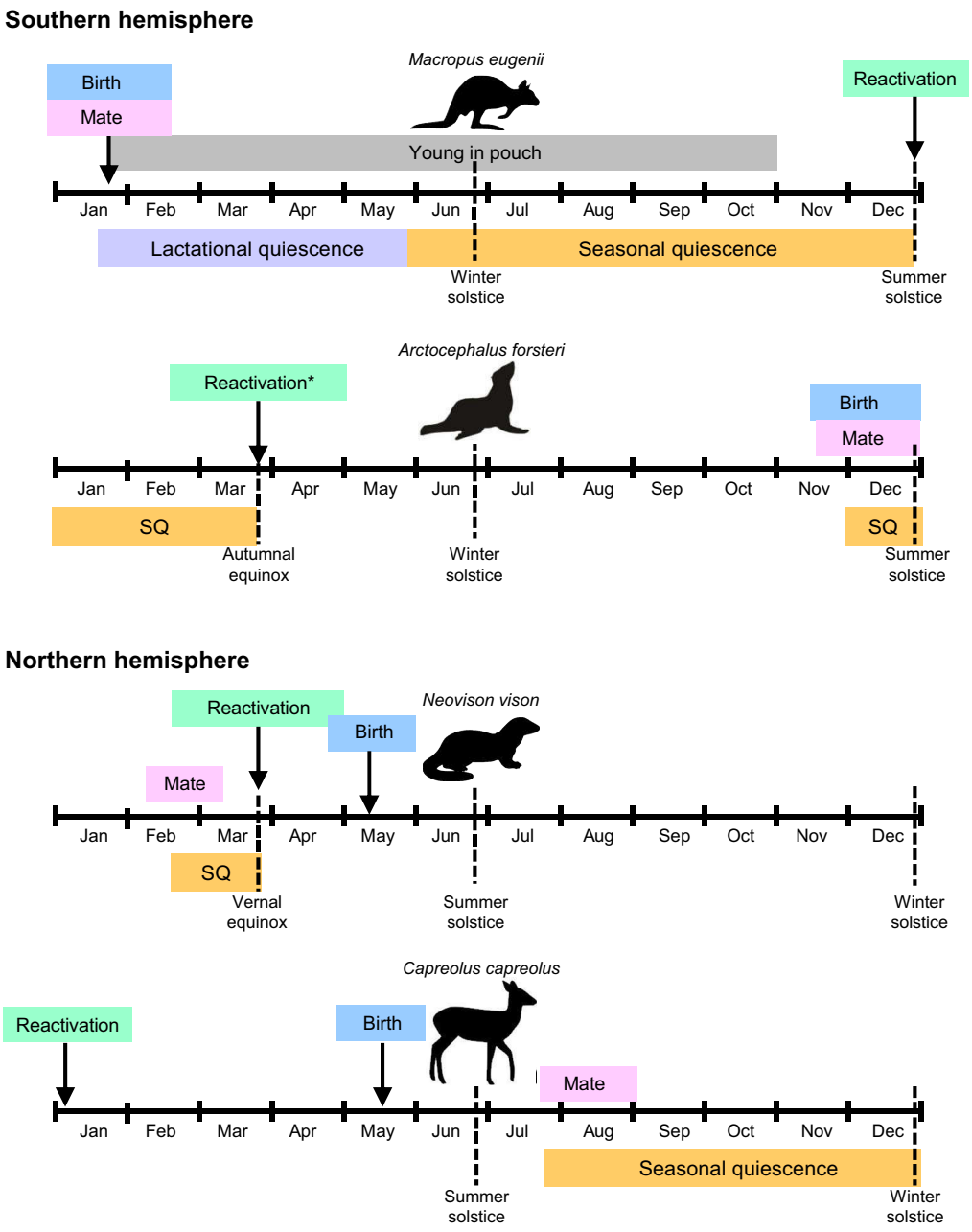

Fig. 3. Lactational and seasonal diapause. Comparison of the annual cycle of two Southern and two Northern hemisphere seasonal diapause mammals: tammar wallaby (*Macropus eugenii*), New Zealand fur seal (*Arctocephalus forsteri*), mink (*Neovison vison*) and roe deer (*Capreolus capreolus*). Note that reactivation in the fur seal is predicted (indicated by asterisk) to take place in response to the autumnal equinox. The advantage of embryonic diapause is that the timing of mating can be separated from the time of birth so that the animal can deliver the young at a time when conditions are optimal for survival. The length and timing of diapause vary greatly and do not depend on geographical location but do depend on reproductive pattern. SQ, seasonal quiescence.

factor β (TGFβ), interleukin 1β (Il1b), bone morphogenetic protein 2 (BMP2) and signalling molecules of the wingless (WNT) family (reviewed by Cha et al., 2012). Suppressive subtractive hybridisation shows that 123 genes are differentially expressed in the mink uterus between diapause and reactivation (Lefèvre et al., 2011a). About 50% of these are secreted factors involved in cell proliferation, homeostasis, protein folding, electron transport, chromatin and tissue remodelling and the innate immune response (Lefèvre et al., 2011a), as exemplified by the secreted glycoprotein SPARC (secreted protein acidic and cysteine-rich) and the expression of HMGN1 (high mobility group nucleosome binding domain 1), a chromatin remodelling factor, both of which increase in the uterine epithelium at reactivation. Similarly, in a proteomic analysis of tammar uterine secretions, 21% of the proteins were secretory proteins including mitogen, hepatoma-derived growth factor and soluble epidermal growth factor receptors (Martin et al., 2016). However, further studies are needed to investigate whether and how these factors and others identified function in the reactivation process.

Two key highly conserved transcription factors that have been implicated in the control of diapause are those encoded by the muscle segment homeobox genes *MSX1* and *MSX2*, both of which are downregulated by LIF (Cha et al., 2013). MSX1 is a transcriptional regulator of uterine implantation factors in mice, and they halt proliferation of the luminal epithelium and so must be downregulated to allow implantation (Daikoku et al., 2011; Cha et al., 2013). A lack of *Msx1* in mice affects uterine receptivity by disrupting signalling through Wnt5a, which promotes proliferation of the stroma and luminal epithelium (Daikoku et al., 2011; Nallasamy et al., 2012; Cha et al., 2013). These transcriptional regulators are present in the endometrium of mice, mink and tammar wallabies during diapause (Cha et al., 2013; Fenelon et al., 2014a;

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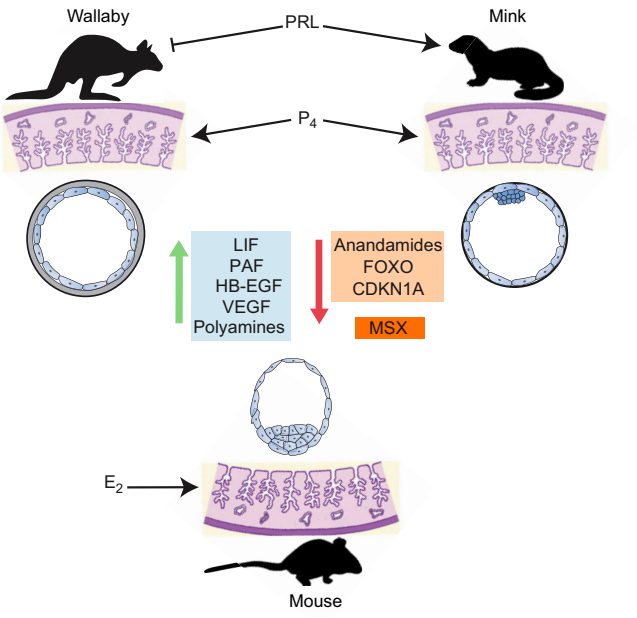

Fig. 4. Uterine-embryo signalling during diapause. The molecular effects of hormones (PRL, P_4 and E_2) on the tammar wallaby, mouse and mink at the time of reactivation after diapause, and the common interactions between the uterus and blastocyst, are summarised. Although there is overlap in the hormones required to stimulate reactivation of the uterus for each of these species, their effects vary depending on species. Both the tammar and the mink respond to prolactin, but it is stimulatory in the mink and inhibitory in the tammar. By contrast, the mouse is not directly affected by prolactin (although it is at high levels during lactation). All three species require progesterone for reactivation but in the mouse progesterone only primes the uterus, and oestradiol alone is the stimulatory hormone. Oestradiol does not directly affect the tammar or mink. Despite these differences, once the uterus is reactivated the molecular factors required for reactivation of the blastocyst appear to be conserved. PRL, prolactin; P_4, progesterone; E_2, oestradiol. Cytokines and growth factors that are upregulated at reactivation are indicated with a green arrow, whereas factors that are downregulated are shown with a red arrow. Muscle segment homeobox (MSX) genes are also downregulated in the uterus before reactivation.

Renfree and Shaw, 2014). Indeed, *Msx1* is transiently expressed early on day 3.5 of undelayed mouse gestation, but it is highly expressed during diapause, and in *Msx1/2*$^{-/-}$ double-knockout mice the blastocysts are less viable (Cha et al., 2013; Cha and Dey, 2014). There is also a feedback loop between *Msx1* and *Lif*, although *Msx1* expression continues in *Lif* knockout mice so the functional relevance of this loop is unclear (Daikoku et al., 2011; Cha et al., 2013). In the tammar, *MSX2* plays a similar role to the *Msx1*/*MSX1* gene in mice and mink, suggesting that these genes might have developed slightly different mechanisms of action in the 160 million years since the divergence of these two groups of eutherian mammals (Luo et al., 2011).

Changes in metabolism may also play a role in inducing or maintaining the diapause state. Indeed, three enzymes involved in polyamine synthesis exhibit reduced expression during diapause, in both the mink embryo and the uterus (Lefèvre et al., 2011a; Fenelon et al., 2016). Polyamines are synthesized from the amino acids ornithine, arginine, proline and methionine, and their regulation is tightly controlled, primarily by the rate-limiting enzyme ornithine decarboxylase 1 (ODC1) (Bachrach, 2010). In both the mink and the mouse, ODC1 inhibition *in vivo* (using DL-α-difluormethylornithine, or DFMO) causes blastocysts to enter into diapause, but if these embryos are flushed they are able to expand and proliferate (Lefèvre et al., 2011b; Fenelon and Murphy, 2017). Furthermore, uterine levels of the polyamine putrescine in the mink increase at reactivation, and the culture of diapause blastocysts with putrescine induces increases in cell proliferation and diameter, which are hallmarks of reactivation (Box 2) (Lefèvre et al., 2011b; Fenelon et al., 2016). However, DFMO treatment is unable to maintain mouse blastocysts in diapause *in vitro* (Fenelon and Murphy, 2017), and it is unknown how a decrease in polyamines can induce or maintain embryonic diapause. In other cell types, polyamines are involved in a multitude of cellular processes including metabolism, cell cycle control and apoptosis, and they can bind to and interact with nucleic acids, proteins and phospholipids (Wallace et al., 2003; Igarashi and Kashiwagi, 2010; Lefèvre et al., 2011c). It is predicted that polyamines would be involved in the control of cell proliferation, but whether they have additional functions at reactivation remains to be seen. In this regard it is interesting that cancer cell lines treated with inhibitors of polyamine biosynthesis (Mamont et al., 1978) or inducers of catabolism (Vujcic et al., 2000; Kee et al., 2004) lose their ability to divide.

The endocannabinoid anandamide might also regulate metabolic changes as well as changes in calcium signalling during diapause. In the mouse, anandamide levels appear to have a significant role in regulating blastocyst reactivation from embryonic diapause (Wang et al., 2003). Low levels of anandamide can activate the blastocyst via the MAPK pathway, whereas high but nonetheless physiological levels do not activate the MAPK pathway and blastocysts remain dormant (Wang et al., 2003). Furthermore, high levels of anandamide inhibit Ca^{2+} signalling, and there is evidence to suggest that anandamide can also regulate LIF levels (Maccarrone et al., 2002; Wang et al., 2003). A number of genes involved in the calcium signalling pathway have been shown to be upregulated in the reactivated mouse embryo (Hamatani et al., 2004), and it is known that inhibiting $[Ca^{2+}]_i$ transients in the early embryo results in a delay or inhibition of development and cell proliferation (Stachecki and Armant, 1996; Armant et al., 2000). Furthermore, the activation of calcium signalling upregulates the expression of arginase, which is an enzyme involved in polyamine synthesis, and that of the myelocytomatosis oncogene (MYC), another factor implicated in the control of blastocyst arrest (see below) (Armant et al., 2000; Wallace et al., 2003). The levels of CB1 (CNR1), a cannabinoid receptor to which anandamide binds, are also upregulated in the blastocyst trophectoderm during diapause and rapidly downregulated at reactivation. Hence, high levels of anandamide may act as an inhibitory factor in the mouse blastocyst and be responsible for maintaining diapause by inhibiting both the MAPK pathway and Ca^{2+} signalling. Reactivation would then require a decrease in anandamide, which is likely to be induced via a decrease in the levels of its receptor, although the mechanism by which this occurs is as yet unknown.

Downstream effects on blastocysts and stem cells

As highlighted above, a number of factors have been implicated in inducing, maintaining or releasing the diapause state. How these factors act on the blastocyst at a molecular level remains unclear, although recent transcriptomic and proteomic studies are beginning to provide some clues. For example, it has been shown that diapause in mice is associated with a decrease in the expression of DNA replication genes, and using microarray analyses of mouse blastocysts it was shown that only 1% of the >20,000 genes examined are differentially expressed, with 80 genes highly expressed during diapause and 149 genes highly expressed at reactivation (Hamatani et al., 2004). Gene ontology (GO) analysis

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Box 2. The hallmarks of blastocyst reactivation

To date, there are no known reliable molecular markers of a blastocyst in diapause, beyond the canonical cell cycle arrest and reduced metabolism. The first signs of reactivation in the blastocyst are renewed mitotic activity and increases in cell proliferation, metabolism, and DNA, RNA and protein synthesis. This is followed by the first signs of embryo expansion and implantation. In mouse these reactivation events occur rapidly, within 4-16 h of the initial reactivation signal (Spindler et al., 1996). However, this is followed closely by the initiation of implantation 18-20 h after hormone injection (Yoshinaga and Adams, 1966; Paria et al., 1993b; Das et al., 1994). By contrast, in the mink and tammar, the events of reactivation occur over an extended time period, with the first signs of resumption of the cell cycle occurring 3-4 days after reactivation has been induced. Similarly, the increases in protein synthesis and metabolism occur slowly in the initial stages of reactivation with a rapid increase later in reactivation (Spindler et al., 1998, 1999; Desmarais et al., 2004). It takes a further 4-5 days before significant increases in embryo expansion are observed, with implantation in the mink occurring a total of 13 days after the initial reactivation. The tammar embryo does not implant per se but attachment to the uterine wall is delayed to ∼18 days after reactivation has been initiated (Renfree, 1973; Denker and Tyndale-Biscoe, 1986; Shaw and Renfree, 1986; Spindler et al., 1998, 1999).

of these 229 genes identified cell cycle, cell signalling, adhesion molecules and metabolic pathways among the major functional categories. Similarly, 91 genes are upregulated in the mink blastocyst at reactivation from diapause (Fenelon et al., 2016). The gene data sets are complemented by a proteomic analysis of mouse blastocysts during diapause and after reactivation (Fu et al., 2014), which identified over 2000 proteins that differentially regulate numerous aspects of biosynthesis, glycolysis, metabolism and chromatin remodelling. It will be interesting to see whether these factors, in both the uterus and the blastocyst, have a role in the control of diapause and reactivation.

One way in which these factors could function is by influencing the cell cycle, causing cells to enter a quiescent state. During embryonic diapause, the tammar embryo maintains a glycolytic, basal level of metabolism, and there is no cell division or differentiation; these features, along with the reversibility of arrest, are hallmarks of classical quiescence. Diapause mouse blastocysts are thought to arrest in the G1 phase of the cell cycle (Sherman and Barlow, 1972; Surani, 1975), based on analyses of the DNA content of rat blastocysts in diapause. However, it is not possible to distinguish between G0 and G1 on DNA content alone and as yet it has not been possible to determine whether this arrest is actually at G0, although this appears likely based on quiescence studies in other cell types (Coller et al., 2006). Furthermore, it is not known how this arrest induces reversible quiescence in the blastocyst rather than terminal differentiation or apoptosis (Coller et al., 2006). Recently, a method using mVenus and a mutant form of p27 (also known as CDKN1B) has been developed to clearly define the quiescent G0 phase of the cell cycle, so it should be possible to characterise the cell cycle stage of not only quiescent or diapausing cells but also of stem cells (Oki et al., 2014).

The downregulation of uterine secretory proteins during diapause may also allow the upregulation of factors in the blastocyst that are able to induce and maintain other aspects of diapause, such as basal metabolism, biosynthesis and pluripotency (Boroviak et al., 2015), while still maintaining its viability. Potential blastocyst factors include members of the forkhead box (FOXO) family and components of the cyclin-dependent kinase inhibitor 1A (CDKN1A, also known as p21) pathway (Hamatani et al., 2004; Fenelon et al., 2017). FOXO proteins are important for maintaining diapause in invertebrates (Box 3) and are crucial in maintaining both hematopoietic and neural stem cell quiescence, as well as embryonic stem cell (ESC) pluripotency in human and mouse (Tothova et al., 2007; Martins et al., 2016). Furthermore, many FOXO transcriptional targets have been evolutionarily conserved from invertebrates to human and include genes involved in metabolism, growth factor signalling and transcriptional regulation pathways (Webb et al., 2016). The activated form of FOXOs have been detected in mouse, tammar and mink diapause blastocysts, although how they function in embryonic diapause in mammals is not yet clear (Fenelon et al., 2017). The CDKN1A cell cycle inhibition pathway might also play a role in inducing mitotic arrest during diapause at the G0 or G1 phase of the cell cycle (reviewed by Lopes et al., 2004). In support of this, it has been shown that uterine secretory proteins of the CDKN1A cell cycle inhibition pathway, which have the potential to induce arrest of the blastocyst in G0, are present in the uterine fluid of the tammar during diapause (Martin et al., 2016), although further studies are needed to confirm this possibility. MicroRNAS are also likely to be involved since, in the mouse, at least 45 microRNAs are differentially expressed between embryonic diapause and reactivation, 38 of which are downregulated at reactivation (Liu et al., 2012). Five of the nine members of the lethal-7 (let-7) tumour suppressor microRNA family, which regulate cell proliferation and inhibit attachment, are also downregulated at reactivation (Liu et al., 2012; Gurtan et al., 2013).

The phenomenon of embryonic diapause in the mouse may be linked to the amenability of this species to ESC derivation under LIF-dependent conditions (Batlle-Morera et al., 2008; Nichols et al., 2001; Hondo and Stewart, 2004). Mouse ESCs are normally derived from the stage at which they can enter diapause. Accordingly, optimal efficiency of ESC derivation is in fact achieved via the use of diapausing blastocysts (Kawase et al., 1994; Brook and Gardner, 1997), and the transcriptomes of self-renewing ESCs and diapause embryos are surprisingly similar (Boroviak et al., 2015). This is an earlier stage of development than that at which the more recently described epiblast-derived stem cells (EpiSCs) are derived (De Miguel et al., 2010). Recently, two downstream signalling factors, namely MYC and mechanistic target of rapamycin (mTOR), were identified as being able to induce reversible arrest in the mouse blastocyst (Bulut-Karslioglu et al., 2016; Scognamiglio et al., 2016). The absence of either factor results in a number of hallmarks of diapause (Box 2), including a reduction in *de novo* protein synthesis and transcriptional repression. Furthermore, this inhibition has a similar effect on mouse ESCs and results in a transcriptomic profile that has a number of similarities with that of arrested mouse blastocysts, including downregulation of metabolism, biosynthesis and gene expression pathways. Interestingly, the inhibition of MYC in ESCs takes ∼96 h to completely inhibit cell proliferation, similar to the 3 day delay observed in diapause mouse blastocysts (McLaren, 1968; Spindler et al., 1996). However, neither MYC nor mTOR inhibition has any effect on ESC pluripotency networks, indicating that other factors are required to maintain this aspect of ESC self-renewal (Bulut-Karslioglu et al., 2016; Scognamiglio et al., 2016). In addition, the inhibition of mTOR extends blastocyst survival *in vitro* for 9-12 days, but only if both mTOR complexes (TORC1 and TORC2) are inhibited. A similar effect is observed in ESCs, which can be maintained for weeks following mTOR inhibition without extensive levels of cell death, with their transcriptomic profile closely corresponding to that of a diapause epiblast. By contrast,

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Box 3. Diapause in non-mammalian species

Embryonic diapause is found in a number of non-mammalian vertebrates including elasmobranchs (sharks and rays), lizards, freshwater turtles and several bony fish species (e.g. annual killifishes, Cyprinodontiformes) (Rafferty and Reina, 2012; Waltrick et al., 2012; Martin and Podrabsky, 2017). In these cases, diapause can be obligate or facultative, predominantly in response to environmental influences (e.g. temperature, rainfall). It should be noted, however, that non-mammalian vertebrates can undergo various forms of embryonic arrest at multiple stages during their development. In ovipositional (egg-laying) reptiles, diapause can occur at pre-oviposition or post-oviposition, and anytime in development between the early embryo through to gastrulation (Rafferty and Reina, 2012; Waltrick et al., 2012).

Information about the molecular control of diapause in non-mammalian vertebrates is limited. Similar to mammals, progesterone appears to maintain diapause in the Australian sharpnose shark (*Rhizoprionodon taylori*), but oestradiol is not involved and testosterone instead appears required for reactivation (Waltrick et al., 2014). Insulin-like growth factor binding protein (IGFBP1), which binds to IGFs, has also been implicated but its exact role is unknown (Rafferty and Reina, 2012).

Embryonic diapause has also been studied extensively in invertebrates, and numerous examples are found in nematodes (e.g. *Caenorhabditis elegans*), insects (e.g. *Drosophila*) and crustaceans (e. g. brine shrimp, *Artemia fransciscana*) (reviewed by Hand et al., 2016). Similar to non-mammalian vertebrates, diapause can occur at many developmental stages depending on species and environmental cues, and this affects which arrest mechanisms are activated. Many of these mechanisms have been characterised and, although the precise factors are not always conserved, there are a number of common themes. These include cell cycle arrest by cyclin-dependent kinases, chromatin/histone modifications, the use of small RNAs and the insulin/FOXO signalling pathway. Current evidence suggests that some of the mechanisms that control invertebrate diapause involve ancient genes with functions that are evolutionarily conserved in mammals. One example is the insulin/FOXO signalling pathway, which is important for both *C. elegans* and insect diapause (Hand et al., 2016). In *C. elegans* and many insects, entry into diapause requires activation of the homologue of mammalian FOXO, and the downregulation of FOXO requires many of the genes involved in the mammalian insulin signalling pathway (Sim and Denlinger, 2013; Mukhopadhyay et al., 2006). In insects, FOXO can trigger the activation of multiple pathways, including stress tolerance, cell cycle, metabolism and circadian clock pathways (Sim et al., 2015). Activation of FOXO might thus act as a master controller to generate the many characteristics of the diapause phenotype (Hand et al., 2016). Insects also utilise other novel strategies during diapause that could be important in mammals, including temporal expression of genes and periodic activation of metabolism (Denlinger, 2002).

inhibition of either translation, histone acetylation or MYC can only extend survival by 1 day, and inhibition of MYC cannot prevent ESC death (Bulut-Karslioglu et al., 2016).

Although it is clear that the blastocyst is able to respond to numerous uterine secreted factors, it should also be noted that it is able to take an active role in its own development. Two of the earliest genes upregulated in the mouse blastocyst at reactivation encode interleukin 1 (IL1), which is able to modulate endometrial cell responsiveness (Bourdiec et al., 2013), and HB-EGF, which can induce its own expression in the endometrium (Lim et al., 1998; Hamatani et al., 2004). The blastocyst may also employ specific processes that help support its survival during diapause. One such process is autophagy, providing a potential mechanism by which metabolic requirements are met via the recycling of vital cell nutrients in the cells of the embryo (Lee et al., 2011). Indeed, survival rates of blastocysts arrested by mTOR inhibition are significantly reduced when blastocysts are cultured with an autophagy inhibitor (Bulut-Karslioglu et al., 2016). Similarly, reactivation of the mouse blastocyst is accompanied by mitochondrial activation and activation of the endosome-lysosome system (Fu et al., 2014). However, diapause in the tammar embryo, which normally has an 11 month quiescence period and has only ∼80 cells at the start of diapause, can be extended to 2 years after ovariectomy (Tyndale-Biscoe and Hearn, 1981), suggesting that autophagy during such lengthy periods is unlikely, at least in this species.

Further investigations into all of these factors, in both the uterus and the blastocyst, are needed to clarify their exact role in the control of diapause and reactivation, and there are likely to be many more, as yet unidentified, endogenous factors that control diapause. In addition, the study of other diapause species, both mammalian and non-mammalian (Box 3), is required to determine the extent to which the molecular control mechanisms and physiological responses have been evolutionarily conserved. Regardless, the molecular mechanisms involved are not only complex but also time dependent.

Conclusion and future directions

Embryonic diapause is widespread in mammals, yet does not seem to obey any taxonomic distribution. It can act as a reproductive isolating mechanism or, in many species, matches the timing of reproduction with an optimal time of the year to ensure survival of the mother and young (Discussion of Enders, 1981). As noted above, seasonal factors such as light, rain and food availability are also controlling factors. One especially interesting strategy is that of the black bear, in which recurrent oestruses allow the relatively solitary bear to gain multiple paternity of her young via sequential ovulations and matings, so that the conceptuses of each mating enter diapause and later reactivate together (Himelright et al., 2014). Thus, the selective advantage of diapause allows species to synchronise their reproduction to benefit fitness and fecundity.

The species distribution of diapause could be interpreted as a remnant of an ancestral mechanism that might once have been more widespread. This idea was tested recently; sheep blastocysts, which are not known to exhibit diapause, were transferred into the uteri of pseudo-pregnant mice in which diapause conditions were induced and were left there for 1 week before retransferring them back into recipient sheep uteri, or placing them into culture (Ptak et al., 2012). Sheep blastocysts transferred to mouse uteri that were quiescent became growth arrested but were still viable since they later resumed development, a small number producing lambs, when placed into activated sheep uteri. When placed into culture instead of back into the sheep uteri, a small number of the ‘delayed’ blastocysts expanded and hatched. These experiments clearly demonstrate that normally non-diapausing sheep blastocysts can survive for at least 1 week in a receptive environment (that may lack the necessary stimulatory factors) but whether they truly enter diapause is less clear. There are currently no definitive markers to identify when a blastocyst is in embryonic diapause in any mammal, an area that is in immediate need of attention. On the basis of their experiments, the authors of this study (Ptak et al., 2012, 2013) suggested that this reproductive strategy has been evolutionarily conserved and was not secondarily acquired, and that embryonic diapause is not found in all mammals today because it is no longer necessary for successful reproduction of a particular species.

It seems unlikely that diapause evolved in all mammals as proposed above, and it is more parsimonious to conclude that the majority of mammalian species (>5400 species without diapause versus 130 with diapause) never had diapause. It is also hard to reconcile the suggestion that humans once had (or still have)

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evidence of embryonic diapause with current knowledge of human reproduction. With the exception of a small number of bat species (Box 4) (Wimsatt, 1975), there are very few diapausing mammals in the tropics, where conditions are relatively stable all year round. It seems that synchrony with particular environmental conditions in each specific habitat might only have become important when species moved to higher latitudes. Only a few species (0.02% of extant mammals) adopted diapause as a strategy; others solved their needs by migration or by delivering young in the spring or summer. The opportunity to extend species' ranges and then to use diapause as a species-isolating mechanism (for example, as the skunk has done from its origins in South America; Mead, 1981, 1993) might have exerted sufficient selection pressure for the evolution of diapause in certain specialised cases. For the majority, it is reasonable to conclude that diapause evolved multiple times in different habitats and under differing selection pressures. This notion is supported by the fact that although the mechanisms controlling diapause are diverse, all are underpinned by variations on a common theme of early development. Many of the factors required for embryo reactivation are also required for ensuring successful embryonic development in all mammals, suggesting that the underlying mechanisms have been conserved. Hence, activation of the inhibitory pathways or the lack of appropriate stimulators is also likely to be effective in all mammalian embryos, regardless of whether diapause has been evolutionarily conserved or not.

Regardless, understanding how diapause puts embryonic cells that are normally highly active to sleep, and how it suppresses the proliferation of these normally rapidly dividing cells, might also provide us with tools to understand the dysregulation of cancer cells and identify novel mechanisms of how to put them to sleep. More importantly, understanding diapause might provide insight into the pluripotent states of ESCs, and what is required for the induction/maintenance of these states in various species. The molecular control of embryonic diapause involves a complex and intricate coordination of multiple factors and signalling pathways in the endometrium and blastocyst, as well as a significant amount of redundancy. As we have discussed in this Primer, multiple promising candidates have been identified that induce, maintain and reactivate the blastocyst from embryonic diapause. The challenge now remains to identify the essential signalling pathways from many more species and to incorporate all of these into a cohesive model. Clearly, we still have much to learn – and gain – from the study of the fascinating phenomenon of embryonic diapause.

Box 4. Bats: a special case

There are three types of reproductive delay patterns in bats (Burns, 1981). The first, delayed fertilisation, is common in most vespertilionid bats. The second type, which corresponds to true diapause or delayed implantation, is seen in a few bat species including *Eidolon* and *Miniopterus*. In these species, seasonal rainfall and temperature rhythms control diapause (Wimsatt, 1975), although there are no obvious reasons why the tropical species should have embryonic diapause. The third type, delayed development, occurs in five families (Emballonuridae, Phyllostomidae, Pteropodidae, Rhinolophidae and Vespertilionidae) and may also take place in a member of the Natalidae family (Rasweiler, 1993; Rasweiler and Badwaik, 1997). Two examples of delayed development are seen in the greater short-nosed fruit bat *Cynopterus* from India (Meenakumari and Krishna, 2005) and the short-tailed fruit bat *Carollia* in Trinidad (Rasweiler, 1993; Rasweiler et al., 2011). Both have a period of embryonic diapause after implantation in which the embryos have slow or delayed development at the early gastrulation/primitive streak stage, which can last from weeks to months. It is especially interesting that delay can alternate with a second non-delayed pregnancy. In *Carollia*, which exhibits a non-delayed gestation of 113-119 days and a delayed pregnancy length of 169-229 days (Rasweiler and Badwaik, 1997), this delay period normally occurs seasonally in the wild but it can also occur in response to stress in captivity (Rasweiler and Badwaik, 1997). *Cynopterus*, like other pteropid bats, can have two pregnancies each year: one with delayed development at the blastocyst stage during November with birth in March after a gestation period of 150 days, and a second, undelayed pregnancy with conceptions in April and birth in late July after a pregnancy of 125 days (Krishna and Bhatnagar, 2011). Progesterone and oestrogen synthesis is low in *Cynopterus* during delayed development, which is likely to be due to suppressed luteal synthesis.

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Competing interests

The authors declare no competing or financial interests.

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