Conserved mechanisms for putting pregnancy on hold in the mouse, mink and tammar wallaby

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Abstract

Embryonic diapause was first identified over 150 years ago, but many questions still remain about how the external and hormonal controls of embryonic diapause translate into how the uterus conveys information to the embryo. Current evidence suggests that the control of diapause is mediated by components of the uterine secretions. However, the identity of the essential signalling molecule(s) is unknown. The mouse (*Mus musculus)*, the mink (*Neovison vison)* and the tammar wallaby (*Macropus eugenii)* are the three most extensively studied mammalian diapause species. Despite differences in the external and hormonal control of diapause between these three species, we have now found that there is conservation of numerous molecular factors around diapause and reactivation. This was first suggested via the conserved expression of various growth factors. The first evidence for a conserved mechanism resulted from a study on the muscle segment homeobox transcription factor (MSX) in the uterus during diapause, whose expression is conserved amongst the mouse, mink and wallaby. Following this was the evidence that inhibition of polyamines induces entry into diapause in both the mink and mouse. Thus, although the signalling mechanisms via which the uterus is induced into diapause vary amongst species, the molecular communication that occurs between the uterus and the embryo to control diapause is conserved. Given that these mechanisms are conserved across varying taxa, this implies a universal mechanism for maintaining embryo health amongst all mammals. New technologies are now allowing us to examine diapause from a global perspective and to increase our knowledge of this enigmatic stage of pregnancy.

Introduction

Embryonic diapause is a common reproductive strategy amongst mammals, found in over 130 mammals in seven different orders. Diapause is a reproductive strategy where a naturally occurring, temporary arrest of the embryo occurs at the blastocyst stage to extend the period between mating and parturition to allow synchrony with environmental or seasonal changes. Whilst the endocrine control of pregnancy has been established in many diapause species, fundamental questions remain about the molecular control of embryonic diapause in all mammals that display this reproductive trait [1]. The three best characterized species for the study of embryonic diapause are the mouse (*Mus musculus*), mink (*Neovison vison*) and wallaby (*Macropus eugenii*) [2]. Furthermore, they represent all three modes of embryonic diapause: the mouse has facultative diapause where diapause is selectively induced due to lactational constraints; the mink has obligate diapause in which diapause is induced in every pregnancy in relation to seasonal changes and the wallaby has a combination of the two modes where lactational control occurs in the first half of the year and obligate (seasonal mode) takes over in the second half of the year

(Table 1). A number of other characteristics of diapause differ amongst these three species including the length of diapause and their hormonal control mechanisms, as detailed below (Table 1).

Mechanisms of mouse diapause

In the mouse, diapause is facultative and results from lactational metabolic stress. It can also be experimentally induced (Cha et al this volume). The mouse exhibits post-partum mating within 14-28h of parturition [3]. The sucking stimulus of the litter then prevents the ovarian oestrogen pulse on day 4 of embryo development that initiates the implantation process. However, the length of diapause is dependent on the number of suckling young [4, 5](Table 1). During diapause, the blastocyst gradually slows its rate of cell proliferation over a period of 72h and its rates of protein synthesis and carbohydrate metabolism are greatly reduced [6, 7]. Mice and rats were the first diapause species to be extensively examined for the control of diapause, partly due to the ease in which diapause can be experimentally induced. The classic model involves ovariectomy before 0900h on d4 of pregnancy and maintenance via daily progesterone (P_4) injections. Alternatively, diapause can be induced by a single injection of an anti-oestrogen (ICI182,780) [8]. Reactivation requires a single injection of oestrogen (E_2) with rapid resumption of the cell cycle, metabolism and DNA, RNA and protein synthesis with implantation occurring within 24h [9-11]. Under experimental conditions, diapause can be induced for up to 10 days, after this time, blastocysts begin to show compromised developmental competence and the proportion that are able to implant decreases (Weitlauf & Greenwald 1968, Lee et al 2011).

Table 1: Comparison of the external and endocrine control of diapause and reactivation between the mouse, mink and wallaby including the time taken for the embryo to implant after reactivation from diapause.

	mouse	mink	wallaby
Type of diapause	facultative (lactational)	obligate (seasonal)	facultative & obligate (lactational & seasonal)
Length of diapause (naturally induced)	1-14 days	6 days-3 weeks	11 months
Time to implantation	24 hours	13-14 days	18 days*
Reactivation hormone	oestrogen	prolactin (progesterone)	progesterone

*Note the wallaby does not implant but hatches from the shell coat at d18 of reactivation (equivalent to day 18 of removal of pouch young, RPY) and then attaches to the uterine wall, 9-10 days before birth.

Mechanisms of mink diapause

In the mink diapause is obligatory, being seasonally induced, and extends for variable durations prior to implantation (Murphy & Fenelon this volume). Briefly, the proximal signal responsible for reactivation of the mink blastocyst from diapause is prolactin secreted from the pituitary gland, under the influence of the vernal equinox, which reactivates the dormant corpus luteum to rapidly increase progesterone synthesis [12]. Exogenous prolactin administered during diapause (d0 PRL)

can induce and synchronize reactivation [12-14]. During diapause, the mink blastocyst maintains an almost complete arrest of the cell cycle and a greatly reduced metabolism, DNA and protein synthesis [15]. The first signs of reactivation from diapause are increased DNA and protein synthesis in the embryo on the third day after prolactin injection (d3 PRL), with the first expansion of the blastocyst occurring at day 4 PRL and implantation between day 13-14 PRL [15].

Mechanisms of wallaby diapause

The tammar wallaby can sequentially undergo a facultative, lactational delay during the first half of the year and a seasonal, obligate diapause after the winter solstice (22nd June, in the Southern hemisphere), thus maintaining the embryo in diapause for up to 11 months of the year [16-18]. The tammar mates 1-3h after birth [19] and it is the neural stimulus of the newly born sucking pouch young that releases prolactin and inhibits the luteal progesterone pulse causing the blastocyst and the corpus luteum to enter into diapause by d8 postpartum [1, 20]. For reactivation, the luteal inhibition needs to be removed for 3 days, induced by removal of the pouch young (d0 RPY), and this results in a progesterone pulse around day 4 [21-24]. This pulse stimulates the production of uterine secretions that reactivate the blastocyst - characterised by a resumption of mitosis and a switch to an oxidative metabolism at d4 RPY with expansion by d8 RPY [25-30]. However, if the pouch young is removed after the winter solstice (June 22nd in the Southern hemisphere), the blastocyst will not reactivate and will remain in seasonal diapause until after the summer solstice (December 22nd). Seasonal diapause is controlled by the pineal gland and release of melatonin in response to the changing length of the night [30-32]. Thus, due to this seasonal synchrony, reactivation is synchronised, and while all tammar wallabies give birth around the end of January, the hottest, driest month, the pouch young are all weaned by October after the spring growth.

Conserved mechanisms

Although there is variation amongst the three species in the mechanisms of diapause and the specific endocrine signals required, each result in the control and regulation of the uterus. The uterine environment then controls whether the embryo enters into, and exits, diapause. It is known that this uterine control requires an intimate crosstalk between the endometrium and the embryo, but the precise molecular signals involved have not been clearly defined. The first indication that the molecular mechanisms of diapause could be conserved amongst mammals came from growth factor expression studies.

A member of the epidermal growth factor (EGF) family, heparin-binding EGF (HBEGF), was the first growth factor to be implicated in blastocyst reactivation from diapause in the mouse. Implantation in the mouse occurs within 24h of the oestrogen signal at 0900h of the day of reactivation (or d4 in the non-diapause model) and HBEGF is first expressed in the uterus at 1600h [33]. This is one of the earliest expressed uterine reactivation factors in the mouse and this expression pattern is similar in both the wallaby and the mink reactivated uterus [34, 35]. Furthermore, members of the EGF family receptors are expressed in the blastocyst at reactivation of the mouse, mink and wallaby [36]

LIF is a member of the IL-6 cytokine family and is essential for reactivation from diapause and implantation in the mouse [37]. In the mouse, LIF is absent during diapause and expressed at reactivation in the uterine luminal epithelium [38]. In the uterus, LIF activates the Jak-Stat pathway by binding to the heterodimeric LIF receptor complex consisting of the LIF receptor (LIFR) and glycoprotein 130 (gp130). LIFR and gp130 are also expressed in the luminal and glandular epithelium and conditional knockout of gp130 results in a failure of implantation [39, 40]. Similar to the mouse, in both the mink and western spotted skunk, another diapause mammal, LIF is absent from the uterus during diapause and increases at reactivation [41, 42] whilst in the tammar, LIF has

very low expression in the uterus during diapause and increases significantly at reactivation (MB Renfree, unpublished results).

As many of these growth factor studies were limited by the examination of expression data only, there is a lack of functional evidence to provide a definitive role for any in controlling embryonic diapause. The first factor to be confirmed as affecting whether embryos enter into diapause or not, and with an expression pattern that was conserved amongst diapause species was MSX (Cha et al, this volume). In brief, MSX is expressed in the uterus of the mouse, mink and wallaby during diapause, and its expression is required to cease for reactivation to occur. Conditional knockout studies in the mouse further showed that the presence of MSX in the uterine luminal epithelium is essential for the maintenance of diapause [8].

Following on from MSX was the discovery that polyamines are essential for reactivation of the embryo from diapause in both the mink and the mouse. Polyamines are small non-aliphatic compounds, essential for many cellular pathways, including proliferation, due to their ability to bind to, and interact with, nucleic acids and proteins [43]. Their synthesis is tightly regulated by a system of enzymes and feedback pathways, primarily via the rate limiting enzyme ornithine decarboxylase 1 (ODC1). The first evidence that polyamines could be involved in embryonic diapause was from studies in the mink which showed that ODC1, and two other polyamine pathway enzymes SAT1 and AZIN1, are significantly increased at reactivation in both the embryo and the uterus [44-46]. Confirmation of the essential role for polyamines came from *in vivo* studies where treatment of females at reactivation with an inhibitor of ODC1, difluromethylornithine (DFMO), caused the blastocysts to re-enter diapause with significant reductions in the concentrations of the polyamines in the uterus [46]. Similarly, treating mice *in vivo* from d4 of pregnancy with DFMO causes the blastocysts to enter into diapause and disrupts expression of a number of implantation-related genes, including HBEGF [47]. Finally, in both the mink and the mouse, *in vivo* DFMO treatment decreases expression of multiple enzymes in the polyamine pathway (Box1).

In the mouse, levels of *Amd1* and *Azin1* increase in the uterus in response to DFMO treatment compared to diapause whilst levels of *Odc1* and *Sat1* remain the same [47]. In contrast, in the mink, *SAT1* was significantly decreased in the DFMO-treated uteri to levels consistent with diapause whilst *ODC1* and *AZIN1* expression only showed a trend towards a decrease, which was not significantly different from either the diapause or control uteri. This could potentially indicate a partial compensation for *ODC1* and *AZIN1* expression in response to the DFMO treatment. Similarly, in the mouse, a number of genes related to implantation are decreased after DFMO treatment compared to the untreated uteri, whilst MSX1 expression was maintained [47]. In the mink, there were no significant differences in any of the non-polyamine pathway genes examined including the hormonal receptors, MSX1 or the EGF family. However, many of these genes are post-translationally regulated and therefore may not necessarily show a significant difference in expression at the gene level.

Polyamines directly affect reactivation of the mink blastocyst. *In vitro* addition of the polyamine putrescine to the mink diapause blastocyst increases both survival rates and proliferation - both hallmarks of reactivation from diapause [44]. Similarly, the addition of putrescine to either a mink trophoblast or inner cell mass (ICM) cell lines increases proliferation whilst DFMO treatment decreases proliferation. In contrast, mouse diapause blastocysts are able to spontaneously reactivate *in vitro* and this includes after DFMO-induced diapause [47]. These results indicate that the requirement of the embryo for polyamines at reactivation is a uterine effect. Our current hypothesis is that DFMO dysregulates the communication between the endometrium and the embryo, indicating the importance of polyamine availability in regulating embryonic diapause*.*

Experimental verification: Downstream effects of polyamine inhibition in the mink

In order to examine in more detail the downstream effects of polyamines in the mink, DFMO treatment of reactivated females was repeated following the protocol in [46]. All other experimental procedures regarding immunofluorescence and qPCR data and analysis were as previously reported [44, 47]. Prolactin (PRL) was administered to all treatment animals to terminate diapause. Briefly, pregnant female mink were treated daily with prolactin and either DFMO or saline from d0 for five days. Mink were then euthanised on d6 PRL and the uteri flushed. A control group at diapause (equivalent to d0 PRL) were also euthanised and the uteri flushed for comparison. When initially flushed from the uterus, mink embryos shrink away from their shell coat but after 24h in culture, the majority are able to recover. Embryos flushed from the saline-treated controls were significantly larger compared to the DFMO-treated embryos (p=0.005) and diapause blastocysts (p<0.0001) based on capsule diameter (Figure 2A). The embryos collected on d6 PRL were unable to recover from their original shrinkage, presumably due to their size (Figure 2A)[61]. After DFMO treatment, mink embryos had significantly reduced rates of proliferation, confirming their diapause state, consistent with the previous study [46]. In uteri of treated animals, the proliferation marker MKI67 expression was decreased to a similar level to the diapause controls compared to the reactivated controls, indicating that the DFMO-treated uterus resembled a diapause-like state (Figure 2B).

We examined the effects of compromised polyamine synthesis *in vivo* after treatment with DFMO. In the uterus, there was a significant difference in expression between diapause and reactivation for the polyamine enzymes *ODC1*, *SAT1*, *AZIN1* and the growth factor *HBEGF* (Figure 3). However, in the DFMO-treated uteri, there was no significant difference in the expression of any of the genes examined, except for *SAT1*, where the levels after DFMO treatment were significantly decreased from reactivation. The expression of the remaining genes examined (*SAMDC*, *PRLR*, *PGR*, *ESR1, MSX1* and *EGFR*) remained constant throughout diapause, reactivation and after DFMO treatment (Figure 3).
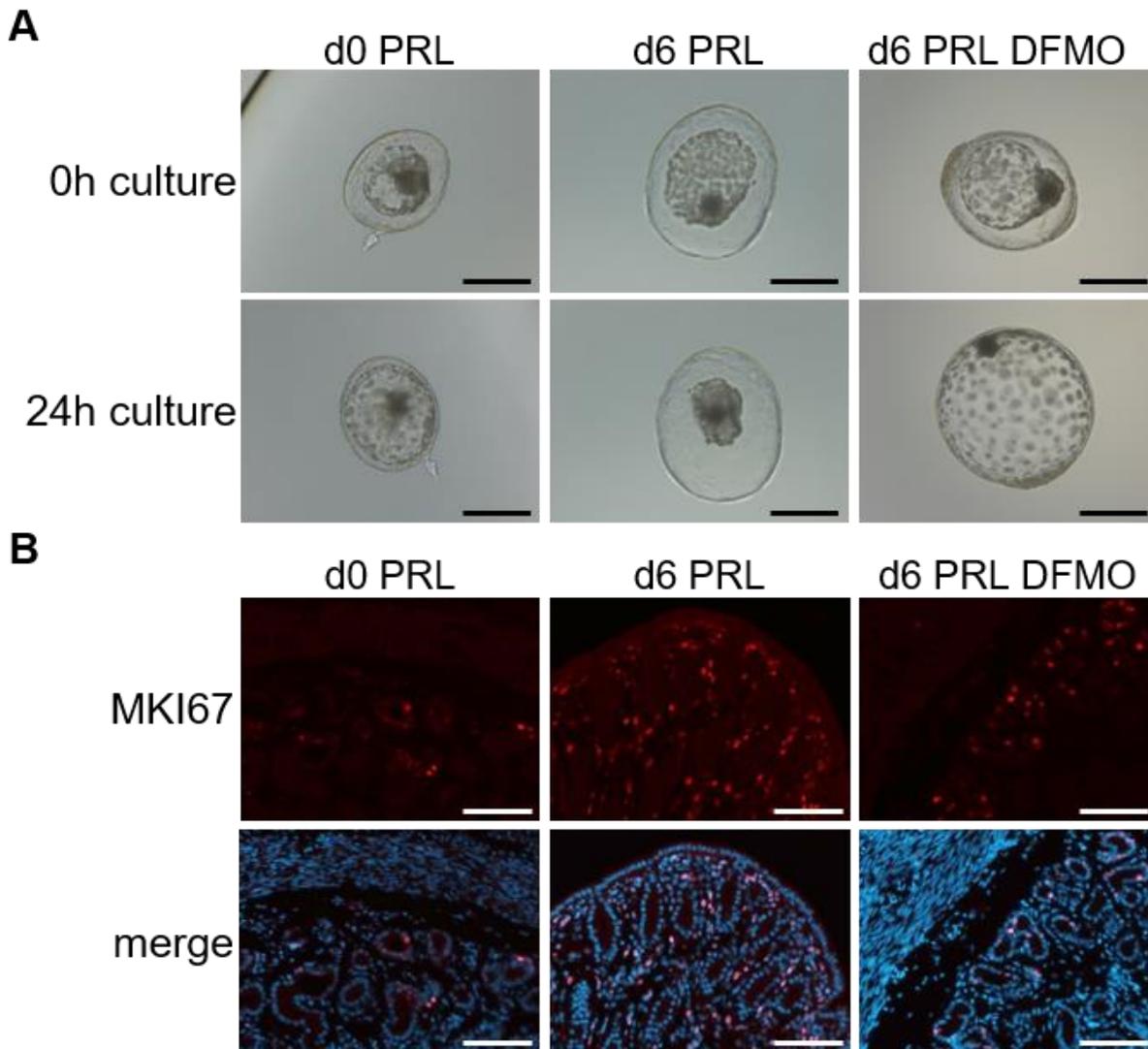
Figure 1: Effect of DFMO treatment in the mink on growth of embryos and the proliferation state of the uterus

A: When first flushed (0h), d6 PRL, embryos were significantly larger than both d0 PRL (diapause) and DFMO treated embryos, but DFMO-treated embryos were also significantly larger than d0 PRL embryos, based on capsule volume. Over 24h in culture, d6 PRL embryos were unable to recover from their original shrinkage, B: MKI67 was detected in the nucleus of the glandular and luminal epithelium at all stages examined and was increased at reactivation. Red represents the staining of the protein of interest, blue indicates DAPI nuclear stain and the merge shows both channels. d0 PRL = diapause control embryo, d6 PRL = vehicle control treated embryo, reactivated, DFMO = d6 PRL DFMO-treated embryo and MKI67: marker of proliferation (Ki-67). All embryo pictures taken at same magnification, scale bar = 200 μm. All immunofluorescence photos taken at the same magnification, scale bar = 100 μm at 20x magnification.
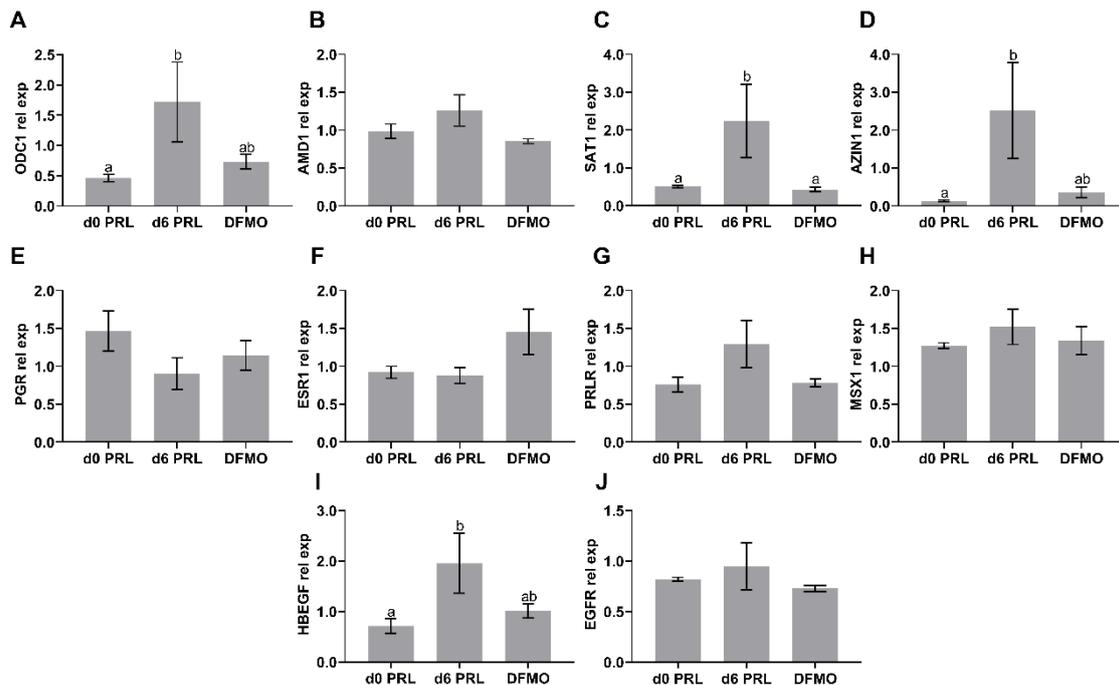
Figure 2: mRNA qPCR expression profile of polyamine pathway genes across diapause (d0 PRL), reactivation controls (d6 PRL) and DFMO treated uteri. Data represent mean mRNA abundance of each gene relative to the expression of the housekeeping genes *RPS18* and *HPRT1*. All values represent mean ⬚ SEM with n=5 samples per stage and means bearing different superscripts are different at p < 0.05. A: ODC1 (ornithine decarboxylase 1); B: AMD1 (adenosylmethionine decarboxylase 1); C: SAT1 (N1-Spermidine/Spermine acetyltransferase); D: AZIN1 (antizyme inhibitor 1); E: PGR (Progesterone receptor); F: ESR1 (Oestrogen receptor 1), G: PRLR (prolactin receptor), H: MSX1 (msh homeobox 1); I: HBEGF (heparin binding epidermal growth factor-like like growth factor); J: EGFR (epidermal growth factor receptor). rel exp: relative expression, diapause: day 0 PRL, react: d6 PRL vehicle control treated, DFMO: d6 PRL DFMO-treated.

New technologies

Numerous other factors have also been implicated in reactivation from diapause (for review refer to [36]). While many of these have similar expression patterns in multiple diapause mammals, a definitive role for any of them is lacking. Furthermore, there is likely to be considerable overlap and redundancy amongst them and confirming whether a factor is essential is laborious and requires independent assessment of each. New technologies have allowed us to now begin to examine diapause from a global perspective and start to identify the conserved downstream pathways involved.

The earliest study to examine diapause from a global view compared diapause versus reactivated mouse embryos and identified 229 differentially expressed genes by microarray analysis [48]. This study confirmed the involvement of predicted pathways (i.e. cell cycle and metabolism) but also highlighted the importance of calcium signalling and chromatin remodelling for reactivation [48]. More recently, the proteome of the diapause and reactivated mouse embryo has been investigated [49]. This study identified 277 proteins specific to the diapause blastocyst and 585 proteins found only in the reactivated blastocysts [49]. Analysis of these proteins revealed that at reactivation the blastocyst requires an increase in aerobic glycolysis, upregulation of nucleotide biosynthesis and activation of mitochondrial function [49]. Thus, the diapause and reactivated blastocyst are each an active unit with their own complex and interacting signalling pathways.

The first global mink studies performed suppressive subtractive hybridisation (SSH) on the mink uteri at reactivation and identified 123 differentially expressed genes that included enrichment for

cell proliferation, cell homeostasis, and was the first study to identify the importance of polyamines [45]. A follow-up SSH study on the diapause and reactivated embryo found upregulation of 216 transcripts at reactivation, including genes in the polyamine pathway [44]. Recently, a more comprehensive study was performed focussing on the transcriptome of the diapause and reactivated mink uterus using RNA-Seq. This study identified 564 genes up-regulated during diapause and 963 genes up-regulated at reactivation [50]. Whilst the most significantly enriched pathways at reactivation were for endometrial morphological changes and functions, there were also increases in genes related to prolactin signalling and downstream pathways of growth factor signalling including G-protein coupled receptor (GPCR) activity and the PI3K-Akt pathway. Finally, a number of genes, including EGF, were identified as critical based on protein-protein interaction network analysis confirming previous work into the importance of these factors during reactivation [50].

Analysis of the uterine fluid proteome has also been examined in the tammar wallaby and the roe deer, another diapause mammal, during diapause and reactivation [35, 51]. In the roe deer 107 differentially expressed proteins were identified comparing the three different stages of diapause (early-, mid- and late-diapause) and reactivation (elongation). This analysis identified a number of clusters that supported embryo development during diapause and cell cycle promoters at elongation, consistent with the requirement for reactivation of the cell cycle after diapause [51]. However, note that the roe deer embryo continues to undergo a slow period of growth during diapause [52]. In the tammar, the majority of the 603 uterine fluid proteins were present at all stages examined, of which 128 were secretory [35]. However, cell cycle regulatory proteins were only present during diapause whilst between days 4-6 RPY (early reactivation) there was an increase in growth factors including soluble transforming growth factor beta (TGF-β), EGFR and heparin binding growth factor (HDGF, previously known as hepatoma-derived growth factor) [35]. In the d0-d3 RPY samples only, there were proteins present that are involved in the p53/p21 cell cycle inhibition pathway, whilst in the reactivation samples the cell proliferation and differentiation markers galectin 1 (LGALS1) and proliferating cell nuclear antigen (PCNA), were present [35]. These studies highlight the importance of communication between the uterus and the embryo in controlling the events of diapause and reactivation, but it is unknown what proportion of these factors came from the uterus, embryo or both.

Finally, in mice there are at least 38 microRNAs (miRNAs) in the blastocyst that are upregulated at diapause compared to reactivation [53]. Similarly, there are 42 miRNAs upregulated in the mouse uterus at diapause [54]. In particular, the lethal-7 (let-7) family of miRNAs appear to have an important role during diapause. Members of this family are upregulated in the mouse at diapause in the blastocyst and the uterus and a partial diapause-like state can be induced by overexpression of let-7a in the embryo [55, 56]. MiRNAs are essential to embryo development in general and it is likely that other miRNAs are also involved in the control of diapause as their primary role is to suppress translation post-transcriptionally [57]. However, a confirmed function for the majority of the miRNAs identified in the mouse during diapause has not been established [53]. No other diapause species have yet been examined.
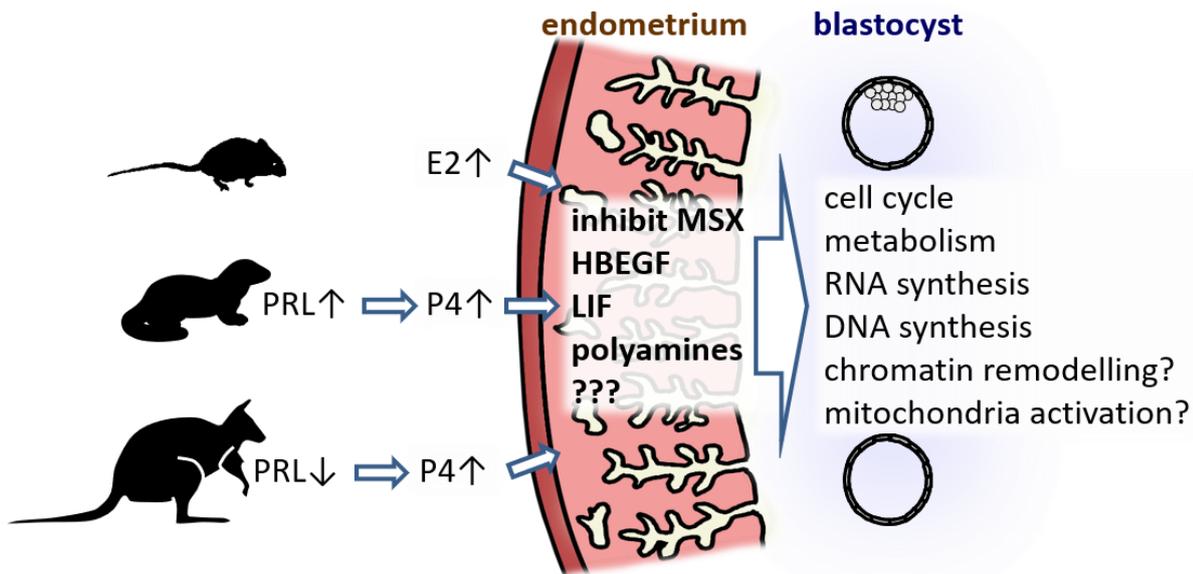
Figure 3: Overview of the evolutionarily conserved processes involved at reactivation from diapause in the mouse, mink and wallaby. Despite the varying hormonal mechanisms required to reactivate the endometrium between the mouse, mink and wallaby, a number of factors have been identified that are conserved amongst all three. At reactivation, these include a decrease in MSX expression and an upregulation of HBEGF, LIF and polyamine related enzymes in the endometrium. However, it is predicted that other factors are also required. These uterine signals then cause reactivation of the blastocyst which requires activation of a number of pathways, for example a resumption of the cell cycle and an increased metabolism. The challenge remains in identifying the essential mechanisms via which this occurs. Note, the tammar blastocyst is unilaminar and it is unknown which cells of the blastocyst form the inner cell mass. E2: oestradiol, PRL: prolactin, P4: progesterone, MSX: muscle segment homeobox transcription factor, HBEGF: heparin-binding epidermal growth factor, LIF: leukemia inhibitory factor.

Future challenges

Despite these extensive new studies, the essential pathways that maintain the diapause state and induce reactivation remain an enigma. Previously it was thought that diapause and reactivation would be controlled by only a few pathways. However, advances in technology are now beginning to highlight the complexity of the processes involved that control diapause and reactivation. What is clear is that the mechanisms of diapause and reactivation require a number of regulatory and secretory pathways in both the uterus and the embryo (Figure 1). In the embryo these include regulation of the cell cycle, control of metabolism and control of DNA and RNA synthesis. Additional processes that may be required at reactivation include chromatin remodelling and activation of mitochondria. Growth factors also appear to be operating in an integrated network between the uterus and embryo, with many activating multiple pathways. This suggests a degree of redundancy in the system. The polyamines appear to be among the essential factors, but it is currently not known how they act. More studies are needed to clarify the complex interactions of the downstream pathways, upon which many of the upstream regulatory factors appear to converge. These include Ca^{2+} signalling, the PI3K-Akt pathway and GPCR signalling. At least some of these pathways appear conserved amongst the species examined to date. Recent work has also identified the importance of the MYC and MTOR signalling pathways as essential to the control of both diapause and stem cell pluripotency, at least in the mouse [58, 59]. There is only limited investigation of miRNAs into the regulation of diapause but the evidence so far suggests they could have an important function in controlling the embryo during diapause [55, 56]. Finally, a recent paper has identified distinct transcript splicing variants in the diapause mouse embryo that could

be important for maintaining the diapause state [60]. Thus, new -omics technologies are now allowing us to gain greater insight than ever before into the mechanisms of diapause and the challenge will be to integrate all this information into a cohesive model by which diapause is controlled.

Declaration of interest

The authors declare no conflicts of interest.

Funding

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Audience Discussion

Susanne Ulbrich: Regarding the initiation of the diapause, are they related to the daylight length or lactation? Do you have any idea whether the general control of melatonin by prolactin being influenced by lactation as the main driver, directly on the uterus or indirectly? What kind of evidence do you have regarding the quiescence of the uterus being imposed by either LH or prolactin or whether you have any indication of estrogen signalling?

Jane Fenelon: One of the things I'm interested in is trying to look more at the control of diapause in the tammar. We don't really understand how they switch from this lactational to seasonal quiescence. We know it is involved with melatonin. There's a crossover period, where fewer and fewer animals will reactivate if you remove the pouch young from the teat. There is only a very small short-lived change in oestrogen after reactivation. In mink, you can't reactivate an embryo with progesterone or estrogen: part of the reason was that because there are no receptors in the uterus.

Bruce Murphy: In preliminary experiments, we made cell lines in the mink and we treated them with prolactin.

Jane Fenelon: If you treat them with prolactin, you do get regulation of the corpus luteum (CL).

Pierre Comizzoli: Marilyn, in your presentation, you said at the beginning that what you call a blastocyst is without a definite inner cell mass (ICM).

Marilyn Renfree: There is no ICM in any marsupial blastocyst.

Pierre Comizzoli: after reactivation you can definitely see in your embryo an embryonic disc, ok, so is it really a blastocyst or is it a morula?

Marilyn Renfree: It`s not a morula, in those early cleavage stages, the cells are all together, but they don`t have tight junctions, they have not formed a morula. The blastocyst is a hollow ball of about 80-100 cells, each one looks identical.