

## Focus on Implantation

### Embryonic diapause and its regulation

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#### Abstract

**Embryonic diapause, a condition of temporary suspension of development of the mammalian embryo, occurs due to suppression of cell proliferation at the blastocyst stage. It is an evolutionary strategy to ensure the survival of neonates. Obligate diapause occurs in every gestation of some species, while facultative diapause ensues in others, associated with metabolic stress, usually lactation. The onset, maintenance and escape from diapause are regulated by cascades of environmental, hypophyseal, ovarian and uterine mechanisms that vary among species and between the obligate and facultative condition. In the best-known models, the rodents, the uterine environment maintains the embryo in diapause, while estrogens, in combination with growth factors, reinitiate development. Mitotic arrest in the mammalian embryo occurs at the G0 or G1 phase of the cell cycle, and may be due to expression of a specific cell cycle inhibitor. Regulation of proliferation in non-mammalian models of diapause provide clues to orthologous genes whose expression may regulate the reprise of proliferation in the mammalian context.**

*Reproduction* (2004) **128** 669–678

#### Introduction

Embryonic diapause, also known as discontinuous development or, in mammals, delayed implantation, is among the evolutionary strategies that ensure successful reproduction. It comprises the uncoupling of mating and fertilization from birth and serves to maintain developmental arrest of the embryo, usually to ensure that postnatal development can be completed under more favorable environmental conditions. Its wide distribution among unrelated taxa, from plants to insects to vertebrates, suggests that it has arisen numerous times during evolution. The defining characteristic of diapause in plants and animals is dramatic reduction or cessation of mitosis in the embryo. Cell cycle arrest can occur at the G0/G1 or G2 phase, depending on the species, and is induced by mechanisms that are poorly understood in virtually every species so far investigated. The exit from diapause can be defined as the resumption of mitotic activity. It is regulated by numerous factors, often specific to the species in question.

Recent comprehensive syntheses of literature have appeared on the evolutionary aspects of diapause (Thom *et al.* 2004), and on the maintenance and termination of diapause (Renfree & Shaw 2000). The molecular regulation of implantation from the uterine perspective has

recently been discussed in depth (Dey *et al.* 2004). In this presentation we address the characteristics of the embryo in diapause and focus on the mechanisms of regulation of this phenomenon, including the environmental and metabolic stimuli that induce and terminate this condition, the hormonal regulatory pathways, and the phenomenon of cell cycle arrest and reactivation.

#### The embryo in diapause

In most mammals displaying discontinuity of development, the progression to the blastocyst stage of the embryo and post-implantation development of the embryo and fetus follow a preordained, species-specific program. There is an arrest in development that initiates diapause occurring at the blastocyst stage in most species. Notable exceptions are found in the bat family, where variation in the rate of post-implantation development has been documented (Rasweiler & Badwaik 1997). Among species displaying diapause at the blastocyst stage there is significant variation in morphology of the arrested embryo. In many species that display pre-implantation delay, including the rodents (Zhao & Dean 2002), the roe deer (Aitken 1975) and the nine-banded armadillo (*Dasypus novemcinctus*; A C Enders, personal communication), the embryo hatches

from its zona pellucida before entering into diapause. The embryo of the roe deer has a modest complement of 30–40 cells (Aitken 1975). The mouse embryo has a similar cell number at hatching, but this number increases to approximately 130 cells within 72 h, and this cell complement is maintained through diapause (Spindler *et al.* 1996). The blastocyst of the armadillo is much larger, consisting of an inner cell mass in excess of 100 cells, and approximately 600 trophoblast cells (Enders 1962). In marsupials, the embryo in diapause comprises 60–100 cells (Smith 1981) surrounded by a glycoprotein investment comprising the zona pellucida of the oocyte, supplemented by two further investments derived from the oviduct (Selwood 2000). The carnivore embryo in diapause consists of 200–400 cells, with a zona that persists until implantation (Desmarais *et al.* 2004). The carnivore zona appears to be supplemented with layers of glycoprotein acquired during the passage of the embryo from the oviduct to the uterus (Enders & Mead 1996). There is evidence from studies of the western spotted skunk (*Spilogale putorius*) and the badger (*Taxidia taxus*) that embryo diameter and the total number of cells in the blastocyst increase during diapause, although this proliferation is restricted to the trophoblast cells (Mead 1993). In other mustelids, the total cell number does not seem to increase during diapause (Mead 1993). In the mink (*Mustela vison*), blastocyst diameter increases and cells proliferate only after reactivation (Desmarais *et al.* 2004). In the tammar wallaby (*Macropus eugenii*), neither the number of cells in the embryo nor its diameter increase during diapause (Renfree 1981). In contrast, a low level of mitosis characterizes pre-implantation delay in the roe deer (Lengwinat & Meyer 1996).

Two variations on the theme of diapause

Two functionally distinct categories of mammalian embryonic diapause are recognized (Table 1). Facultative diapause, best known in rodents and marsupials, is the developmental arrest induced by environmental conditions related to the survival of the dam and her ability to nourish developing embryos. Facultative diapause can be

produced experimentally in rodents by ovariectomy of the female soon after fertilization, followed by progesterone treatment (Paria *et al.* 2002). In contrast, obligate diapause is present during every gestation of a species, and is believed to be a mechanism for synchrony of parturition with environmental conditions favorable to neonatal survival. While common in mustelid carnivores, it is also found in the roe deer and some bats (Sandell 1990). In some species, there is seasonal diapause superimposed on diapause resulting from metabolic factors or lactation (Renfree & Shaw 2000).

Given the selective advantages of diapause in temperate climates, it is somewhat surprising that species that are closely related do not always express the trait. Examples can be found in the mustelids, where gestation undergoes an ordered progression without evidence of diapause in the European ferret, while in the mink, every pregnancy includes a period of pre-implantation delay. Almost every other aspect of reproduction (induced ovulation, post-implantation gestation, etc.) is identical between these species. There are examples in which obligate diapause is restricted to subspecies of animals that are geographically isolated, most notably the spotted skunk (Mead 1993). There is similar selectivity in the occurrence of facultative diapause; it is found in rodents of the subfamily Sigmodontinae in North America (e.g. *Peromyscus* spp.), while completely absent in South American species of this subfamily. Lindenfors *et al.* (2003) argue for a single evolutionary origin of embryonic diapause in carnivores, followed by loss of the trait in some subgroups. It is possible to induce diapause in species where it does not normally occur; for example, blastocysts from the ferret transplanted to the mink uterus cease development (Chang 1968). A pre-implantation delay can be induced in ferrets by experimental manipulation of either pituitary (Murphy 1979) or ovarian (Foresman & Mead 1978) endocrine function. Evidence based on the appearance of chorionic gonadotropin secretion suggests that diapause or developmental delay can occur in human embryos (Tarin & Cano 1999). The anecdotal data, then, allow the speculation that many mammalian species might be capable of expressing diapause under appropriate conditions.

Table 1 Characteristics of facultative vs obligate diapause of mammalian embryos.

| Characteristic                                 | Facultative diapause  | Obligate diapause   |
|--|---|---|
| Distribution                                   | Rodents, marsupials   | Mustelid, ursid and phocid carnivores, roe deer, some bats and armadillos |
| Developmental status of blastocyst in diapause | Hatched in rodents; encapsulated in marsupials                | Encapsulated in carnivores, hatched in roe deer and armadillo             |
| Mitotic activity in the embryo in diapause     | None  | Minor proliferation in some species restricted to trophoblast             |
| Stimulus for entry into diapause               | Lactation and metabolic stress                                | Developmental stage in all gestations                                     |
| Exogenous stimulus for exit from diapause      | Weaning (photoperiod in some marsupials)                      | Photoperiod   |
| Endogenous stimulus for exit from diapause     | Ovarian estrogen (rodents); prolactin withdrawal (marsupials) | Prolactin secretion, unknown ovarian factors                              |

## Regulation of diapause by external factors

Environmental regulation of diapause, including its onset, maintenance and termination, is imposed directly on the exposed embryo in invertebrates. In contrast, it is regulated by means of the maternal organism in viviparous vertebrates. Most mammals that have survived in temperate and variable climates have evolved a pattern of seasonal breeding to maximize their reproductive success. The most common environmental cue that synchronizes both estrus and male reproductive competence in mammalian species is photoperiod. Nonetheless, there are numerous examples of species whose reproductive cycle is dictated by the availability of nutrients, often secondary to rainfall, and by environmental temperature.

### Photoperiod and temperature

Reduced ambient temperature is one of the principal factors inducing diapause in invertebrates (Kostal *et al.* 2000). Low temperature will induce diapause in some reptiles (Shanbhag *et al.* 2003). In mammals, the role of temperature is best known in the regulation of delayed development in bats (Mead 1993). There is, nonetheless, evidence that elevated temperature induces facultative diapause in rodents (Marois 1982), and that low temperatures can prolong obligate diapause in some carnivores (Canivenc & Bonnin 1979). The physiological mechanisms in mammals are currently unknown.

It was recognized early that photoperiod played an important role in termination of diapause and the consequent induction of implantation (Pearson & Enders 1944). In mustelids, diapause is terminated during lengthening photoperiod, and the lengthening of days prior to and after the vernal equinox influences the timing of implantation in numerous species, including the spotted skunk (Mead 1971) and the mink (Murphy & James 1974). In the seal family, implantation occurs under a regime of decreasing day length (Atkinson 1997). Day length – or, more precisely, a regime of photoperiod in which mink are exposed to light during a critical period from 12 to 16 h after dawn – provides a facultative signal that induces implantation (Murphy & James 1974). Studies in both mink and skunks indicate that the requirement for long days is not absolute, as implantation occurs in animals maintained in constant dark as well as in blinded animals (Mead 1993). The pineal gland was first implicated in studies in which its denervation by cervical sympathetic ganglionectomy disrupted photoperiodic regulation of the termination of diapause (Murphy & James 1974), later confirmed by pinealectomy and melatonin replacement (Bonfond *et al.* 1990). While chronic melatonin treatment of mink does not interfere with puberty, ovulation or blastocyst formation in mink, it prevents termination of diapause and implantation (Murphy *et al.* 1990). Implantation can be rescued by exogenous prolactin in this species, suggesting a single mechanism for photoperiod induction of implantation.

In some macropod marsupials, seasonal regulation of diapause is superimposed on lactational diapause, and long days associated with the summer solstice are the cue that reinitiates embryo development (Renfree & Shaw 2000). Diapause can be terminated by denervation of the pineal in marsupials, implicating melatonin as the effector (Renfree *et al.* 1981). The environmental cues and their translation into physiological events are less well studied and more difficult to discern in species such as the roe deer, where diapause is terminated during short days (Sempere *et al.* 1992) or, as in the case of the ursids, when implantation occurs during hibernation (Harlow & Beck 2002).

### Metabolic stress and lactation

There is evidence to suggest, at least in the European badger (*Meles meles*), that reduced nutrition of the dam lengthens diapause (Ferguson *et al.* 1996). In the classic paradigm of facultative diapause in rodents, mating occurs at a postpartum estrus, and implantation is delayed by the presence of suckling young, with larger litters causing a longer delay (Weichert 1940). In marsupials, the presence of suckling young, independent of number, represents the stimulus for entry and for maintenance of diapause, and removal of pouch young results in rapid reactivation of the embryo and consequent implantation (Renfree & Shaw 2000). Social stress, including crowding or introduction of new males, will induce facultative diapause in rodents (Marois 1982).

## Regulation by endogenous factors

### Maternal control

Rodent blastocysts survive, but do not implant when transferred to the uterus of ovariectomized, progesterone-treated adult females (Weitlauf & Greenwald 1968) or the oviducts of intact, immature females (Papaioannou & Ebert 1986). Under both conditions, embryos retain their capability to implant and develop normally, indicating that the maternal environment is the crucial factor that maintains diapause. Evidence that the uterus inhibits the renewal of embryonic development in obligate diapause comes from transplant experiments where blastocysts from the ferret (a non-diapause species) were arrested in development when transferred to the mink uterus, while mink blastocysts reinitiated embryogenesis in the ferret uterus (Chang 1968). Mink embryos in diapause co-cultured with conspecific uterine cell lines displayed the capacity for reprise of embryonic development *in vitro*, providing further evidence that the uterus maintains diapause in this species (Moreau *et al.* 1995).

### Control by the pituitary gland

Regulation of embryonic diapause via hypophyseal prolactin demonstrates the principle that existing hormones have been co-opted for variable, often diametrically

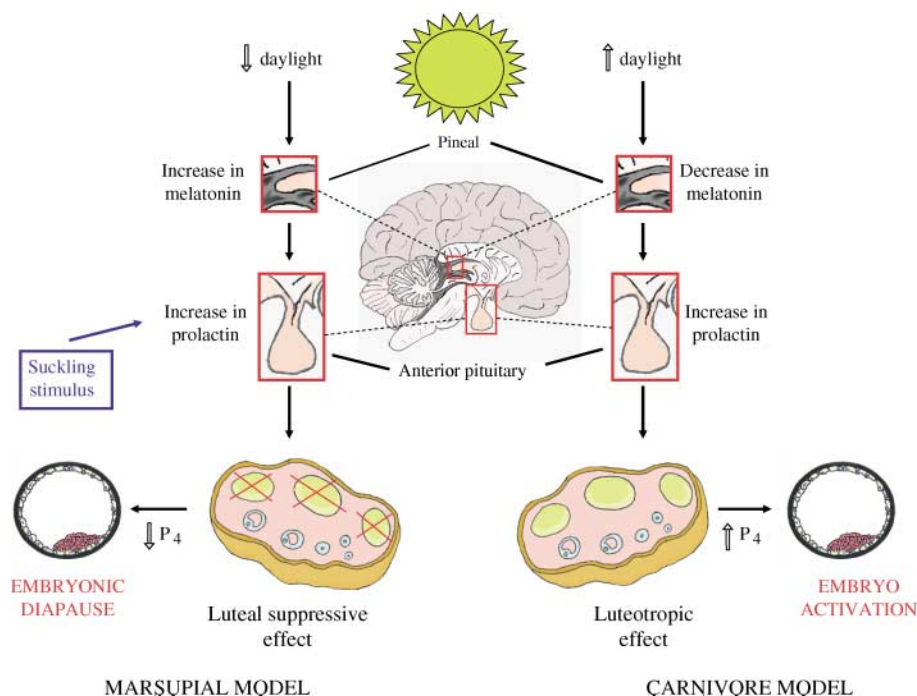
opposed, uses during evolution (Fig. 1). Prolactin is the key factor essential for embryo implantation in mustelid carnivores. Its circulating concentrations increase some days prior to implantation in both the mink (Murphy & Rajkumar 1985) and the spotted skunk (Mead 1993). Treatment of mink in diapause with prolactin precociously terminates diapause, while dopamine agonists, at doses that prevent prolactin secretion, prevent implantation (Papke *et al.* 1980). Withdrawal of the dopamine agonist (Papke *et al.* 1980) or administration of dopamine antagonists (Murphy 1983) terminates diapause in mink. Indeed, prolactin alone induced implantation in hypophysectomized mink (Murphy *et al.* 1981), as did administration of prolactin to animals in protracted diapause due to chronic melatonin treatment (Murphy *et al.* 1990). In macropod marsupials, prolactin plays an inhibitory role. In these species, hypophysectomy terminates diapause, and it has been shown that suckling-induced prolactin secretion during lactational delay prevents implantation (Renfree & Shaw 2000). During the seasonal delay in marsupials, a pulse of prolactin secretion is necessary for inhibition of implantation, and this pulse can be blocked by long photoperiods (Renfree & Shaw 2000).

Rodent facultative diapause is terminated by a short-lived surge of estrogen from the ovary (Dey *et al.* 2004). Hypophyseal secretion of luteinizing hormone (LH) is essential for this release (Macdonald *et al.* 1967). It is of

interest that both LH and prolactin will activate the corpus luteum (CL) in the long-fingered bat (*Miniopterus schreibersii*), but only prolactin terminates diapause (Bernard & Bojarski 1994).

### Ovarian events

Following ovulation in mustelids displaying an obligate diapause, the ovarian follicle collapses and forms the CL (Hanssen 1947). The CL body undergoes a remarkable structural reduction in size as diapause ensues, all the time secreting low levels of progesterone (Mead 1993, Murphy *et al.* 1993). In contrast to the pattern of terminal differentiation that characterizes CL development in most species, the mink CL retains its mitotic potential during the period of diapause (Douglas *et al.* 1998). In response to the pituitary prolactin signal that terminates diapause, the CL is rejuvenated, a process characterized by a several-fold increase in volume and in progesterone output (Murphy *et al.* 1993). In contrast to models of facultative delay, it has not been possible to terminate diapause in carnivores by steroid administration. Studies in the ferret (Foresman & Mead 1978) and mink (Murphy *et al.* 1983) indicated that a luteal protein in combination with progesterone are required for successful implantation. A credible candidate protein, glucose-6-phosphate isomerase also known as autocrine motility factor, has recently been shown to be



**Figure 1** Strategies for photoperiodic modulation of diapause employ melatonin and prolactin for contrasting purposes. In the marsupial model, both suckling stimulus and increased melatonin secretion associated with nocturnal periods in excess of the summer solstice upregulate prolactin, which then inhibits luteal activation, thereby initiating and maintaining diapause. In the carnivore model, photoperiod associated with the vernal equinox decreases melatonin secretion, releasing prolactin from inhibition. Prolactin activates the CL, provoking release of progesterone and other factor(s) that terminate diapause. P<sub>4</sub> (luteal progesterone).

secreted by the ferret CL during the appropriate pre-implantation window (Schulz & Bahr 2004) and to be required for implantation (Schulz & Bahr 2003). In mink, the activity of glucose-6-phosphate isomerase in circulation is low during diapause and increases with activation of the CL, and is elevated at the time of implantation (R D Bennett & B D Murphy, unpublished observations), suggesting that it might also play a role in reactivation of the mink embryo in diapause.

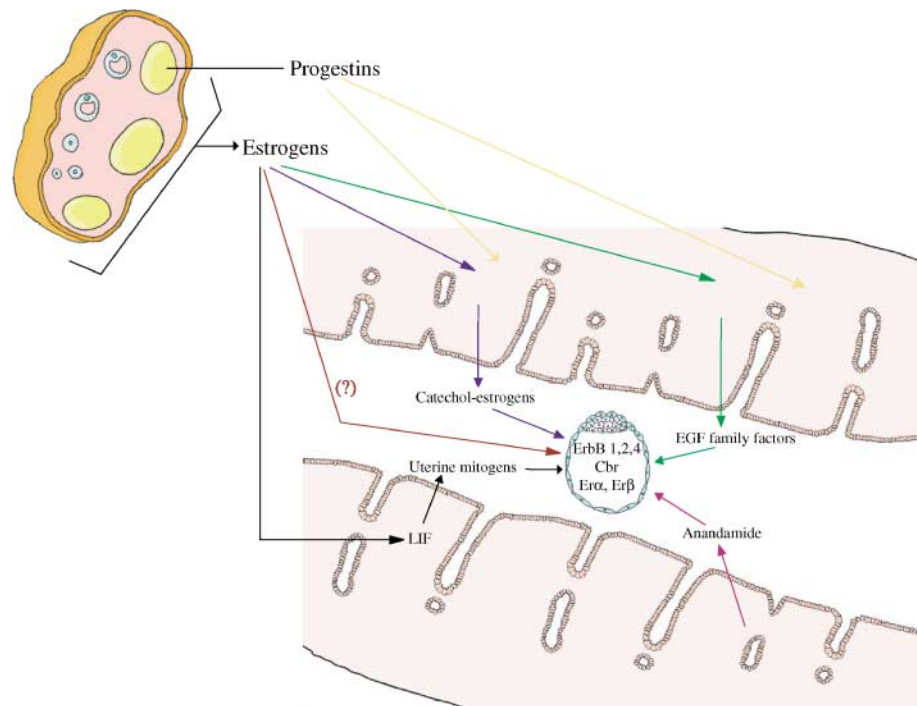
In marsupials, as exemplified by the wallaby, the CL develops during the estrous cycle, only to be inactivated by lactational or seasonal prolactin secretion (Renfree & Shaw 2000). Reactivation ensues when this inhibitory influence is removed. In rodents, it is the absence of an ovarian estrogen pulse that maintains diapause, but the ovarian structure (CL or follicle) from which the steroid issues has not been resolved.

The most unusual manifestation of ovarian regulation of diapause is found in the nine-banded armadillo. In this species, there appears to be no regression of the CL associated with delay, as indicated by plasma progesterone concentrations (Peppler & Stone 1980). Nonetheless, implantation is induced some 14 days after ovariectomy (Mead 1993), suggesting ovarian inhibition of nidation. The basis for this inhibition remains undiscovered.

### Uterine factors

As noted above, reciprocal embryo transfers have demonstrated that the maternal uterine environment induces and maintains the embryo in its developmental arrest. An important question is whether diapause is due to the absence of uterine factor(s) necessary for development beyond the blastocyst, or whether the uterus actively maintains diapause by inhibition of development. Support for the former view can be found in studies that have shown large-scale increase in uterine protein synthesis and secretion concurring with the termination of obligate diapause (Mead 1989, Lambert *et al.* 2001). Furthermore, cascades in the synthesis of several classes of proteins, including adhesion factors, cytokines and growth factors, follow the estrogen pulse that induces mouse implantation (Dey *et al.* 2004). A simplified view of uterine and ovarian regulation of diapause in the embryo is presented in Fig. 2.

The requirement for uterine expression of the cytokine leukemia inhibitory factor (LIF) for implantation has been demonstrated by targeted mutation in mice (Dey *et al.* 2004). In this species LIF injection can replace the nidatory estrogen pulse (Sherwin *et al.* 2004), indicating an important role in termination of diapause. LIF transcripts are detected in the uterus of carnivores during the early



**Figure 2** Summary of uterine influences that could be acting on the dormant embryo in the rodent model to terminate the mitotic arrest of diapause. Against a background of luteal progesterone ( $P_4$ ), an ovarian pulse of estradiol-17 $\beta$  is the proximal stimulus for developmental recrudescence of the embryo. It may have direct mitogenic effects on the dormant embryo via its nuclear receptors, ER $\alpha$  and ER $\beta$ . It can be replaced by LIF acting on the uterus to induce secretion of factor(s) mitogenic to the dormant embryo. Estradiol-17 $\beta$  provokes expression of members of the EGF family, including EGF, heparin-binding EGF and amphiregulin, acting through the EGF receptors ErbB1 and ErbB4 to reactivate mitotic activity of the embryo in diapause. Catechol estrogens derived from uterine conversion of estradiol-17 $\beta$  act via an unknown receptor to activate the embryo via the mitogen-activated protein kinase pathway. Finally, anandamide, an endogenous cannabinoid from the endometrium, acts in low concentrations on its cognate receptors (Cbr), to stimulate the reprise of embryo development.

stages of embryo reactivation (Song *et al.* 1998, Hirzel *et al.* 1999), rendering it a candidate for a uterine factor that terminates mitotic arrest in the embryo. Evidence is lacking for a direct stimulatory role of LIF on the embryo to reinitiate development in any species, indeed, mouse embryos bearing inactivating mutation of the LIF receptor develop beyond the blastocyst stage and successfully implant (Ware *et al.* 1995).

Epidermal growth factor (EGF) is a potent mitogen, and thus a candidate for a uterine paracrine or autocrine factor regulating embryo mitosis. It can terminate diapause in ovariectomized rats in the absence of the estrogen pulse (Johnson & Chatterjee 1993), and members of the EGF family of growth factors, including heparin-binding EGF (hbEGF) and amphiregulin are expressed in overlapping patterns by the uterus during rodent implantation (Dey *et al.* 2004). The expression pattern of hbEGF at sites of implantation prior to embryo activation implicates it as a uterine factor effecting termination of diapause in rodents (Das *et al.* 1994). DNA microarray comparison of dormant and activated mouse blastocysts indicates that activation is associated with the expression of the gene encoding hbEGF, as well as the EGF receptor isoforms ErbB1 and ErbB4 (Hamatani *et al.* 2004). Further, hbEGF expression is induced in the uterus by estrogen (Zhang *et al.* 1998), the proximal signal for the termination of diapause. In addition, EGF receptors are present in dormant carnivore embryos, and their signaling activity is increased associated with escape from diapause (Paria *et al.* 1994). It is therefore reasonable to speculate that estrogen-induced expression of EGF and EGF-like factors from the uterus and embryo, acting on cognate receptors in the blastocyst, reinitiates development.

Microarray analysis comparing the mouse uterus before and after the nidatory estrogen pulse indicates upregulation of other growth factor-related transcripts (Reese *et al.* 2001). A pentraxin family protein (PTX3) is expressed at nearly fourfold greater intensity in the post-delay uterus. This protein is involved in complement binding and in innate immune responses, and is secreted in response to inflammatory cytokines (Fulop *et al.* 2003). PTX3 gene deletion disrupts ovarian function by interfering with cumulus formation (Fulop *et al.* 2003). While there are no investigations of its role in termination of diapause, its expression pattern and its known role in glycoprotein synthesis identify it as a potential downstream target of the growth factor and cytokine cascade that terminates embryo arrest.

There is evidence to suggest that the uterus actively inhibits development of the embryo, thereby inducing and maintaining diapause. Flushings from the uteri of ovariectomized, progesterone-treated mice (the delayed implantation model) contain protein fractions that inhibit DNA synthesis of embryos *in vitro* (Weitlauf 1978). Recent studies have revealed that the endogenous cannabinoid, anandamide, at high, but nonetheless physiologically relevant, concentrations inhibits mouse embryo development (Wang *et al.* 2003). Low levels of anandamide, in stark

contrast, activate the dormant mouse blastocyst via mitogen-activated kinase pathways. Thus, differential expression of cannabinoids may regulate facultative diapause.

Uterine microarray analysis indicates that several interferon- $\gamma$  induced genes are downregulated in the activated, relative to the delayed mouse uterus (Reese *et al.* 2001), suggesting that this cytokine might play a role in induction or maintenance of mitotic quiescence of the embryo.

## Regulation of diapause by cellular factors

### Cell cycle arrest

The mammalian embryo develops from the zygote by cell division and differentiation. The common theme in diapause is the inhibition of the mitotic cell cycle in embryonic cells, such that proliferation ceases or is greatly reduced. Cells enter a quiescent state, and apoptosis is prevented by the maintenance of the basal metabolism, with protein and RNA synthesis, as well as oxygen consumption (Renfree & Shaw 2000). Entry into dormancy occurs first in the trophoblast sub-population of the mouse blastocyst, followed by a more gradual entry of the inner cell mass cells (Given 1988). In insects, the mitotic arrest most commonly occurs at the G0/G1 stage of the cell cycle, but there are examples of G2 arrest in some species (Tammariello 2001). Quantification of DNA (Sherman & Barlow 1972) suggests that the arrest in mammalian embryos occurs prior to the S phase of the cell cycle. The absence of 5-bromo-2-deoxyuridine uptake by mink embryos in diapause (Desmarais *et al.* 2004) supports the case for G0/G1 arrest in this species.

By definition, the quiescent embryonic cells also retain the ability to resume the cell cycle when diapause terminates (Renfree & Shaw 2000). In the mouse, proliferation is initiated first in the inner cell mass of the blastocyst, almost immediately after the estrogen signal, and follows 6–12 h later in the trophoblast (Given & Weitlauf 1981). An intriguing new report suggests that reactivation of development in the trophoblast compartment of the spotted skunk embryo engenders endocycles, resulting in endopolyploidy (Isakova & Mead 2004). The significance of this finding to the termination of diapause awaits further investigation.

Cell cycle arrest has not been extensively studied in the mammalian embryo in diapause. Given the conservation of genes during evolution, investigations of diapause in invertebrate and sub-mammalian vertebrate models might be expected to provide insight into the maintenance of mammalian diapause. It has been shown that, in the fruit fly (*Drosophila melanogaster*), the developmental arrest during embryogenesis can be attributed to the *dacapo* gene, homolog of the mammalian p21, an inhibitor of cyclin E–cdk2 complex activation (Lane *et al.* 1996). This is consistent with inhibition in G1, as cyclin E–cdk2 complex formation is necessary for entry into S phase. Other candidate genes for inhibition of the cell cycle in diapause

have been derived from cDNA microarray and subtractive hybridization comparisons of embryos in diapause with their activated counterparts. In insects, proliferating cell nuclear antigen (PCNA), a factor associated with DNA synthesis and regulated by p21 (Fotedar *et al.* 2004), is not expressed during diapause (Denlinger 2002). These findings are consistent with new information from the mouse embryo where dormancy is associated with the increased expression of p21<sup>cip1/WAF1</sup> and concomitant decrease in a number of DNA replication genes (Hamatani *et al.* 2004). These studies also demonstrated that an inhibitor of G0/G1 transition, the B cell translocation gene 1 (Btg1 (Rouault *et al.* 1992)) is upregulated in the embryo during facultative diapause, providing a mechanism for maintenance of cell arrest. Expression of the classic cell cycle inhibitor p53 and associated genes did not differ between dormant and activated mouse embryos, suggesting that this common effector of cell cycle arrest is not involved in diapause (Hamatani *et al.* 2004).

### **Regulation of the cell cycle in diapause and reactivation**

Given the variation among mammalian groups displaying embryonic diapause, there may be no single mechanism of reactivation of mitosis. In non-mammalian models, several different proximal signals (temperature, photoperiod, nutrient supply) regulate cell cycle arrest and reactivation. In insects, reduction in ambient temperature induces a decline in ecdysteroid concentrations that in turn signals the initiation of diapause (Denlinger 2002). The earliest intracellular response detected to environmental stimuli that terminate diapause, including increasing temperature, is upregulation of synthesis of ecdysone and expression of its nuclear receptor (Denlinger 2002). In the nematode, *Caenorhabditis elegans*, multiple signals inducing the dauer diapause converge on daf-12, a nuclear receptor for a yet-unknown sterol ligand (Gerisch & Antebi 2004). In this species, termination of diapause engenders downregulation of daf-9, a P450 hydroxylase that catalyzes formation of the ligand. Thus, a common theme emerges of termination of diapause in invertebrates by cholesterol or its derivatives, acting through classic nuclear receptor pathways.

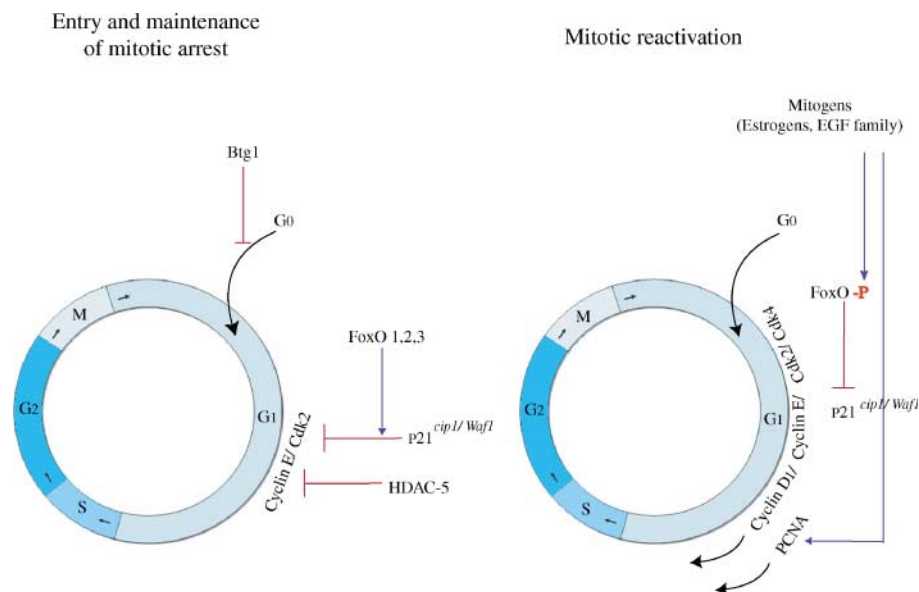
Vertebrates have evolved to employ specific cholesterol derivatives, the steroids, in the regulation of reproduction. In all known examples of mammalian diapause, with the possible exception of the armadillo, ovarian progesterone is essential for the termination of delay (Mead 1993). Further, a single estrogen injection terminates diapause in rodents (Dey *et al.* 2004). Treatment of carnivores in obligate (Murphy *et al.* 1982) or marsupials in seasonal (Fletcher *et al.* 1988) delay with estrogen does not induce reactivation of the embryo. Nonetheless, estrogens have pleiotropic mitogenic and mitotic effects on target tissues, mediated through classic nuclear receptors, membrane estrogen receptors and actions of multiple intracellular

effectors (Frasor *et al.* 2003) and may, in the appropriate concentration and temporal sequence, reactivate embryos in diapause. Estrogen receptors (ER) are expressed in all cell types of the dormant and activated mouse blastocyst (Hou *et al.* 1996) and both nuclear receptor subtypes, ER $\alpha$  and ER $\beta$ , have been identified in the cells of the blastocyst in diapause (Paria *et al.* 1998). Treatment of mice in delay of implantation with estradiol-17 $\beta$  resulted in S-phase activity in the embryos at the earliest time tested, 6 h (Given & Weitlauf 1981), and a detectable increase in the cell number within 12 h (Spindler *et al.* 1996). Paria *et al.* (1998) report that the principal mammalian estrogens, estradiol-17 $\beta$ , estrone and estriol, do not directly activate the dormant mouse embryo. This was concluded because estradiol-17 $\beta$  failed to induce the expression of EGF binding to the embryo, the hallmark of embryo activation, and blastocysts treated with estrogen *in vitro* failed to implant. These findings notwithstanding, it remains likely, based on the temporal sequence of occurrence of the S phase after estrogen treatment, that estrogens function as mitogens to terminate cell cycle arrest. The effects of estrogens may not be mediated through the classic nuclear receptors. Paria *et al.* (1998) make a case for embryo activation (EGF signaling) by uterus-derived catechol estrogens, signaling via a non-genomic pathway. EGF is a potent mitogen, and there is evidence for non-genomic signaling between estrogen- and EGF-mediated cellular events (Driggers & Segars 2002). A plausible hypothesis is that mitosis is reinstated in the embryo by EGF as a downstream event induced by primary estrogen or catechol estrogen signaling.

Clues to the intracellular events in mitotic renewal can be derived from comparison of the transcriptome between dormant and activated mouse embryos (Hamatani *et al.* 2004). There is upregulation of the estrogen-responsive target, *Brca1*, a gene that promotes proliferation in other tissues (Deans *et al.* 2004). Dormant embryos have greater abundance of the histone deacetylase-5 (HDAC-5) transcript, a gene whose expression is associated with attenuation of proliferation, and is independent of p53 mechanisms (Huang *et al.* 2002). The mitotic stimulus downregulates this chromatin modifier, allowing for histone acetylation and consequent transcription of previously silenced genes.

Comparative models provides some insight into potential regulation of mitotic re-initiation at the end of diapause. The FoxO genes are the mammalian orthologs of the *C. elegans* Daf genes that regulate diapause (Hosaka *et al.* 2004). Among the roles played by FoxO transcription factors in *C. elegans* is the induction of p21 expression and consequent mitotic arrest (Seoane *et al.* 2004). Indeed, overexpression of FoxO transcription factors induces cell cycle arrest at the G1 phase in mammalian cells *in vitro* (Burgering & Kops 2002). Although null mutation of FoxO1, 2 or 3 does not interfere with early embryo development or implantation in mammals, ovarian function is disrupted in FoxO3a null mice, with a phenotype of proliferation of the granulosa component of an abnormal





**Figure 3** Some of the potential cell cycle regulatory mechanisms controlling the entry of the mammalian embryo into diapause by mitotic arrest and consequent developmental recrudescence by re-initiation of mitotic activity. Entry and maintenance of diapause result from expression of cell cycle inhibitors of the p21 family that interfere with cyclin E–cdk2 complex formation necessary for progression through G1. Transcription factors of the FoxO family upregulate p21 expression, to initiate and maintain this effect. In addition, HDAC-5 is upregulated in the dormant embryo, which can prevent chromatin modification necessary for transcription of cell cycle genes. Further, dormant embryos express Btg1, a factor that prevents entry from G0 to G1, providing a further mechanism for cell cycle arrest. Embryo reactivation occurs due to mitogens from the ovary (estrogens) and from the uterus (EGF family and other factors). These have pleiotropic effects on the mitotic cycle: first by upregulation of PCNA; second by phosphorylating FoxO transcription factors, thereby preventing their translocation to the nucleus and consequent upregulation of p21; and third by direct stimulation of several components of G1 regulation, including cyclins D and E and cdk2 and cdk4.

number of follicles and consequent precocious depletion of the follicle population (Hosaka *et al.* 2004). Thus, FoxO-induced cell cycle inhibition may be an important mechanism in the maintenance of diapause. Recent studies have further defined a mechanism of escape from FoxO-induced inhibition of proliferation. Phosphorylation of FoxO genes occurs in response to mitogens, including estrogen (Birkenkamp & Coffe 2003). This modification restricts their translocation to the nucleus, thereby abrogating their cytostatic effects (Seoane *et al.* 2004). Further investigation is required to verify this hypothesis.

### Summary and conclusions

Embryonic diapause is an intriguing biological mechanism that has been employed by species in numerous taxa to ensure successful reproduction. In mammals, its onset, maintenance and termination are under maternal control, and are influenced by environmental factors and lactation. Reduction or cessation of mitotic activity in the embryo most likely results from the absence of uterine and ovarian mitogens necessary for development of the embryo beyond the blastocyst stage. Members of the EGF family of uterine origin are the best current candidates for induction of mitotic reprise in the embryo. Ovarian estrogen may also act directly to induce embryo mitosis. Cessation of development occurs before the S phase of the cell cycle in mammals, and may be due to expression of cell cycle inhibitors of the p21 family. Little is known about

cell cycle regulation upon reactivation of the dormant embryo (Fig. 3). Based on non-vertebrate models, a case is made for transcriptional regulation of cell cycle inhibitors by the forkhead family of transactivators, inactivated by mitogenic stimulation.

### Acknowledgements

The investigations described from the laboratory of BDM have been funded by generous and consistent support from the Natural Sciences and Engineering Research Council of Canada.

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Received 2 August 2004

First decision 13 August 2004

Accepted 26 August 2004