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Embryonic diapause in mammals and dormancy in embryonic stem cells with the European roe deer as experimental model

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Abstract. In species displaying embryonic diapause, the developmental pace of the embryo is either temporarily and reversibly halted or largely reduced. Only limited knowledge on its regulation and the inhibition of cell proliferation extending pluripotency is available. In contrast with embryos from other diapausing species that reversibly halt during diapause, embryos of the roe deer *Capreolus capreolus* slowly proliferate over a period of 4–5 months to reach a diameter of approximately 4 mm before elongation. The diapausing roe deer embryos present an interesting model species for research on preimplantation developmental progression. Based on our and other research, we summarise the available knowledge and indicate that the use of embryonic stem cells (ESCs) would help to increase our understanding of embryonic diapause. We report on known molecular mechanisms regulating embryonic diapause, as well as cellular dormancy of pluripotent cells. Further, we address the promising application of ESCs to study embryonic diapause, and highlight the current knowledge on the cellular microenvironment regulating embryonic diapause and cellular dormancy.

Keywords: dormancy, embryonic diapause, embryonic stem cells, European roe deer *Capreolus capreolus*.

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Embryonic diapause

The time between fertilisation and embryo implantation varies greatly among species. In mice, preimplantation development comprises no more than 5 days (Davidson and Coward 2016). The time until embryo implantation is around 8 days in humans (Niakan *et al.* 2012), around 18–22 days in cattle (Lonergan *et al.* 2016) and around 40 days in the mare (Allen and Stewart 2001). This period is prolonged in species that exhibit embryonic diapause, including the European roe deer *Capreolus capreolus*, in which preimplantation embryo development lasts 4–5 months (Aitken 1974).

Embryonic diapause is a temporary and reversible arrest of embryo development that was discovered in 1854 in the roe deer (Bischoff 1854). To date, embryonic diapause has been described in more than 100 different mammalian species as an evolutionary conserved mechanism (Fenelon *et al.* 2014; Renfree and Fenelon 2017). In mammals exhibiting diapause, implantation can be delayed from several days to several months (Renfree and Fenelon 2017). This delay is characterised by a decelerated developmental pace at the hatched blastocyst stage and allows the progeny to be born under the most favourable conditions. The roe deer is the only known ungulate exhibiting embryonic diapause. In contrast with other well-studied diapausing species (e.g. mouse, mink and tammar wallaby), developmental delay in the roe deer has unique features. To date, it remains unknown when and in response to which signal diapause in the roe deer commences. Around Day 14 after fertilisation, expanded blastocysts, hatching and from the zona pellucida completely hatched blastocysts have been found in the uterine horns of roe deer (Drews *et al.* 2020). From early August until late December, the hatched blastocysts remain free floating in the uterus (Lambert *et al.* 2001), exhibiting a comparatively long period of diapause (Renfree and Fenelon 2017). Strikingly, development is not completely halted, as shown in many other species. Rather, it features gradual proliferation at a considerably slower pace (Rüegg *et al.* 2020). The diameter of the blastocyst increases from 0.5 mm in September to approximately 4.0 mm in December (Fig. 1; Drews *et al.* 2019; Rüegg *et al.* 2020; van der Weijden and Ulbrich 2020*a*, 2020*b*). The end of diapause in the roe deer is marked by a rapid resumption of developmental pace, which morphologically coincides with embryo elongation, a feature shared across ungulates. Because

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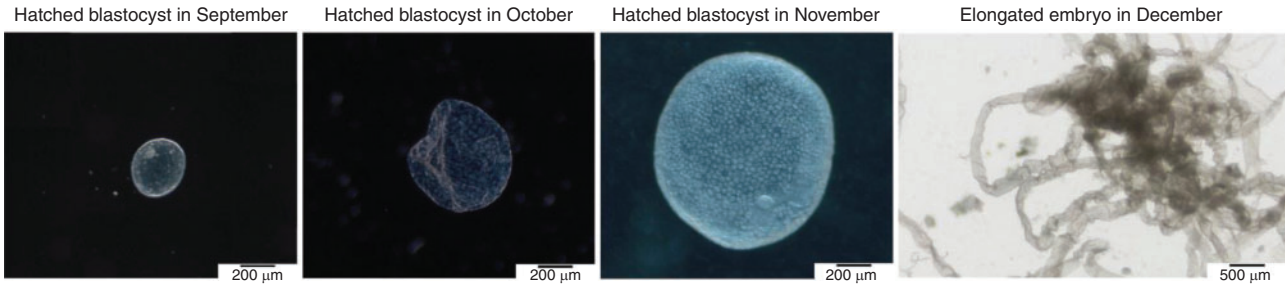

Fig. 1. Representative images of roe deer embryos at different stages of development during diapause and elongation. Embryo size increases during diapause, and elongation takes place after a 4- to 5-month period of embryonic diapause (Vera A. van der Weijden, Anna B. Rüegg, Sandra M. Bernal-Ulloa and Susanne E. Ulbrich, unpubl. data).

embryo elongation and the following epitheliochorial cotyledonary placentation are comparable, preimplantation development in the roe deer shows morphological similarities to bovine preimplantation development (Short and Hay 1966; Badinga *et al.* 1994).

Diapause in mice can be induced by ovariectomy (Weitlauf 1974) or reversibly provoked by lactation (McLaren 1968). Moreover, an interspecies embryo transfer experiment, in which sheep embryos were transferred to the mouse uterus, provided evidence that the diapausing mouse uterine environment temporarily induced a proliferation arrest in non-diapausing sheep embryos (Ptak *et al.* 2012). Thereby, the intrauterine environment received attention as a regulator of decelerated development.

The optimal environment for implantation and establishment of pregnancy depends on the interaction between a developmentally competent embryo and a receptive endometrium. Most embryo losses occur during the peri-implantation period. However, despite the long duration of the peri-implantation period in roe deer, high fertility and little evidence of early embryo losses have been reported (Gaillard *et al.* 1993; Andersen and Linnell 1996; Focardi *et al.* 2002; van der Weijden and Ulbrich 2020*a*). Like the high pregnancy rates in many wildlife species, the estimated implantation rates in roe deer are 92% (van der Weijden and Ulbrich 2020*a*), and field observations, as well as observations with captive roe deer, have shown a mean of 1.4–2.0 implanted fetuses and live-born fawns per doe, indicating predominantly twin pregnancies (Gaillard *et al.* 1993; Andersen and Linnell 1996; Focardi *et al.* 2002). The roe deer is an interesting model species because it can be used to study the regulation of the pace of embryo development and preimplantation embryo development in slow motion.

Embryonic stem cells

After fertilisation, the zygote undergoes several rounds of cleavage to form a blastocyst. Embryonic genome activation (EGA) takes place soon after fertilisation, occurring at the 2-cell stage in murine embryos, the 4-cell stage in porcine embryos, the 4- to 8-cell stage in human embryos and the 8-cell stage in bovine embryos (Flach *et al.* 1982; Arrell *et al.* 1991; Niakan *et al.* 2012; Graf *et al.* 2014). Following EGA, the embryonic cells show first signs of cellular commitment. The morula and subsequent blastocyst, consisting of the inner cell mass (ICM) and the surrounding trophoblast cells (TB), are formed. During early embryo development, extensive and efficient reprogramming takes place (Fulka *et al.* 2008). The ICM contains pluripotent stem cells (Evans and Kaufman 1981; Niwa 2007) that retain the ability to unrestrictedly develop into all somatic and germ cells (Wu *et al.* 2016). Although different to mice, there are similarities in embryo size and the expression of pluripotency markers in bovine and human preimplantation embryos (Simmet *et al.* 2018*a*, 2018*b*). Whether there are similarities between human and roe deer preimplantation embryos remains to be determined.

Embryonic stem cells (ESCs) are derived from the ICM of blastocysts and exhibit a remarkable proliferative capacity, allowing expansion of established cultures *in vitro* (Evans and Kaufman 1981). Almost 40 years ago, the first ESCs were isolated from diapausing blastocysts derived from ovariectomised mice (Evans and Kaufman 1981; Martin 1981). In 2015, it was shown that proliferating murine ESCs and diapausing mouse blastocysts exhibit highly similar transcriptome profiles (Boroviak *et al.* 2015). The proliferation of ESCs has subsequently been studied in further detail. The cell cycle machinery of ESCs differs from that of other mammalian cell lines. Cell cycle genes including the S-phase, M-phase and checkpoint genes are essential for the growth of human ESCs (Viner-Breuer *et al.* 2019). However, ESCs are capable of proliferation in the absence of all G_1 cyclins, which are involved in the transition from the G_1 to S phase of the cell cycle (Liu *et al.* 2017). A reduction in the length of both the G_1 and the S/G_2/M phases was recently related to an increased proliferation rate in mouse ESCs (Waisman *et al.* 2019). Differences in the regulation of the cell cycle between somatic cells and ESCs may explain why many commonly known cell cycle regulators have not been confirmed to induce diapause in blastocysts. To further study cellular dormancy in a cell culture model, a discrete set of markers serving as dormancy indicators is required.

Pluripotent stem cells in dormancy are thought to exit the cell cycle to survive in a quiescent state (i.e. a reversible state of growth arrest; Vera-Ramirez and Hunter 2017; van Velthoven and Rando 2019). *In vitro*, cellular dormancy can be induced by starvation or inhibition of key factors involved in proliferation, such as the mammalian target of rapamycin (mTOR) and MYC (Boroviak *et al.* 2015; Bulut-Karslioglu *et al.* 2016;

Scognamiglio *et al.* 2016*a*; Renfree and Fenelon 2017). The use of ESCs as a cellular dormancy model can facilitate the identification of molecular factors regulating embryonic diapause (Bulut-Karslioglu *et al.* 2016; Scognamiglio *et al.* 2016*b*).

Cellular microenvironment driving embryonic diapause and cellular dormancy of pluripotent stem cells

The limited availability and cell numbers of diapausing embryos *in vivo* and the absence of adequate culture systems mimicking dormancy *in vitro* have made it challenging to study molecular signalling pathways governing embryonic diapause. Nevertheless, cells within a completely reversibly arrested blastocyst during diapause showed reduced metabolic activity and RNA transcription and no signs of cell division or differentiation (Fu *et al.* 2014; Renfree and Fenelon 2017). Based on the DNA content, mouse and rat diapausing blastocysts are proposed to be arrested at the G_1 phase of the cell cycle (Sherman and Barlow 1972; Surani 1975; Kamemizu and Fujimori 2019). The mVenus-p27K$^-$ fusion protein can be used to visualise the G_0–G_1 transition (Oki *et al.* 2014), thereby allowing the investigation of the nature of embryonic diapause and cellular dormancy. It is hypothesised that the cellular microenvironment governs embryonic diapause, as well as cellular dormancy. The mTOR signalling pathway, which is highly conserved in mammals (Wolfson and Sabatini 2017), comprises two complexes, namely the mammalian target of rapamycin complex (mTORC) 1 and mTORC2. Via Ras homolog enriched in brain (RHEB), mTORC1 is controlled by nutrients, such as glucose and amino acids (Sancak *et al.* 2010; Jewell and Guan 2013). Although individual amino acids have been shown to have little effect on mTORC1 activity (Dyachok *et al.* 2016), mTORC1 signalling is downregulated by the removal of leucine and arginine (Avruch *et al.* 2009). Further studies defined a set of mTORC1 priming and activating amino acids (Dyachok *et al.* 2016; Meng *et al.* 2020), providing evidence that the amino acids in the cellular microenvironment affect cell proliferation. In mouse blastocysts, the inhibition of the expression of the glutamine transporter solute carrier family 38 member 1 (SLC38A1) was found to block the mTOR-dependent diapause state (Hussein *et al.* 2020). These diapausing embryos had a higher content of free fatty acids and phosphatidylcholine than the non-diapausing control preimplantation blastocysts, but a lower content of triacylglycerol and acylcarnitine (Hussein *et al.* 2020). Similarly, autophagy, the generation of nutrients required for cell survival during starvation, was activated during diapause (Lee *et al.* 2011). Autophagy has been shown to be regulated by mTOR (Kim and Guan 2015). Collectively, this indicates that constituents in the cellular microenvironment play a decisive role in regulating embryonic diapause.

Hormone involvement and molecular mechanisms regulating embryonic diapause and cellular dormancy

Circulating hormones are involved in regulating the uterine microenvironment in diapausing mammals (Paria *et al.* 1998). Neither progesterone nor estrogens altered embryonic metabolism in cultured mouse and rat blastocysts (Weitlauf 1974). However, steroid hormones act on the endometrial cells, which, in turn, secrete a multitude of factors into the uterine lumen (Renfree and Fenelon 2017). Generally, diapause was proposed to be stimulated by the presence or absence of maternal factors in the uterine fluid at the blastocyst stage of development. These factors remain to be identified in many species (Ptak *et al.* 2012). In mouse, mink and tammar wallaby, implantation-related factors have been found to play a decisive role during blastocyst reactivation. Leukaemia inhibitory factor (LIF) was among the first identified diapause regulating factors. In its absence, blastocysts fail to implant and enter a diapause-like state (Nichols *et al.* 1996, 2001; Rosario and Stewart 2016). The administration of LIF to ovariectomised mice results in embryo implantation (Chen *et al.* 2000). The muscle segment homeobox (MSX) genes *MSX1* and *MSX2* are downregulated by *LIF* (Cha *et al.* 2013), and have been suggested to inhibit embryo implantation in mouse, mink, and tammar wallaby. Studies comparing diapausing and reactivated mouse blastocysts reported higher expression of p21 family members in diapausing embryos and higher expression of heparin-binding epidermal growth factor-like growth factor (*HB-EGF*) in reactivated blastocysts (Hamatani *et al.* 2004). *HB-EGF* and erb-b2 receptor tyrosine kinase 4 (*ERBB4*) are also present during the reactivation of mink and tammar wallaby blastocysts (Fenelon *et al.* 2017). In addition, FOXO transcription factors and cyclin-dependent kinase inhibitor 1A (*CDKN1A*) were identified in diapausing mink and tammar wallaby blastocysts, suggesting their role in inhibiting developmental progression (Fenelon *et al.* 2017). A transcriptome analysis of diapausing mink blastocysts identified an upregulation of genes involved in polyamine synthesis after reactivation (Lefèvre *et al.* 2011). The importance of polyamines, and especially the rate-limiting enzyme ornithine decarboxylase 1 (ODC1), for diapause entry and reactivation was also confirmed in mice (Fenelon and Murphy 2017). Although an ODC1 inhibitor had no direct effect on cultured blastocysts, the treatment of pregnant mice with an ODC1-inhibitor caused a diapause-like state in blastocysts (Fenelon and Murphy 2017).

Most recently, the inhibition of Myc and its upstream regulator mTOR was shown to induce a diapause-like state in mouse blastocysts cultured *ex vivo* (Foster *et al.* 2010; Bulut-Karslioglu *et al.* 2016; Scognamiglio *et al.* 2016*b*). A short, reversible diapause-like state of cellular dormancy lasting 18 h was induced in ESCs and mouse blastocysts using 10058-F4, an inhibitor of the transcription factor c-Myc (Scognamiglio *et al.* 2016*b*). Myc is one of four transcription factors capable of causing adult cells to re-differentiate into pluripotent stem cells (Takahashi and Yamanaka 2006), and has been suggested to be involved in oocyte meiotic resumption (Miles *et al.* 2012). This hints towards a role of Myc in controlling the entry into and exit from cellular dormancy. The inhibition of mTORC1 and mTORC2 with INK128 induced a reversible diapause-like state for up to 30 days and ESC dormancy (Bulut-Karslioglu *et al.* 2016). However, a recent report stated that this mTOR inhibitor can only induce a diapause-like state in naïve mouse ESC, and that the process was not linked to cellular translation or inhibition of the mTOR pathway (Sousa *et al.* 2020). In addition, microRNAs (e.g. let-7, which regulates MYC) have been reported as being more abundant during the diapause period

(Chang *et al.* 2009; Cheong *et al.* 2014; Fenelon *et al.* 2014). To date, the identification of the molecular factors regulating diapause has been predominantly based on the association of differential gene expression of embryonic diapause versus reactivation. The biological relevance of differentially expressed genes has only been shown for a small subset of candidates. The reversibly inducible dormancy in ESCs offers a promising approach to further our understanding of pluripotent cell dormancy and the molecular signalling pathways involved (Bulut-Karslioglu *et al.* 2016).

Future directions

The roe deer embryo undergoing diapause has important features to serve as model species in which to investigate the mechanisms driving the pace of embryo development. Maternal factors involved in governing the cell cycle may be elucidated. They may further our understanding of molecular factors in the uterine microenvironment that play a role in stem cell pluripotency (Drews *et al.* 2019; van der Weijden *et al.* 2019*a*, 2019*b*, 2019*c*). The findings may be translatable to other species, including bovine and human.

Because proliferation arrest has been shown to be inducible in non-diapause species (Ptak *et al.* 2012), further findings on the regulation of embryonic diapause may offer novel perspectives. These include insights into the developmental competence of preimplantation embryos, that may enhance our understanding of early embryo mortality in livestock species. As molecular factors inducing dormancy most likely affect the cell cycle by leading to either reduced proliferation or complete cell cycle arrest, the physiological regulation underlying diapause and dormancy may have implications for cell proliferation inhibition to extend pluripotency, induce senescence and to suppress proliferation.

Conflicts of interest

The authors declare no conflicts of interest.

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