The mTOR pathway in reproduction: from gonadal function to developmental coordination

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Abstract

Reproduction depends on many factors, from gamete quality to placenta formation, to fetal development. The mTOR pathway is emerging as a major player that integrates several cellular processes in response to a variety of environmental cues that are relevant in many aspects of reproduction. This review provides a general overview, summarizing the involvement of the two mTOR complexes (mTORC1 and mTORC2) in integrating signaling pathways, sensing environmental status, and managing physiological processes inherent to successful reproductive outcomes and pluripotent stem cell function. As a well-known governor of multiple cellular functions, it is not surprising that mTOR has a key regulatory role in determining cell quiescence or differentiation. In the gonads mTOR helps maintain spermatogonial stem cell and follicle identity and tightly regulates differentiation in both systems to ensure proper gamete production. The mTOR pathway is also known to prevent premature follicle exhaustion, while also controlling the blood–testis barrier in the male gonad. In stem cells mTOR again seems to have a role in controlling both pluripotency and differentiation, mirrored by its *in vivo* roles in the embryo, notably in regulating diapause. Finally, although there are clearly more complex systems intertwined in placental function, mTOR seems to serve as an early checkpoint for development progression and successful implantation.

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Introduction

Gamete production involves a series of complex meiotic processes, which occur in parallel with extensive morphological and physiological changes. Following fertilization, the one-cell zygote undergoes mitotic divisions culminating in blastocyst formation, accompanied by constant alterations in metabolism (Gardner & Harvey 2015), signaling pathways, chromatin arrangement and cell fate decisions (Bedzhov *et al.* 2014, Weinberger *et al.* 2016). In the blastocyst, the two first lineages are already segregated into the inner cell mass (ICM) and the trophectoderm (TE). The former will contribute to the embryonic tissues, and the latter, upon implantation, will contribute to the placenta that dynamically supports further development. In all these processes there is a constant need for tightly controlled cell divisions and precise environmental sensing, to ensure the quality of development.

The TOR protein was discovered in 1991 in the budding yeast, *Saccharomyces cerevisiae*, while uncovering the mechanism of action of rapamycin (Heitman *et al.* 1991), an efficient antiproliferative drug. A few years later, the mammalian analog of TOR was isolated and named mammalian Target Of Rapamycin (mTOR; Brown *et al.* 1994, Sabatini

et al. 1994) or mechanistic Target Of Rapamycin. As a major player in cell signaling, mTOR coordinates several fundamental processes such as cytoskeletal organization (Jacinto *et al.* 2004, Sarbassov *et al.* 2004), cell survival (Stambolic *et al.* 1998, Hirose *et al.* 2014), autophagy (Nicklin *et al.* 2009, Koren *et al.* 2010), lipid synthesis (Li *et al.* 2011, Peterson *et al.* 2011), protein synthesis (Ma & Blenis 2009, Laplante & Sabatini 2012) and the cell cycle (Fingar *et al.* 2004, Gao *et al.* 2004, Chatterjee *et al.* 2015), among others (Fig. 1). More importantly, outcomes of these distinct pathways depend on key environmental cues, including the presence/absence of growth factors (Laplante & Sabatini 2012), oxygen/hypoxia (Brugarolas *et al.* 2004, DeYoung *et al.* 2008), energy status (Inoki *et al.* 2003a), nutrients (Kim *et al.* 2002, 2008, Smith *et al.* 2005) and other stressors (Fig. 1). This complex integration of diverse stimuli has an important role in cell homeostasis, proliferation and differentiation. Consequently, mTOR signaling impairment can lead to severe physiological dysfunction at many levels. Considering the global nature of this signaling pathway, this review aims to integrate the role of mTOR in reproduction, providing a brief overview of its importance, from gonads and gametes, to embryos, stem cells and the placenta.

Figure 1 The integrative role of the mTOR pathway on cellular homeostasis. mTORC1 and mTORC2 assemble differently, relying on particular allies to respond to distinct stimuli and accordingly regulate specific cellular outcomes.

mTOR: more complex than just a protein

mTOR is a highly conserved protein that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family. The ability of this serine/threonine kinase to sense different cues and interact with particular cellular targets is dependent on its ability to interact with other proteins, forming two multimeric complexes – mTORC1 and mTORC2. RAPTOR is mandatory for the assembly of the mTORC1 complex (Kim *et al.* 2002, Sancak *et al.* 2007). On the other hand, the assembly of the mTORC2 complex relies on mSIN1 and RICTOR (Sarbassov *et al.* 2004) and on RICTOR association with PROTOR 1/2 (Pearce *et al.* 2007, 2011). Regardless of being assembled with the partners that characterize mTORC1 or mTORC2, the mTOR protein is capable of binding to mLST8, DEPTOR and the Tti1/Tel2 complex (Laplanche & Sabatini 2012).

mTORC1 is sensitive to growth factors, hypoxia, energy status (via AMPK activity) and nutrients. Consequently, mTORC1 activity promotes translation and protein synthesis, mainly through two mTORC1 substrates: ribosomal protein S6 kinases (S6Ks) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1; Ma & Blenis 2009, Laplanche & Sabatini 2012). Furthermore, mTORC1 promotes lipid biosynthesis by modulating the activity of SREBP1/2 (Li *et al.* 2011, Peterson *et al.* 2011) and PPARG (Kim & Chen 2004, Zhang *et al.* 2009). On the other hand, autophagy is negatively regulated by mTORC1 via several mechanisms (Laplanche & Sabatini 2012), namely, by activating the autophagy suppressor DAP1 (Koren *et al.* 2010), and also by regulating TFEB and preventing lysosome biogenesis (Settembre *et al.* 2012). Additionally, mTORC1 regulates glucose metabolism

and mitochondrial function by modulating HIF1A and PGC1A (Brugarolas *et al.* 2003, Cunningham *et al.* 2007, Düvel *et al.* 2010).

Growth factors activate PI3K- or Ras-dependent signaling pathways that, in turn, can regulate mTORC1, leading to TSC1 and TSC2 complex phosphorylation, inactivating its GAP activity (Inoki *et al.* 2002, Manning *et al.* 2002, Tee *et al.* 2003). This triggers the Rheb GTPase, which in its GTP-bound state (Inoki *et al.* 2003b) directly activates mTORC1, via phosphorylation (Inoki *et al.* 2002, Manning *et al.* 2002, Potter *et al.* 2002, Long *et al.* 2005, Sancak *et al.* 2007). On the other hand, AMPK is sensitive to the AMP:ATP ratio, making AMPK a major cellular energy sensor (Shackelford & Shaw 2009, Hardie *et al.* 2012). Accordingly, when this ratio increases, AMPK also activates mTORC1, and other stressors such as DNA damage may also regulate mTORC1 via the regulation of REDD1 (Brugarolas *et al.* 2004, DeYoung *et al.* 2008). Moreover, amino acid availability was shown to regulate mTORC1 similar to its inhibitor rapamycin (Hara *et al.* 1998). Amino acid withdrawal leads to dephosphorylation of direct mTORC1 targets S6K1 and 4EBP1, inhibiting mRNA translation (Hara *et al.* 1998), an effect that is reversed upon amino acid reconstitution in cell culture media. Although the exact mechanism of how amino acid availability activates mTORC1 is still unclear, pathways involving Rag GTPases (Kim *et al.* 2008) or biogenic polyamines have been proposed (Zeng *et al.* 2013, Ray *et al.* 2015). However, it should be noted that not all amino acids display the same efficiency in stimulating mTORC1 (Hara *et al.* 1998, Long *et al.* 2005). Finally, impairment in the transport or the lack of amino acids also affects cell proliferation in an mTOR -dependent

Figure 2 Upstream signals that affect mTOR regulation. Different stimuli, namely, growth factors and hormones, insulin, energy stress (AMP/ATP ratio), hypoxia and nutrient availability, are known to modulate mTORC1 and mTORC2 differently, acting through different signaling cascades.

manner (Smith & Proud 2008, Nicklin *et al.* 2009, González *et al.* 2012) (Fig. 2).

In contrast to mTORC1, mTORC2 is less well understood, although it is known to be activated in a PI3K-dependent fashion and thus is also responsive to growth factors. Furthermore, its main targets are AKT, SGK1 and PKCA, thus regulating cell survival and growth (Stambolic *et al.* 1998, Laplante & Sabatini 2012), ion transport (Potter *et al.* 2002, García-Martínez & Alessi 2008) and cell shape through cytoskeletal reorganization (Jacinto *et al.* 2004, Sarbassov *et al.* 2004). mTORC2 has also an indirect role in metabolism, not only in terms of mitochondrial physiology (Betz *et al.* 2013) but also by being able to control glucose metabolism in AKT-dependent (Hagiwara *et al.* 2012, Roberts *et al.* 2013, Masui *et al.* 2014) and FOXO-dependent (Hagiwara *et al.* 2012, Masui *et al.* 2013) manners. Finally, mTORC2 is also able to modulate the hexosamine biosynthetic pathway (Moloughney *et al.* 2016), thus affecting protein and lipid glycosylation, as well as glutamine metabolism (Boehmer *et al.* 2003, Moloughney *et al.* 2016), both involved in anabolic processes.

It should be noted that both mTOR complexes can intrinsically regulate each other via a feedback mechanism that has not been completely characterized. Of the known mTORC2 targets, AKT is the most intensively studied, followed by mTORC2-mediated serine 473 phosphorylation. Further, AKT targets TSC2, resulting in mTORC1 activation (Laplante & Sabatini 2012). In contrast, mTORC1 can limit mTORC2 activity by stimulating S6K1, that can, in turn, inactivate mTORC2 by direct phosphorylation of mSIN1 (Liu *et al.* 2013) and RICTOR (Julien *et al.* 2010, Fig. 2).

(Sper)mTOR: mTOR activity in testicular function

Infertility is classically defined as the inability to conceive after frequent and unprotected sexual intercourse for over a year (WHO 2010). It affects about 10% of couples worldwide (Mascarenhas *et al.* 2012), and approximately 50% of all cases are attributed to the male partner (WHO 2010, Miyamoto *et al.* 2012). During spermatogenesis, spermatogonia undergo a few mitotic divisions and then differentiate into primary spermatocytes that undergo meiotic divisions to yield haploid spermatids, which differentiate to mature spermatozoa (Holstein *et al.* 2003, Cheng & Mruk 2012). Unsurprisingly, this process is highly controlled by a wide range of signaling pathways, including mTOR.

The first evidence of a possible impact of mTOR on male fertility was related to the clinical report of an immune-compromised patient due to sirolimus (rapamycin) treatment, showing a dramatic decrease in sperm quality, namely, sperm count (oligospermia), percentage of normal-shaped sperm (teratospermia) and sperm motility (asthenospermia). Importantly, these negative effects of rapamycin treatment were completely reversible (Bererhi *et al.* 2003). In line with these effects on sperm quality, several case reports subsequently showed that rapamycin treatment led to decreased testosterone levels and increased levels of FSH and LH, reinforcing the hypothesis that this mTOR inhibitor could be having an adverse effect on testicular function (Fritsche *et al.* 2004, Lee *et al.* 2005). These patients also had severe oligoasthenospermia and in some cases complete azoospermia (complete lack of sperm in the ejaculate), which, again, was reversible

after the treatment ended (Deutsch *et al.* 2007, Skrzypek & Krause 2007, Zuber *et al.* 2008, Boobes *et al.* 2010).

In agreement with these reports, rats exposed to rapamycin presented lower testicular weight and a decreased seminiferous tubule area. Additionally, in the testes of these animals, only spermatogonia were found but not spermatocytes or spermatids, suggesting that mTOR inhibition blocked spermatogenesis at the spermatogonial level (Rovira *et al.* 2012). Rapamycin-treated rats presented not only smaller and vacuolated seminiferous tubules but also decreased sperm production and blocked spermatogonial cell cycle progression (with increased percentage of spermatogonial cells in G1), suggesting inhibited spermatogonia proliferation that could explain the impaired sperm production, although entry into meiosis might also be affected (Xu *et al.* 2016).

Indeed, in mice, rapamycin decreased the expression of meiotic markers, suggesting that mTOR inhibition maintains spermatogonia in the undifferentiated state (Busada *et al.* 2015, Sahin *et al.* 2018). More importantly, a spermatogonial-specific over-activation of the mTORC1 complex led to testicular development defects, sperm count reduction and increased germ cell apoptosis. In addition, mTORC1 activation induced spermatogonial differentiation, which, combined with increased germ cell apoptosis, resulted in the depletion of the germ cell pool (Hobbs *et al.* 2010, Zhou *et al.* 2015, Wang *et al.* 2016). It is therefore unsurprising that a conditional germ cell mTOR knockout (cKO) resulted in smaller testes with abundant spermatogonia and no apparent spermatocytes, confirming impairment in germ cell differentiation. Interestingly, a subset of undifferentiated spermatogonia in the mTOR cKO mouse could still self-renew, suggesting that mTOR is dispensable for the maintenance of undifferentiated spermatogonia but necessary for differentiation and spermatogenesis (Serra *et al.* 2017). In agreement with this observation, differentiation-prone spermatogonia have elevated mTORC1 activity, when compared to their undifferentiated stem cell counterparts, suggesting that mTORC1 may negatively regulate spermatogonial stem cell self-renewal (Hobbs *et al.* 2015).

It is clear that mTOR is highly involved in spermatogenesis; however, the exact mechanism by which it impacts the spermatogonial pool is still under discussion, as some studies have shown that mTOR modulation impacts spermatogonial proliferation and apoptosis, while others suggest that it is not a major player in self-renewal but is crucial for spermatogonial differentiation. Another important fact to consider is that the various methodological approaches used in different papers resulted in different rates of modulation for each of the mTOR complexes, leading to distinct outcomes. Nonetheless, a germ cell-specific *Rictor* cKO mouse model presented significant spermatogenesis impairment, with some tubules exhibiting the loss of germ cell hierarchy including elongated spermatids

at the basal membrane, while other tubules were germ cell-depleted, probably due to an increase in preleptotene and leptotene spermatocyte apoptosis. Importantly, the loss of *Rictor* caused an upregulation of cell adhesion and migration-associated genes in Sertoli cells, suggesting that mTOR has a role in not only the regulation of spermatogonial proliferation but also the integrity of the blood–testis barrier (BTB; Bai *et al.* 2018).

The BTB is formed by adjacent Sertoli cells at the basement membrane of the seminiferous tubules with intercellular spaces sealed by tight junctions (TJs) at the apical region, while ectoplasmic specialization (ES) junctions, namely, adherens junctions (AJs), provide links not only between Sertoli cells but also between Sertoli and germ cells (Mok *et al.* 2013a). In addition, the basal region of the BTB depends on desmosomes and gap junctions (GJs) for its cohesion (Cheng & Mruk 2012, Mok *et al.* 2013a).

The BTB undergoes cyclic reconstruction during spermatogenesis, to allow the transit of preleptotene spermatocytes into the adluminal region during stages VIII to XI of the testicular epithelial cycle (Mok *et al.* 2013a,b). The mTOR protein is highly expressed at the BTB, suggesting an important role in its modulation, and over-activation by conditional deletion of *Tsc1* led to mislocalization of several proteins involved in testicular junctional complexes (Tanwar *et al.* 2012). Furthermore, phosphorylated pS6, a downstream target of mTORC1, is expressed specifically at late stages VII and XI of the spermatogenic cycle and is co-localized with several proteins of the BTB, namely, ZO-1 (a TJ-associated protein), N-cadherin (an ES protein) and F-actin, in a stage-specific manner (Mok *et al.* 2012, 2015). Importantly, the inhibition of mTORC1 by rapamycin and S6 Knockdown (cKD), not only strengthened the BTB by increasing the expression of TJ-associated proteins but also increased the proportion of F-actin bundle alignments (Mok *et al.* 2012). This suggests that the activation of the mTORC1 complex is necessary to weaken the BTB and allow the transit of preleptotene spermatocytes to the adluminal region of the seminiferous tubules (Mok *et al.* 2012, 2015).

On the other hand, the mTORC2 complex protein, RICTOR is partially co-localized with TJ proteins, and its expression shifts with the seminiferous cycle stage. Although both RICTOR and mTOR are highly expressed at stages V to VI, at stage VII, RICTOR expression starts to decline until it is barely detectable at stages VIII to IX. To note, the high mTOR expression throughout these late stages supports the hypothesis that mTORC2 assembly is required in the first stages of the spermatogenic cycle to maintain the integrity of the BTB but is dispensable and downregulated during stages VIII and IX, when the barrier restructuring takes place (Mok *et al.* 2013b). Interestingly, when *Rictor* was conditionally knocked down in Sertoli cells, there was no downregulation of TJ proteins, but these proteins were mis-localized

from the Sertoli–Sertoli interface. Moreover, F-actin bundles were disorganized, suggesting that mTORC2 assembly is important for the maintenance of a cohesive BTB (Mok *et al.* 2013b). In accordance, *Rictor* cKO mice had severe testicular defects, as they had no seminiferous lumen or elongated spermatids and, more importantly, presented a loss of germ cell hierarchy in the seminiferous epithelium (Dong *et al.* 2015) that could explain the resulting azoospermia. In agreement, Mok *et al.* (2013b) reported that *Rictor* ablation did not change the protein levels of occluding (TJ protein), N-cadherin or actin-related protein complex 2 (Arpc2) but altered their localization, weakening the BTB (Dong *et al.* 2015). Interestingly, *mTOR* cKO in Sertoli cells produced a similar disorganization of the seminiferous epithelium, which seemed to be due to a redistribution of the BTB gap junction protein 1 (GAP-1) (Boyer *et al.* 2016). Thus, the mTOR pathway is a major regulator of the BTB permeability, given that a timely activation of both mTORC1 and 2 is required to maintain a cohesive and flexible barrier.

In summary, the mTOR pathway is involved in spermatogenesis, by regulating spermatogonial germ cell proliferation and apoptosis and by tightly controlling BTB function. However, further research is still needed to evaluate the effect, if any, of mTOR on the differentiated functional sperm cell (Silva *et al.* 2019). It is intriguing to speculate that an effect of mTOR on male fertility may be in some way related to energy sensing and nutrient availability, which could condition male investment in gamete production, depending on environmental cues. Considering that after ejaculation sperm must travel through the female reproductive tract to fertilize the oocyte and that the mTOR pathway is regulated by hypoxia and extracellular pH, it would be interesting to assess how the female reproductive tract environment could modulate sperm function.

mTOR in female reproduction: keep it slow or speed it up

Following the migration and establishment of primordial germ cells (PGCs) in the developing ovary, a process named primordial follicle assembly takes place, in which PGCs are surrounded by somatic cells (namely, squamous granulosa cells (GCs)). When squamous GCs assume a cuboid morphology, the primary follicle is formed. The GCs start to proliferate and recruit steroidogenic cells (theca cells), leading to the secondary follicle formation. The antral follicle is characterized by the appearance of a cavity filled with follicular fluid (antrum), which divides two populations of GCs: the more external mural GCs and the cumulus GCs. This follicle growth will give rise to the pre-ovulatory Graafian follicle. Female meiosis is initiated during fetal development, and the oocytes enclosed in primordial and primary follicles are arrested

in diplotene of prophase I, also known as the germinal vesicle (GV) stage. After primary follicle activation, GV envelope breaks down (GVBD) and meiosis progresses until it is once again arrested at metaphase II. It is at this stage that oocytes are ovulated in most mammals, and meiosis is completed only if fertilization takes place.

The PI3K/AKT/mTOR pathway regulates events that involve follicle quiescence, activation, development, proper oocyte maturation and ovulation. Because this pathway responds to several growth factors and hormones, it is not surprising that it is sensitive to LH signaling in theca cells (Palaniappan & Menon 2010, 2012) and FSH signaling in GCs (Kayampilly & Menon 2007, Sirotkin *et al.* 2015). Several studies suggest that mTOR is a positive regulator of GC proliferation (Kayampilly & Menon 2007, Yaba *et al.* 2008, Yu *et al.* 2011); follicular survival, activation and development (Cheng *et al.* 2015, Zhang *et al.* 2017a, Li & Liu 2018); and translation of maternal transcripts (Chen *et al.* 2013). Furthermore, its deregulation is associated with follicular atresia and ovarian function impairment (Choi *et al.* 2014, Chen *et al.* 2015a). For those outcomes, communication between oocytes and GCs is of utmost importance. In oocytes the disruption of the negative regulators of mTORC1 TSC1 (Adhikari *et al.* 2010) or TSC2 (Adhikari *et al.* 2009) led to the over-activation of primordial follicles and infertility. This reinforces the notion that mTOR activity through oocyte–GC communication is crucial to maintain primary follicles in a quiescent state. On the other hand, *Rictor* cKO on oocytes showed that mTORC2 activity seems to be mostly associated with follicle survival in an oocyte-dependent fashion (Chen *et al.* 2015a). Moreover, the fact that *mTOR* cKO in primordial oocytes causes a shift in GCs morphology to an immature Sertoli cell-like phenotype, including the expression of genes characteristic of this testicular cell type (Guo *et al.* 2018), suggests that the communication between oocytes and the surrounding GCs is crucial to sustain cellular identity, although not much is known about how exactly mTOR is regulating this.

Given these results, mTOR has emerged as a putative target to ameliorate either fertility dysfunction related to premature follicle pool loss or exhaustion of the oocyte pool, and therefore in modulating aging-specific effects. Thus, several rodent models of premature ovarian failure (POF) and primary ovarian insufficiency (POI) conditions, characterized by premature follicle depletion, revealed that mTOR deregulation led to anomalous primary follicle activation (Adhikari *et al.* 2009, 2010, 2013, Sherman *et al.* 2014). Furthermore, rapamycin restricts the transition from primordial to developing follicles in rodent models, reducing follicular atresia and extending ovarian lifespan by downregulating mTOR activity and increasing SIRT activity, similar to caloric restriction (Luo *et al.* 2013, Zhang *et al.* 2013). However, although prolonged rapamycin exposure delays follicle pool

exhaustion, it has detrimental consequences on murine ovarian function and thus on the reproductive cycle, which may be overcome by transient treatments of rapamycin (Dou *et al.* 2017), although possible persistent side effects require further attention.

Furthermore, in terms of putative effects on gametogenesis, it is important to mention that mTOR disruption has been reported to cause errors during meiotic and mitotic chromosome disjunction (Lee *et al.* 2012, Yu *et al.* 2012, Dou *et al.* 2017). In interphase GCs mTOR has a predominant cytoplasmic distribution (as is the case for its phosphorylated form) but is also found in the nuclei. In contrast, during prophase, its localization shifts to the vicinity of chromosomes and becomes more intense in the spindle during metaphase and cytokinesis (Kogasaka *et al.* 2013). Interestingly, RAPTOR, part of mTORC1, co-localizes with mTOR in all these phases of the mitotic process (Yu *et al.* 2011, Kogasaka *et al.* 2013), while mTORC2-specific RICTOR seems to be dissociated from mTOR throughout mitosis (Kogasaka *et al.* 2013). This indicates that mTORC1 and mTORC2 are differentially active, not only during mitosis in GCs but also during meiosis in oocytes. In mature metaphase II oocytes mTOR localization at the spindle (Lee *et al.* 2012, Kogasaka *et al.* 2013) may be related to its repressive effect on autophagy, given that low levels of autophagy are observed in oocytes, increasing dramatically upon fertilization (Tsukamoto *et al.* 2008). Despite all the correlative evidence concerning mTOR localization during meiosis, it is still unknown if the effects are directly related to mTOR function *in situ* or are a consequence of its activity in previous phases of oocyte development.

mTOR and early embryo development

After fertilization the totipotent zygote undergoes mitosis, and the 8- to 16-blastomere embryo experiences a process known as compaction (Ziomek & Johnson 1980, Fierro-González *et al.* 2013). Accordingly, an outer layer of polarized and non-polarized blastomeres is defined, surrounded by more internal cells containing non-polar blastomeres (Yamanaka *et al.* 2010, McDole *et al.* 2011). In the 32-blastomere-compacted morula, there is evidence that cells are already more committed to one of the two first embryo cell lineages: the outer cells will give rise to the trophoctoderm (TE), and the inner cells to the pluripotent ICM (McDole *et al.* 2011, Goolam *et al.* 2016, Posfai *et al.* 2017). As development proceeds, pluripotency genes are gradually restricted to internal non-polarized cells via TEAD4 activity (Yagi *et al.* 2007, Nishioka *et al.* 2008). Thus, TE cells will be specified by *Cdx2* (Nishioka *et al.* 2009, Schrodde *et al.* 2013) and *Gata3* (Ralston *et al.* 2010, Schrodde *et al.* 2013) expression, whereas the ICM will be specified by the expression of *Oct4*, also known as *Pou5f1* (Nichols *et al.* 1998, Nichols & Smith 2012), *Nanog* (Chambers

et al. 2003, Mitsui *et al.* 2003) and *Sox2* (Avilion *et al.* 2003).

Within the compacted morula a cavity full of fluid (the blastocoel) forms, giving rise to the blastocyst, in which TE cells are arranged side to side in a more external layer of the embryo, surrounding both the ICM and the blastocoel (Bedzhov *et al.* 2014). The transition from early to late blastocyst comprises the spatial segregation of the epiblast (EPI) composed of pluripotent stem cells (PSCs) – also known as embryonic stem cells (ESCs) – and the hypoblast composed of primitive endoderm (PrE) cells, allocating PrE cells between the EPI and blastocoel (Yamanaka *et al.* 2010, Leung & Zernicka-Goetz 2015). All the three cell types of the pre-implantation blastocyst have different developmental destinations. In the conceptus, the EPI cells will originate the fetus, while the PrE will form the yolk sac, and the TE cells will contribute to the placenta (Schrodde *et al.* 2013, Leung & Zernicka-Goetz 2015).

In the mouse homozygous mutations for mTOR are lethal, and blastocysts fail to develop, displaying proliferation flaws of both embryonic and extraembryonic tissues (Murakami *et al.* 2004). Moreover, proliferation of both ICM and trophoctoderm cells from these embryos was impaired *in vitro*, which was also observed in mutants lacking amino acid residues on the mTOR C terminus, critical for its kinase activity (Murakami *et al.* 2004).

To better understand the role of the mTOR pathway in both embryo development and in ESCs, studies have targeted the specific binding partners and upstream regulators of mTOR complexes. Thus, the lack of PI3K or Akt family members or subunits, both upstream regulators of mTOR activity, resulted in lethality or developmental defects (Peng *et al.* 2003, Tschopp *et al.* 2005, Yang *et al.* 2005, Foukas *et al.* 2006, Zhou *et al.* 2011, Yoshioka *et al.* 2012). Mutants of the direct regulators of mTORC1, *Tsc1* (Kobayashi *et al.* 2001), *Tsc2* (Rennebeck *et al.* 1998) and *Rheb* (Goorden *et al.* 2011) died approximately at the same developmental time window, with ectodermal and mesodermal lineage development defects. Interestingly, mutants of *Raptor* (mTORC1) become non-viable at the same developmental time as that of mTOR mutants, while *Rictor* (mTORC2) mutants die later due to fetal cardiovascular and nervous system abnormalities (Guertin *et al.* 2006, Shiota *et al.* 2006), indicating that each complex is involved in different developmental processes.

mTOR was also identified as a master regulator of embryonic diapause (ED – Box 1; Bulut-Karslioglu *et al.* 2016). In fact, culturing embryos in the presence of a pharmacological mTOR inhibitor arrested embryo development for 9–12 days at the blastocyst stage, an effect that was reversible upon the inhibitor removal. Moreover, this work was extended to PSCs, inducing a novel state of paused pluripotency in which cells presented a hypotranscriptome, proliferating less in

Box 1: Embryonic Diapause

Diapause is a very conserved phenomenon across nature, from plants to mammals (MacRae 2010), though it does not happen equally in all living systems. ED is achieved when embryo development is very slow or even temporarily arrested (Fenelon & Renfree 2018). Two forms of ED are currently known: obligatory or facultative. Obligatory diapause is species-specific and happens at a well-defined stage of development or in response to environmental stimuli such as photoperiod (Renfree & Fenelon 2017). It occurs in some bats, bears and marsupials (Fenelon *et al.* 2014). Facultative diapause is a consequence of adverse conditions such as lactation or metabolic stress (e.g. starvation), when further embryo development could be risky (Lopes *et al.* 2004). This seems to be a very sophisticated mechanism to ensure that proper conditions for development to occur are available, optimizing offspring survival.

Facultative ED is frequent in some mammals such as mice. The hormonal regulation during lactation makes the uterus a hostile environment for implantation (Renfree & Fenelon 2017); however because female mice can mate instantaneously after giving birth, ED is a strategic mechanism to guarantee the survival of the next generation without jeopardizing the current litter. Consequently, embryos develop until the blastocyst stage, and when there is contact with the uterine wall (apposition), they become dormant. During ED the rate of proliferation and growth becomes slower, accompanied by a decrease in DNA, RNA and protein synthesis. Furthermore, a hypometabolic state and an improvement of stress resistance are established (MacRae 2010, Fenelon *et al.* 2014). As soon as the proper conditions arise or upon estrogen administration, development is resumed and embryo implantation quickly takes place (Renfree & Fenelon 2017). Thus, communication between the embryo and the uterine tissue appears to be critical in regulating diapause (Nichols *et al.* 2001, Fenelon *et al.* 2014).

The role of ovarian hormones on endometrial receptivity is clear, given that embryos from ovariectomized mice cannot implant and that estrogen and progesterone are important for uterine receptivity (Chen *et al.* 2000). Furthermore, estrogen is also essential for uterine LIF expression, which, in turn, is fundamental for implantation (Stewart *et al.* 1992, Chen *et al.* 2000). Female LIF-deficient mice generate viable embryos that cannot implant unless they are transferred to a pseudo-pregnant recipient (Stewart *et al.* 1992), and diapause embryos lacking part of the LIF receptor heterodimer are unable to develop (Nichols *et al.* 2001) demonstrating the importance of LIF in this process.

the presence of the inhibitor. The pausing effects were reversible without any negative impact on pluripotency. Earlier the same year the myelocytomatosis oncogene (*Myc*) had already been suggested as a central player promoting ED, since transcriptional factor family members are under-expressed in paused embryos and KO or pharmacological inhibition of *Myc* members in mouse embryos, and mESCs also lead to a reversible diapause-like state in both pluripotent cells and embryos (Scognamiglio *et al.* 2016). However, this stage was sustained for only a few hours in culture, pinpointing that an upstream signal would be a best candidate to control developmental pausing, which is plausible given that *Myc* acts downstream of mTOR (Masui *et al.* 2013).

Considering the high plasticity of the embryo, the mTOR pathway may be somewhat redundant, and compensatory mechanisms could assure viability until the embryo develops to the blastocyst stage, where mTOR activity is, in fact, crucial for further development. Considering that the KO of mTOR regulators and binding partners display different embryonic phenotypes, it is not clear if the role of mTOR at this level is solely dependent on its kinase activity, as suggested by Murakami *et al.* (2004), or if it regulates cellular processes during earlier embryonic events that may be required for further blastocyst development (e.g. epigenetic alterations).

mTOR and pluripotency regulation

Pluripotency is a transient state during embryo development, but different pluripotent states can be recapitulated *in vitro* with defined media formulations (Nichols & Smith 2012, De Los Angeles *et al.* 2015, Weinberger *et al.* 2016), allowing for the study of pluripotency regulation *in vitro* and comparisons to *in vivo* counterparts (Box 2). Changes in pluripotency

and cell fate commitment involve not only metabolic and chromatin remodeling (Boroviak *et al.* 2014, Zyllicz *et al.* 2015, Mathieu & Ruohola-Baker 2017) but also signaling pathways (Illich *et al.* 2016, Weinberger *et al.* 2016).

Serum/LIF cultured PSCs represent a transient state between the naive and the primed state, since cells in this culture system are highly heterogeneous. Although the core pluripotency transcription factors are expressed, genes involved in naive cell self-renewal are heterogeneously expressed in serum/LIF cells (Chambers *et al.* 2007, Niwa *et al.* 2009), becoming more vulnerable to differentiation. Yet cells that downregulate these genes also seem to be able to re-express them (Chambers *et al.* 2007) and therefore are not completely committed.

Pluripotent mouse embryonic stem cells (mESC) were derived from the ICM of diapause blastocysts and cultured *in vitro* for the first time in 1981 (Evans & Kaufman 1981, Martin 1981). These cells share many features with the epiblast (EPI) cells of the pre-implantation embryo (Boroviak *et al.* 2014), and they were later tagged as “naive” PSCs. There is another pluripotent state, described as the “primed” state, involving mEpiSCs, which resemble the post-implantation EPI cells of the embryo from where they were derived (Brons *et al.* 2007, Tesar *et al.* 2007, Nichols & Smith 2012, Boroviak *et al.* 2014).

Stem cells can undergo an undefined number of divisions in culture, always maintaining the stem cell pool (self-renewal) without losing their ability to differentiate into specific lineages according to their potential (De Los Angeles *et al.* 2015). PSCs can differentiate into cells from the three germ layers, and this potential can be assessed using teratoma and chimera generation assays, which in theory should give rise to characteristic mesoderm, endoderm and ectoderm cell types

Box 2: Pluripotency Regulation

Naive mESCs resemble ICM cells and express *Oct4*, *Sox2* and *Nanog*, which are core pluripotency establishment and maintenance genes, acting through positive-feedback loops that promote their own expression and suppressing the expression of genes that trigger differentiation (Young 2011).

Other genes such as *Klf4*, *Klf2*, *Esrrb*, *Tfcp2l1*, *Tbx3* and *Gbx2* are involved in self-renewal and naive pluripotency preservation (Tai & Ying 2013, Boroviak *et al.* 2014, Weinberger *et al.* 2016), and in some cases their expression is promoted by LIF signaling (Niwa *et al.* 2009, Tai & Ying 2013, Martello *et al.* 2013, Ye *et al.* 2013) and the activity of the GSK3B inhibitor CHIR (also known as CHIR99021) (Wray *et al.* 2011, Martello *et al.* 2012). Moreover, using PD03, a MAPK/Erk inhibitor also enhances naive state propagation avoiding Erk-mediated chromatin modulation to promote the transcription of lineage-commitment genes (Tee *et al.* 2014).

mESC culture conditions have been improving throughout the years with the development of a medium supplemented with LIF and the two specific inhibitors noted earlier. This 2i media is sufficient to maintain naive pluripotency and self-renewal. Even though LIF signaling is dispensable for naive cell maintenance, its presence is beneficial for proliferation (Ying *et al.* 2008). On the other hand, mEpiSCs downregulate the expression of many of the genes from the pluripotency circuitry controlling naive cells pluripotency, such as *Gbx2*, *Tbx3*, *Esrrb*, *Rex1* (Weinberger *et al.* 2016), while maintaining the expression of *Oct4* and *Sox2* (Tesar *et al.* 2007, Guo *et al.* 2009). At the same time, primed cells heterogeneously upregulate the expression of lineage-commitment genes, such as *T-brachyury* and *Fgf5* (Tesar *et al.* 2007, Guo *et al.* 2009). Naive and primed cells differ not only in their developmental origin. EpiSCs, as mentioned, can be derived from the post-implantation embryo and can also be obtained by the differentiation of mESC by culturing cells in the presence of Fgf2/Activin A (Guo *et al.* 2009, Weinberger *et al.* 2016). Interestingly, mEpiSCs can differentiate into cells of the three germ layers in embryonic bodies and teratoma assays but inefficiently contribute toward chimeras (Brons *et al.* 2007) when injected in the ICM of pre-implantation embryos, probably due to their developmental incompatibility with the ICM of the pre-implantation embryo.

(Bradley *et al.* 1984, Huang *et al.* 2012, De Los Angeles *et al.* 2015).

In vivo the mTOR pathway is known to be involved in suppressing mesendoderm commitment (McLean *et al.* 2007, Zhou *et al.* 2009, Yu *et al.* 2015, Jung *et al.* 2016). While mTORC1 disruption appears to trigger early differentiation, mTORC2 seems to be important during later stages, rather than in early lineage priming (Zhao *et al.* 2014, Lu *et al.* 2017, Zheng *et al.* 2017a,b). mTOR activity has also been reported to enhance ectoderm differentiation (Freund *et al.* 2008, Jung *et al.* 2016), which could possibly be a consequence of preventing cell differentiation toward other germ layers.

In cultured ESCs mTOR has been associated with self-renewal and pluripotency maintenance while preventing differentiation (Zhou *et al.* 2009, Ryu and Han 2011, Betschinger *et al.* 2013, Cho *et al.* 2014, Jung *et al.* 2016). In accordance, rapamycin, which targets mTORC1, can disrupt pluripotency by increasing differentiation rates in mesoderm/endoderm through a transient suppression of this complex (Zhou *et al.* 2009, Lu *et al.* 2017). However, this effect may not be solely mediated by mTORC1, given that prolonged exposure to rapamycin also disrupts mTORC2 (Sarbassov *et al.* 2006), raising the question of which mTOR complex plays the more crucial role in pluripotency. In contrast, other reports show that phosphorylated levels of S6K1 (a direct target of mTORC1) are reduced in hESCs when compared to differentiated cells (Easley *et al.* 2010), as are overall translation rates (Sampath *et al.* 2008, Easley *et al.* 2010). This may be closely associated with the balance between pluripotency and the translation of differentiation factors. Moreover, the mTOR-driven increase of S6K1 activity induces differentiation (Easley *et al.* 2010) and transient suppression of mTORC1 activity, with rapamycin enhancing the efficiency of pluripotency induction (Wang

et al. 2013), indicating that mTORC1 inhibition favors both pluripotency maintenance and induction. Furthermore, the expression of DEPTOR, an intrinsically negative regulator of both mTORC1 and mTORC2, is increased in ESCs and downregulated during differentiation (Agrawal *et al.* 2014), modulating pluripotency by stabilizing *Oct4*, *Nanog* and *Zfp42* expression.

mTOR activity has also been linked to glucose metabolism regulation downstream of LIN28, facilitating the transition from a naive to a primed pluripotency state by supporting high glycolysis flux and nucleotide metabolism (Zhang *et al.* 2016). Studies using other cell types revealed that mTORC1 is involved in the regulation of nucleotide synthesis (Düvel *et al.* 2010, Ben-Sahra *et al.* 2016) by controlling the expression of pentose phosphate pathway-related genes (Düvel *et al.* 2010) and glycolysis-related gene expression mediated by HIF1A (Li *et al.* 2014, Saxton & Sabatini 2017). In cancer cells, mTORC2 indirectly regulates c-MYC availability by promoting its mRNA translation (Masui *et al.* 2013) and c-MYC is a well-known key regulator, not only of metabolic reprogramming of cancer cells but also of PSC metabolism. Likewise, AKT activation in a mTORC2-dependent fashion can also modulate glucose metabolism (Deprez *et al.* 1997, Hagiwara *et al.* 2012, Roberts *et al.* 2013, Masui *et al.* 2014), thus linking energy metabolism to biosynthetic processes relevant for differentiation. Considering the metabolic resemblance between PSCs and cancer cells, it would be interesting to determine the relevance of mTOR in the regulation of the metabolic pathways relevant in PSC identity. However, although there is some literature on mTOR and pluripotency, the findings are inconsistent and mechanisms involved in regulating the expression of the pluripotency or differentiation-related networks are poorly understood.

Figure 3 mTOR in the reproductive cycle. The impact of the mTOR pathway on gamete production, embryo and placenta development and pluripotent stem cell maintenance and differentiation.

mTOR and placenta establishment and function

The importance of nutrients such as amino acids, as well as oxygen, on embryo metabolism and redox state is clear. By promoting survival and morphological changes, adaptation to stress, regulating epigenetic and transcriptional events, signaling and biosynthetic pathways, these nutrients are able to modulate development potential (Bazer *et al.* 2015, Eckert *et al.* 2015, Gardner & Harvey 2015, Puschek *et al.* 2015). Notably maternal metabolic status has a relevant role in supporting initial embryogenesis and implantation and may even affect fetal development and postnatal life (Bazer *et al.* 2015, Eckert *et al.* 2015, Puschek *et al.* 2015).

In the majority of mammalian species, including rodents, ovine and porcine species, the attachment of blastocysts to the uterine luminal epithelium triggers mural TE cell differentiation into primary TE giant cells (TGCs), which in turn are able to invade the maternal tissue to reach the endometrial stroma, initiating placentation. mTOR has been shown to regulate trophoblast cell outgrowth in the conceptuses (the embryonic/fetal and extraembryonic tissues) of these species in an amino acid- and glucose-dependent manner (Gwatkin 1966, Murakami *et al.* 2004, Bazer *et al.* 2012, González *et al.* 2012, Kim *et al.* 2013, Zeng *et al.* 2013, Wang *et al.* 2015), as well as cell migration (Correia-Branco *et al.* 2018) and metabolic gene expression (Rosario *et al.* 2019). In line with these observations, TE proliferation and migration are blocked by rapamycin *in vitro* (Murakami *et al.* 2004, González *et al.* 2012, Correia-Branco *et al.* 2018), and endometrial invasion is consequently decreased (Knuth *et al.* 2015). Given that amino acids are sensed

by mTORC1 (Hara *et al.* 1998) and that rapamycin targets this complex, these studies suggest not only the prevailing role of mTORC1 rather than mTORC2 in TE cells but also that this pathway may act as an early checkpoint for development progression and successful implantation. In fact, *Rictor* ablation in mouse embryos results in abnormal placental histology, with smaller and thinner placentas that have an increased number of trophoblast giant cells with anomalous distribution and fewer maternal–fetal interfaces (Shiota *et al.* 2006).

Throughout the implantation process, endometrial nutrient supply is crucial for mTOR activity, together with TE cell crosstalk. Importantly, the availability of glucose, amino acids, fatty acids and other nutrients such as folate increases mTOR activity (Roos *et al.* 2009, Jansson *et al.* 2012, Lager *et al.* 2014, Rosario *et al.* 2017a,b), coupling the expression of placental nutrient transporters as well as their trafficking into the plasma membrane, which is also mTOR dependent (Roos *et al.* 2009, Rosario *et al.* 2011, 2013, 2016, Chen *et al.* 2015b). The physical link between embryonic and maternal tissues is essential to support further development events, given that the placenta is a dynamic organ and an exchange mediator between the mother and the fetus (Burton & Jauniaux 2018). Low-protein diets and caloric restriction in pregnant females result in depressed placental levels of mTOR activation, affecting nutrient transport (Rosario *et al.* 2011, Kavitha *et al.* 2014). Furthermore, placental dysfunctions, fetal growth restriction and preeclampsia can result from aberrant TE invasion and are characterized by placental insufficiency and decreased mTOR activity (Roos *et al.* 2007, Yung *et al.* 2008, Arroyo *et al.* 2009, Chen *et al.* 2015b, Rosario *et al.* 2017b), increased autophagy (Zhang *et al.* 2017b)

and altered nutrient availability, including amino acid transport (Vaughan *et al.* 2017). On the other hand, the placentas of both obese and diabetes mellitus-affected mothers have increased mTOR activation (Gaccioli *et al.* 2013, Jansson *et al.* 2013, Capobianco *et al.* 2016, Villalobos-Labra *et al.* 2017), which is related to the increased expression and abnormal function of nutrient transporters (Gaccioli *et al.* 2013, Lager *et al.* 2014). These effects culminate in fetal overgrowth, which is typical of animal models for obesity. These conditions jeopardize fetal development and are often associated with higher mobility and mortality (Wadhwa *et al.* 2009, Johnsson *et al.* 2015). However, there are unique biochemical and morphology characteristics across placental mammals (Roberts *et al.* 2016), and defining a good animal model for human placental-associated pathologies is difficult. Moreover, most of the human studies are performed on term or spontaneous preterm labor placentas, but considering that it is a highly dynamic organ throughout the gestation period, some of the effects of maternal metabolic dysfunctions on mTOR status and the coupled outcomes are hard to study *in vivo* or to accurately capture using *in vitro* models.

Conclusion and future perspectives

Published literature strongly supports an important role for mTOR function, involving both mTORC1 and mTORC2, in reproductive function, preserving the necessary homeostasis for adjusted gamete production and regulating the stem cell and follicle pool, essential for sperm and oocyte production. It is also important in embryo and placenta development, as well as in PSC maintenance and differentiation (Fig. 3). However, much work is needed to uncover the specific mTOR contributions, but, as discussed here, the key aspects are related to the control of metabolic quiescence and biosynthesis. Given the role of mTOR in energy sensing and nutrient availability, future work on this key pathway could include several parallel perspectives. On the one hand, if disruption of mTOR signaling could result in subfertility, via both quiescence of the stem cell pool and its premature exhaustion, mTOR manipulation might be useful in ovarian and pre-pubertal testicular tissue preservation in oncofertility procedures, avoiding stem cell loss. This could also be extended to both embryo culture and manipulation if diapause can be pharmacologically induced in human embryos, and to control placental development to both ensure implantation and avoid overgrowth in specific cases. Finally, mTOR could also be relevant in manipulating PSC fate, with possible implications for modifications and differentiation toward specific fates.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

BC and JRS developed the original idea. BC and MIS performed literature search and analysis and provided figures. BC wrote the main version of the manuscript, revised by JRS. All authors approved the final manuscript.

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