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Mammalian target of rapamycin (mTOR): a central regulator of male fertility?

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Abstract

Mammalian target of rapamycin (mTOR) is a central regulator of cellular metabolic phenotype and is involved in virtually all aspects of cellular function. It integrates not only nutrient and energy-sensing pathways but also actin cytoskeleton organization, in response to environmental cues including growth factors and cellular energy levels. These events are pivotal for spermatogenesis and determine the reproductive potential of males. Yet, the molecular mechanisms by which mTOR signaling acts in male reproductive system remain a matter of debate. Here, we review the current knowledge on physiological and molecular events mediated by mTOR in testis and testicular cells. In recent years, mTOR inhibition has been explored as a prime strategy to develop novel therapeutic approaches to treat cancer, cardiovascular disease, autoimmunity, and metabolic disorders. However, the physiological consequences of mTOR dysregulation and inhibition to male reproductive potential are still not fully understood. Compelling evidence suggests that mTOR is an arising regulator of male fertility and better understanding of this atypical protein kinase coordinated action in testis will provide insightful information concerning its biological significance in other tissues/organs. We also discuss why a new generation of mTOR inhibitors aiming to be used in clinical practice may also need to include an integrative view on the effects in male reproductive system.

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Introduction

Biological homeostasis depends on the balance among cell growth, proliferation, and death. These processes are highly coordinated and regulated by several factors, including growth factors, hormones, nutrients, and many others. Among the several signaling pathways that integrate those signals, the network of the mammalian Target of Rapamycin (mTOR) kinase has emerged as a central regulator (for review see Laplante & Sabatini, 2012a,b). Indeed, mTOR regulates the signaling pathway that mediates energy supply and protein synthesis as well as many other events related to accumulation of biomass or actin cytoskeleton organization. In order to fulfill its function as a central signal transducer, mTOR interplays with different signaling pathways which thus add onto the complexity in studying its biological significance. TOR is a conserved large Ser/Thr protein kinase of approximately 290 kDa, which associates with various other proteins and generates two structurally and functionally distinct complexes. Interestingly, both complexes have different sensitivities to TOR inhibitors and mediate different cellular events in response to environmental cues (for review, see Laplante & Sabatini, 2009a,b). However, they form a functionally interactive and connected network to achieve the proper functioning.

mTOR is required for normal development and growth. Experiments in mice have shown that homozygous *mTOR*^{-/-} embryos die shortly after implantation in a manner similar to those starved of amino acids (Gangloff et al., 2004; Martin & Sutherland, 2001; Murakami et al., 2004). In addition, current research highlights that mTOR is a crucial regulator of cellular homeostasis and metabolism, controlling several processes such as amino acid synthesis, glucose metabolism, cytoskeleton organization and many other functions. Metabolism is pivotal to spermatogenesis and thus, determines the fertility of males (for review see Alves et al., 2014; Rato et al., 2012). Spermatogenesis is a complex process that takes place in the testis, specifically across the seminiferous tubule epithelium. The formation of a competent spermatozoon involves the production of a large number of germ cells in testis and several maturation steps that occur in the subsequent ducts. During the entire process, it is vital to establish adequate environments not only for the production of spermatozoa but also for providing a means of transport for spermatozoon during its development (for review see Rato et al., 2010). In the seminiferous tubules, there is a metabolic cooperation established between the somatic Sertoli cells and the developing germ cells. The latter including primary spermatocytes and haploid spermatids are localized behind the blood–testis barrier (BTB) and, thus, are dependent on the nutritional and the paracrine support of Sertoli cells, which produce lactate from several energy sources (for review see Rato et al., 2016). In fact, developing germ cells are unable to use glucose (Boussouar & Benahmed, 2004) and rely on the lactate produced from Sertoli cells. Thus, the mechanisms that control those events are pivotal to determine the reproductive potential of the males. During spermatogenesis, the seminiferous epithelium is organized in stages, which vary in number between 14 in rats and 7 in humans, and are related to the different

development stages of germ cells and their association with Sertoli cells (Hess & Renato de Franca, 2008). During those stages, germ cells have to be transported across the seminiferous epithelium and reach the luminal edge of the seminiferous tubule, where spermiation occurs. This event of spermatocyte and spermatid transport is synchronized with restructuring of BTB. Any alteration of those events, which are tightly regulated and precisely coordinated, perturbs spermatogenesis, leading to infertility. The metabolic dependence of germ cells and BTB dynamics during the process of sperm production are the two key events that clearly fit the overall reported and suggested action for mTOR.

Dysregulation of mTOR signaling is associated to several pathologies including cancer and metabolic diseases. Indeed, treatment with mTOR inhibitors has been associated with increased incidence of hyperglycemia and even with increased onset of diabetes (for review see Bhattacharjee *et al.*, 2016; Verges & Cariou, 2015). Impaired insulin secretion and insulin resistance are suggested to mediate those effects. Recently, it was also discussed the therapeutic potential of mTOR inhibitors to treat hypoglycemia and hyperinsulinemia (for review see Shah *et al.*, 2016). Interestingly, it has been widely known that both, insulin and glucose, play pivotal roles during spermatogenesis (Martins *et al.*, 2016; Meneses *et al.*, 2016). Yet, only a handful of studies have been performed to unveil the relevance of mTOR on male reproduction. Here, we present an up-to-date discussion of the latest findings concerning the role of mTOR in male reproduction, with particular emphasis on the clinical findings in men using rapamycin (and its analogs), on testis physiology and the molecular mechanisms by which mTOR signaling may control Sertoli cells function.

Emerging biological significance of the mTOR kinase

Multi-cellular organisms survive and grow in environments in which the availability of nutrients/energy is variable. This is only possible because cells have developed mechanisms for an efficient transition between anabolic and catabolic states. The target of rapamycin (TOR) is a protein that has evolved to integrate this nutritional need. It is a well-conserved serine/threonine kinase that plays an essential role in the signaling network that controls cell growth and metabolism accordingly to environmental and physiological cues (Laplane & Sabatini, 2012a,b). Accordingly to its name, TOR is the target of a molecule named rapamycin (or sirolimus). Rapamycin is an anti-fungal antibiotic produced by *Streptomyces Hygroscopicus* bacteria (Vezina *et al.*, 1975) that has gained attention due to its broad anti-proliferative properties, making this molecule a great tool to study cell growth control. In the early 1990s, yeast genetic screens allowed the identification of TOR as one mediator of the toxic effects of rapamycin (Cafferkey *et al.*, 1993; Heitman *et al.*, 1991; Kunz *et al.*, 1993). Shortly after, the mTOR was purified by biochemical approaches and identified as the physical target of rapamycin in mammals (Brown *et al.*, 1994; Sabatini *et al.*, 1994; Sabers *et al.*, 1995).

mTOR is a serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family. It can form two distinct multiprotein complexes to execute its functions, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), by associating with different binding protein partners (Figure 1) (Guertin & Sabatini, 2007; Zoncu *et al.*, 2011). The different protein compositions of the mTOR complexes confer differences not

only in the sensitivities to rapamycin, in the upstream signals that they integrate, but also in the downstream molecules that they regulate and in the biological processes they control (Laplante & Sabatini, 2009a,b). mTORC1 has five and mTORC2 six known protein components beyond the mTOR catalytic domain (Figure 1). mTORC1 is a homodimer (Takahara *et al.*, 2006; Wang *et al.*, 2006; Yip *et al.*, 2010; Zhang *et al.*, 2006) with two specific components: regulatory-associated protein of mTOR (raptor) (Hara *et al.*, 2002; Kim *et al.*, 2002), proline-rich Akt substrate 40 kDa (PRAS40) (Sancak *et al.*, 2007; Thedieck *et al.*, 2007; Vander Haar *et al.*, 2007; Wang *et al.*, 2007); and shares with mTORC2 the components: DEP-domain-containing mTOR-interacting protein (Deptor) (Peterson *et al.*, 2009), mammalian lethal with sec-13 protein 8 (mLST8, also known as GβL) (Jacinto *et al.*, 2004; Kim *et al.*, 2003), and the Tti1/Tel2 complex (Figure 1) (Kaizuka *et al.*, 2010). The exact role for most mTOR-interacting proteins in mTOR complexes still remains undisclosed. Previous studies have characterized PRAS40 and Deptor as different negative regulators of mTORC1 (Peterson *et al.*, 2009; Sancak *et al.*, 2007; Vander Haar *et al.*, 2007; Wang *et al.*, 2007). Notably, when PRAS40 and Deptor are engaged to mTORC1, the activity of the complex is reduced suggesting that there is an inhibition of mTORC1. It is also proposed that PRAS40 regulates mTORC1 kinase activity by direct inhibition of substrate binding (Figure 1) (Wang *et al.*, 2007). Upon activation, mTOR component of mTORC1 phosphorylates PRAS40 and Deptor, reducing their physical interaction with the complex which further activates mTORC1 signaling (Peterson *et al.*, 2009; Wang *et al.*, 2007). Similar to what is described for mTORC1, Deptor also negatively regulates mTORC2 activity (Laplante & Sabatini, 2009a,b), being so far the only characterized endogenous inhibitor of mTORC2. Previous studies reported that raptor affects mTORC1 by mediating the assembly of the complex, recruiting kinase substrates, such as eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), in sensing amino acids and regulating the complex activity and subcellular localization (Hara *et al.*, 2002; Kim *et al.*, 2002; Sancak *et al.*, 2008). The role of mLST8 in mTORC1 function remains a matter of debate, since knockdown of this protein does not affect *in vivo* activity of mTORC1 (Guertin *et al.*, 2006). However, mLST8 is essential for mTORC2 function, as its deletion severely reduces the stability and the activity of that complex (Guertin *et al.*, 2006). The Tti1/Tel2 complex was found to be important for the stability and assembly of the mTOR complexes, as the knockdown of either Tti1 or Tel2 causes disassembly of mTORC1 and mTORC2 (Kaizuka *et al.*, 2010).

mTORC2 is also composed by rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSIN1) and protein observed with Rictor-1 (Protor-1, also known as PRR5) (Figure 1). Some studies report that Rictor and mSIN1 stabilize each other, forming the structural basis of mTORC2 (Frias *et al.*, 2006; Jacinto *et al.*, 2006). Rictor also interacts with Protor-1. However, the physiological relevance of this interaction still need to be clarified (Thedieck *et al.*, 2007; Woo *et al.*, 2007). Despite the identification of many binding partners on mTOR complexes, more studies will be necessary to enlighten all functions of these proteins in mTOR signaling.

mTOR complexes have different sensitivities to rapamycin, as well as upstream inputs and downstream outputs (Figure 1). mTORC1 integrates inputs from at least five major signals: growth factors, energy status, oxygen, DNA damage, and amino acids; and positively regulates cell growth, cell-cycle progression and proliferation, by promoting many anabolic

processes, such as biosynthesis of proteins, lipids and organelles, and by negatively regulating catabolic processes such as autophagy (Figures 1 and 2). Much of what we currently know about mTORC1 functions arises from the use of the bacterial macrolide rapamycin. Rapamycin forms a gain-of-function complex, binding to the intra-cellular FK506-binding protein of 12 kDa (FKBP12) (Brown *et al.*, 1994; Sabatini *et al.*, 1994), which interacts with the FKBP12-rapamycin binding domain (FRB) of mTOR inhibiting mTORC1 functions (for review, see Guertin & Sabatini, 2007). However, the mechanisms by which the interaction of FKBP12-rapamycin to mTORC1 inhibits its activity are still a matter of debate. The mTORC1 structural integrity may be compromised by rapamycin (Kim *et al.*, 2002; Yip *et al.*, 2010), which may allosterically reduce the specific activity of mTORC1 kinase domain (Brown *et al.*, 1995; Brunn *et al.*, 1997; Burnett *et al.*, 1998).

mTORC2 integrates inputs from growth factors and regulates cell survival and metabolism, beyond cytoskeleton organization. Contrastingly to mTORC1, acute exposure to rapamycin does not perturb mTORC2 activity and FKBP12-rapamycin is not able to physically interact with mTORC2, being originally thought that this mTOR complex was rapamycin-insensitive (Jacinto *et al.*, 2004; Sarbassov *et al.*, 2004). Thus, mTORC1 and mTORC2 were initially described as rapamycin-sensitive and rapamycin-insensitive complexes, respectively. However, this was not shown to be accurate, since long-term treatment with rapamycin inhibits mTORC2 signaling in some, but not all, cell types by suppressing mTORC2 assembly (Phung *et al.*, 2006; Sarbassov *et al.*, 2006). The reason for this cell type-specific mTORC2 assembly sensitivity to rapamycin remains a matter of debate. Many proteins of mTOR complexes have been described, and different sensitivities of mTOR-containing complexes to rapamycin are explored. mTORC1 still remains as the better characterized of the two mTOR complexes. As mentioned, mTORC1 senses at least five major intra- and extracellular signals to regulate cell growth promoting processes. All those inputs regulate mTORC1 activity by modulating the activity of tuberous sclerosis complex (TSC1/2) (Figure 1), with the exception of amino acids that independently act on TSC1/2 (Smith *et al.*, 2005). However, the mechanistic processes responsible for mTORC1 to sense changes in the levels of intracellular amino acids are still unknown.

TSC1/2, a heterodimer that comprises tuberous sclerosis 1 (TSC1, also known as harmatin) and tuberous sclerosis 2 (TSC2, also known as tuberin), is an important sensor and upstream regulator of mTORC1. TSC1/2 plays a role as a GTPase-activating (GAP) protein for the Ras homolog enriched in brain GTPase (Rheb), which in the GTP-bound form directly interacts with mTORC1 and stimulates its kinase activity (Figure 1) (Long *et al.*, 2005). The exact mechanism by which Rheb activates mTORC1 is still unclear. As a Rheb GTPase, TSC1/2 negatively regulates mTORC1 by converting Rheb into its inactive GDP-bound state (Inoki *et al.*, 2003; Tee *et al.*, 2003). Studies suggested a negative regulation of TSC1/2 over mTORC1 since mutations or loss of heterozygosity of TSC1/2 give rise to tuberous sclerosis, a disease associated with the presence of numerous benign tumors that are composed of enlarged and disorganized cells (for review see Crino *et al.*, 2006).

TSC1/2 acts on mTORC1, transmitting upstream signals coming from phosphoinositide 3-kinase (PI3K) and Ras signaling pathways, due to stimulation by growth factors, such as insulin and insulin-like growth factor 1 (IGF1) (Figure 1). Stimulation of these pathways

directly leads to phosphorylation of TSC by the effector kinases protein kinase B (PKB, also known as Akt) (Inoki *et al.*, 2002; Manning *et al.*, 2002; Potter *et al.*, 2002), by extracellular-signal-regulated kinase 1/2 (ERK1/2) (Ma *et al.*, 2005) and by p90 ribosomal S6 kinase 1 (RSK1) (Roux *et al.*, 2004). Phosphorylated Akt (pAkt) (at Ser473) phosphorylates TSC2 and inactivates it, preventing its association with TSC1 and the inhibition of Rheb. The phosphorylation of TSC1/2 leads to its inactivation, allowing the conversion of Rheb into its active GTP-bound state and thus leading to the activation of mTORC1 (Figure 1). Akt is also able to activate mTORC1 in a TSC1/2-independent way by phosphorylating PRAS40, causing its dissociation from mTORC1 and increasing its kinase activity (Sancak *et al.*, 2007; Sini *et al.*, 2010; Vander Haar *et al.*, 2007; Wang *et al.*, 2007).

TSC1/2 is also a target for many stresses, such as low energy and oxygen levels, as well as DNA damage. TSC2 can be phosphorylated by adenosine monophosphate-activated protein kinase (AMPK), which is a vital sensor of intracellular energy status, in response to mild hypoxia (Arsham *et al.*, 2003; Liu *et al.*, 2006) or low energy levels (Inoki *et al.*, 2003). This phosphorylation increases the TSC2 GAP activity toward Rheb, which in turn reduces mTORC1 activation (Figure 1). In low-energy conditions, AMPK can directly phosphorylate Raptor, reducing mTORC1 activity (Gwinn *et al.*, 2008). These studies clearly illustrate that AMPK is a key energetic sensor that regulates mTORC1 activity. Hypoxia also induces the expression of transcriptional regulation of DNA damage response 1 (REDD1), which activates TSC2 function in a still poorly understood mechanism (Figure 1) (Brugarolas *et al.*, 2004; DeYoung *et al.*, 2008; Reiling & Hafen, 2004). DNA damage signals also mediate mTORC1 activity through p53-dependent transcription, which induces the expression of *TSC2* (Feng *et al.*, 2005) and of phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) (Stambolic *et al.*, 2001). The activation of *TSC2* and *PTEN* downregulates the entire PI3K-mTORC1 axis and activates AMPK, which phosphorylates TSC2 (Figure 1) (Budanov & Karin, 2008; Feng *et al.*, 2005).

In contrast to mTORC1, little is known concerning mTORC2 signaling and its up- and downstream regulators. However, the deletion of components from this complex causes early lethality in mice (Guertin *et al.*, 2006). Unfortunately, the absence of specific mTORC2 inhibitors has complicated the study of this mTOR complex. Yet, from what is currently known, mTORC2 signaling is insensitive to nutrients but responsive to growth factors, such as insulin. Although the underlying mechanisms remain poorly understood, one of them suggests that ribosomes are necessary to mTORC2 activation and this complex binds to them in a PI3K-dependent manner (Figure 1) (Zinzalla *et al.*, 2011).

mTOR and cell metabolism: brief overview

As mentioned, mTORC1 positively regulates cell growth and cell-cycle progression and proliferation by (1) promoting anabolic processes, including biosynthesis of proteins, lipids, and organelles; (2) negatively regulating catabolic processes such as autophagy (Figure 2). Biosynthesis of proteins is the best-characterized process controlled by mTORC1. It is a costly energy biologic process that requires huge amounts of ATP and the production of a large number of ribosomes. mTORC1 is responsible for the regulation of this translational machinery activity. It promotes protein synthesis by direct phosphorylation of translational

regulators of eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1) (for review see Ma & Blenis, 2009). The phosphorylation of 4E-BP1 prevents its binding to eIF4E, enabling this cap-binding protein to join the eIF4F complex, which is required for the initiation of cap-dependent translation (Figure 2) (for review, see Richter & Sonenberg, 2005). The phosphorylation of S6K and its activation by mTORC1 leads to an increase in mRNA biogenesis, cap-dependent translation and elongation, and the translation of ribosomal proteins, such as ribosomal protein S6 (Figure 2) (for a review, see Ma & Blenis, 2009).

mTORC1 also regulates the biosynthesis of lipids, which are required for cell growth and proliferation, such as for the assembly of biomembranes (for review see Laplante & Sabatini, 2009a,b). mTORC1 positively regulates the activity of sterol binding regulatory element-binding protein 1 (SREBP1) (Porstmann *et al.*, 2008) and peroxisome proliferator-activated receptor- γ (PPAR γ) (Kim & Chen, 2004). These transcription factors control the expression of genes implicated in fatty acid and cholesterol synthesis (Figure 2). mTORC1 also promotes cell metabolism and ATP production by activating translation, in a 4E-BP-dependent manner, of the alpha subunit of hypoxia-inducible factor (HIF α) (Brugarolas *et al.*, 2003; Duvel *et al.*, 2010; Hudson *et al.*, 2002), which is a positive regulator of many glycolytic genes (Figure 2). A recent study reported that HIF α knockdown resulted in reduced mRNA levels of Glut1 (glucose transporter 1) and Pfkfb (phosphofructokinase) (Duvel *et al.*, 2010). Mitochondrial metabolism and biogenesis are also controlled by mTORC1 (Figure 2). mTORC1 was shown to form a complex with outer-membrane protein B-cell lymphoma-extra-large (Bcl-xl) and voltage-dependent anion-selective channel protein 1 (VDAC1), and this complex inhibition by rapamycin reduced mitochondrial function, being the energy production preferentially enhanced via aerobic glycolysis in detriment of mitochondrial respiration (Ramanathan & Schreiber, 2009). The inhibition of mTORC1 by rapamycin was also reported to decrease mitochondrial membrane potential, oxygen consumption, and cellular ATP levels while also altering the mitochondrial phosphoproteome (Schieke *et al.*, 2006).

As briefly summarized above, mTORC1 promotes cell growth by positively regulating many anabolic processes, but it also promotes growth by negatively regulating catabolic processes, such as autophagy. That is a central process in cells, where intra-cellular components are sequestered within autophagosomes posteriorly degraded by lysosomes, to recycle organelles and protein. The stimulation of mTORC1 reduces this process (for a review, see Codogno & Meijer, 2005) by phosphorylating and suppressing the activity of unc-51-like kinase 1 (ULK1) and autophagy-related gene 13 (ATG13), which are components of a kinase complex required for autophagy (Figure 2) (Ganley *et al.*, 2009; Hosokawa *et al.*, 2009; Jung *et al.*, 2009). Moreover, mTORC1 also regulates death-associated protein 1 (DAP1), which is a suppressor of autophagy (Koren *et al.*, 2010).

Regarding mTORC2, less is known when compared with mTORC1, since the signaling pathways that promote mTORC2 activation are not yet well characterized. However, mTORC2 plays a key role in cell survival-related processes, metabolism, proliferation, and cytoskeleton organization. Akt phosphorylates various effectors, positively regulating cell surveillance, growth, metabolism, and proliferation (for a review, see Manning & Cantley,

2007). For full activation of Akt, it is required its phosphorylation at Thr308 and Ser473. Akt phosphorylation at Thr308 occurs upon growth factors action with phosphatidylinositol-3,4,5-triphosphates (PIP3), which is phosphorylated by PI3K (PIP2->PIP3) by binding to N-terminal pleckstrin homology (PH) domain. Under these conditions, 3-phosphoinositide-dependent protein kinase-1 (PDK1) is also recruited through the PH domain and phosphorylates Akt at Thr308 (Alessi *et al.*, 1997; Stephens *et al.*, 1998). Phosphorylation of Akt at Ser473 is reported to result from the kinase activity of mTORC2 (Sarbasov *et al.*, 2005). The inhibition of Akt following mTORC2 knockdown reduces phosphorylation and subsequently activation of the forkhead box O1/3a (FoxO3a) transcription factors (Guertin *et al.*, 2006; Jacinto *et al.*, 2006), which are involved on controlling the expression of stress resistance, metabolism, cell-cycle arrest, and apoptosis-related genes (for a review, see Calnan & Brunet, 2008). Other Akt targets such as TSC2 and glycogen synthase kinase 3- β (GSK3- β) remain unaffected, suggesting that Akt activity is not completely abolished in cells lacking mTORC2 (Guertin *et al.*, 2006; Jacinto *et al.*, 2006). Besides Akt, mTORC2 controls several members of the AGC subfamily of kinases including, serum- and glucocorticoid-induced protein kinase 1 (SGK1), and protein kinase C- α (PKC- α) (Figure 2). In contrast to Akt, SGK-1 activity is completely blocked by the loss of mTORC2. Since SGK1 also controls FoxO1/3a phosphorylation on similarly residues phosphorylated by Akt, loss of SGK1 activity is probably responsible for the reduction in FoxO1/3a phosphorylation in mTORC2-depleted cells (Guertin *et al.*, 2006). Furthermore, mTORC2 affects the cytoskeleton organization, since various studies have reported that depletion of mTORC2 components affects actin polymerization and cell morphology (Jacinto *et al.*, 2004; Sarbasov *et al.*, 2004). The activation of PKC- α by mTORC2 regulates cell shape in cell type-specific fashion by affecting the actin cytoskeleton (Figure 2) (Jacinto *et al.*, 2004; Sarbasov *et al.*, 2004).

mTOR and male fertility

mTOR controls cellular growth and metabolism in response to nutrients, growth factors, and energy status. As such, it is usually dysregulated in cancer and metabolic disorders. Rapamycin is an allosteric inhibitor of mTOR and in 1999 was approved as immunosuppressant to prevent allograft rejection in kidney transplant recipients under the name of sirolimus, Rapamune ® (for review, see Benjamin *et al.*, 2011). After transplantation, it is important to ensure long-term patient survival but also to offer the recipients an opportunity to achieve and sustain a good quality of life, including normal fertility and pregnancy. In 2003, it was reported the first case of sirolimus-associated infertility from a male renal-transplant recipient (Table 1) (Bererhi *et al.*, 2003). After the failed attempt of pregnancy for his wife, a subsequent sperm analysis has revealed low sperm count and a decrease in motility, vitality, and the percentage of normal sperm. The switch of therapy to calcineurin inhibitors (CNI), another immunosuppressant with recognized safety profile during pregnancy, was followed by a complete normalization of the transplant recipient's sperm parameters. In another study with two treated heart-transplant recipients groups ($n = 66$), one with SRL and other with a CNI-based immunosuppression, a negative impact of sirolimus on sex hormone levels was reported (Kaczmarek *et al.*, 2004). The heart-transplanted recipients treated with SRL presented lower free testosterone levels

and an increase on the levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The negative effect of sirolimus on male sex hormone levels was further confirmed in other studies with renal-transplant recipients, where SRL treatment was associated with lower testosterone (Fritsche *et al.*, 2004; Lee *et al.*, 2005; Tondolo *et al.*, 2005) and higher serum FSH and LH (Table 1) (Fritsche *et al.*, 2004; Lee *et al.*, 2005). Furthermore, it was demonstrated in a retrospective observational study a difference in sperm count (oligozoospermia), motility (asthenozoospermia) and rate of fathered pregnancies, in male kidney-transplant patients who were treated with SRL versus those treated without (Table 1) (Zuber *et al.*, 2008). Similar results were described in a case report where a male heart-lung transplant recipient under SRL treatment also developed oligozoospermia and showed marked improvement on sperm exam when SRL was switched to mycophenolate mofetil (Table 1) (Deutsch *et al.*, 2007). In 2010, Boobes *et al.* (2010) reported their clinical experience with SRL-induced gonadal dysfunction and infertility in both male and female kidney-transplant patients, where two male patients developed severe oligozoospermia and two other had azoospermia (Table 1). These clinically orientated reports provided strong evidence that the use of mTOR inhibitors has a negative impact on male fertility and thus, mTOR signaling may be a regulator of male reproductive potential.

More recently, a few studies were focused to unravel the origin of the problems observed in the reproductive function of males after the use of mTOR inhibitors. Chen *et al.* (2013) evaluated the impact of commonly used immunosuppressants on the male reproductive system of rats in a physiological and clinically relevant manner. The drugs were orally administrated and applied in a proportional manner to the therapeutic used for post-renal transplanted patients. Administration of tacrolimus (FK506) to rats subjected to unilateral nephrectomy (UN) induced mild changes on spermatogenesis, without causing any alteration on body weight gain and testicular development. There was no evidence of testicular injury, although testosterone levels were reduced and elevated levels of LH were noted. In UN rats treated with sirolimus, major histological changes of testicular structure were detected along with severe impairment of testicular development and spermatogenesis, as well as male gonadal dysfunction. Those effects corroborate with previous works showing significant testicular toxicity associated with sirolimus, including sexual hormone dysfunction, seminiferous tubule dystrophy, and spermatogenesis blockade (Rovira *et al.*, 2012). Fortunately, sirolimus-associated testicular toxicity and infertility are potentially reversible (Rovira *et al.*, 2012; Skrzypek & Krause, 2007). In 2007, Skrzypek & Krause (2007) reported a case in which the switch of a 10-year therapy from sirolimus to tacrolimus of a post-renal transplant recipient man who developed azoospermia, allowed the recovery of spermatogenesis and normalized the hormone levels of the patient (Table 1). Although no further studies were performed to confirm and unveil the molecular mechanisms responsible for such outcome, it provides evidence that the deleterious effects of the clinical use of mTOR inhibitors to male reproduction may be reverted.

The mechanisms by which mTOR inhibitors cause gonadal dysfunction and infertility remain largely unknown. The c-kit-encoded transmembrane tyrosine kinase receptor for stem cell factor (SCF or KITL) (c-kit or KIT) is required for normal hematopoiesis, melanogenesis, and gametogenesis (Besmer *et al.*, 1993; Galli *et al.*, 1994; Lyman & Jacobsen, 1998). This SCF/c-kit system is one of the most important hormone-receptor

signaling pathways required for proper development and maturation of functional germ cells and maintenance of fertility (Besmer *et al.*, 1993; Kissel *et al.*, 2000; Loveland & Schlatt, 1997). The c-kit belongs to a family of platelet-derived growth factor (PDGF) hormone receptors that are predominantly expressed in type A spermatogonial cells. SCF is expressed by neighboring Sertoli cells in the seminiferous epithelium (Loveland & Schlatt, 1997), under FSH stimulation, and causes c-kit dimerization and autophosphorylation. SCF/c-kit is involved in various crucial functions in the testis including germ cell migration, cell adhesion, cellular proliferation, and anti-apoptotic actions in the testis (Mauduit *et al.*, 1999). Notably, Feng *et al.* (2000) demonstrated that the SCF/c-kit system uses the rapamycin sensitive PI3K/Akt/p70S6K/cyclin D3 pathway to promote spermatogonial proliferation. It activates the PI3K intracellular signaling pathway in spermatogonia which is a necessary trigger for their entry into meiosis (Blume-Jensen *et al.*, 2000; Kissel *et al.*, 2000). Thus, it suggests that mTOR pathway may also be involved in the control of meiosis in spermatogonia. Moreover, it also supports the hypothesis that sirolimus can arrest spermatogonial cell-cycle progression and growth, together with the fact that high levels of FKBP12 (that binds to both sirolimus and tacrolimus) have been identified in male reproductive tissues (Walensky *et al.*, 1998). This study also showed that recombinant FKBP12 enhanced the curvilinear velocity of immature sperm, suggesting a role for FKBP12 in the initiation of sperm motility. Therefore, this mechanism is also expected to be relevant in the development of gonadal dysfunction. Nevertheless, the gonadal dysfunction may be caused by an upstream effect on the spermatogenic event.

mTOR and testicular cells

The process of germ cell development is under tight control of various signaling pathways, including the PI3k-Akt-mTOR pathway. Notably, several studies reported how the various components of this pathway have sex-specific roles. Foxo1 is specifically expressed in undifferentiated spermatogonia, and the three Foxos together regulate multiple steps of spermatogenesis comprising stem cell maintenance and differentiation (Figure 3) (Goertz *et al.*, 2011; Tarnawa *et al.*, 2013). Pten is also required for spermatogenesis and its inactivation with the germ line-specific Vasa-Cre (Tg(Ddx4-cre)1Dcas) (Gallardo *et al.*, 2007) results in severe defects in spermatogonial maintenance and differentiation, in part through Foxo1 (Goertz *et al.*, 2011). Other study has suggested that mTORC1 acts as a regulator of spermatogenesis and spermatogonial stem cell maintenance (Hobbs *et al.*, 2010). Promyelocytic Leukemia Zinc Finger (PLZF) is expressed by spermatogonial progenitor cells (SPCs) and is needed in a cell autonomous fashion for maintenance of the germ lineage. Gonad hypoplasia has been reported in a male patient with biallelic PLZF loss-of-function (Fischer *et al.*, 2008), suggesting a crucial role for PLZF in germ cell biology. Moreover, Hobbs *et al.* identified in 2010 a role for PLZF-mediated Redd1 expression in inhibiting mTORC1 activation in SPCs (Figure 3).

As discussed herein, Rheb is a small GTPase and a critical component for mTORC1 activation. In 2014, Baker *et al.* studied the role of Rheb in male germ cell line and showed that conditional knockout of Rheb resulted in severe oligoasthenoteratozoospermia, a condition that includes oligozoospermia (low number of sperm), asthenozoospermia (poor sperm movement), and teratozoospermia (abnormal sperm morphology) and male sterility

(Figure 3). This study documented a male infertile phenotype associated with multiple defects in meiotic and post-meiotic stages of spermatogenesis, evidenced by an increase in abnormal sperm morphology. Although Rheb was reported to be dispensable for spermatogonial self-renewal and mitotic proliferation (Hobbs *et al.*, 2010), it seems necessary for spermiogenesis, as Rheb knockout males exhibited severe reduced epididymal sperm numbers, and the few sperm present were immotile and exhibited grossly abnormal head morphology (Figure 3) (Baker *et al.*, 2014). A delay in meiotic progression was also evident, with spermatocytes counts being significantly increased at the age of 2 months (Baker *et al.*, 2014). Another study showed that mTOR is positively correlated with spermatogenesis, through the detection of mTOR expression and its downstream targets p70S6K, rpS6 and 4E-BP1 at different developmental stages. Phosphorylated p70S6K (p-p70S6K), rpS6 (p-rpS6), and 4E-BP1 (p-4E-BP1) levels were reported to be independent and gradually downregulated with age (Xu *et al.*, 2016). It has been reported that upon mTOR inactivation by rapamycin, both *in vitro* and *in vivo*, the number of spermatozoa decreased and spermatogonia proliferation was blocked (Figure 3). The levels of p-p70S6K and p-rpS6 were downregulated, but the levels of p-4E-BP1 did not change, except during treatment with a specific PI3K inhibitor (LY294002). These results lead to the suggestion that mTOR plays an important role in spermatogenesis by regulating p70S6K activation and that 4E-BP1 is either directly or indirectly regulated by PI3K (Xu *et al.*, 2016).

Retinoic acid is also a key regulator of spermatogonial differentiation and has been implicated in the regulation of meiotic initiation via retinoic acid stimulated gene 8 (Stra8) (Koubova *et al.*, 2006). This gene is expressed in type A and type B spermatogonia and preleptotene spermatocytes in adult mouse testes (Zhou *et al.*, 2008). Indeed, Stra8 is required for spermatogenic cells to undergo the morphological changes that define meiotic prophase and for these cells to exhibit the molecular hallmarks of meiotic chromosome cohesion, synapsis, and recombination. Sahin *et al.* (2014) studied the role of mTOR pathway *ex vivo* in adult mouse spermatogenesis using cultured seminiferous tubule and observed a decrease in the expression levels of p-p70S6k, p-4EBP1, and STRA8 in the presence of rapamycin, suggesting that mTOR signaling may have a role in the proliferation and stimulation of meiotic initiation in spermatogonial stem cells. It was also reported that retinoic acid signaling operated via PI3K/Akt/mTOR pathway to induce the efficient translation of mRNAs for c-kit, which are present but not translated in undifferentiated spermatogonia (Busada *et al.*, 2015a,b). As discussed, the ligand of c-kit is SCF, which is expressed by neighboring Sertoli cells in the seminiferous epithelium.

mTOR and sertoli cells: a link in male (in)fertility?

Sertoli cells are highly polarized mesoepithelial cells (Weber *et al.*, 1983) that extend from the basement membrane to the lumen of the seminiferous tubule and control the tubular fluid secretion in the seminiferous epithelium (Alves *et al.*, 2015b; Jesus *et al.*, 2014). The long apical extensions of Sertoli cells are permanently in contact with differentiating germ cells, directing their migration towards the lumen of the seminiferous tubule. The loss of these extensions leads to premature germ cell loss (Tanwar *et al.*, 2011). Sertoli cells have also an extensive network of microtubules organized in a spoke-like pattern that is also required for germ cell migration. Disruption of this microtubular network using microtubule-specific

toxins by genetic alterations also leads to germ cell loss (Correa *et al.*, 2002; Tanwar *et al.*, 2011). Liver Kinase B1 (LKB1) is a known regulator of cell polarity and microtubular assembly (Hezel & Bardeesy, 2008; Mihaylova & Shaw, 2011). LKB1 is activated after forming a heterotrimeric complex to regulate the activity of 14 different kinases, including AMPK (Hezel & Bardeesy, 2008; Shackelford & Shaw, 2009). The LKB1-AMPK signaling cascade negatively regulates mTOR by phosphorylating and activating Rheb-GTPase activity and by phosphorylating and inhibiting Raptor (Mihaylova & Shaw, 2011). Important information concerning the relevance of LKB1-AMPK signaling was obtained from patients with Peutz-Jeghers syndrome. Peutz-Jeghers syndrome is a hereditary autosomal dominant, cancer prone disease (Hezel & Bardeesy, 2008; Jeghers *et al.*, 1949) related to inactivating mutations in the LKB1/Serine Threonine Kinase 11 (LKB1/STK11) gene (Hemminki *et al.*, 1998; Jenne *et al.*, 1998). Patients with Peutz-Jeghers syndrome have increased prevalence of cancers in various organs including reproductive tract cancers, such as testicular cancers (Hezel & Bardeesy, 2008). Moreover, Peutz-Jeghers syndrome patients have defective spermatogenesis and often develop Sertoli cell tumors (Ulbright *et al.*, 2007; Venara *et al.*, 2001; Young *et al.*, 1995). Histological examination of those patient's testes revealed serious loss of germ cells, vacuolated Sertoli cell cytoplasm and a Sertoli cell-only seminiferous tubular phenotype (Figure 4) (Ulbright *et al.*, 2007), suggesting that Sertoli cell functions are compromised in those patients.

Sertoli cells are the only somatic cells present inside the seminiferous tubules of mammalian testes, assuring the necessary microenvironment for normal germ cell development and self-renewal of spermatogonial stem cells (Oatley & Brinster, 2008) and are highly sensitive to toxicants that compromise male fertility (for a review, see Reis *et al.*, 2015). In 2012, Tanwar *et al.* reported that Sertoli cell-specific loss of Lkb1 caused severe defects in spermatogenesis including a Sertoli cell only tubule phenotype. TSC1/2-mTOR signaling is negatively regulated by loss of LKB1 and causes upregulation of mTOR (Shaw *et al.*, 2004). Loss of Lkb1 causes defects in Sertoli cell polarity and testicular junctional complexes by regulating multiple kinases, including AMPK and mTOR (Tanwar *et al.*, 2012). Also the deletion of Tsc1 and Tsc2 phenocopies Lkb1cko mice, demonstrating the significance of mTOR activation in the development of the Lkb1 mutant phenotype (Figure 4). This shows the critical need for homeostatic LKB1-mTOR pathway signaling in testicular physiology and also for understanding the pathogenesis of testicular defects in Peutz-Jeghers syndrome patients (Tanwar *et al.*, 2012).

Spermatogenesis is a very tightly regulated process where developing germ cells have to cross the seminiferous epithelium, from basal to the adluminal compartment, and reach the luminal border at spermiation. This timely translocation of germ cells through seminiferous epithelium is synchronized with a series of cyclic junctional restructuring events at the Sertoli-Sertoli and Sertoli-germ cell interface (Cheng & Mruk, 2010, 2012). These events are accurately regulated and coordinated. Their failure can perturb spermatogenesis, leading to infertility. During spermatogenesis, spermatocytes have to cross a blood-tissue junctional barrier, the BTB. The BTB is located near the basement membrane and is formed by adjacent Sertoli cells. It separates the basal from the adluminal compartment and is one of tightest blood-tissue barriers, due its constitution composed by coexisting tight junction, basal ectoplasmic specialization, and other adhesion junctions structures (Cheng & Mruk,

2012; Wong & Cheng, 2005). The majority of above adhesion junctions are all connected to the actin cytoskeleton, especially basal ectoplasmic specialization. Those junctions possess tightly packed actin filament bundles that lie perpendicular to the Sertoli cell plasma membrane and are sandwiched between cisternae of endoplasmic reticulum and the opposing Sertoli cell plasma membrane. This ultrastructure is what gives to BTB its unusual adhesive strength (Cheng & Mruk, 2010; Mruk *et al.*, 2008) making it an immunological barrier that protects the developing spermatocytes and spermatids from the systemic circulation, preventing the development of immune responses against the haploid germ cells that reside at the adluminal compartment (Fijak *et al.*, 2011; Meinhardt & Hedger, 2011).

There was a longstanding idea about the coexistence of an “old” and a “new” BTB that enclose the spermatocytes transposing the BTB and it was designated the intermediate compartment (Russell, 1977). That was shown in a lanthanum study using electron microscopy (Yan *et al.*, 2008). As different types of junctions at the BTB are attached to actin cytoskeleton, BTB restructuring can be regulated via cyclic reorganization of F-actin network, utilizing different actin-regulating proteins. Accumulating evidence suggests that mTOR is also responsible for the extensive reorganization of F-actin network to assist BTB restructuring during the epithelial cycle of spermatogenesis (Mok *et al.*, 2012, 2013a,b). mTOR and crucial subunits that compose mTORC1 (e.g. raptor) and mTORC2 (e.g. rictor) are localized in the seminiferous epithelium near the basement membrane, which is consistent with their localization at the BTB. Notably, the stage-specific expression of raptor and rictor during the epithelial cycle, with raptor being the highest and rictor at its lowest at late stages of epithelial cycle in rat seminiferous epithelium, suggests that mTORC1 and mTORC2 exert different effects on BTB restructuring (Mok *et al.*, 2012, 2013a,b). These new findings support a novel theory regarding the “yin” and “yang” effects of mTORC1 and mTORC2 signaling on the BTB dynamics and their action in regulating BTB restructuring during the seminiferous epithelial cycle of spermatogenesis (for a review, see Mok *et al.*, 2013a,b). The activated form of rpS6, p-rpS6 is highly expressed at the BTB and co-localized with putative BTB proteins zonula occludens-1 (ZO-1) (adaptor proteins that connect tight junctions to actin cytoskeleton), but restrictive to late stage, coinciding with the time of BTB restructuring to facilitate the transit of preleptotene spermatocytes (Mok *et al.*, 2012). This timely upregulation in the phosphorylated form of rpS6 at the BTB suggests that it may act in the “opening” of the BTB (Figure 4). To confirm this, rpS6 phosphorylation was abolished by inactivating mTORC1 signaling in cultured rat Sertoli cells, either by treatment of cells with rapamycin or by knockdown of rpS6 with RNAi. Both experiments were shown to promote Sertoli cell tight junction barrier by making BTB “tighter”, more actin filaments were found at Sertoli cell–cell interface, after a blockade rpS6 activation or its knockdown (Mok *et al.*, 2012).

Rictor, a key binding protein of mTORC2, was shown to be highly expressed at early stages of the seminiferous epithelial cycle and progressively downregulated at late stages, where high expression of p-rpS6 is observed. This suggests that mTORC2 may be involved in maintaining the BTB integrity during all stages of the epithelial cycle of spermatogenesis, excepting later stages when it is downregulated, concurring with BTB restructuring (Figure 4) (Mok *et al.*, 2013a,b). Following rictor knockdown in Sertoli cells by RNAi, the tight junction proteins and ZO-1 were reallocated from the cell–cell interface and moved into

cytosol (Mok *et al.*, 2013a,b), thereby weakening cell adhesion and leading to Sertoli cell tight junction barrier disruption.

The Sertoli cells are also known as nurse cells since they provide physical and nutritional support for spermatogenesis (Alves *et al.*, 2013; Boussouar & Benahmed, 2004). Indeed, as discussed, the Sertoli cell is responsible for the production of lactate, in a Warburg-like metabolism (for review, see Oliveira *et al.*, 2015a), and other metabolites (Alves *et al.*, 2012) that are then exported to the intratubular fluid to be used by the developing germ cells. Thus, the mechanisms that control Sertoli cell metabolism are pivotal for male reproductive potential. Recently, it was reported that mTOR controls the glycolytic profile of cultured primary human Sertoli cells. Rapamycin-exposed human Sertoli cells increased the consumption of glucose and presented altered mitochondrial bioenergetics, particularly by decreasing complex III levels. It was also suggested that mTOR pathway may control the activity of glucose transporters and rate-limiting glycolytic enzymes. Notably, similar to cancer cells (Nogueira & Hay, 2013), rapamycin treatment also sensitized human Sertoli cells to oxidative stress, particularly to lipid peroxidation (Jesus *et al.*, 2016). Finally, the chronic treatment of human Sertoli cells with rapamycin resulted in a partial inhibition of mTOR phosphorylation at Ser-2448. However, it was reported that during that period, the phosphorylated 4E-BP1 levels, which is a downstream effector, remained unchanged, suggesting its rephosphorylation. This was a first assessment on the effect of rapamycin in isolated human Sertoli cells, but the mechanisms by which mTOR may affect the nutritional support of spermatogenesis remain undisclosed, although it may also be a way by which mTOR controls spermatogenesis. Interestingly, alterations in Sertoli cells physiology and function have been suggested as pivotal for the development of subfertility or infertility associated with metabolic diseases (Alves *et al.*, 2014, 2016a,b; Martins *et al.*, 2015; Oliveira *et al.*, 2015a). Indeed, mTOR has also been reported to be involved in the establishment of metabolic diseases (Laplane & Sabatini, 2012a,b) thus, it would be important to unveil the possible role of mTOR in subfertility or infertility induced by those pathological states.

Concluding remarks

mTOR is proposed to function as a multichannel processor that receives multiple stimuli from distinct signals (e.g. growth factors, nutrients), activating a controlled response through different downstream effectors. The fact that it is active only as part of the two multiprotein complexes, mTORC1 and mTORC2, also increases complexity in studying mTOR functions. The protein–protein interactions that occur during the assembly of those complexes may also be very important to understand the biological role of mTOR. Inhibition of mTOR was shown to enhance aerobic glycolysis and reduce mitochondrial function in leukemic cells (Ramanathan & Schreiber, 2009). Indeed, it was shown that inhibition of mTOR shifts glucose metabolism from mitochondria to lactate production. It was also reported that mTOR/S6K cascade controls cell damage when an excess of nutrients occurs or there is a scarcity of growth factors (Panieri *et al.*, 2010). That occurs in several pathologies that have been associated with arrest of spermatogenesis, including Klinefelter syndrome (Alves *et al.*, 2016a,b), diabetes mellitus (Alves *et al.*, 2015a; Dias *et al.*, 2014), or obesity (Martins *et al.*, 2015; Rato *et al.*, 2014). mTOR inhibition has been investigated as a

key to manage several diseases, including cancer, diabetes mellitus, and its complications (Sataranatarajan *et al.*, 2007), extension of lifespan or controlling age-related health problems (Nacarelli *et al.*, 2015). Ongoing clinical trials indicate that several mTOR inhibitors may be used for the treatment of these and other diseases in a near future, but there are several unanswered questions. It is imperative to fully understand the pathways that regulate mTOR function and the complete functions of this protein. The possible negative feedback loops that may occur in downstream effectors of mTOR signaling must also be cautioned and well-studied. Moreover, mTORC1 and mTORC2 share upstream regulators and signaling, which highlights the need for an integrative analysis of the outcomes.

Oxidative stress and apoptosis also play a key role during spermatogenesis (Aitken *et al.*, 2015; Dias *et al.*, 2013; Oliveira *et al.*, 2015b). mTOR inhibition was shown to alleviate the levels of ROS induced by acrolein, to protect mitochondrial membrane and to increase the Bcl2/Bax ratio in mice male germ cells, illustrating a protection against apoptosis and an involvement in mitochondrial function in these cells (Webber *et al.*, 2014). Nevertheless, the molecular mechanisms by which that occurs remain a matter of debate. *In vivo* inhibition of mTORC1 by rapamycin was also shown to block mice spermatogonial differentiation, resulting in a clear accumulation of undifferentiated spermatogonia (Busada *et al.*, 2015a,b), while retinoic acid treatment, which is required for spermatogonial differentiation and subsequent entry into meiosis, is accompanied by increased phosphorylation of mTOR (Busada *et al.*, 2015a,b). These results suggest a role for mTOR in deciding spermatogonial cells fate.

The actin cytoskeleton dynamics (Tang *et al.*, 2016) and insulin signaling (Alves *et al.*, 2012; Oliveira *et al.*, 2012) are also pivotal for spermatogenesis. Those events are regulated by mTORC2 in several cellular systems and thus, there is an arising number of studies suggesting that mTORC2 may be essential for BTB dynamics to support sperm formation. Yet, there is still much to unveil concerning the action of mTORC2 in those events and its relevance for male fertility. It is also important to note that the rapamycin sensitivity of testicular cells is still unknown. The clinical use of rapamycin and its analogs is gaining much attention, but their exact effect in testis and particularly in the different testicular cells deserves special attention in the future. Studies are needed to explore the mechanism of mTOR action in testicular cells physiology beyond spermatogonial development and sperm formation. There is a consensus that mTOR pathways control key steps of spermatogenesis and thus, disturbances in those pathways lead to abnormalities. Nevertheless, the available information is not conclusive regarding a definitive positive or negative role for mTOR-mediated signaling in male reproductive health. As discussed above, independent studies clearly highlight that both, positive and negative effects, arise from distinct actions over mTOR and the upstream and downstream effectors. For instance, depletion of mTOR upstream suppressors such as PTEN, results in impaired spermatogonial maintenance and differentiation as well as Sertoli cells defects, respectively. On the contrary, loss of mTORC1 activity leads to male sterility in animal model indicating a positive role for mTORC1 in male reproductive health. These apparently conflicting roles for mTOR on male reproduction highlight the complex interaction between the several intervenient of these pathways. Negative and positive feedback must be operating while the assembly of each complex, mTORC1 and mTORC2, may reveal several operators of the network that may

have distinct positive or negative roles according to the signals received. More studies will be needed to confirm and understand the role of mTOR, and its upstream and downstream effectors and suppressors, in male reproduction.

Although it has been shown that mTOR is involved in many physiological processes, its role in male reproduction is still far from being completely unveiled. The synergistic or antagonistic action of mTOR with other proteins essential for spermatogenesis deserves more attention in the years to come. Inhibition of mTOR is currently being actively investigated for the treatment of certain cancers and other diseases and thus, positive health benefits for male reproduction may also arise.

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Figure 1.

mTOR-signaling pathway: mTORC1 and mTORC2 complexes and the key signaling nodes that regulate mTORC1 and mTORC2. Critical inputs regulating mTORC1 include growth factors, DNA damage, energy status, and oxygen. mTORC2 is activated by growth factors and ribosomes in a poorly understood mechanism. Akt: protein kinase B; AMPK: adenosine monophosphate-activated protein kinase; ERK1/2: extracellular-signal-regulated kinase 1/2; Grb2: growth factor receptor-bound protein 2; IRS: insulin receptor substrates; LKB1: Liver Kinase B1; MEK: mitogen-activated protein kinase kinase; PI3K: phosphoinositide 3-kinase; PTEN: phosphatase and tensin homolog; PDK1: 3-phosphoinositide-dependent protein kinase-1; raptor: regulatory-associated protein of mTOR; rictor: rapamycin-insensitive companion of mTOR; REDD1: regulation of DNA damage response 1; RSK1: p90 ribosomal S6 kinase 1; TSC1/2: tuberous sclerosis 1/2; Rheb: Ras homolog enriched in brain GTPase; SOS: Ras-guanine exchange factor. (see color version of this figure at www.informahealthcare.com/bmg).

Figure 2.

Key outputs of the mTORC1 and mTORC2 pathways. mTORC1 regulates many biological processes through phosphorylation of several proteins, positively regulating anabolic processes, such as protein and lipid synthesis and energy metabolism, and negatively regulating catabolic processes such as autophagy. S6K1 and 4E-BP1 are the best-characterized substrates of mTORC1. mTORC2 regulates cell metabolism/survival and the cytoskeleton through the phosphorylation of many AGC kinases including Akt, SGK1, and PKC- α . 4E-BP1: translational regulators eukaryotic translation initiation factor 4E binding protein 1; Akt: protein kinase B; ATG13: autophagy-related gene 13; DAP1: death-associated protein 1; eIF4E: translational regulators eukaryotic translation initiation factor 4E; FoxO3a: forkhead box O1/3a; HIF α : alpha subunit of hypoxia-inducible factor; PKC- α : protein kinase C- α ; PPAR γ : peroxisome proliferator-activated receptor- γ ; S6K1: S6 kinase 1; SGK1: serum- and glucocorticoid-induced protein kinase 1; rpS6: ribosomal protein S6; SREBP1: sterol binding regulatory element-binding protein 1; ULK1: unc-51-like kinase 1. (see color version of this figure at www.informahealthcare.com/bmg).

Figure 3.

Effects of alterations in mTOR signaling pathway on spermatogenesis. Pten inactivation with the germ line-specific Vasa-Cre (Tg(Ddx4-cre)1Dcas) results in severe defects in spermatogonial maintenance and differentiation, in part through Foxo1. It was identified a role for PLZF-mediated Redd1 expression in inhibiting mTORC1 activation in spermatogonial progenitor cells. Conditional knockout of Rheb resulted in severe oligoasthenoteratozoospermia, defects in meiotic and post-meiotic stages of spermatogenesis and decreased epididymal sperm numbers. mTOR inactivation by rapamycin downregulated phosphorylated p70S6K (p-p70S6K) and rpS6 (p-rpS6). Studies, *in vitro* and *in vivo*, reported that upon mTOR inactivation by rapamycin, the number of sperms significantly decreased and spermatogonia proliferation was blocked. SCF/c-kit system also uses the rapamycin sensitive PI3K/Akt/p70S6K/cyclin D3 pathway to promote spermatogonial proliferation. PLZF: Promyelocytic Leukemia Zinc Finger; c-kit: c-kit-encoded transmembrane tyrosine kinase receptor for stem cell factor; PI3K: phosphoinositide 3-kinase; Pten: Phosphatase and tensin homolog; Foxo1: forkhead box O1; Rheb: Ras homolog enriched in brain GTPase; S6K1 or p70S6K: S6 kinase 1; rpS6: ribosomal protein S6; SCF: stem cell factor. (see color version of this figure at www.informahealthcare.com/bmg).

Figure 4.

Role of mTOR complexes in Sertoli cells. Effects on male reproduction resulting from alterations on mTOR signaling: loss of Lkb1 causes severe defects in spermatogenesis including a Sertoli cell only tubule phenotype. The deletion of Tsc1 and Tsc2 causes similar defects. Rapamycin-exposed human Sertoli cells increased the consumption of glucose, presented altered mitochondrial bioenergetics and increased lipid peroxidation. There is a “yin” and “yang” effect of mTORC1 and mTORC2 signaling on the blood–testis barrier (BTB) dynamics in regulating BTB restructuring during the seminiferous epithelial cycle of spermatogenesis. Stage-specific expression of raptor and rictor during the epithelial cycle, with raptor being the highest and rictor at its lowest at stage IX of epithelial cycle (of rat seminiferous epithelium), suggests that mTORC1 and mTORC2 have differential effects on BTB restructuring. Timely upregulation of phosphorylated form of rpS6 at the BTB suggests that rpS6 may act in the “opening” of the BTB. Rictor, a key binding protein of mTORC2, was shown to be highly expressed at early stages of the epithelial cycle and progressively downregulated at subsequent stages. LKB1: Liver Kinase B1; TSC1/2: tuberous sclerosis 1/2; raptor: regulatory-associated protein of mTOR (component of mTORC1); rictor: rapamycin-insensitive companion of mTOR (component of mTORC2); rpS6: ribosomal protein S6. (see color version of this figure at www.informahealthcare.com/bmg).

Clinically orientated studies associating mTOR inhibitors and male reproductive dysfunction.

Table 1

Organ pathology	Number of patients	Treatment	Duration	Reported effects on male reproduction after treatment	Study
Kidney	1	Sirolimus	3 years	Oligozoospermia, asthenozoospermia and teratozoospermia	Bererhi <i>et al.</i> (2003)
Heart	132	Group 1: Sirolimus + MMF/TAC ($n = 66$); Group 2: CNI ($n = 66$)	2 years	The group treated with Sirolimus showed: ↓ free testosterone levels and ↑ LH, FSH	Kaczmarek <i>et al.</i> (2004)
Kidney	56	Group 1: no Sirolimus ($n = 28$); Group 2: Sirolimus ($n = 28$)	n.d.	The group treated with sirolimus showed: ↓ free testosterone levels and ↑ LH, FSH	Fritsche <i>et al.</i> (2004)
Kidney	59	Group 1: CNI ($n = 15$); Group 2: Sirolimus ($n = 15$); Group 3: Sirolimus + CNI ($n = 29$)	3 months	The group with Sirolimus showed the lowest testosterone level	Tondolo <i>et al.</i> (2005)
Kidney	66	Sirolimus	n.d.	↓ Testosterone and ↑ FSH and LH	Lee <i>et al.</i> (2005)
Kidney	1	Sirolimus	n.a. (10 years)	Azoospermia	Skrzypek & Krause (2007)
Heart-Lung	1	Sirolimus	n.a. (3,3 years)	Oligozoospermia	Deutsch <i>et al.</i> (2007)
Kidney	89	Group 1: Sirolimus throughout the post-transplant period ($n = 19$); Group 2: no Sirolimus ($n = 67$); Group 3: intermittent Sirolimus ($n = 30$)	n.d.	Group 1 presented oligozoospermia and asthenozoospermia	Zuber <i>et al.</i> (2008)
Kidney	4	Sirolimus	5–12 months	Two patients developed severe oligozoospermia and the other two had azoospermia	Boobes <i>et al.</i> (2010)

n.d.: not defined; n.a.: not applicable.