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mTOR pathway is a novel regulator of male fertility
Tito Teles Jesus

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List of Publications

Publication included in this thesis

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2. **Jesus TT**, Oliveira PF, Sousa M, Cheng CY, Alves MG (2017) Mammalian target of rapamycin (mTOR): a central regulator of male fertility? *Critical Reviews in Biochemistry and Molecular Biology*. 52(3):235-253. (DOI: 10.1080/10409238.2017.1279120)
3. **Jesus TT**, Oliveira PF, Barros A, Sousa M, Cheng CY, Alves MG. Differential response of lactate production by human Sertoli cells to glucose and essential or non-essential amino acids stimulation. (Submitted)
4. **Jesus TT**, Almeida SP, Barros A, Sousa M, Oliveira PF, Alves MG. Short-term human sperm preservation in media supplemented with mTOR activator MHY 1485 improves sperm quality without compromising DNA integrity and capacitation. (Submitted)

Abstract

Infertility is a health problem that affects millions of couples worldwide. One third of the cases results from a male factor alone. Spermatogenesis is a complex event that occurs in the testes, specifically in the seminiferous epithelium. In this tubular structure, Sertoli cells (SCs) and developing germ cells establish an intimate association. In fact, the occurrence of spermatogenesis depends on the metabolic cooperation established between SCs and germ cells, where SCs supply the developing germ cells with the essential metabolic substrates. These events are modulated by numerous factors, including metabolic substrates availability. The action of hormones and other endogenous or exogenous factors is also pivotal and accounts with the involvement of multiple signaling pathways. The understanding of SC energy metabolism and its regulation may help to identify and support new therapeutic approaches for cases of subfertility or infertility caused by pathological conditions, since spermatogenesis is in the basis of male fertility.

We hypothesized that the mammalian target of rapamycin (mTOR) signaling pathway can play a role in the control of human SC (hSC) metabolism and the nutritional support of spermatogenesis as well as sperm physiology. We firstly evaluated the effect of mTOR multiprotein complex 1 (mTORC1) inhibition by rapamycin in the glycolytic and oxidative profiles as well as in the mitochondrial function of hSCs. Our results show that exposure of hSCs to rapamycin induces an increase in glucose consumption, without altering the production of lactate and acetate. Secondly, and since only recently it has been unveiled how amino acid levels modulate the function of mTORC1, we studied the role of amino acids stimulation in hSCs mTOR pathway. Specifically, we subjected hSCs to stimulation with different carbon sources: glucose (5.5 and 17.5 mM); leucine (5 mM) and glutamine (5 mM). After that, the metabolic and oxidative profiles of hSCs were determined, as well as the expression of mitochondrial complexes and also of specific players of the mTOR signaling pathway. We observed that glutaminolysis correlates with mTORC1 activation and that it promotes a rapid accumulation of acetate, which latter enhances the synthesis and export of pyruvate, without altering lactate production. When comparing the results

obtained after culturing hSCs with two distinct amino acids, it seems that glutamine (a non-essential amino acid), and thus stimulation of glutaminolysis, may be more relevant as a metabolic substrate for the testicular metabolic cooperation than leucine (an essential amino acid). This process appears to be mediated by mTOR since the timeframe of our experiments promotes mTOR phosphorylation at Ser²⁴⁴⁸, with the phosphorylated ribosomal protein S6 (rpS6) levels remaining elevated within the first 30 min. After 6 hours there is a dephosphorylation of both targets, suggesting transient activation and latter desensitization of this pathway. Finally, to assess the role mTOR in a clinical relevant setting related with sperm physiology, and as mTOR acts as a metabolic modulator, we hypothesized that supplementation of a commercial sperm preservation medium with an mTOR enhancer (MHY 1485) could modulate sperm quality after short-term storage. This work was the ground for the patent NP110231: “mTOR enhancers and uses thereof to improve sperm quality and function during storage”. We observed that sperm preservation in a medium supplemented with MHY 1485 increases sperm metabolic activity without inducing oxidative damages. Remarkably, the supplementation of preservation media with MHY 1485 also improved sperm viability and motility, while decreasing DNA fragmentation and maintaining spermatozoa binding capacity. Our data suggests that mTOR is not only involved in sperm quality, but also that the use of an enhancer of this protein in preservation media improves spermatozoa quality. In sum, we report that mTOR signaling pathway contributes for glucose and amino acids homeostasis in hSCs and regulates the redox balance in these cells unveiling a new role for mTOR on the nutritional support of spermatogenesis. Also the activation of mTOR during sperm preservation improves its quality even after 2 hours. Overall, we show that mTOR signaling pathway is a novel regulator of male fertility.

Resumo

A infertilidade é um problema de saúde que afeta milhões de casais mundialmente. Um terço dos casos resulta apenas de problemas do fator masculino. A espermatogénese trata-se de um processo complexo que ocorre no testículo, mais especificamente no epitélio dos túbulos seminíferos. É nestas estruturas tubulares que células de Sertoli (SCs) e células germinativas em diferentes estados de desenvolvimento se encontram estreitamente relacionadas. A ocorrência normal da espermatogénese depende da cooperação metabólica estabelecida entre as SCs e as células germinativas, onde as primeiras fornecem os substratos metabólicos essenciais ao desenvolvimento das segundas. Este processo é modulado por inúmeros fatores, incluindo a disponibilidade de nutrientes. A ação de hormonas e outros fatores endógenos e/ou exógenos é fundamental e conta com o envolvimento de múltiplas vias de sinalização. A compreensão do funcionamento do metabolismo energético da célula de Sertoli e dos mecanismos que o regulam podem ajudar na identificação de novas abordagens terapêuticas para os casos de subfertilidade ou infertilidade causados por condições patológicas, uma vez que a espermatogénese é um processo chave na fertilidade masculina.

A hipótese que colocámos foi se a via de sinalização do *mammalian target of rapamycin* (mTOR) pode regular o metabolismo das células de Sertoli humanas (hSCs) e o suporte nutricional da espermatogénese, assim como a fisiologia do espermatozoide. Primeiro estudámos o efeito da inibição, com rapamicina, do complexo multiproteico 1 do mTOR (mTORC1) nos perfis glicolíticos e oxidativos, assim como na função mitocondrial, das hSCs. Os nossos resultados mostraram que a exposição das hSCs à rapamicina induz o aumento do consumo de glucose sem, no entanto, alterar a produção de lactato e acetato. Em segundo, e uma vez que apenas recentemente foi descrito como os aminoácidos modulam a função do mTORC1, analisámos o papel destes na estimulação da via do mTOR nas hSCs. Mais especificamente, submetemos hSCs à estimulação de diferentes fontes de carbono: glucose (5.5 and 17.5 mM); leucina (5 mM) e glutamina (5 mM). Após os tratamentos, foram estudados os

perfis metabólico e oxidativo, assim como a expressão de complexos mitocondriais e de proteínas da via de sinalização do mTOR. Observámos que a glutaminólise relaciona-se com a ativação do mTORC1 e promove a acumulação rápida de acetato. Esta promoveu a síntese e exportação de piruvato sem alterar a produção de lactato. Quando comparados os resultados obtidos após a estimulação de hSCs com os dois aminoácidos distintos, observámos que a glutamina (um aminoácido não essencial), e consequente estimulação da glutaminólise, aparenta ser um substrato mais relevante para a cooperação metabólica testicular do que a leucina (um aminoácido essencial). Este processo parece ser mediado pelo mTOR uma vez que durante o intervalo de tempo da nossa experiência promoveu a fosforilação do mTOR no resíduo Ser²⁴⁴⁸, com os níveis de proteína ribossomal S6 (rpS6) fosforilada a manterem-se elevados nos primeiros 30 minutos. Após 6 horas, verificou-se a desfosforilação de ambas as proteínas, sugerindo a ativação transiente e posterior dessensitização desta via de sinalização. Em último, no sentido de estudar o papel do mTOR numa aplicação clínica relevante relacionada com a fisiologia do espermatozoide, e uma vez que o mTOR atua como um modulador do metabolismo, colocámos a hipótese da suplementação de um meio de preservação de espermatozoides comercial com um ativador do mTOR (o MHY 1485) com vista a manutenção ou melhoria da qualidade espermática após um período curto de conservação. Este trabalho serviu de base para a patente NP110231: “mTOR enhancers and uses thereof to improve sperm quality and function during storage”. Observámos que a preservação de espermatozoides em meio suplementado com MHY 1485 aumenta a atividade metabólica dos espermatozoides sem indução de danos oxidativos nestes. É de realçar que esta suplementação do meio com MHY 1485 melhorou a viabilidade e vitalidade dos espermatozoides, enquanto que a fragmentação de DNA diminui e a capacidade de capacitação se manteve. Os nossos resultados sugerem que o mTOR não está só envolvido na manutenção da qualidade dos espermatozoides, mas que o uso de um ativador desta proteína no meio de preservação melhora a qualidade destes.

Resumindo, observámos que a via de sinalização do mTOR contribui para a homeostase da glucose e aminoácidos em hSCs e que regula o balanço redox

nestas células, revelando um papel desta proteína no suporte nutricional da espermatogénese. Também que a ativação do mTOR durante a preservação de espermatozoides melhora a sua qualidade mesmo após duas horas. Assim, apontamos a via de sinalização do mTOR como um regulador da fertilidade masculina.

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List of Abbreviations

4E-BP1 - translational regulators eukaryotic translation initiation factor 4E binding protein 1

4-HNE - 4-hydroxynonenal

Akt - protein kinase B (also known as PKB)

AMPK - adenosine monophosphate-activated protein kinase

ART - assisted reproductive technologies

ATG13 - autophagy-related gene 13

BTB - blood-testis barrier

c-kit (or KIT) - c-kit-encoded transmembrane tyrosine kinase receptor for stem cell factor (SCF or KITL)

CNI - calcineurin inhibitors

DAP1 - death-associated protein 1

Deptor - DEP-domain-containing mTOR-interacting protein

DMEM: Ham's F12 - Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12

EDs - efferent ducts

eIF4E - translational regulators eukaryotic translation initiation factor 4E

ERK1/2 - extracellular-signal-regulated kinase ½

FK506 – tacrolimus

FKBP12 - intracellular FK506-binding protein of 12 kDa

FoxO3a - forkhead box O1/3a

FRB - FKBP12-rapamycin binding domain

FSH - follicle-stimulating hormone

GLUT - glucose transporters

Grb2 - Growth factor receptor-bound protein 2

GSK3 - glycogen synthase kinase 3

GβL - G protein beta subunit-like

HIF α - alpha subunit of hypoxia-inducible factor

IGF1 - insulin-like growth factor 1

IRS – insulin receptor substrates

IVF – *in vitro* fertilization

LDH - lactate dehydrogenase

LH - luteinizing hormone

LKB1 - liver kinase B1

MCT - monocarboxylate transporter

MEK - mitogen-activated protein kinase kinase

mLST8 - mammalian lethal with sec-13 protein 8 (also known as G β L)

mSIN1 - mammalian stress-activated protein kinase interacting protein

mTOR - mammalian target of rapamycin

mTORC - mammalian target of rapamycin multiprotein complex

NT - nitro-tyrosine

PBS – phosphate buffered saline

PDK1 - 3-phosphoinositide-dependent protein kinase-1

PFK - phosphofructokinase

PI3K - phosphoinositide 3-kinase

PKC- α - protein kinase C- α

PLZF - Promyelocytic Leukemia Zinc Finger

PPAR γ - peroxisome proliferator-activated receptor- γ

PRAS40 - proline-rich Akt substrate 40 kDa

Protor-1 - protein observed with Rictor-1 (also known as PRR5)

PTEN - phosphatase and tensin homolog

raptor - regulatory-associated protein of mTOR

REDD1 - regulation of DNA damage response 1

Rheb - Ras homolog enriched in brain GTPase

rictor - rapamycin-insensitive companion of mTOR

ROS - reactive oxygen species

rpS6 - ribosomal protein S6

RSK1 - p90 ribosomal S6 kinase 1

RSK1 - p90 ribosomal S6 kinase 1

S6K1- S6 kinase 1 (also known as p70S6K)

SC - Sertoli cell

SGK1 - serum- and glucocorticoid-induced protein kinase 1

SH – sulfhydryl

SOS - Ras-guanine exchange factor

SREBP1 - sterol binding regulatory element-binding protein 1

SRL – sirolimus (also known as rapamycin)

TSC1/2 - tuberous sclerosis 1/2

ULK1 - unc-51-like kinase 1

ZO-1 - zonula occludens-1

Chapter 1

Introduction

This chapter was adapted from the published work:

Jesus TT, Oliveira PF, Sousa M, Cheng CY, Alves MG (2017) Mammalian target of rapamycin (mTOR): a central regulator of male fertility? Critical Reviews in Biochemistry and Molecular Biology. 52(3):235-253.

1. Introduction

Infertility affects millions of couples worldwide and nearly one third of the cases results from male factor alone (Hamilton and Ventura, 2006; Lutz, 2006). In addition, a decline in male reproductive health has been observed in European men, with studies identifying a high prevalence of low sperm counts in young men (<40 years) (Fernandez et al., 2012; Jorgensen et al., 2001; Jorgensen et al., 2002; Nordkap et al., 2012). Many factors have been suggested to contribute to this problem including the current lifestyle in industrialized countries (diet, smoking, alcohol, drugs) and the exposure to environmental substances, which can negatively affect testicular function to a greater degree than genetic factors (Bonde and Storgaard, 2002; Bustos-Obregón and Hartley, 2008; Goulis and Tarlatzis, 2008; Mah and Wittert, 2010; Mathur and D'Cruz, 2011; Sharpe, 2010). Furthermore, systemic diseases can directly or indirectly affect the male reproductive axis at multiple levels and irreversibly impair spermatogenesis (Karagiannis and Harsoulis, 2005; Sartorius and Handelsman, 2010; Suehiro et al., 2008).

The molecular mechanisms that control male fertility are of extreme relevance to counteract the deleterious effects promoted by several diseases. When male factor is the responsible for the infertility observed in the couple, the treatment is not usually directed to a specific identified cause because a clear diagnosis is not established for the observed alterations in the reproductive health of the male. Likewise, the male is apparently clinically normal although suffering from abnormalities in sperm quality or quantity (including decreased sperm counts, low sperm motility and poor sperm-oocyte interactions) (Hirsh, 2003; Pacey, 2009). In those cases, the treatment is usually associated with assisted reproductive technologies (ART), which is often applied in an empirical manner to maximize the chances to conceive but without an in-depth study on the causes of the condition. Thus, it is imperative a deeper commitment upon basic and clinical research on the causes of male infertility.

The male reproductive tract is composed of highly heterogeneous tissues, including the testes, the efferent ducts, the epididymis, and the vas deferens

(Figure 1.1). Spermatozoa are produced within the testes in a process known as spermatogenesis. However, spermatozoa maturation occurs along the excurrent ducts and the epididymis. In fact, the formation of a competent spermatozoon is a complex process that involves the production of a large number of germ cells in the testes and several maturation steps that occur in the subsequent ducts. During the entire process, the establishment of the adequate environments, with the secretion of the numerous components of the different fluids throughout the male reproductive tract, is vital for the production of spermatozoa and for providing a means of transport for spermatozoon during its development (Rato et al., 2010). Among the several testicular cells, the somatic Sertoli cells play a crucial role in those processes.

Sertoli Cell: physiology and function

The mammalian testes are paired complex organs. Each testis is divided in compartments, called testicular lobules, which are separated by fibrous inward extensions of the tunica albuginea (septum) (Figure 1.1) (Saladin, 2003). Each lobule encloses coiled seminiferous tubules (Shubhada et al., 1993; Walker and Cheng, 2005). These lobules are avascular and no nerves penetrate through their walls (Setchell, 1986). The interstitial space of the testis comprises all the spaces between the seminiferous tubules, containing the blood and lymphatic vessels, which are essential for the movement of hormones and nutrients into and out of the testes (O'Donnell et al., 2001). In this space we can also find the nerves, the Leydig cells, which are the primary sites of steroidogenesis in the testis, and a significant population of macrophages (Setchell, 1986).

The testes perform essentially two major tasks: the biosynthesis of steroid hormones and the formation of haploid germ cells. These functions are primarily regulated by pituitary gonadotropins, with luteinizing hormone (LH) acting on the testosterone-producing Leydig cells located in the interstitium, and follicle-stimulating hormone (FSH) acting on Sertoli cells (SC) in the seminiferous tubules (Griswold, 1998; Walker and Cheng, 2005). Spermatogenesis is the process by

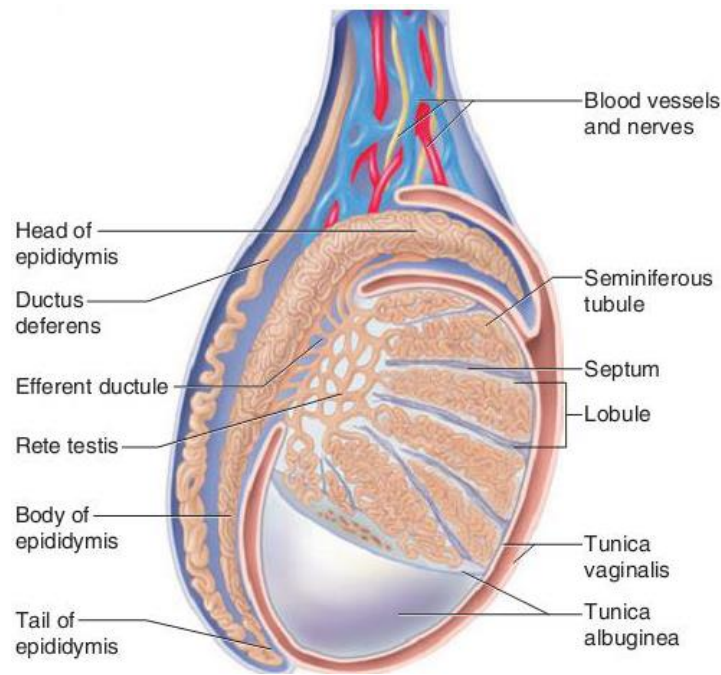


Figure 1.1 - Schematic representation of the mammalian testis and epididymis. The testis is encapsulated by two layers: tunica vaginalis (the most outer tunic) and tunica albuginea. Extensions from tunica albuginea (septum) divide testis in lobules where the seminiferous tubules are located. Seminiferous tubules converge to the rete testis that is connected to the efferent ducts (EDs). The head of the epididymis is linked to the testis by various EDs. Adapted from (Saladin, 2003).

which immature germ cells undergo division, differentiation and meiosis to give rise to haploid spermatids. This process takes place within the seminiferous tubules epithelium where epithelial somatic SCs and germ cells in different stages of development are in close association. A chain of complex local interactions involving the various testicular cell types, such as germ, Sertoli, peritubular myoid and Leydig cells, are responsible for the control of spermatogenesis (Shubhada et al., 1993; Walker and Cheng, 2005). In this process, SCs play a pivotal role. They are highly polarized epithelial cells that extend upwards from the basement membrane of the seminiferous tubule to its open lumen, and directly interact with the developing germ cells (Mruk and Cheng, 2004). These are irregularly shaped, columnar cells with large dimensions and an enormous surface area, which allow each one of them to support a vast but controlled number of developing germ cells (Mruk and Cheng, 2004). This is an essential feature for spermatogenesis and germ cell movement (Mruk and Cheng, 2004). From Enrico Sertoli works in 1865, later confirmed by several authors, emerged the concept that SCs function

as “nurse cells” since they provide structural and nutritional support, for the maintenance of developing germ cells (Foley, 2001; Griswold, 1995). SC functions go from establishing a barrier to germ cell phagocytosis, hormone production (inhibin and cytokines) and fluid secretion (Foley, 2001). Between adjacent SCs, located near the basement membrane, there are specialized junctions, which include tight junctions, basal ectoplasmic specializations, basal tubulobulbar complex gap junctions and desmosome-like junctions (Cheng and Mruk, 2009; Cheng et al., 2010). These Sertoli-Sertoli junctions form the “blood-testis barrier” (BTB) that divides the seminiferous epithelium in basal and adluminal (apical) compartments. Spermatogonia and immature spermatocytes are present within the basal compartment, while beyond the BTB, in the adluminal compartment, lay the developing germ cells, e.g. the different stages of meiotic spermatocytes, round spermatids, elongated spermatids and spermatozoa (Mruk and Cheng, 2004; Su et al., 2011). The BTB is one of the tightest blood–tissue barriers in mammalian tissues and the central structural element in testicular physiology (Setchell, 1980; Su et al., 2011; Wong and Cheng, 2005), responsible for conferring polarity to SCs (Wong and Cheng, 2005). When the BTB is dysfunctional, germ cell differentiation is arrested (Toyama et al., 2003). Generation and maintenance of the BTB is assured by somatic SCs and its molecular composition has long been a matter of debate (Cheng and Mruk, 2009; Cheng et al., 2010; Lui and Cheng, 2007; Waites and Gladwell, 1982; Wong and Cheng, 2005). Nevertheless, it has been established that a fully functional BTB consists of three components: (1) an anatomical/physical barrier restricting entry of molecules and germ cells from the basal compartment, which is in close contact with blood vessels and lymphatic endothelium, into the adluminal compartment of the seminiferous tubules; (2) an immunological barrier that regulates the movement of immune cells and the level of cytokines in the seminiferous epithelium; and (3) a physiological barrier, composed by transporters and membrane channels, that is highly dynamic to encounter the needs of both developing germ cells and SCs (Mital et al., 2011; Sikka and Wang, 2008). Despite the complex composition of the BTB, it undergoes highly dynamic restructuring at specific stages of the spermatogenic cycle, in order to allow developing germ cells to cross the BTB into the adluminal compartment (Russell, 1978), in a such a well-coordinated process that immune privilege is maintained

(Wong and Cheng, 2005). Throughout this process, germ cells continue to be tightly anchored to SCs (Siu and Cheng, 2008). Together, these components are essential to the function of the BTB, creating a microenvironment that allows the establishment of luminal fluid within the seminiferous tubule and responsible for the proper development of germ cells into fully functional spermatozoa (Setchell, 1980; Su et al., 2011; Wong and Cheng, 2005). Thus, the developing germ cells that are present above the BTB in the adluminal compartment become effectively protected from direct access to plasma constituents and are highly dependent of SCs nurturing and protection (Mruk and Cheng, 2004; Rato et al., 2012; Sharpe, 1994; Sharpe et al., 2003; Walker, 2010). Indeed, SCs are responsible for providing energy and nutritional support to developing germ cells. It is imperative that germ cells receive an adequate level of energy substrates, otherwise they will degenerate and enter the apoptotic pathway (Boussouar and Benahmed, 2004; Jutte et al., 1981). Developing germ cells have specific metabolic requirements, preferentially using lactate as a substrate for ATP production (Grootegeod et al., 1986). The SCs are responsible for the production of the lactate used by developing germ cells, through the metabolization of various substrates, particularly glucose (Robinson and Fritz, 1981). Thus, SCs ensure the nutritional support of germ cells by secreting nutrients or metabolic intermediates, such as amino acids, carbohydrates, lipids, vitamins, and metal ions (Mruk and Cheng, 2004; Robinson and Fritz, 1981).

Glucose transport across the cell membrane of SCs is an important event for the spermatogenic event. Glucose is hydrophilic and, as a result, cannot diffuse freely through the lipid bilayer. Therefore, there are specific carrier proteins to facilitate its diffusion along a concentration gradient. Glucose transporters (GLUTs) are a super-family of structurally related transport facilitators glycoproteins, with 13 family members, distributed in a wide variety of species (Joost and Thorens, 2001), responsible for mediating passive glucose transport through membranes (Klip et al., 1994). Different members of this protein family are expressed in the testis: GLUT1 (Ulisse et al., 1992), GLUT2 (Kokk et al., 2004), GLUT3 (Burant and Davidson, 1994), GLUT5 (Burant et al., 1992) and GLUT8 (Doege et al., 2000). More specific, until now, the GLUT isoforms GLUT1, GLUT2, GLUT3, and GLUT8, have been identified in SC (Carosa et al., 2005; Galardo et al., 2008;

Kokk et al., 2004; Martins et al., 2015; Ulisse et al., 1992). Nevertheless, one must highlight that not all isoforms are expected to have a preponderant role on the import of glucose to SC. For instance, GLUT8 has not been localized at the plasma membrane, excluding its role in glucose transport from the extracellular milieu (Kokk et al., 2004; Piroli et al., 2002; Reagan et al., 2001), while GLUT1, GLUT 2 and GLUT3 are present at the plasma membrane, and therefore it may be assumed that they mediate the majority of glucose incorporation into SC. Studies with cultured mammalian cells suggest that glucose itself may regulate its own transport, since glucose absence or deprivation leads to a compensatory increase in the glucose uptake to optimize the utilization of this sugar and maintain energy levels (Klip et al., 1994). In SC, when glucose was removed from culture medium, an increase in GLUT1 and a decrease in GLUT3 expression levels were reported (Riera et al., 2009), leading to the deduction that changes of glucose levels in the extracellular milieu may represent a signal for SCs upregulate their extracellular transport system in a way to ensure the appropriate lactate production for germ cells development. In a study from our group a prediabetic state, achieved by a high-energy diet, was reported to alter the testicular glycolytic profile of rats with the expression of both GLUT1 and GLUT3 being increased (Rato et al., 2013). Furthermore, in a study where SCs were cultured in conditions of insulin deprivation it was observed altered glucose consumption and lactate production together with a modulation of GLUT1 and GLUT3 mRNA expression (Oliveira et al., 2012).

Lactate is produced from pyruvate following lactate dehydrogenase (LDH) catalysis. The LDH enzyme family is responsible for the interconversion of pyruvate into lactate, with the concomitant oxidation/reduction of NADH to NAD⁺ (Everse and Kaplan, 1973), essential for the continued production of ATP by glycolysis (Kreisberg, 1980). LDH isozymes are formed by random association of four subunits to make homo- or heterotetramers. The subunits are encoded by three loci in mammals: *Ldha*, *Ldhb* and *Ldhc*. In testis, LDHA subunit is highly and the predominantly expressed isoform (Hawtrey and Goldberg, 1968). Interestingly, *Ldhc* is expressed exclusively in tumors (Koslowski et al., 2002) and in the testis (Goldberg, 1990). Germ cells specifically express a unique isozyme of LDH (Coonrod et al., 2006; Goldberg, 1985), LDHC, which is abundant in

spermatids and spermatozoa (Goldberg, 1985; Goldberg et al., 2010; Li et al., 1989). Studies in mouse testis showed that the appearance of LDHC coincides and correlates with its mRNA, with both C-subunit polypeptide (Li et al., 1989) and its mRNA (Alcivar et al., 1991) being first detectable in preleptotene spermatocytes. This unique characteristic of germ cells is related with their unique dependence on lactate and, as expected, the targeted disruption of *Ldhc* gene results in male infertility due to a progressive decrease in sperm motility, failure in the capacity to develop the hyperactivated motility, vital for fertilization, and a decline in ATP levels (Odet et al., 2008). Koslowski et al. (2002) reported that LDHC escapes from transcriptional repression, resulting in significant expression levels in virtually all tumor types tested. In cancer, LDHC activation may provide a metabolic rescue pathway in tumor cells by exploiting lactate for ATP delivery and, although germ cells are not tumor cells, it is liable that these active cells might use lactate for ATP delivery also, as both cells express this isozyme to metabolize it. Following glucose uptake by SCs and the conversion of pyruvate to lactate by LDH, it is crucial that this product of glycolysis becomes available for the developing germ cells. This event is mediated by specific proton/monocarboxylate transporters (MCTs) that transport lactate through the plasma membrane of SCs (Boussouar and Benahmed, 2004; Oliveira et al., 2012; Oliveira et al., 2011; Riera et al., 2009). In most cells, MCTs are largely responsible for the transport of L-lactate and other monocarboxylates across the plasma membrane (Halestrap and Price, 1999). Until recently, 14 members of MCT family have been described in several tissues and cells (Bonen et al., 2006) but only MCTs 1-4 have been functionally characterized as proton-linked MCTs and proven to transport monocarboxylates (Halestrap, 2012; Pellerin, 2003). MCT1, MCT2 and MCT4 are widely expressed in all tissues, while MCT3 is specifically expressed in retina (Brauchi et al., 2005). MCT2 can be found in elongated spermatids (Goddard et al., 2003) and Galardo et al. (2007) confirmed the presence of MCT1 and MCT4 in SC. MCT1 has a higher affinity and a major role in lactate import from the extracellular milieu (Bonen, 2001), and was also identified in germ cells (Goddard et al., 2003), while MCT4, which has a lower affinity for lactate, is primarily a lactate exporter (Bonen, 2001; Galardo et al., 2007), being mostly expressed in cells with high glycolytic capacity (Bonen, 2001; Bonen et al., 2006; Galardo et al., 2007) and seems to play an important role in

SC (Bonen, 2001; Galardo et al., 2007; Oliveira et al., 2012; Oliveira et al., 2011; Rato et al., 2012). Recently our group also described that, besides lactate, SC also produce high amounts of acetate (Alves et al., 2012). The physiological relevance for the acetate produced by SC is still a matter of debate, however it has been suggested that it may be essential to sustain the high rate of lipid synthesis and remodeling by developing germ cells (Bajpai et al., 1998; Oresti et al., 2010).

Hormones are key regulatory factors of SC functioning. Spermatogenesis is dependent on the presence of a suitable intratesticular level of sex steroid hormones (androgens and estrogens). SCs express androgen receptors, whereas germ cells lack these receptors (Lyon et al., 1975). Most of the effects of androgens in SCs are mediated by androgen receptors and by 5 α -reduced metabolites of testosterone, such as DHT, which present biological activities greater than testosterone (Alves et al., 2013). Our group showed recently that SC metabolism is also under the control of DHT. DHT was reported to increase glucose consumption by SCs, without increasing the production of lactate. The mRNA levels of *Ldha* and *Mct4* were decreased after exposure to DHT, illustrating that androgens may control SC metabolism, particularly the production and export of lactate (Oliveira et al., 2012; Rato et al., 2012). Moreover, it was suggested that exposure of SCs to DHT shifts metabolism from lactate production, as a final metabolic outcome, to Krebs cycle (Martins et al., 2013). Estrogens are also modulators of SCs metabolism (Martins et al., 2013; Oliveira et al., 2011; Rato et al., 2012). Treatment with 17 β -estradiol altered the expression of glycolysis-related transporters and enzymes (Martins et al., 2013), illustrating that estrogens are also important modulators of SC metabolism.

The regulation these metabolic processes is vital and could have an influence on male fertility. Thus, modulation of metabolic pathways in SCs is likely to be determined by multiple factors including metabolic substrates availability and the action of hormones (Alves et al., 2012; Galardo et al., 2008; Hall and Mita, 1984; Oliveira et al., 2011; Rato et al., 2012; Riera et al., 2001) and other endogenous or exogenous factors that contribute to the progression of the spermatogenic event. The understanding of SCs energy metabolism may help to identify and

support new therapeutic approaches for cases of subfertility or infertility caused by pathological conditions since spermatogenesis is in the basis of male fertility.

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

Biological homeostasis depends on the balance between 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 (Laplane and Sabatini, 2012)). 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 (Laplane and Sabatini, 2009)). 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 and Sutherland, 2001; Murakami et al., 2004). 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, the establishment of the adequate environments is vital for the production of spermatozoa and 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 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 and 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 and 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. 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 mammalian Target of Rapamycin (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 these nutritional needs. 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 (Laplante and Sabatini, 2012). 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 mammalian TOR (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 2) (Guertin and 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 and Sabatini, 2009). mTORC1 has five and mTORC2 six known

protein components beyond the mTOR catalytic domain (Figure 1.2). 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 G β L) (Jacinto et al., 2004; Kim et al., 2003), and the Tti1/Tel2 complex (Figure 1.2) (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 1.2) (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 and Sabatini, 2009), 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).

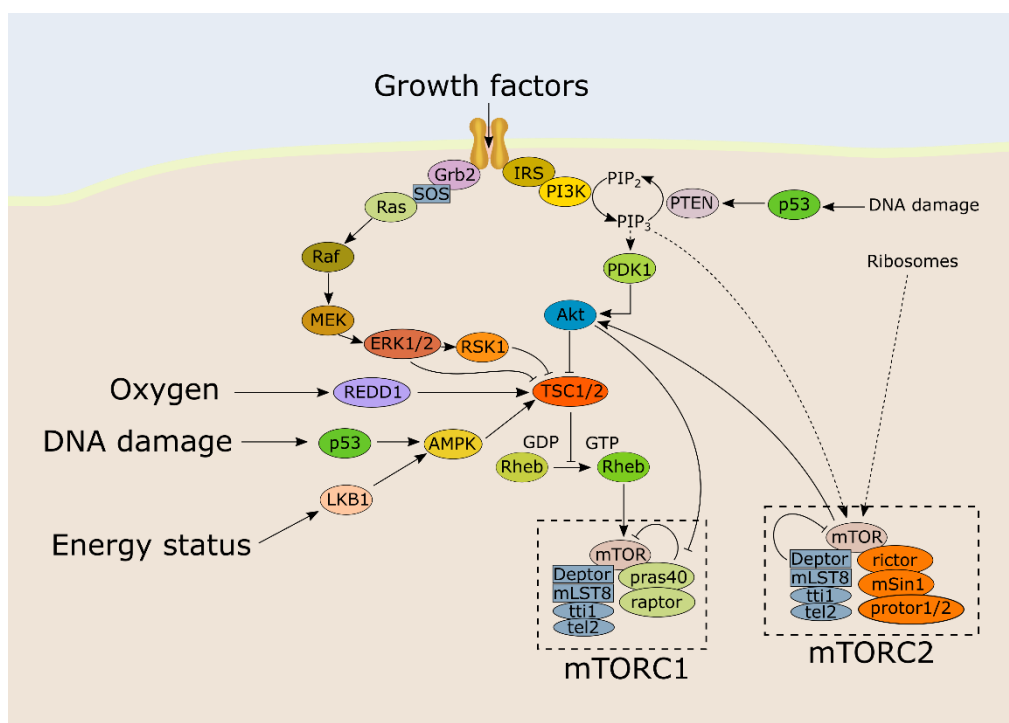


Figure 1.2. mTOR signaling pathway: mTORC1 and mTORC2 complexes and the key signaling nodes that regulate mTORC1 and mTORC2. Critical inputs regulating mTORC1 include growth factors, DNA damage, energy status, and oxygen. mTORC2 is activated by growth factors and ribosomes in a poorly understood mechanism. Abbreviations: 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.

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 1.2). 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 needs 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 1.2). 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 1.2 and 1.3). 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 intracellular 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 (reviewed by Guertin and 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 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 1.2), 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 level of intracellular amino acids is still unknown.

TSC1/2, a heterodimer that comprises tuberous sclerosis 1 (TSC1, also known as hamartin) 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 1.2) (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 a 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 1.2). 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 1.2). 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 towards Rheb, which in turn reduces mTORC1 activation (Figure 1.2). 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 1.2) (Brugarolas et al., 2004; DeYoung et al., 2008; Reiling and 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 1.2) (Budanov and 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 1.2) (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 1.3). 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 a review, see Ma and 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 3) (for a review, see Richter and Sonenberg (2005)). The phosphorylation of S6K and its activation by mTORC1 lead to an increase in mRNA biogenesis, cap-dependent translation and its activation by mTORC1 lead to an increase in mRNA biogenesis, cap-dependent translation of ribosomal proteins, such as ribosomal protein S6 (Figure 1.3) (for a review, see Ma and 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 a review, see Laplante and Sabatini (2009)). 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 and Chen, 2004). These transcription factors control the expression of genes implicated in fatty acid and cholesterol synthesis (Figure 1.3). 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 3). 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

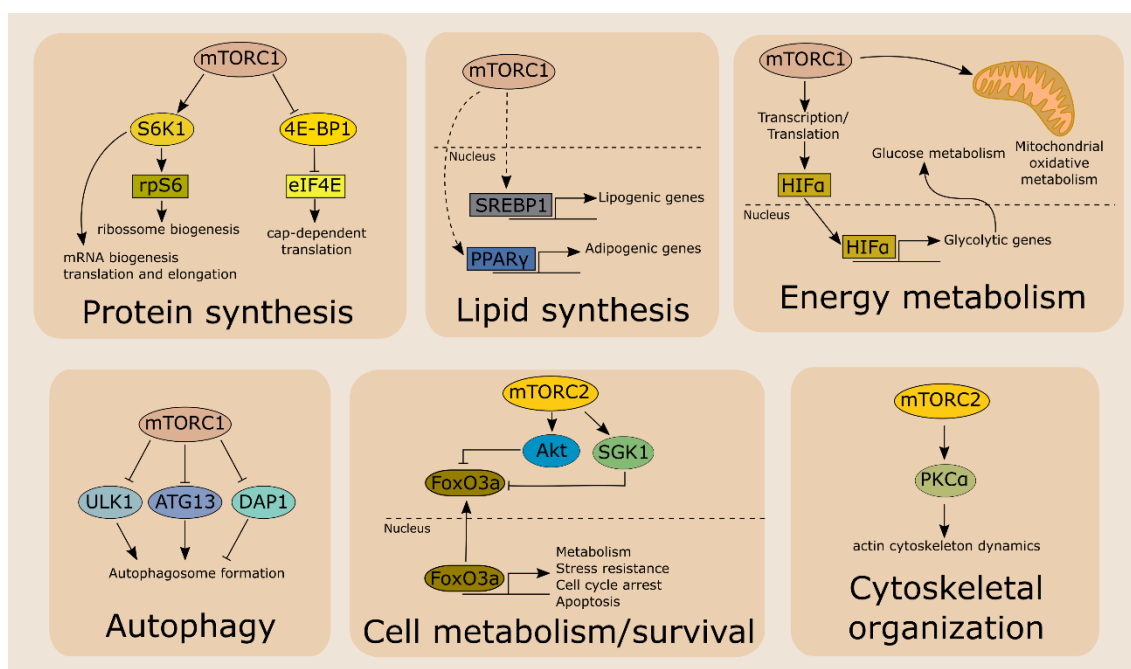


Figure 1.3. Key outputs of the mTORC1 and mTORC2 pathways. mTORC1 regulates many biological processes through phosphorylation of several proteins, positively regulating anabolic processes, such as protein and lipid synthesis and energy metabolism, and negatively regulating catabolic processes such as 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 . Abbreviations: 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.

biogenesis are also controlled by mTORC1 (Figure 3). 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 the inhibition by rapamycin of this complex reduced mitochondrial function, being the energy production preferentially enhanced via aerobic glycolysis in detriment of mitochondrial respiration (Ramanathan and 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 and 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 1.3) (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 to 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 and 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 and 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 1.3). 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; Sarbassov et al., 2004). The activation of PKC- α by mTORC2 regulates cell shape in cell type-specific fashion by affecting the actin cytoskeleton (Figure 3) (Jacinto et al., 2004; Sarbassov 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 (SLR), Rapamune® (for a 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 SLR-associated infertility from a male renal-transplant recipient (Table 1.1) (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 LH and FSH. The negative effect of SLR 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 patient who were treated with SRL versus those treated without (Table 1.1) (Zuber et al., 2008). Similar results were described in a case report where a male heart-lung transplant recipient under SRL treatment developed also oligozoospermia and showed marked improvement on sperm exam, when SRL was switched to mycophenolate mofetil (Table 1.1) (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 patient, where two male patients developed severe oligozoospermia and two other had azoospermia (Table 1.1). These clinically-orientated reports provided strong evidence that the use of mTOR inhibitors have 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

Table 1.1. Clinically-orientated studies associating mTOR inhibitors and male reproductive dysfunction

Pathology	Number of patients	Treatment	Duration	Reported effects on male reproduction after treatment	Study
Renal	1	Sirolimus	3 years	Oligozoospermia, asthenozoospermia and teratozoospermia	(Bererhi et al., 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 et al., 2004)
Renal	56	Group 1: no Sirolimus (n=28); Group 2: Sirolimus (n=28)	n.d.	The group treated with SRL showed: ↓ free testosterone levels and ↑ LH, FSH	(Fritsche et al., 2004)
Renal	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 et al., 2005)
Renal	66	Sirolimus	n.d.	↓ testosterone and ↑ FSH and LH	(Lee et al., 2005)
Renal	1	Sirolimus	n.a. (10 years)	Azoospermia	(Skrzypek and Krause, 2007)
Heart-Lung	1	Sirolimus	n.a. (3,3 years)	Oligozoospermia	(Deutsch et al., 2007)
Renal	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 et al., 2008)
Renal	4	Sirolimus	5-12 months	Two patients developed severe oligozoospermia and the other two had azoospermia	(Boobes et al., 2010)

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

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 and Krause, 2007). In 2007, Skrzypek and 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 1.1). 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 and 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 and Schlatt, 1997). The c-kit belongs to a family of platelet-derived growth factor (PDGF) hormone receptors that is predominantly expressed in type A spermatogonial cells. SCF is expressed by neighboring Sertoli cells in the seminiferous epithelium (Loveland and 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, in 2000, Feng, Ravindranath et al. 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 1.4) (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, Seandel et al. identified in 2010 a role for PLZF-mediated Redd1 expression in inhibiting mTORC1 activation in SPCs (Figure 1.4).

As discussed herein, Rheb is a small GTPase and a critical component for mTORC1 activation. In 2014, Baker, Ezzati 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

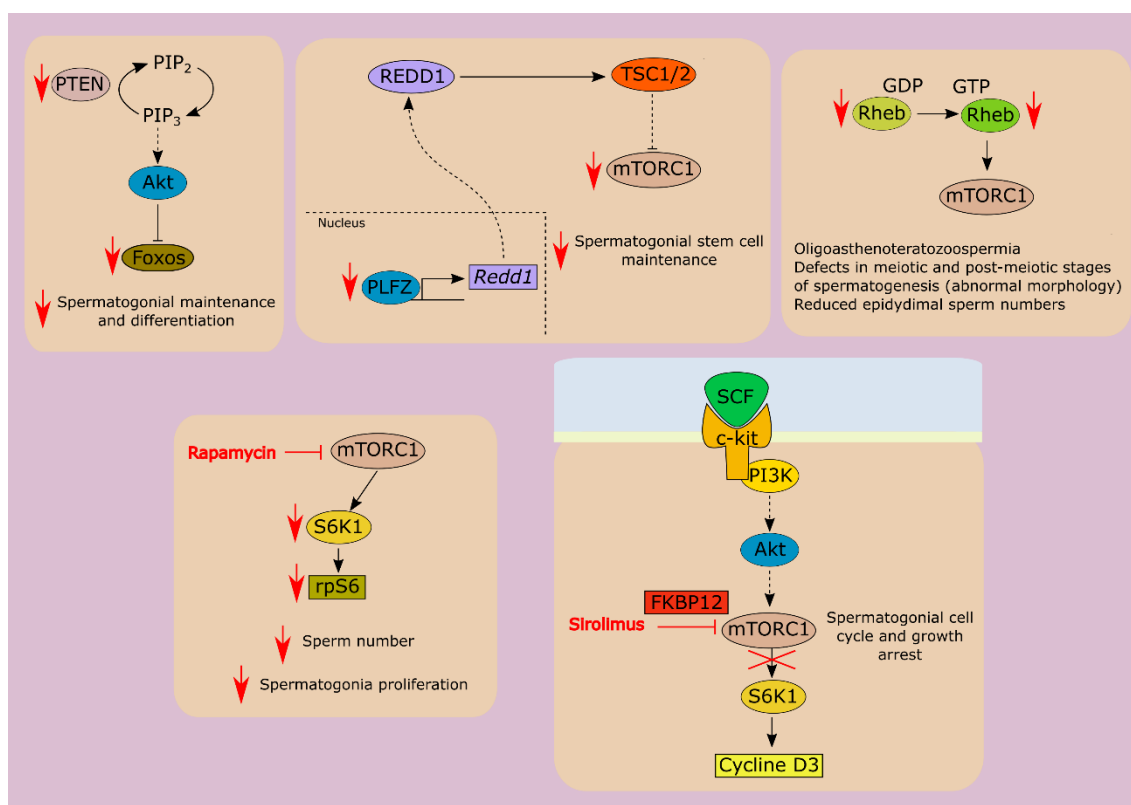


Figure 1.4. Effects of alterations in mTOR signaling pathway on spermatogenesis.

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 sperm 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. **Abbreviations:** 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.

number of sperm), asthenozoospermia (poor sperm movement), and teratozoospermia (abnormal sperm morphology) and male sterility (Figure 1.4). 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 1.4) (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 1.4). 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. In 2014, Sahin et al. 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., 2015). 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., 2015; 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 and Bardeesy, 2008; Mihaylova and Shaw, 2011). LKB1 is activated after forming a heterotrimeric complex to regulate the activity of 14 different kinases, including AMPK (Hezel and Bardeesy, 2008; Shackelford and Shaw, 2009). The LKB1-AMPK signaling cascade negatively regulates mTOR by phosphorylating and activating Rheb-GTPase activity and by phosphorylating and inhibiting Raptor (Mihaylova and 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 and 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 and 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 1.5) (Ulbright et al., 2007), suggesting that SC 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 and Brinster, 2008) and are highly sensitive to toxicants that compromise male fertility (for a review see (Reis et al., 2015)). In 2012, Tanwar, Kaneco-Tarui 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 *Lkb1*cko mice, demonstrating the significance of mTOR activation in the development of the *Lkb1* mutant phenotype (Figure 1.5). 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 and 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 and Mruk, 2012; Wong and 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 the BTB the unusual adhesive strength (Cheng and 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 and 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., 2013; Mok et al., 2012). 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., 2013; Mok et al., 2012). 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., 2013)). 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 1.5). 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 suggest 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 1.5) (Mok et al., 2013). 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., 2013), 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 and Benahmed, 2004). Indeed, as discussed, the Sertoli cell is responsible for the production of lactate, in a Warburg-like metabolism (for a review see (Alves et al., 2015)), 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 and Hay, 2013),

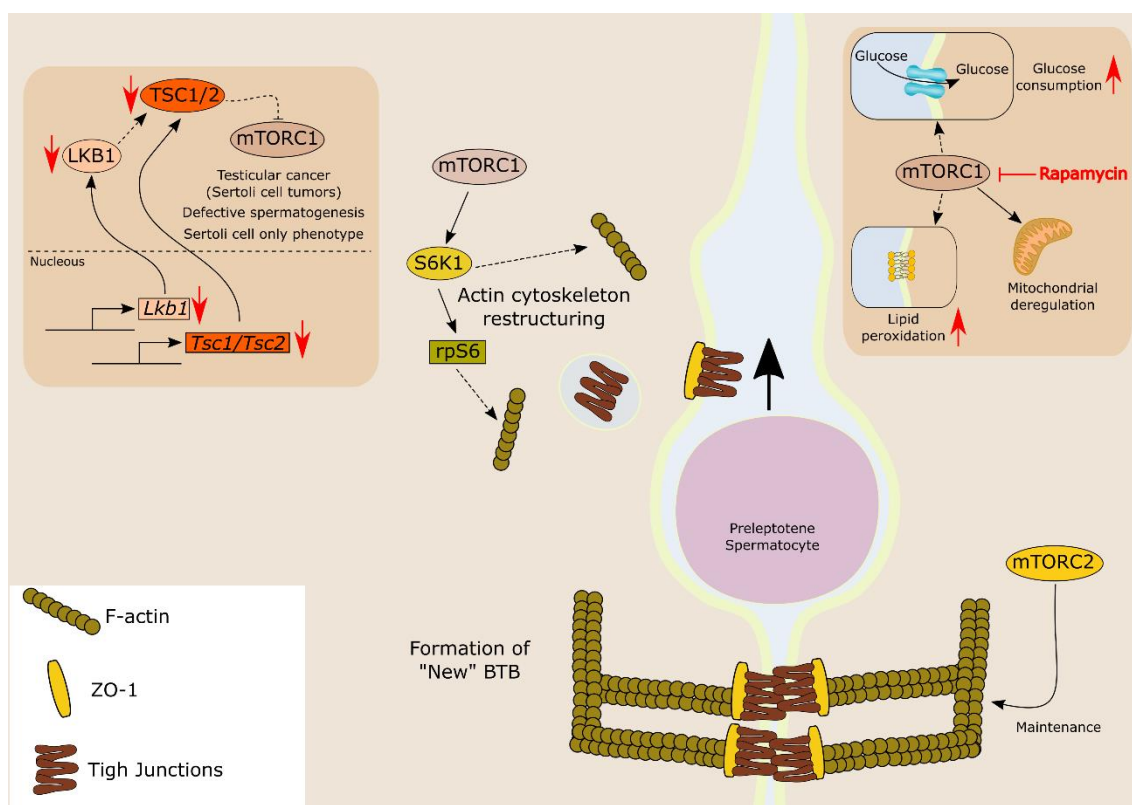


Figure 1.5. Role of mTOR complexes in Sertoli cells. Effects on male reproduction of alterations on mTOR signaling: loss of Lkb1 causes severe defects in spermatogenesis including a Sertoli cell only tubule phenotype. The deletion of Tsc1 and Tsc2 causes similar defects on male reproduction. Rapamycin-exposed 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. **Abbreviations:** 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.

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, though 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; Alves et al., 2016; Martins et al., 2015; Oliveira et al., 2015). Indeed, mTOR has also been reported to be involved in the establishment of metabolic diseases (Laplante and Sabatini, 2012) thus, it would be important to unveil the possible role of mTOR in subfertility or infertility induced by those pathological states.

Final 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 of 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 and 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., 2016), diabetes mellitus (Alves et al., 2015; 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., 2015). 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, remains 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., 2015), 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., 2015). These results suggest a role for mTOR in deciding spermatogonial cells fate.

The actin cytoskeleton dynamics (Tang et al., 2016) and insulin signaling 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 sensitivity to rapamycin 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.

Though 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 deserve 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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Chapter 2

Objectives

2. Objectives

Herein, we aimed to study the regulation of the metabolic processes that occur in somatic Sertoli cells and sperm, since they are vital for determining male fertility. The modulation of metabolic pathways in testicular somatic cells, particularly in Sertoli cells (SCs), is likely to be determined by multiple factors including metabolic substrates availability, and the action of hormones and other endogenous or exogenous factors that could have a contribution in the progression of the spermatogenic event. All these signals are integrated in signaling pathways that coordinate the response. In the last years, some signaling pathways have emerged as essential in the control of male fertility. In this work, we hypothesized that mTOR is a novel regulator of male fertility. For that, we studied mTOR action in human Sertoli cells (hSCs) function. Specifically, we evaluated the effect of mTORC1 inhibition by rapamycin in the glycolytic and oxidative profiles as well as in the mitochondrial function of hSCs. Next, since only recently has been unveiled how amino acid levels module the function of mTORC1, we studied the role of amino acids stimulation in mTOR pathway in hSCs. Specifically, the SCs were subjected to stimulation with different carbon sources and the metabolic and oxidative profiles were determined, as well as mitochondrial complexes and mTOR expression.

Finally, to determine a more specific role for mTOR in a clinical relevant setting, and since this molecule is also a metabolic modulator, we hypothesized that supplementation of a commercial sperm preservation medium with an mTOR enhancer could improve sperm quality after short-term storage. To further understand how these effects were mediated, we focused on sperm metabolic activity and oxidative damages during storage.

Overall, we evaluated if mTOR is a key player in male reproduction, from its action in testicular cells (particularly Sertoli cells, which are the responsive for the nutritional support of spermatogenesis) to spermatozoa physiology and their storage for further use in Assisted Reproductive Technologies.

Chapter 3

Mammalian target of rapamycin controls glucose consumption and redox balance in human Sertoli cells

This chapter was adapted from the published work:

Jesus TT, Oliveira PF, Silva J, Barros A, Ferreira R, Sousa M, Cheng CY, Silva BM, Alves MG (2016) Mammalian target of rapamycin controls glucose consumption and redox balance in human Sertoli cells. Fertility and Sterility. 105(3):825–833

3. Mammalian target of rapamycin controls glucose consumption and redox balance in human Sertoli cells

Running title: mTOR and Sertoli cell metabolism

Abstract

Objective: To study the role of mammalian target of rapamycin (mTOR) in the regulation of human Sertoli cell (hSC) metabolism, mitochondrial activity and oxidative stress.

Design: Experimental study

Setting: University research center and private IVF centers.

Patients/Animals: Six men with anejaculation (psychological, vascular, neurologic) and conserved spermatogenesis.

Intervention: Testicular biopsies from patients under treatment for recovery of male gametes were used. Primary hSCs cultures were established from each biopsy and divided in: a control group and one treated with rapamycin, the inhibitor of the mammalian target of rapamycin (mTOR), for 24 hours.

Main Outcome Measure(s): Cytotoxicity of hSCs to rapamycin was evaluated by sulforhodamine B assay. hSCs glycolytic profile was assessed by Proton Nuclear Magnetic Resonance and by studying protein expression of key glycolysis-related transporters and enzymes. Expression of mitochondrial complexes and citrate synthase activity were determined. Protein carbonylation, nitration, lipid peroxidation and sulfhydryl protein groups content were quantified. mTOR signaling pathway was studied.

Results: Rapamycin increased glucose consumption by hSCs maintaining lactate production. Alanine production by rapamycin-exposed hSCs was affected resulting in an unbalanced intracellular redox state. Rapamycin-exposed hSCs had decreased expression of mitochondrial complex III and increased lipid peroxidation, while other oxidative stress markers remained unaltered. Treatment of hSCs with rapamycin down-regulated phospho-mTOR (Ser-2448) levels, illustrating an effective partial inhibition of mTORC1. Protein levels of downstream

signaling molecule p-4E-BP1, were not altered, suggesting that during treatment it became rephosphorylated.

Conclusion: We show that mTOR regulates the nutritional support of spermatogenesis by hSCs and redox balance in these cells.

Key words: mTOR; Sertoli cells; Spermatogenesis; Testis; Rapamycin

Introduction

Spermatogenesis is a highly regulated process that takes place in the seminiferous epithelium, where Sertoli cells (SCs) directly interact with developing germ cells (Mruk and Cheng, 2004). The somatic SCs form the blood-testis barrier (BTB) and establish an adequate luminal environment within the seminiferous tubules for the occurrence of the process of sperm cells development (Rato et al., 2010). SCs are known as “nurse cells” due to their functions in the physical and nutritional support of spermatogenesis (Cheng and Mruk, 2010; Griswold, 1998; Mruk and Cheng, 2004). Indeed, SC metabolism is essential for the normal occurrence of spermatogenesis (Oliveira et al., 2015), since developing germ cells are incapable of utilizing glucose for energy metabolism and are dependent on the lactate produced by SCs (Boussouar and Benahmed, 2004).

The mammalian target of rapamycin (mTOR) is a Ser/Thr protein kinase that plays an essential role on several cellular events such as cell growth and proliferation (Weichhart, 2012; Zoncu et al., 2011). It is also a key regulator for sensing and integrating various environmental cues, including growth factors and nutrients (Laplane and Sabatini, 2012). mTOR can form two complexes to execute its functions, mTORC1 and mTORC2, by associating with different binding partners (Dazert and Hall, 2011; Laplane and Sabatini, 2012). mTORC1 is composed of mTOR, regulatory associated protein of mTOR (raptor) and other factors. It mediates the mTOR action by modulating protein synthesis. This complex is sensitive to rapamycin, which acts as an allosteric inhibitor of mTORC1 by dissociating raptor from mTOR (Sengupta et al., 2010; Zhou and

Huang, 2010). The key binding partner of mTORC2 is rictor (rapamycin-insensitive companion of mTOR), which according with its name is insensitive to rapamycin.

Recently, new functions have been described for mTOR in the male reproductive system. It was shown that mTOR is involved in BTB restructuring during the epithelial cycle of spermatogenesis (Mok et al., 2013; Mok et al., 2012), illustrating that this molecule may have a preponderant role in the control of male fertility. Hence, we hypothesized that mTOR can play an essential role in the control of SC metabolism and in the nutritional support of spermatogenesis. Moreover, since mitochondria function is also crucial for spermatogenesis and regulates SC metabolism (Rato et al., 2014), we also hypothesized that mTOR signaling could modulate mitochondrial activity in hSCs. To test our hypotheses, we examined the effect of exposure to rapamycin, the inhibitor of mTORC1, in the glycolytic profile of human Sertoli cells (hSCs) and mitochondrial function.

Material and Methods

Chemicals

Tris-Base and NZYColour Protein Maker II were purchased from NZYTech (Lisboa, Portugal). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Patient selection, ethical issues and testicular biopsies

Patient's selection and clinical study was performed at the Center for Reproductive Genetics Professor Alberto Barros (Porto, Portugal) after approval by the Local Ethics Committee. The studies followed the Guidelines of the Local, National and European Ethical Committees and the Declaration of Helsinki. Testicular biopsies were obtained from patients seeking for fertility treatment, after informed written consent. Only cells left in culture plates after fertility treatment were used. hSCs were isolated from testicular biopsies from patients

with anejaculation (psychological, vascular, neurologic) and with conserved spermatogenesis.

Sertoli cells culture

Testicular biopsies were washed using a routine method (Oliveira et al., 2011). hSCs primary cultures were obtained using routine methods (Oliveira et al., 2009). The resulting pellet was suspended in SC culture medium (DMEM:Ham's F-12 1:1, containing 15 mM HEPES, 50 U/ml penicillin and 50 mg/ml streptomycin sulfate, 0.5 mg/ml fungizone, 50 µg/ml gentamicin and 10% heat inactivated fetal bovine serum). Cells were plated and incubated at 32-34°C, 5% CO₂ in air. Cultures were examined and those with contaminants below 5% were used. Cultures purity was tested by immunoperoxidase detection of Anti-Mullerian hormone and Vimentin specific markers (Steger et al., 1996).

Experimental groups

hSCs were allowed to grow (90–95% of confluence). Then, cells were washed and cultured in serum-free medium (supplement DMEM:F12 1:1, pH 7.4). The medium was supplemented either with insulin-transferrin-sodium selenite (ITS) (5 µg/mL–5 µg/mL–5 ng/mL, respectively) [control group], or with ITS plus rapamycin (ITS+R) (100 ng/mL) [rapamycin group]. Cells were maintained for 24 hours. After treatment, culture media were collected, the cells were detached using a Trypsin–EDTA solution and collected. Total protein in each group was quantified using Pierce™ BCA Protein assay kit (Pierce Biotechnology, Rockford, USA). A viability test was performed on the cells of the different experimental groups using the Trypan Blue Exclusion Test. Viability averaged 85–90%, always with values higher than 85%.

Cytotoxicity Assay

The cytotoxicity of hSCs to rapamycin was determined by the colorimetric sulforhodamine B (SRB) assay (Vichai and Kirtikara, 2006). In brief, cells were seeded and cultured during 24 hours in ITS or ITS+R medium. After treatment, cells were washed twice in phosphate buffered saline (PBS) solution and fixed overnight in 1% acetic acid methanol. Cells were then incubated with 0.5% (w/v) SRB in 1% of acetic acid for 1 h at 37°C. The unbound dye was removed by washing with 1% acetic acid solution. Dye bound to cells was extracted with 10 mM Tris solution (pH 10). Optical densities of the media were measured at 540 nm. No cytotoxicity was observed for the concentration of rapamycin used in this work during 24 hours (Fig. 3.1).

NMR spectroscopy

¹H-NMR spectroscopy was performed using routine methods used by our team (Alves et al., 2011). Sodium fumarate (final concentration of 2 mM) was used as an internal reference (6.50 ppm) to quantify metabolites in solution (multiplet, δ , ppm); lactate (doublet, 1.33); alanine (doublet, 1.45); acetate (single, 1.91); pyruvate (singlet, 2.36); H1- α glucose (doublet, 5.22). Spectra analysis was performed offline. The relative areas of ¹H-NMR resonances were quantified using the curve-fitting routine using the NutsPro™ NMR spectral analysis program (Acorn, NMR Inc., Fremont, CA, USA).

Western blot

Western-Blot was performed using standard methods (Alves et al., 2012). In brief, protein samples (75-100 μ g) were fractionated on a 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked and incubated with primary antibodies (Table 3.1) overnight at 4°C.

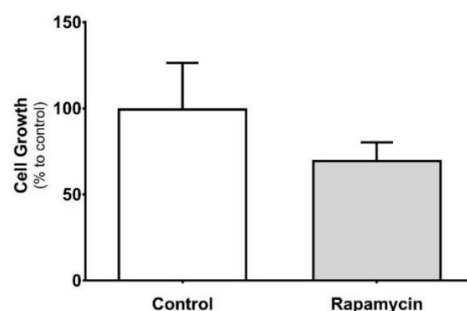


Figure 3.1 – Cytotoxicity of rapamycin in human Sertoli cells (hSCs). Cell density was determined, based on the Sulforhodamine B assay. Results are presented in percentage of cell growth as compared to control. Results are expressed as mean $\pm$ SEM (n = 6 for each condition). *Significantly different results relative to control ($p < 0.05$);

Mouse anti- β -tubulin was used as the protein loading control, except for Total OXPHOs, in which mouse anti- β -actin was used as loading control. The immune-reactive proteins were detected separately with secondary antibodies. Membranes were reacted with ECF detection system (GE Healthcare, Freiburg, Germany) and visualized with the BioRad GelDoc XR (Bio-Rad, USA). The densities from each band were obtained using the Quantity One Software (Bio-Rad, Hemel Hempstead, UK).

Slot blot

The levels of carbonyl and nitro-tyrosine (NT) protein groups and of 4-hydroxynonenal (4-HNE) were measured by the slot-blot technique. Protein carbonyl group levels were determined as described previously (Rato et al., 2014). For determination of NT and 4-HNE levels, 5 μ g of protein were diluted in PBS and transferred to PVDF membranes using a Hybri-slot manifold system (Biometra, Göttingen, Germany). The membranes were then blocked and incubated with the respective primary antibodies (Table 3.1) overnight at 4°C. The immune-reactive proteins were detected separately with secondary antibodies. Membranes were reacted and visualized as described above. Densities were quantified using the Quantity One Software.

Table 3.1: List of Antibodies used in this study

Antibody	Source	Molecular Weight (kDa)	Dilution	Vendor	Catalog #
GLUT1	Rabbit	46	1:5000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-7903
GLUT2	Rabbit	61	1:1000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-9117
GLUT3	Goat	46	1:500	Santa Cruz Biotechnology, Heidelberg, Germany	sc-7582
PFK-1	Rabbit	85	1:500	Santa Cruz Biotechnology, Heidelberg, Germany	sc-67028
LDH	Rabbit	37	1:5000	Abcam, Cambridge, MA, USA	ab52488
MCT4	Rabbit	43	1:1000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-50329
PI3K	Rabbit	85	1:2500	Milipore, Darmstadt, Germany	Cat.#06-195
GSK3	Mouse	51(alfa)	1:1000	Milipore, Darmstadt, Germany	Cat.#05-412
P-mTOR	Rabbit	289	1:1000	Milipore, Darmstadt, Germany	Cat.#09-213
P-4EB-P1	Rabbit	20	1:1000	Cell Signaling Technology, Beverly, MA, United States	#2855
Total OXPHOS	Mouse	20, 30, 40, 48, 55	1:1000	Abcam, Cambridge, MA, USA	ab110413
β-Tubulin	Mouse	55	1:5000	Thermo Fisher Scientific, Rockford, IL, USA	BT7R
β-Actin	Mouse	42	1:5000	Thermo Fisher Scientific, Rockford, IL, USA	BA3R
Mn SOD	Mouse	43	1:1000	ENZO Life Sciences, Farmingdale, NY, USA	ALX-804-265-C100
DNP	Rabbit	---	1:5000	Sigma-Aldrich, Roedermark, Germany	D9656
4-HNE	Goat	---	1:5000	EMD Millipore, Temecula, CA, USA	AB5605
Nitro-Tyrosine	Rabbit	---	1:2500	Cell Signaling Technology, Danvers, MA, USA	9691S
Mouse	Goat	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A3562
Rabbit	Goat	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A3687
Goat	Monkey	---	1:5000	Santa Cruz Biotechnology, Heidelberg, Germany	SC-2020

GLUT1- Glucose transporter 1; GLUT2- Glucose transporter 2; GLUT3- Glucose transporter 3; PFK-1- Phosphofructokinase 1; MCT4- Monocarboxylate transporter 4; LDH- Lactate dehydrogenase; PI3K- Phosphatidylinositol 3-kinase; GSK3- glycogen synthase kinase 3; P-mTOR- phosphorylated mammalian target of rapamycin; P-4EBP-1- phosphorylated Eukaryotic translation initiation factor 4E-binding protein 1; OXPHOS - oxidative phosphorylation; Mn SOD - manganese superoxide dismutase; DNP- Dinitrophenyl; 4-HNE- 4-Hydroxynonenal;

Citrate synthase activity

Citrate synthase (CS) activity was measured according to Coore and colleagues (Coore et al., 1971) using a spectrophotometric assay (412 nm), in which the amount of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) that reacted with coenzyme A (CoASH) (released from the reaction of acetyl-CoA with oxaloacetate) was determined by measuring the appearance of 5-thio-2-nitrobenzoic acid (TNB) (molar extinction coefficient $\epsilon = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$). Results were expressed in nanomoles per minute per microgram of protein.

Sulfhydryl protein groups

Sulfhydryl protein groups were spectrophotometrically determined as described by Hu and colleagues (Hu, 1994). The absorbance of the samples was determined at 412 nm against a blank, after reaction of 5 μl of protein extract in phosphate buffer (pH 7.4) with 1 μl of DTNB (10 mM) in a medium containing 15 μl of Tris buffer (0.25 M Tris, 20 mM EDTA) and 79 μl of methanol. Sulfhydryl content was calculated measuring the appearance of TNB (using the molar extinction coefficient $\epsilon = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$) and expressed in nanomoles per milligram of protein.

Statistical analysis

All data are shown as mean $\pm$ SEM ($n = 6$ for each condition). Statistical differences between experimental groups were assessed by unpaired t test using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered significant.

Results

Rapamycin increases glucose consumption by human Sertoli cells

SCs metabolize the extracellular glucose and ensure the production of metabolic precursors necessary for developing germ cells (for review (Alves et al., 2013)). After treatment with rapamycin, glucose consumption by hSCs was higher, when compared with cells cultured in control conditions (383 ± 19 vs 219 ± 62 nmol of glucose/ μ g of protein) (Fig. 3.2A). Glucose enters into hSCs through glucose transporters (GLUTs), particularly GLUT1, GLUT2 and GLUT3 (Alves et al., 2014; Carosa et al., 2005; Galardo et al., 2008; Martins et al., 2014). There were no

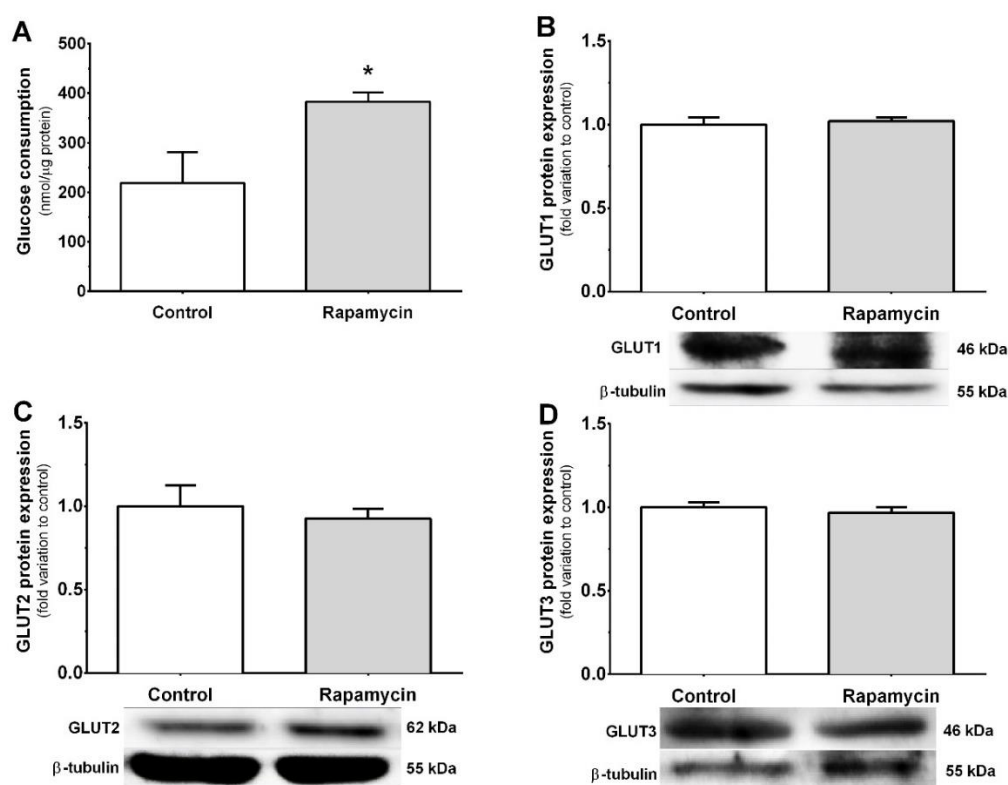


Figure 3.2 – Effect of rapamycin in glucose uptake and consumption by human Sertoli cells (hSCs). The consumption of glucose by hSCs was determined (Panel A) and is represented in nmol/ μ g of protein. The expression of GLUT1 (Panel B); GLUT2 (Panel C); GLUT3 (Panel D) are also represented, as well as illustrative western blot bands. Results are expressed as mean $\pm$ SEM ($n = 6$ for each condition). *Significantly different results relative to control ($p < 0.05$).

alterations in the expression levels of GLUT1, GLUT2 or GLUT3, as determined by Western blot, in hSCs exposed to rapamycin (Fig. 3.2B, C and D).

Production of monocarboxylates by human Sertoli cells is insensitive to rapamycin treatment

Glucose is metabolized in a sequence of multi-step reactions. Phosphofructokinase (PFK) mediates the first rate-limiting step in glycolysis (Chehtane and Khaled, 2010) and plays a crucial regulatory role in the control of SC metabolism (Martins et al., 2014; Martins et al., 2013). Nevertheless, exposure to rapamycin was not able to alter the protein expression of this enzyme, as measured by Western Blot (data not shown). Also, the consumption of pyruvate by hSCs was not altered by exposure to rapamycin (Fig. 3.3A).

The pyruvate originated from glycolysis can: (1) be converted to alanine by alanine aminotransferase; (2) be converted to lactate by lactate dehydrogenase (LDH), with the simultaneous oxidation/reduction of NADH to NAD⁺; (3) or be converted to acetyl-CoA, which enters mitochondria to be used in the Krebs cycle, or is converted into acetate. In hSCs, the majority of pyruvate taken from the media or derived from glycolysis is converted to lactate. When hSCs were exposed to rapamycin, we did not detect (by Western Blot) alterations in LDH protein levels (data not shown) or in lactate production (Fig. 3.4A) when

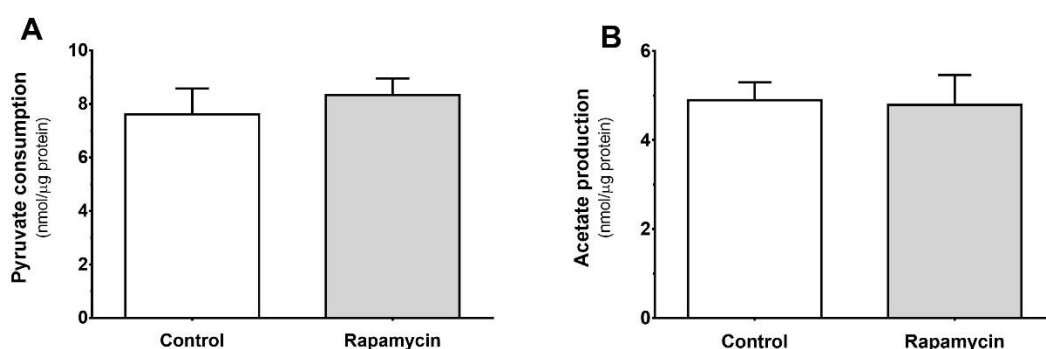


Figure 3.3 – Effect of rapamycin in intracellular glucose metabolism. The production of pyruvate (Panel A) and acetate (Panel B) by human Sertoli cells (hSCs) was determined. The results are expressed as mean ± SEM (n = 6 for each condition) in nmol/μg of protein. *Significantly different results relative to control (p < 0.05);

compared with cells cultured in control conditions. The production of acetate by hSCs exposed to rapamycin was also not altered when compared with hSCs in control conditions (Fig. 3.3B). However, treatment of hSCs with rapamycin induced a decrease in alanine production when compared with cells in control conditions (1.8 ± 0.8 vs 4.3 ± 0.9 nmol of alanine/ug of protein) (Fig. 3.4B).

Rapamycin promotes intracellular redox unbalance and stimulates lipid peroxidation in human Sertoli cells

Exposure of hSCs to rapamycin augmented the lactate/alanine ratio (1.0 ± 0.4), when compared with cells cultured in control conditions (2.6 ± 0.6) (Fig. 3.4C). The NAD^+/NADH ratio reflects the oxidative status of the cell. Our results suggested an unbalanced redox status in rapamycin-exposed hSCs. Reactive oxygen and nitrogen species may oxidize protein side chains, generating the formation of carbonyl group and nitrotyrosine groups (Dalle-Donne et al., 2003). Rapamycin-exposed hSCs did not present altered levels of protein carbonylation (dinitrophenylhydrazine reacts with free carbonyl groups on proteins, leading to adduction of dinitrophenyl groups) or nitration when compared to non-exposed cells (Fig. 3.4D and E). The protein sulfhydryl (SH) groups are also susceptible to oxidation (Frei et al., 1988; Halliwell and Gutteridge, 1989; Halliwell and Gutteridge, 1990; Wayner et al., 1987). We observed no differences in total SH groups in hSCs exposed to rapamycin compared with cells cultured in control conditions (Fig. 3.4F). 4-HNE is a byproduct formed during oxidation of unsaturated fatty acids (Shiraishi and Naito, 2007). The levels of 4-HNE were higher in cultured hSCs exposed to rapamycin than in non-exposed cells (Fig. 3.4A).

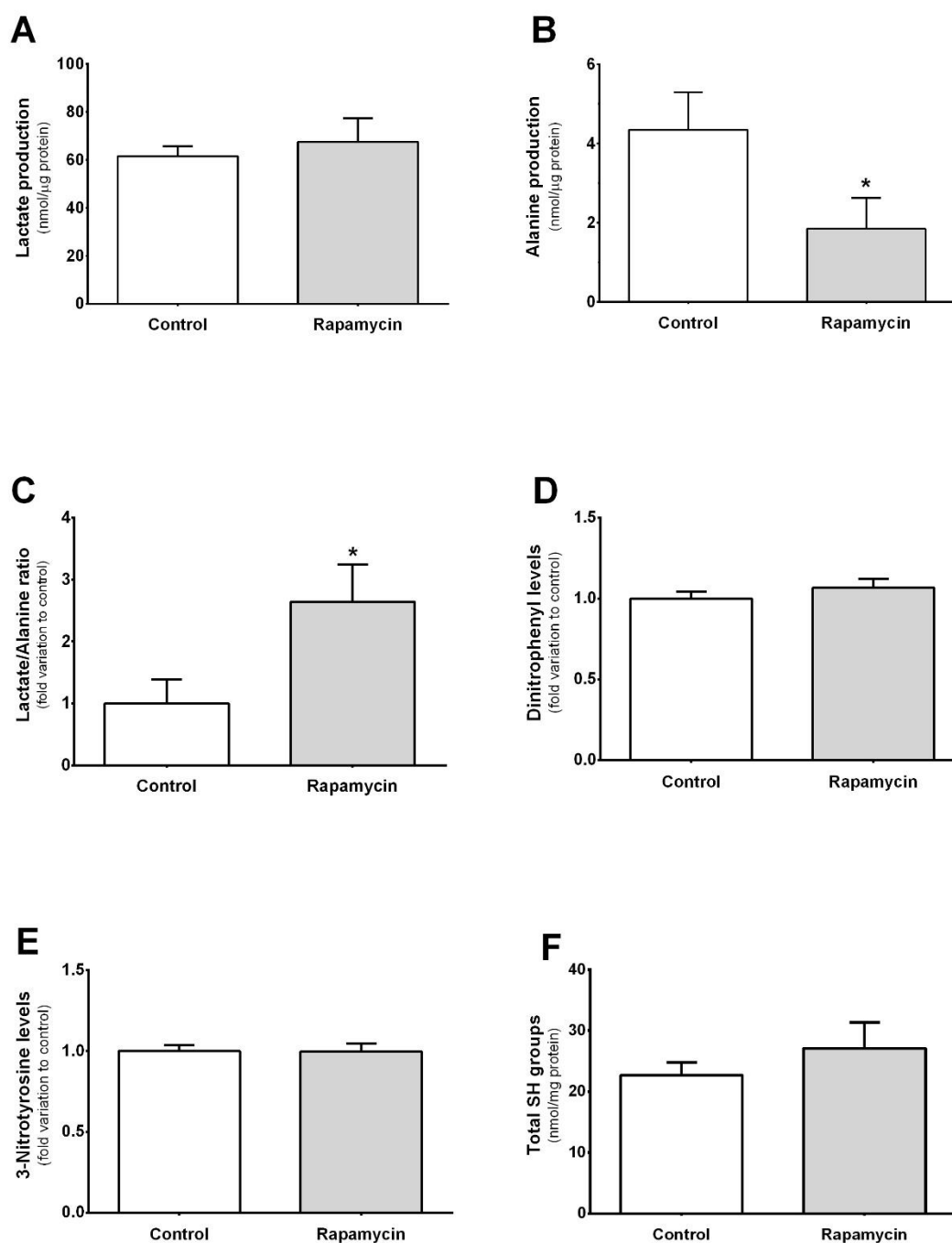


Figure 3.4 – Effect of rapamycin in intracellular glucose metabolism and in oxidative profile of human Sertoli cells (hSCs). The production of lactate (Panel A) and alanine (B) by human Sertoli cells (hSCs) was determined. The results are expressed as nmol/μg of protein. The lactate/alanine ratio (Panel C) is represented as an indicator of intracellular redox state. Oxidative damages were measured in proteins carbonylation (Panel D) and nitration (Panel E) and by sulfhydryl groups content (Panel F). Results are expressed as mean ± SEM (n = 6 for each condition). *Significantly different results relative to control (p < 0.05);

Exposure to rapamycin decreases mitochondrial complex III expression in human Sertoli cells

Mitochondria are responsible for ATP synthesis by oxidative phosphorylation. The electrons resultant from oxidation of substrates are guided through redox carriers (mitochondrial complexes I, II, III and IV) and linked with proton pumping by an F-ATPase (mitochondrial complex V). Rapamycin-exposed hSCs presented no alterations in the expression of the mitochondrial complexes II, IV and V, compared to non-exposed cells (Fig. 3.5B, D and E). However, a decrease was detected (from 1.00 ± 0.17 to 0.50 ± 0.04) in rapamycin-exposed hSCs expression of mitochondrial complex III, when compared to non-exposed cells (Fig. 3.5C). The enzyme citrate synthase stands as a pace-making enzyme for Krebs cycle (Wiegand and Remington, 1986) and is used as a quantitative marker for intact mitochondria. The activity of citrate synthase was not altered in hSCs exposed to rapamycin when compared to non-exposed cells (Fig. 3.5F).

Phosphorylation of mammalian target of rapamycin is inhibited in human Sertoli cells exposed to rapamycin

Phosphatidylinositol 3-kinase (PI3K) is an upstream regulator of mTORC1, which is phosphorylated and activated upon the binding of insulin to its receptors (Zoncu et al., 2011). Rapamycin treatment did not alter PI3K expression in hSCs (Fig. 3.6A). PIP₃ is a second messenger, phosphorylated by PI3K. Akt is recruited by PIP₃ and, besides being an upstream regulator of mTORC1, it phosphorylates glycogen synthase kinase 3 (GSK-3) (Leary et al., 2013). The expression of GSK-3 is not altered in hSCs exposed to rapamycin (Fig. 3.6B). However, as expected, treatment of hSCs with rapamycin caused a decrease in the expression of phosphorylated mTOR (P-mTOR) when compared with non-exposed cells (Fig. 3.6C). The eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) is a downstream signaling molecule of mTORC1 (Dazert and Hall, 2011; Laplante and Sabatini, 2012). Notably, the expression of phosphorylated 4E-BP1 (P-4E-BP1) is not altered in hSCs exposed to rapamycin (Fig. 3.6D).

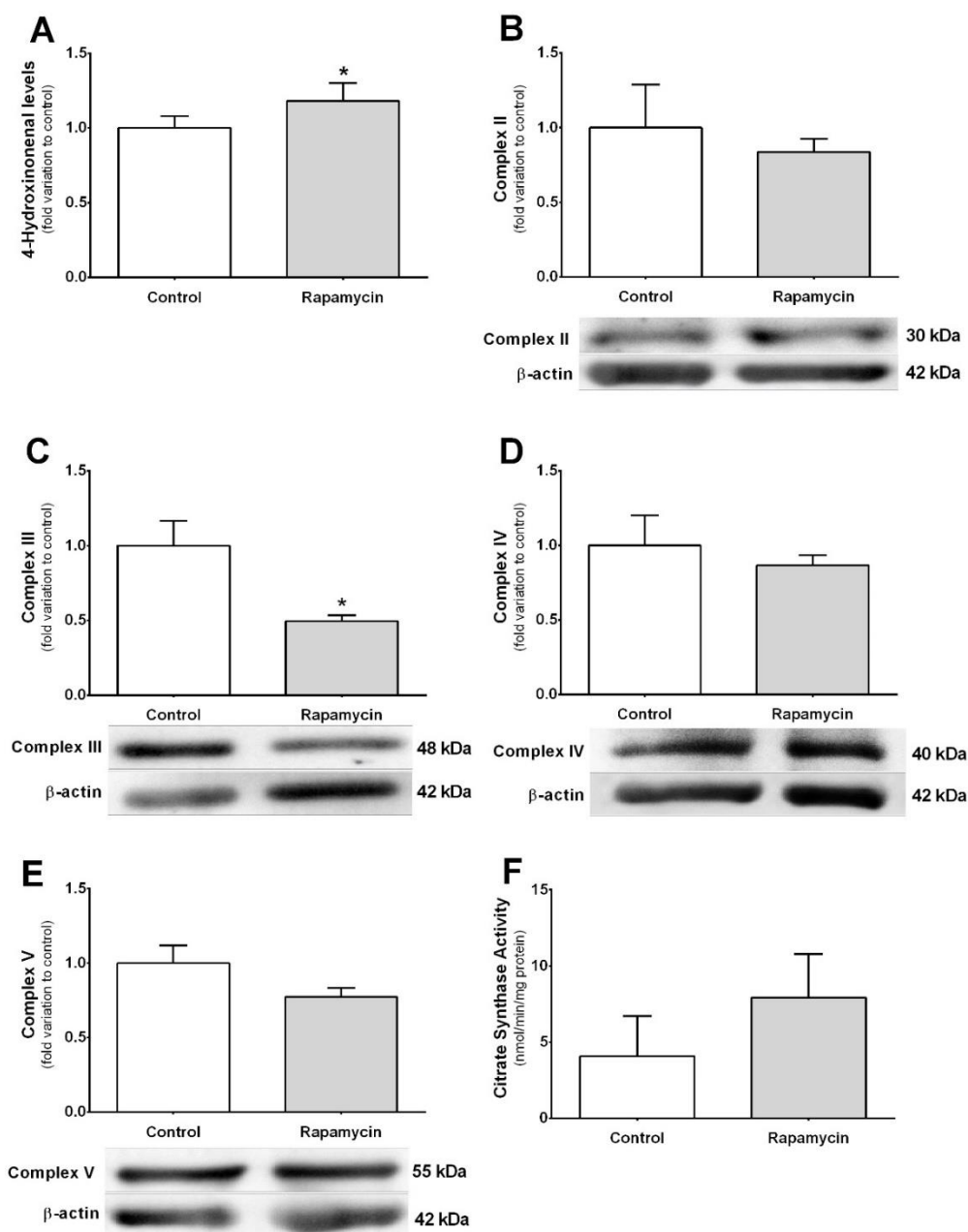


Figure 3.5 – Effect of rapamycin in the oxidative profile, complexes proteins levels and in mitochondrial integrity of human Sertoli cells (hSCs). Oxidative damages were measured in lipid peroxidation (Panel A). The protein levels of mitochondrial complex II (Panel B), complex III (Panel C), complex IV (Panel D) and complex V (Panel E) are represented as well as illustrative western blot bands. The bands were divided by the respective actin and the results are represented in fold variation to control. The presence of/intact mitochondria was measured by citrate synthase activity (Panel F). The results are expressed as nmol of CoASH/min/mg of protein. Results are expressed as mean $\pm$ SEM ($n = 6$ for each condition). * Significantly different results relative to control ($p < 0.05$).

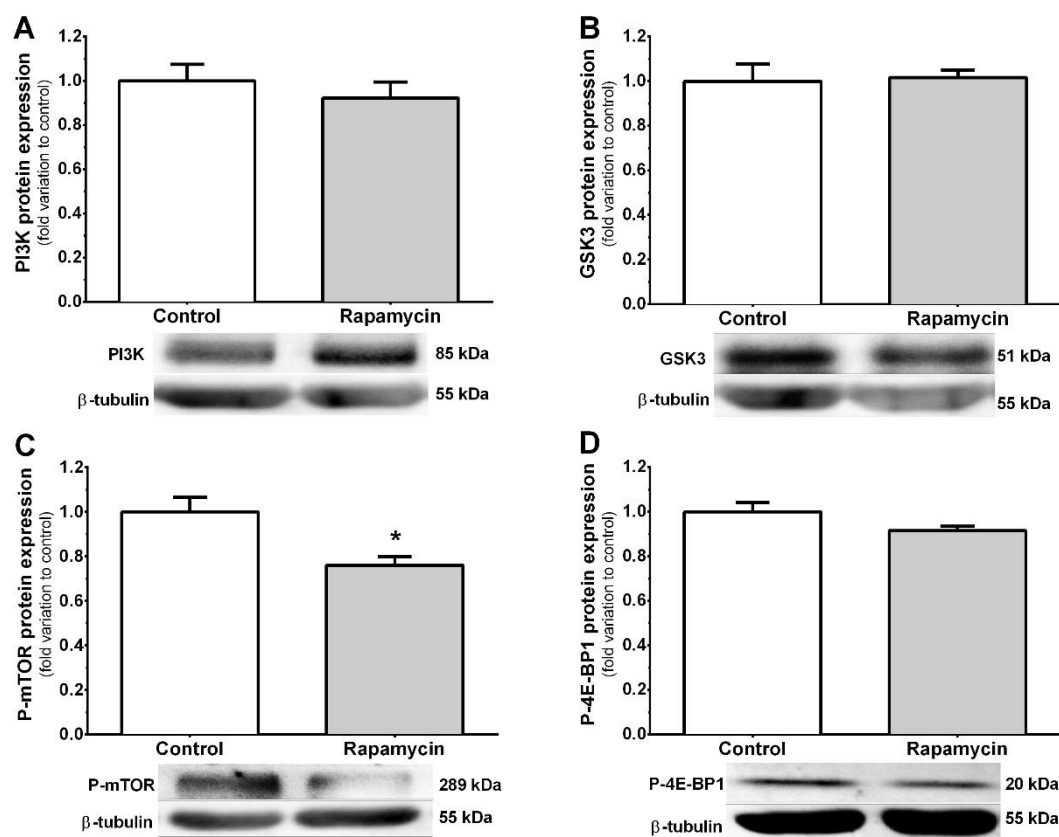


Figure 3.6 – Effect of rapamycin in the protein expression levels of target of rapamycin (TOR) signaling pathway. The protein expression levels of PI3K (Panel A); GSK3 (Panel B); P-mTOR (Panel C) and P-4E-BP1 (Panel D) in hSCs were determined. Results are expressed as mean $\pm$ SEM ($n = 6$ for each condition). * Significantly different results relative to control ($p < 0.05$). PI3K - phosphatidylinositol 3-kinase; GSK3 - glycogen synthase kinase 3; mTOR - mammalian target of rapamycin; 4E-BP1 - eukaryotic translation initiation factor 4E-binding protein 1.

Discussion

Sertoli cells (SCs) create the BTB and are responsible for the physical and nutritional support of developing germ cells (Cheng and Mruk, 2010; Griswold, 1998; Mruk and Cheng, 2004). Thus, SC metabolism is an essential event for the normal occurrence of spermatogenesis (Oliveira et al., 2015). Compelling evidence showed that mTOR is pivotal in the establishment and reorganization of BTB. However, other functions for mTOR signaling pathway in male reproductive system remain to be unveiled. Thus, we aimed to evaluate the effect of an mTORC1 inhibitor (rapamycin) in the nutritional support of spermatogenesis

by hSCs. We determined the effect of rapamycin in hSCs carbohydrate metabolism, metabolite secretion/consumption, oxidative status, the involvement of mTOR signaling pathway and alterations in mitochondrial complexes expression.

Our data shows that mTOR is a novel regulator of hSCs glycolytic and oxidative profiles. Cultured hSCs use glucose to produce lactate at high concentrations, in a typical Warburg-like metabolism (Oliveira et al., 2015). Interestingly, glucose consumption was increased in rapamycin-treated cells though lactate, pyruvate and acetate production remained unchanged. SCs display abnormally elevated glycolytic activity, under normal conditions, and a high metabolic plasticity to sustain lactate production (Oliveira et al., 2012; Rato et al., 2012). Rapamycin, the inhibitor of mTORC1 pathway, is a regulator of the glycolytic flux in mammalian cells (Edinger et al., 2003). mTORC1 pathway controls glucose metabolism and glycolysis through regulation of glucose transporters and rate-limiting glycolytic enzymes (Finlay et al., 2012; Li et al., 2013). In human skeletal muscle, rapamycin administration augmented glucose uptake in the presence of combined hyperaminoacidemia and prandial-like peripheral hyperinsulinemia (Krebs et al., 2007). Under unfavorable conditions, such as insulin deprivation (Alves et al., 2012; Oliveira et al., 2012), hSCs sustain lactate production by adapting their metabolism. Inhibition of the mTORC1/S6K pathway by rapamycin induces an increase in glucose consumption to produce the same amount of lactate and acetate as control cells. This was not followed by alterations in GLUTs, PFK or LDH protein levels suggesting that mTORC1 pathway regulates glucose transporters and rate-limiting glycolytic enzymes activity in hSCs.

The mTOR signaling pathway is also linked to amino acids metabolism (Tremblay and Marette, 2001). Cells cultured in conditions of amino acids deprivation show a rapid decline in the phosphorylation of proteins involved in mTOR pathway (Hara et al., 1998). In addition, amino acids activate ribosomal protein S6 kinase (S6K), the downstream signaling of mTOR pathway. Pyruvate produced by hSCs can be either converted to alanine or lactate through reactions coupled with the NAD^+/NADH equilibrium. Since rapamycin-exposed hSCs decrease the production of alanine, a higher lactate/alanine ratio was detected. Concurrently,

the cellular NAD⁺/NADH ratio was increased in rapamycin-exposed hSCs, which led us to hypothesize that oxidative stress and/or mitochondrial dysfunction could occur. We analyzed specific markers for oxidative stress such as protein carbonylation, nitration, SH groups and lipid peroxidation levels. Our data shows that rapamycin-exposed SCs present a higher lipid peroxidation. Rapamycin is known to sensitize cells with a high glycolytic activity, namely cancer cells, to oxidative stress (for review (Nogueira and Hay, 2013)). Indeed, rapamycin hyperactivates Akt *via* inhibition of a negative feedback loop induced by mTORC1 to inhibit Akt activity (Bhaskar and Hay, 2007). This has been suggested as a strategy to selectively kill cells treated with rapamycin, particularly cancer cells (Nogueira et al., 2008). hSCs present some remarkable metabolic similarities with cancer cells (for review (Oliveira et al., 2015)) and cellular glucose metabolism is closely related to oxidative stress. Thus, our data suggests that rapamycin treatment sensitizes hSCs to oxidative stress, particularly lipid peroxidation.

Mitochondrial dysfunction is a major contributor for lipid peroxidation (Cummins et al., 1994). Moreover, an interplay between mitochondrial function and mTOR signaling has been shown, since mTOR pathway plays a critical role in oxygen consumption and oxidative capacity in mammalian cells (Schieke et al., 2006). The inhibition of mTOR signaling by rapamycin in Jurkat cells promoted the shift of glucose metabolism from mitochondrial respiration to glycolysis (Ramanathan and Schreiber, 2009). It was also suggested that mTOR activity may be sensitive to cells redox status, which is concomitant with our data concerning lipid peroxidation. mTOR was also shown to bind to the mitochondrial outer membrane and alter mitochondrial genes expression (Cunningham et al., 2007; Ramanathan and Schreiber, 2009). Our data shows that exposure to rapamycin decreases mitochondrial complex III protein levels in hSCs, illustrating that mTOR signaling is pivotal for maintenance of normal bioenergetics. Although hSCs present a clear Warburg-like metabolism, under detrimental conditions, such as insulin deprivation, mitochondrial-mediated apoptosis is activated (Dias et al., 2013), thus mitochondrial bioenergetics are also pivotal. This is crucial since SCs are only capable to sustain a limited number of germ cells (Weber et al., 1983; Wong and Russell, 1983) and male reproductive potential is often associated with the number and function of SCs. Further studies will be needed to enlighten the

relevance of mTOR in oxidative stress, mitochondrial physiology and apoptosis in these cells.

The treatment of hSCs with rapamycin during 24h led, as expected, to a decrease in mTOR phosphorylation at Ser-2448 illustrating an effective partial inhibition of mTORC1 by rapamycin. Interestingly, there were no differences in PI3K, GSK3 and P-4E-BP1 levels. We aimed to determine the impact of rapamycin on hSCs metabolism and thus, we selected a period of 24 h in order to allow changes in metabolic pathways to occur, while not long enough to induce mTORC2 inhibition. mTORC1 inhibition is reported to stimulate Akt signaling (Bhaskar and Hay, 2007), which can upregulate mTOR pathway. Moreover, rapamycin is not able to completely inhibit mTORC1 phosphorylation/pathway. 4E-BP1 is a downstream signaling molecule of mTORC1 (Beretta et al., 1996). However, 4E-BP1 is rephosphorylated and is refractory in exposure to rapamycin during longer periods (Choo et al., 2008; Thoreen et al., 2009). A 24 h treatment of hSCs with rapamycin partially inhibits mTOR phosphorylation at Ser-2448, though downstream P-4E-BP1 levels remain unchanged, probably due to rephosphorylation.

mTOR signaling was shown to regulate BTB dynamics (Mok et al., 2013; Mok et al., 2014; Mok et al., 2013; Mok et al., 2012) but few studies have been performed to unveil other mechanisms by which mTOR can regulate spermatogenesis. Rapamycin inhibits apoptosis promoted by reactive oxygen species inducers by alleviating mitochondrial dysfunction (He et al., 2014). Moreover, rapamycin treatment in mice led to reduced testicular size and arrest of germ cells development, illustrating that mTORC1 is required for mice spermatogonial differentiation *in vivo* (Busada et al., 2015). Nevertheless, the effect of rapamycin in isolated testicular cells and human male reproductive system remained undisclosed. Herein we show that inhibition of mTORC1 in hSCs increases glucose consumption and decreases alanine production, promoting an unbalanced redox state. This redox unbalance is reflected in an increased lipid peroxidation that may be associated with decreased mitochondrial complex III expression.

Overall, our data shows that mTOR signaling, particularly mTORC1, alters the glycolytic and oxidative profile of hSCs. Still, our report presents some limitations. The testicular tissue includes several types of cells and this work was done using an *in vitro* approach, with isolated hSCs. Being so, it was not possible to fully expand the conclusions to an *in vivo* situation, and extrapolate these results in terms of whole testis environment, in which the role of mTORC1 remains to be unveiled. Further studies will be needed to disclose the full involvement of mTOR in the control of human male reproductive potential, particularly in individuals suffering from metabolic diseases, aged men, those with low sperm counts and other fertility problems.

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Chapter 4

Differential response of lactate production by human Sertoli cells to glucose and essential or non-essential amino acids stimulation

This chapter was adapted from the submitted work:

Jesus TT, Oliveira PF, Barros A, Sousa M, Cheng CY, Alves MG. Differential response of lactate production by human Sertoli cells to glucose and essential or non-essential amino acids stimulation.

4. Differential response of lactate production by human Sertoli cells to glucose and essential or non-essential amino acids stimulation

Running title: Substrate availability and lactate production by human Sertoli cells

Abstract

Objective: To study the differential lactate production by human Sertoli cells (hSCs) promoted by glucose and essential or non-essential amino acids stimulation.

Design: Experimental study

Setting: University research center and private IVF centers.

Patients/Animals: Six men with anejaculation (psychological, vascular, neurologic) and conserved spermatogenesis.

Intervention: Testicular biopsies from patients under treatment for recovery of male gametes were used. Primary hSCs cultures were established from each biopsy and subjected to stimulation with different carbon sources: glucose (5.5 and 17.5 mM); leucine (5 mM) and glutamine (5 mM) during 30 minutes and 6 hours.

Main Outcome Measure(s):

¹H-NMR was performed to detect metabolite consumption and production by hSCs. In addition, mitochondrial complexes expression was evaluated in cells, as well as the levels of 4-hydroxynonenal, a marker of lipid peroxidation. Specific markers of mTOR pathway were determined.

Results: Increased glucose availability increases glucose uptake without stimulation of pyruvate export. Notably, physiological levels of glucose further stimulated lactate production than higher glucose levels, suggesting that glucose homeostasis is essential for lactate production by hSCs. When leucine was the only carbon source in the media, pyruvate export was decreased, as well as lactate production by hSCs, when compared with physiological concentrations of

glucose. Glutamine enhanced pyruvate and acetate export by hSCs. Glutamine also decreased the expression of mitochondrial complexes II and V in hSCs, while inducing a decrease in lactate production, when compared with physiological concentrations of glucose. Finally, all substrates transiently induced phosphorylation of S6 ribosomal protein (rpS6) in hSCs.

Conclusion: We report a differential response of lactate production by hSCs to glucose and amino acids stimulation, transiently coordinated by mTOR pathway. This study unveils a new role for mTOR in male fertility and highlights the contribution of glucose and amino acids homeostasis for spermatogenesis.

Key words: Sertoli cells; Spermatogenesis; leucine; glutamine; amino acids

Introduction

Sertoli cells (SCs) reside in the of seminiferous epithelium, side by side with developing germ cells at different stages of development (Mruk and Cheng, 2004). Adjacent SCs establish the blood-testis barrier (BTB), or Sertoli cell barrier, in order to maintain an adequate environment for the occurrence of spermatogenesis (Rato et al., 2010). These testicular somatic cells are known as “nurse cells” because they are responsible for the physical and nutritional support of spermatogenesis (Cheng and Mruk, 2010; Griswold, 1998; Mruk and Cheng, 2004). Germ cells depend upon the production and export of specific metabolites by SCs. For instance, lactate is essential as energy source for developing germ cells (Boussouar and Benahmed, 2004), being pivotal for the normal occurrence of spermatogenesis (Oliveira et al., 2015; Rato et al., 2016).

The mammalian target of rapamycin (mTOR) is a Ser/Thr protein kinase (Hsu et al., 2011; Yu et al., 2011) that belongs to a cell signaling pathway with the same name. This signaling pathway integrates an enormous number of environmental cues and exerts functions on numerous cellular events including cell growth/proliferation and metabolism (Weichhart, 2012; Zoncu et al., 2011). mTOR can be incorporated into two different multiprotein complexes: mTORC1 and mTORC2 (Dazert and Hall, 2011; Laplante and Sabatini, 2012). While

mTORC2 promotes cell proliferation and survival (Laplante and Sabatini, 2012; Saxton and Sabatini, 2017), mTORC1 is generally associated with cell growth (Laplante and Sabatini, 2012; Saxton and Sabatini, 2017). mTOR is a key regulator for sensing and integrating various environmental cues, including nutrients (Laplante and Sabatini, 2012; Saxton and Sabatini, 2017), such as amino acids. For a decade it became clear that amino acids are essential to mTORC1 activation. However, precisely how this input was integrated remained a matter of discussion (Efeyan et al., 2012; Jewell et al., 2013). mTORC1 promotes growth in response to nutrients availability, such as amino acids, which drive mTORC1 to its activation site at the lysosomal surface. Only recently it was shown that amino acid levels communicate with mTORC1 after the discovery of lysosome-based signaling systems (Bar-Peled and Sabatini, 2014).

In male reproductive system, new functions for mTOR signaling have been described. It was demonstrated that mTOR is involved in BTB restructuring during the seminiferous epithelial cycle (Mok et al., 2013; Mok et al., 2012) and, more recently, it was reported that mTOR regulates glucose consumption of human SCs (hSCs) and the redox balance in these cells (Jesus et al., 2016). mTOR seems to regulate the nutritional support of spermatogenesis by integrating signals. Thus, we hypothesized that mTOR can have an important role in the control of SC metabolism and in the nutritional support of spermatogenesis. To test this hypothesis, and since only recently it has been unveiled how amino acid levels are communicated to mTORC1, we deprived hSCs from glucose and fed them with specific amino acids only, an essential (leucine) or a non-essential (glutamine) amino acid, and compared to hSCs exposed to physiological levels of glucose (found in plasma) and high-levels of glucose, and examined the effect of these conditions in the glycolytic profile of hSCs and mitochondrial function.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Patient selection, ethical issues and testicular biopsies

Patient's selection and clinical study was performed at the Center for Reproductive Genetics Professor Alberto Barros (Porto, Portugal) in accordance with the Guidelines of the Local, National and European Ethical Committees. Testicular biopsies were obtained from patients seeking for fertility treatment, after attaining informed written consent. Only cells left in the culture plates after fertility treatment were used. All studies were performed according to the Declaration of Helsinki. Human Sertoli cells (hSCs) were isolated from testicular biopsies from patients with anejaculation (psychological, vascular, neurologic) and conserved spermatogenesis.

Sertoli cell culture

Testicular biopsies were washed twice in HBSSf (Hanks Balanced Salt Solution without Ca^{2+} or Mg^{2+} ; Sigma-Aldrich) with centrifugation at 500.g at room temperature, using a routine method (Oliveira et al., 2011). hSCs primary cultures were also obtained using routine methods (Oliveira et al., 2009). The resulting pellet was suspended in SC culture medium (DMEM:Ham's F-12 1:1, containing 15 mM HEPES, 50 U/ml penicillin and 50 mg/ml streptomycin sulfate, 0.5 mg/ml fungizone, 50 $\mu\text{g}/\text{ml}$ gentamicin and 10% heat inactivated FBS). The concentration of cells was adjusted to 5000 cells/ml in SC culture medium, plated and incubated at 32-34°C, 5% CO_2 in air. Cultures were examined by phase contrast microscopy and only those with contaminants below 5% were used. The

purity of the cultures was tested by immunoperoxidase detection of Anti-Mullerian hormone and Vimentin specific markers (Steger et al., 1996).

Experimental groups

hSCs were allowed to grow until reaching 90–95% of confluence. Then, cells were thoroughly washed and medium replaced by serum-free saline Krebs-Henseleit solution (in mM: NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.8, KH₂PO₄ 1.8, NaHCO₃ 25, HEPES 25, pH 7.4). To evaluate the effects of stimulation of different amino acids in the glycolytic profile of hSC five different treatment groups were established: (1) Krebs-Henseleit solution only (No Glucose); or Krebs-Henseleit solution supplemented with: (2) Glucose 5.5 mM (physiological levels found in human plasma); (3) Glucose 17.5 mM (high glucose levels); (4) Leucine 5 mM; or (5) Glutamine 5 mM. Cells were maintained in these conditions for two different time points: 30 minutes and 6 hours. After treatment, culture media was collected for ¹H-NMR analysis and the cells detached using a Trypsin–EDTA solution and collected. A viability test was performed on the cells of the different experimental groups using the Trypan Blue Exclusion Test. Viability averaged 85–90%, always with values higher than 85%.

Total protein fraction was extracted using Mammalian Protein Extraction Reagent (supplemented with 1% protease inhibitor cocktail and 100 mM sodium orthovanadate) following the manufacturer's instructions. Protein concentration of samples was quantified using Pierce™ BCA Protein assay kit (Pierce Biotechnology, Rockford, USA).

NMR spectroscopy

¹H-NMR spectroscopy was performed to determine lactate and acetate production, glucose consumption and variations in other substrates, such as pyruvate and alanine, during the incubation of hSCs in the different experimental conditions, as routinely done by our team (Alves et al., 2011). Sodium fumarate

(final concentration of 2 mM) was used as an internal reference (6.50 ppm) to quantify metabolites in solution (multiplet, δ , ppm); leucine (triplet, 0.95); lactate (doublet, 1.33); alanine (doublet, 1.45); acetate (single, 1.91); pyruvate (singlet, 2.36); glutamine (triplet, 3.75); H1- α glucose (doublet, 5.22). Spectra analysis was performed offline. The relative areas of ^1H -NMR resonances were quantified using the curve-fitting routine using the NutsProTM NMR spectral analysis program (Acorn, NMR Inc., Fremont, CA, USA).

Western blot

Western-Blot was performed using standard methods (Alves et al., 2012). In brief, protein samples (75-100 μg) were fractionated on a 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked and incubated with primary antibodies overnight at 4°C as indicated in Table 4.1. Mouse anti- β -tubulin was used as the protein loading, except for Total OXPHOs, which used mouse anti- β -actin. The immune-reactive proteins were detected separately with secondary antibodies at room temperature (Table 4.1). Membranes were reacted with ECF detection system (GE Healthcare, Freiburg, Germany) and read with the BioRad GelDoc XR (Bio-Rad, USA). The densities from each band were obtained using the Quantity One Software (Bio-Rad, Hemel Hempstead, UK).

Slot blot

The levels of 4-hydroxynonenal (4-HNE) were measured by Slot blot. In brief, for 4-HNE levels, 5 μg of protein were diluted in PBS and transferred to PVDF membranes using a Hybri-slot manifold system (Biometra, Göttingen, Germany). The membranes were then blocked and incubated with the respective primary antibody (Table 4.1) overnight at 4°C. The immune-reactive proteins were detected separately with secondary antibodies (Table 4.1) at room temperature. Membranes were reacted with ECF detection system (GE Healthcare, Freiburg,

Table 4.1: List of Antibodies used in this study

Antibody	Source	Molecular Weight (kDa)	Dilution	Vendor	Catalog #
LDH	Rabbit	37	1:5000	Abcam, Cambridge, MA, USA	ab52488
P-mTOR	Rabbit	289	1:1000	Milipore, Darmstadt, Germany	Cat.#09-213
P-rps6	Rabbit	32	1:1000	Cell Signaling Technology, MA, USA	#2215
Total OXPHOS	Mouse	20, 30, 40, 48, 55	1:1000	Abcam, Cambridge, MA, USA	ab110413
β-Tubulin	Mouse	55	1:5000	Thermo Fisher Scientific, Rockford, IL, USA	BT7R
β-Actin	Mouse	42	1:5000	Thermo Fisher Scientific, Rockford, IL, USA	BA3R
4-HNE	Goat	---	1:5000	EMD Millipore, Temecula, CA, USA	AB5605
Mouse	Goat	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A3562
Rabbit	Goat	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A3687
Goat	Donkey	---	1:5000	Santa Cruz Biotechnology, Heidelberg, Germany	SC-2020

LDH- Lactate dehydrogenase; P-mTOR- phosphorylated mammalian target of rapamycin; P-rps6- phosphorylated S6 ribosomal protein; OXPHOS - oxidative phosphorylation; 4-HNE- 4-Hydroxynonenal

Germany) and read using the BioRad GelDoc XR (Bio-Rad, Hemel Hempstead, UK). Densities from each band were quantified using Quantity One software (Bio-Rad, Hemel Hempstead, UK).

Statistical analysis

Statistical differences between experimental groups were assessed by one-way ANOVA test. All data are shown as mean ± SEM (n = 6 for each condition). Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). p < 0.05 was considered significant.

Results

Increased glucose availability drives hSCs to a higher glucose uptake that did not translate into an increased pyruvate export

One major function of SCs is to metabolize the extracellular glucose and ensure the production of the metabolic precursors necessary for developing germ cells (for review (Alves et al., 2013)). In fact, carbon-deprived hSCs did not export any pyruvate to the extracellular medium but they were able to sustain lactate production after 30 min (0.35 ± 0.16 pmol/cell) and 6 h (1.06 ± 0.20 pmol/cell) even if cultured in the absence of glucose, leucine or glutamine (Fig. 4.1B).

After 30 minutes, hSCs exposed to media with higher concentration of glucose showed an increased glucose consumption (61.8 ± 2.4 pmol/cell) comparing to hSCs exposed to media with physiological concentration of glucose (25.9 ± 6.2 pmol/cell) (Fig. 4.1A). This, however, was not translated on an increase on pyruvate, lactate and acetate export (Fig. 4.2A, B and C).

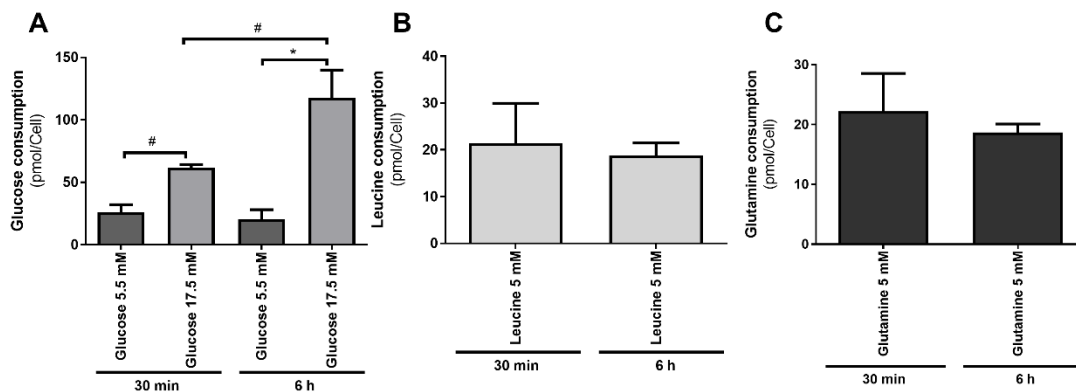


Figure 4.1 – Glucose uptake and metabolites consumption by human Sertoli cells (hSCs). The consumption of glucose (Panel A), leucine (Panel B) and glutamine (Panel C) by hSCs was determined and represented in pmol/cell. Results are expressed as mean $\pm$ SEM ($n = 6$ for each condition). Legend: # Significantly different results relative to glucose 17.5 mM - 30 min ($p < 0.05$); * Significantly different results relative to glucose 5.5 mM - 6 h ($p < 0.05$).

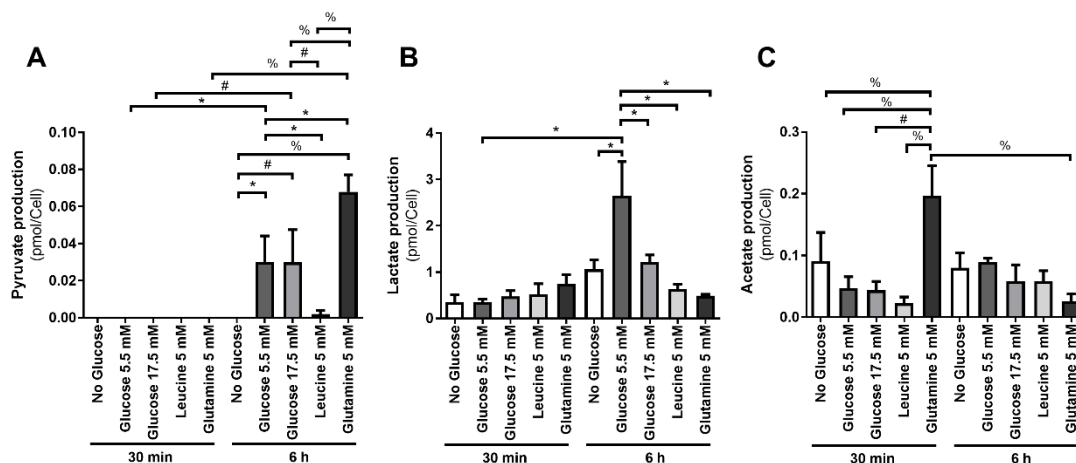


Figure 4.2 – Metabolite production by human Sertoli cells (hSCs). The production of pyruvate (Panel A), lactate (Panel B) and acetate (Panel C) by hSCs was determined and represented in pmol/cell. Results are expressed as mean $\pm$ SEM ($n = 6$ for each condition). Legend: # Significantly different results relative to glucose 17.5 mM - 30 min & 6h ($p < 0.05$); * Significantly different results relative to glucose 5.5 mM – 6 h ($p < 0.05$); % Significantly different results relative to glutamine 5 mM – 6 h ($p < 0.05$).

Physiological glucose levels stimulate more production of lactate by hSCs than high glucose levels

After 6 hours, the difference on glucose consumption between hSCs cultured with high levels of glucose (117.7 ± 22.2 pmol/cell) and the ones cultured under physiological levels of glucose (20.5 ± 7.6 pmol/cell) was significantly increased, as could be expected. Though even in the absence of extracellular glucose, hSCs were able to sustain a basal production of lactate, after 6 hours of culture, there was a higher lactate production by hSCs exposed to 5.5 mM glucose (2.65 ± 0.74 pmol/cell), compared to hSCs cultured in the absence of glucose (1.06 ± 0.20 pmol/cell) or even with 17.5 mM glucose (1.22 ± 0.15 pmol/cell) (Fig. 4.2B). On contrast, no differences were observed on pyruvate export between the cells cultured with different levels of glucose (5.5 and 17.5 mM), while those cultured without glucose did not export any pyruvate to the extracellular medium (Fig. 4.2 A).

Pyruvate export is decreased in hSCs cultured with leucine as the only energy source

Human SCs cultured during 6 hours with leucine (5 mM) as the only carbon source showed a decreased pyruvate export (0.002 ± 0.002 pmol/cell), comparing to hSCs cultured with physiological (5.5 mM) (0.030 ± 0.014 pmol/cell) or high (17.5 mM) (0.030 ± 0.018 pmol/cell) levels of glucose (Fig. 4.2A). A significantly lower production of lactate was also observed on these cells (0.63 ± 0.11 pmol/cell) comparing to those cultured with physiological levels of glucose (2.65 ± 0.74 pmol/cell) (Fig. 4.2B). Contrastingly, no differences were observed on acetate export when hSCs were cultured with leucine 5 mM (0.058 ± 0.017 pmol/cell), as compared with cells cultured with the different concentrations of glucose (values for 0 mM 0.080 ± 0.02 pmol/cell, 5.5 mM 0.090 ± 0.006 pmol/cell and 17.5 mM glucose 0.059 ± 0.026 pmol/cell, respectively) and glutamine 5 mM (0.026 ± 0.012 pmol/cell) (Fig. 4.2C).

Culture of hSCs with glutamine as the only energy source promotes acetate and pyruvate export

When hSCs were cultured with 5 mM glutamine as the only carbon source during 30 minutes, no export of pyruvate to the extracellular medium was observed (Fig. 4.2A). Moreover, lactate export was not altered (0.74 ± 0.21 pmol/cell), when compared with the cells of the other experimental groups (values for 0 mM 0.35 ± 0.16 pmol/cell, 5.5 mM 0.35 ± 0.068 pmol/cell and 17.5 mM glucose 0.48 ± 0.12 pmol/cell and leucine 5 mM 0.52 ± 0.23 pmol/cell, respectively) (Fig. 4.2 B). Contrastingly, a significantly higher production of acetate was observed on these cells (0.20 ± 0.05 pmol/cell), comparing to hSCs cultured in the absence of glucose (0.09 ± 0.05 pmol/cell), with 5.5 mM glucose (0.05 ± 0.02 pmol/cell), 17.5 mM glucose (0.04 ± 0.01 pmol/cell) or 5 mM Leucine (0.02 ± 0.01 pmol/cell) (Fig. 4.2C).

After 6 hours, hSCs cultured with 5 mM glutamine showed an increased pyruvate export (0.07 ± 0.01 pmol/cell), comparing to hSCs cultured without glucose

(0.00 ± 0.00 pmol/cell), with physiological levels of glucose (5.5 mM) (0.03 ± 0.01 pmol/cell) or high levels of glucose (17.5 mM) (0.03 ± 0.02 pmol/cell), or with 5 mM leucine (0.00 ± 0.01 pmol/cell) (Fig. 4.2A). No alteration on lactate export was observed in these cells (0.49 ± 0.03 pmol/cell), when compared with the cells cultured with the absence of glucose (1.06 ± 0.20 pmol/cell), with high levels of glucose (0.47 ± 0.12 pmol/cell) or to 5 mM leucine (0.63 ± 0.11 pmol/cell) (Fig. 4.2 B). However, when comparing lactate production of cells cultured with 5 mM glutamine with that of cells cultured with physiological levels of glucose (5.5 mM) (2.64 ± 0.74 pmol/cell) a significant reduction was observed (Fig. 4.2B).

No differences were observed on acetate export when hSCs were cultured with glutamine 5 mM (0.026 ± 0.012 pmol/cell), as compared with cells cultured with the different concentrations of glucose (values for 0 mM: 0.080 ± 0.024 pmol/cell;

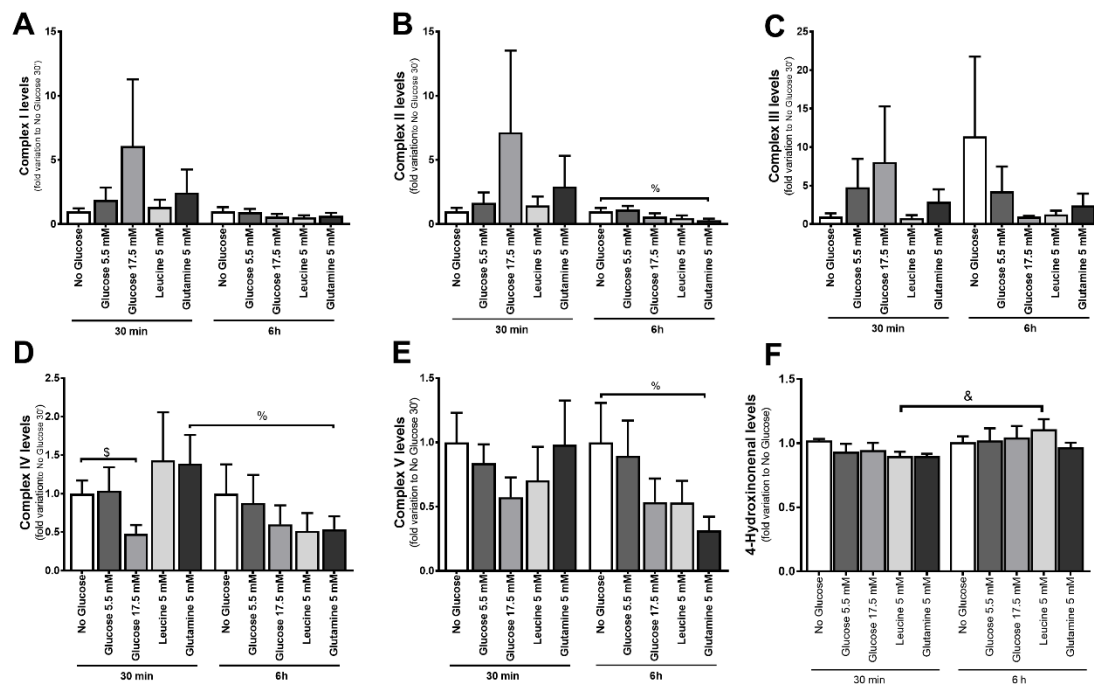


Figure 4.3 – Mitochondrial complexes protein expression levels in human Sertoli cells (hSCs) and effect of amino acids exposure to the oxidative profile of hSCs. The protein levels of mitochondrial complex I (Panel A), complex IIIIII (Panel C), complex IV (Panel D) and complex V (Panel E). Oxidative damages were measured in lipid peroxidation. Results are expressed as mean $\pm$ SEM (n = 6 for each condition). \$ Significantly different results relative to no glucose - 30 min (p < 0.05); # Significantly different results relative to glucose 17.5 mM - 30 min (p < 0.05); & Significantly different results relative to leucine 5 mM - 30min (p < 0.05); % Significantly different results relative to glutamine 5 mM - 6h (p < 0.05);

5.5 mM: 0.090 ± 0.006 pmol/cell; and 17.5 mM: 0.059 ± 0.026 pmol/cell, respectively) or with leucine 5 mM (0.058 ± 0.017 pmol/cell) (Fig. 4.2C). Curiously, there was a reduction of acetate content on the extracellular medium from 30 min to 6 h, when hSCs were cultured with 5 mM glutamine as the only carbon source.

Glutamine as only energy source decreases the expression of mitochondrial complexes II and V in hSCs

The culture of hSCs with 5 mM glutamine during 6 hours decreased the expression of mitochondrial complexes II (0.52 ± 0.20 -fold variation to no glucose conditions) and V (0.31 ± 0.11 -fold variation to no glucose conditions) (Fig. 4.3B and E), as compared to cells cultured without glucose (1.00 ± 0.26 -fold variation to no glucose conditions for complex II; 1.00 ± 0.31 -fold variation to no glucose conditions for complex V).

A similar trend was observed when we evaluated the expression of the mitochondrial complex IV in hSCs cultured with 5 mM glutamine as the only carbon source. The expression levels of this mitochondrial complex were decreased from 30 min (1.39 ± 0.37 -fold variation to no glucose conditions) to 6 hours (0.54 ± 0.17 -fold variation to no glucose conditions) (Fig. 4.3D).

Moreover, after 30 min culture with high levels of glucose, the protein expression of mitochondrial complex IV was decreased in hSCs (0.37 ± 0.03 -fold variation to no glucose conditions) when comparing cells cultured in the absence of glucose (1.00 ± 0.17 -fold variation to no glucose conditions) (Fig. 4.3C).

Glucose and amino acids quickly and transiently modulate the phosphorylation of S6 ribosomal protein (rpS6) in hSCs

The use of glucose (5.5 mM and 17.5 mM), leucine (5 mM) or glutamine (5 mM) as energy source in the culture media of hSCs induced an increase on rpS6

phosphorylation at Ser240/244 (1.26 ± 0.09 ; 1.29 ± 0.10 ; 1.39 ± 0.11 ; 1.28 ± 0.10 -fold variation to no glucose conditions, respectively), as soon as 30 min, when comparing to hSCs treated with no glucose (1.00 ± 0.08 -fold variation to no glucose conditions). This effect was reverted and we did not observe any alteration on the phosphorylation levels (Ser240/244) of rpS6 after 6 hours of culture with glucose (5.5 mM and 17.5 mM), leucine (5 mM) or glutamine (5 mM), when comparing to hSCs cultured without glucose.

Contrastingly, we observed a decrease on the phosphorylation levels of rpS6 in hSCs cultured with 5 mM leucine for 30 minutes (1.39 ± 0.11 -fold variation to no glucose conditions) and those cultured during 6 hours (1.07 ± 0.13 -fold variation to no glucose conditions) (Fig. 4.4B). This decrease was concurrent with an increase on 4- HNE levels (Fig. 4.3F). 4-HNE is an indicator marker for cellular oxidative damages, as it is a byproduct formed during oxidation of unsaturated fatty acids (Shiraishi and Naito, 2007). Its levels were increased when hSC were cultured with leucine (5 mM) during 6 hours (1.10 ± 0.09 -fold variation to no glucose conditions), when compared to 4-HNE levels of cells cultures with leucine (5 mM) during 30 minutes (0.89 ± 0.04 -fold variation to no glucose conditions).

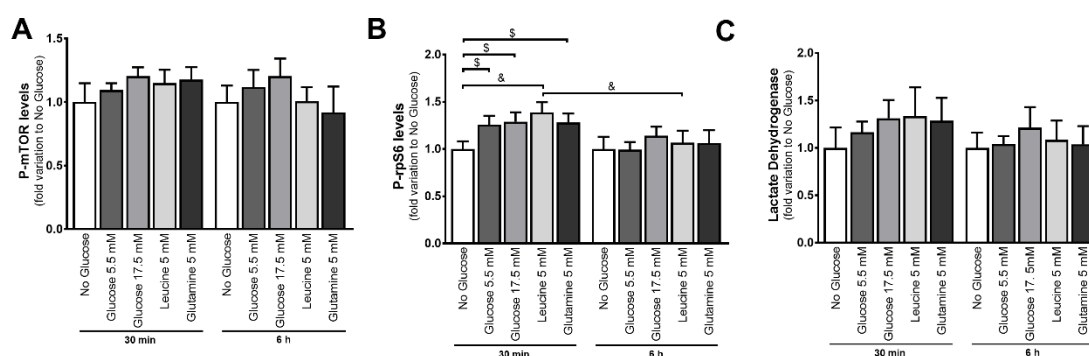


Figure 4.4 - Protein expression levels of target of rapamycin (TOR) signaling pathway molecules and lactate dehydrogenase. The protein expression levels of P-mTOR (Panel A); P-rpS6 (Panel B); and lactate dehydrogenase (Panel C) in human Sertoli cells were determined. Results are expressed as mean $\pm$ SEM (n = 6 for each condition). Legend: \$ Significantly different results relative to no glucose - 30 min (p < 0.05); & Significantly different results relative to leucine 5 mM – 30 min (p < 0.05); P-mTOR – phosphorylated mammalian target of rapamycin; P-rpS6 – phosphorylated ribosomal S6 protein.

Discussion

Sertoli cells have long been known for their metabolic plasticity (for review (Oliveira et al., 2015)), presenting a high glycolytic flux in order to ensure the production of lactate required for spermatogenesis (Boussouar and Benahmed, 2004; Rato et al., 2012). An enhanced aerobic glycolysis, or a Warburg-like metabolic behavior, has long been established as essential for the metabolic cooperation established between the SC and the developing germ cells (Oliveira et al., 2015). In the last years mTOR has emerged as crucial in the control of SC metabolism (Jesus et al., 2016). Still, the role of mTOR is complex and its function in male reproductive system remains to be unveiled. In addition, most of the current data was generated in *in vitro* systems using human and animal cell cultures that aim to be representative of a physiological state (Alves et al., 2012; Boussouar and Benahmed, 2004; Jesus et al., 2016; Oliveira et al., 2011). These culture systems contain a diversity of metabolic fuels (including carbohydrates, fatty acids and amino acids) usually present at concentrations either within the physiological range or, as it happens in the case of glucose (and sometimes glutamine), at concentrations three-fold higher than the normal range, not always representing a physiological situation. Hence, we aimed to evaluate how different carbon sources availability (and at distinct concentrations) communicate with mTORC1 signaling. We evaluated the glycolytic profile (including lactate production) and mitochondrial complexes expression of hSCs by placing these cells under distinct glycemic conditions or feeding them with an essential and a non-essential amino acid (leucine and glutamine, respectively). We further examined if the metabolic profile for each condition was followed by specific changes in mTORC1 signaling pathway players.

We observed that carbon ratio between glucose consumption and lactate production in hSCs is altered after culture in media supplemented with distinct glucose concentrations. Exposure to elevated glucose levels (17.5 mM), which we might correlate with a hyperglycemic status (315 mg/dL), lead to a reduction of lactate production, although the consumption of glucose was higher, as compared to the cells cultured with 5.5 mM glucose (100 mg/dL). It has been described that SCs cultured under physiological levels of glucose convert the vast

majority of glucose (around 95%) to lactate (Oliveira and Alves, 2015; Robinson and Fritz, 1981). When hSCs were exposed for a longer period (6 hours) to elevated glucose levels (17.5 mM) a transition from a Warburg-like effect (converting most glucose to lactate) to a non-glycolytic mode (decrease in lactate production) was observed. A similar trend has been described when cancer cells were cultured under high glucose levels, in which lactate accumulation seem to virtually block net lactate generation (Xie et al., 2014).

We also observed that, at least during the 6 hours while the cells were monitored, hSCs were able to accumulate lactate in the extracellular medium even when they were cultured in carbon-deprived conditions. As extracellular lactate accumulation by hSCs deprived of exogenous carbon sources occurs mostly during the first 30 min of culture we suggest that its synthesis is due to the use of metabolic pathways that are still active in these cells. Due to the physiological significance of testicular glycogen, particularly for SCs (Franz et al., 2013; Rato et al., 2015), one may speculate that this alternative endogenous glucose source may be used as a basal mechanism that allows hSCs to maintain lactate production even in adverse conditions. Although it is known that lipid metabolism plays an important role on SCs function, it is not expectable that it participates on lactate production as reported previously (Jutte et al., 1985).

It is known that mTORC1 signalling pathway participates in the regulation of the glycolytic flux in mammalian cells (Edinger et al., 2003), particularly in SCs (Jesus et al., 2016). The trend enhancement of mTOR (by phosphorylation of Ser²⁴⁴⁸) after only 30 minutes on hSCs for all culture conditions in the presence of glucose is concomitant with the effective activation (by phosphorylation of Ser^{240/244}) of its downstream effector rpS6 within the same timeframe. rps6 is one of mTORC1 best known downstream effectors. Phosphorylation of S6 ribosomal protein correlates with an increase in translation of specific mRNA transcripts (Jesus et al., 2017). Studies have shown that mTORC1 signaling pathway is involved in the regulation of glucose homeodynamics (Pusapati et al., 2016), and also regulates the metabolic support of spermatogenesis by hSCs (Crisóstomo et al., 2018; Jesus et al., 2016; Jesus et al., 2017). However, it is known that the phosphorylation of mTOR and mTORC1 downstream effectors may be refractory

(Holz and Blenis, 2005). It seems that the timeframe of our experiments promotes mTOR phosphorylation at Ser²⁴⁴⁸, with the phosphorylated rpS6 levels remaining elevated within the first 30 min, but after 6 hours there is a dephosphorylation of both targets, suggesting transient activation and latter desensitization of this pathway, as reported previously (Nicklin et al., 2009). The essential role of amino acids on mTORC1 activation in several cellular systems has also become clear over the last few years (Efeyan et al., 2012; Jewell et al., 2013), but exactly how this input was integrated in testicular cells, and particularly hSCs, remains unknown. We observed that both glutamine and leucine activate mTORC1 signaling in hSCs in a similar timeframe as seen in the distinct conditions of glucose availability. mTORC1 promotes growth in response to nutrients availability, such as amino acids, which drive mTORC1 activation (Bar-Peled and Sabatini, 2014).

Glutamine (through glutaminolysis) contributes with the anabolic carbons for the biosynthesis of distinct metabolic building blocks, and also accounts for lactate production (Zielke et al., 1980), when glucose is insufficient for the metabolic demands of the cells (DeBerardinis and Cheng, 2010). Exposing hSCs to glutamine as the only carbon source was able to cause a dramatic increase in acetate production as soon as 30 minutes of culture, comparing to hSCs cultured in the absence of glucose, with different concentrations of glucose or with 5 mM leucine. This accumulation of acetate on the extracellular medium was reverted in cells cultured for 6 hours with glutamine. However, at this time point there was an accumulation of pyruvate in the media of hSCs cultured with glutamine as the only energy source. This accumulation seems to result from both, a metabolization of acetate and decreased expression of some mitochondrial complexes. This is concurrent with the literature that suggests that acetate and glutamine provide alternative carbon sources when access to glucose-derived acetyl-CoA is impaired by hypoxia or mitochondrial dysfunction (Metallo et al., 2011; Mullen et al., 2011; Schug et al., 2015; Wise et al., 2011). Interestingly, we observed that glutaminolysis correlates with mTORC1 activation in the 30 min time frame and that glutaminolysis promotes a rapid accumulation of acetate, which latter enhances the synthesis and export pyruvate, without altering lactate production.

On the other hand, when hSCs were cultured with leucine as the only carbon source, no alteration was detected in the accumulation of extracellular metabolites during the first 30 min of culture, as compared with the cells maintained with glucose. After 6 hours, hSCs cultured with leucine exported much less pyruvate than cells from all other conditions and less lactate than cell cultured under physiological levels of glucose (5.5 mM). It has been reported that leucine metabolism is directly linked with glycolysis and oxidative phosphorylation, although this subject remains a matter of debate. While some reported that inhibition of leucine uptake leads to the reduction of oxygen consumption and glycolysis (Ananieva-Stoyanova et al., 2016; Papathanassiou et al., 2017), others report that leucine supplementation induces an energy metabolism switch from oxidative phosphorylation (OXPHOS) towards glycolysis, reducing then ROS levels (Hu et al., 2017). In our work, we observed that culturing hSCs with leucine promotes a dramatic reduction on the export of the key endpoints of SCs glucose metabolism (lactate and pyruvate) as compared to those cells cultured with physiological levels of glucose, both at 30 min or after 6 h. These observations, together with the fact that leucine is being consumed, suggest that this amino acid might be promoting an enhancement of oxidative phosphorylation. In fact, this may be the reason for the concurrent increase on oxidation of unsaturated fatty acids. When comparing the results obtained after cultivating hSCs with two distinct amino acids, it seems that glutamine (a non-essential amino acid), and a stimulation of glutaminolysis, may be more relevant as a metabolic substrate for the testicular metabolic cooperation than leucine (an essential amino acid).

In sum, our study showed the plasticity of SC metabolism with different nutrients availability conditions. Our results suggest a differential response of lactate production by human Sertoli cells to glucose and amino acids stimulation. This is of major relevance since the nutritional support of spermatogenesis is dependent on how SC proceed to keep sustaining the metabolic needs of developing germ cells.

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Chapter 5

Short-term human sperm preservation in media supplemented with mTOR activator MHY 1485 improves sperm quality without compromising DNA integrity and capacitation

This chapter was adapted from the submitted work:

Jesus TT, Almeida SP, Barros A, Sousa M, Oliveira PF, Alves MG. Short-term human sperm preservation in media supplemented with mTOR activator MHY 1485 improves sperm quality without compromising DNA integrity and capacitation.

This work was the ground for the patent NP110231: “mTOR enhancers and uses thereof to improve sperm quality and function during storage”.

5. Short-term human sperm preservation in media supplemented with mTOR activator MHY 1485 improves sperm quality without compromising DNA integrity and capacitation

Running title: Sperm preservation with mTOR activator improves sperm quality

Abstract

Study Question: Can supplementation of commercial sperm preservation media with an mTOR enhancer improve spermatozoa quality after short-term storage?

Summary Answer: We show for the first time that short-term human sperm preservation in a medium supplemented with mTOR enhancer MHY 1485 improves sperm quality without compromising DNA integrity and capacitation.

What is known already: There is an increasing number of couples seeking for assisted reproductive technologies (ART) that rely on sperm collection. After collection, sperm quality rapidly declines. Several signaling pathways are expected to be involved, but to date there is no evidence of a way to improve sperm quality during preservation. The use of substances that could improve sperm quality during preservation for use in ART would be a decisive step in the treatment of infertility.

Study design, size, and duration: Male gametes were obtained at the Center for Reproductive Genetics Professor Alberto Barros (Porto, Portugal) from 15 non-smokers patients with normal sperm parameters and a minimum sperm concentration of 20 million spermatozoa/mL. One million spermatozoa were placed in a commercial medium (Sperm Preparation Medium – SPM – from Origio, Måløv, Denmark) and the same amount of spermatozoa were placed in SPM supplemented with rapamycin (110 nM) or MHY1485 (5 µM). Subsequently,

sperm suspensions were kept in an incubator at 37°C during 30, 120 and 180 minutes.

Participants/materials, setting and methods: Spermatozoa suspensions were processed at each time interval. Sperm quality was assessed using WHO guidelines, particularly motility, viability and capacitation. The percentage of sperm DNA fragmentation was determined by the Halotech® Halosperm® G2 Kit. Sperm capacitation was evaluated by determining the levels of phosphotyrosine. Sperm metabolic profile was assessed by Proton Nuclear Magnetic Resonance and the damages caused by oxidative stress were detected by measuring the levels of protein carbonylation and lipid peroxidation.

Main results and the role of chance: Our results show that the supplementation of a commercial sperm preservation medium with the mTOR enhancer MHY 1485 improves the percentage of viable and motile spermatozoa and decreases sperm DNA fragmentation after short-term storage. In addition, this supplementation with MHY 1485 increased lactate production without changing glucose consumption illustrating a stimulation of spermatozoa metabolic activity. Interestingly, this was not followed by any change in the levels of oxidative damages. Thus, activation of mTOR during preservation improves sperm quality, which may be crucial to improve the outcomes of ART or prolong the time for storage without spermatozoa losing their most relevant qualities.

Limitations, reasons for caution: Although sperm physiology and quality is normal after supplementation of a commercial sperm preservation medium with mTOR enhancer MHY 1485, it is essential to further expand this work by doing fertilization and pre-implantation assays to determine if storage of spermatozoa in the supplemented medium does not promote deleterious effects in the production of the viable embryos.

Wider implications of the findings: mTOR is being studied as a pharmacological target for the treatment of several diseases. We report that activation of mTOR during short-term sperm preservation allows improved preservation of sperm quality for ART, which may have wider implications to prolong the time for storage without spermatozoa losing their most relevant qualities.

Keywords: Sperm preservation; MHY 1485; mTOR; Capacitation; Sperm DNA integrity

Introduction

Infertility is a health problem that already affects an estimated 48.5 million couples worldwide (Mascarenhas et al., 2012). This increasing trend is accompanied by an augmented number of individuals seeking for assisted reproduction techniques (ART) in the last decades (Elder and Dale, 2010). Indeed, recent statistics highlight that more than 15% of the couples are infertile, being that ART become their most secure hope. In addition, male infertility is the only factor responsible for up to 30% of infertility cases and contributes to 50% of the cases overall (Sharlip et al., 2002). Thus, sperm quality plays a crucial role in the *in vitro* fertilization (IVF) laboratory as treatment options for the male factor are mainly based on sperm-quality improvement techniques (WHO, 2010). For that, sperm collection and preservation in good physiological conditions is pivotal for the success of ART. There are several sperm preparation media that can be used but after collection there is a rapid decline in sperm quality (Anbari et al., 2016; Nabi et al., 2014), which not only compromises the success of ART, but can also hamper the use of the sample. Thus, great efforts have been made to extend the viability of stored male gametes to improve the success of ART (Jackson et al., 2010). Cryopreservation and refrigeration of sperm have been widely debated and the maintenance of male gametes during short-term periods should avoid the fast decline of sperm viability after storage in a refrigerated environment (Sato and Ishikawa, 2004). Thus, the formulation of an optimal composition for sperm storage media is of extreme relevance since these cells highly depend on the supply of exogenous substrates and, due to their high metabolic rates, produce large amount of ROS (Sato and Ishikawa, 2004).

In the last years several signaling pathways have been reported as pivotal to sperm physiology (Ijiri et al., 2012), providing optimism for a way to control sperm quality. Among those pathways, the mammalian target of rapamycin (mTOR) is a highly conserved multifunctional serine/threonine protein kinase that regulates

several cellular events, such as energy supply (for review (Jesus et al., 2017; Laplante and Sabatini, 2012; Saxton and Sabatini, 2017)). It also interplays with other signaling pathways, which adds a great complexity to its biological significance. Thus, in recent years, this pathway has been studied and explored by several research groups and by pharmaceutical industry to develop novel therapeutic strategies to treat cancer, cardiovascular disease, metabolic disorders and many others (Lineham et al., 2017; Wang et al., 2017). However, only recently it has been suggested and shown that mTOR integrates and mediates signals throughout the male reproductive system (for review (Jesus et al., 2017; Oliveira et al., 2017)). For instance, we recently showed that mTOR signaling controls glucose consumption and redox balance in human Sertoli cells and thus, it appears to play a central role in the nutritional support of spermatogenesis (Jesus et al., 2016). Herein we hypothesized that supplementation of a commercial sperm preservation medium with an mTOR enhancer could improve sperm quality after short-term storage, without changing DNA integrity and their capacitation. To further understand how these effects are mediated, we focused on sperm metabolic activity and oxidative damages during storage. This is the first report focused on a possible role for mTOR during short-term sperm preservation.

Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Patient selection, ethical issues and sperm samples

Patient's selection and clinical study was performed at the Center for Reproductive Genetics Professor Alberto Barros (Porto, Portugal) in accordance with the Guidelines of the Local, National and European Ethical Committees. Male gametes were obtained from 15 non-smokers patients with a minimum 20 million sperm /mL and with normal motility and morphology. All studies were performed according to the Declaration of Helsinki.

Experimental groups

The number of spermatozoa was determined using a hemocytometer, and 1×10^6 spermatozoa were placed in 500 μ L of a control medium (Sperm Preparation Medium from Origio, Måløv, Denmark). The same amount of spermatozoa was placed in two other media, with the same composition of the control group but supplemented with an mTOR inhibitor (Rapamycin, 110 nM) or an mTOR activator (MHY1485, 5 μ M). Subsequently, sperm suspensions were kept in an incubator at 37°C during 30, 120 and 180 minutes. These sperm suspensions were then processed and used to determine sperm motility, viability, capacitation, DNA fragmentation, as well as metabolic and oxidative profiles.

Assessment of sperm quality

Sperm quality was assessed using the guidelines published in the WHO laboratory manual for the examination and processing of human semen (WHO, 2010). In brief, sperm motility was evaluated by placing a drop of sperm suspension (20 μ L) in a warmed slide (37°C) and motile sperm percentage was assessed in 10 random fields, using an optical microscope ($\times 100$ magnification), and the average value was used as the total sperm motility. To assess sperm viability, an eosin/nigrosin staining was used because it is effective, simple, and, in addition to allowing sperm to be readily visualized, permits assessment of

sperm membrane integrity. This method was performed with slight modifications of a method previously described (Lopes et al., 2009). In brief, a total of 5 μL of the sperm suspensions obtained as described above were mixed with 10 μL of 0.5% eosin/nigrosin stain and placed on a glass microscope slide. Samples were analyzed at 30 and 120 min of incubation. The percentage of viable and nonviable spermatozoa was determined by counting a total of 333 spermatozoa per slide in continuous random fields under a light microscope, with oil immersion ($\times 1000$ magnification), to determine the percentage of viable sperm, as previously described (Rato et al., 2013). Live sperm remained white, whereas dead sperm stained pink, because the integrity of their plasma membrane was compromised, causing an increase in membrane permeability and to the uptake of the dye. For sperm capacitation assay, sperm preserved in media from the different groups were incubated at 37°C on a capacitation medium (ALLGrad Wash, FGWG-100, LifeGlobal group, Guilford, CT, USA) during 180 min. Sperm suspensions were centrifuged and the supernatant media discarded.

Slot blot

Sperm suspensions were centrifuged and the supernatant media was collected for ^1H -NMR analysis. The pellets were then lysated with a Tris Buffer (2 M) with 1% (v/v) Triton X-100, 1 mM Sodium Orthovanadate and 1% (v/v) Proteases Inhibitors Cocktail. Total protein was quantified using Pierce™ BCA Protein assay kit (Pierce Biotechnology, Rockford, USA).

The levels of protein carbonyl groups, 4-hydroxynonenal (4-HNE), and phospho-tyrosine were measured by Slot blot. Protein carbonyl groups levels were determined as described (Rato et al., 2014). For determination of 4-HNE and phospho-tyrosine levels, 5 μg of protein were diluted in PBS and transferred to PVDF membranes using a Hybri- Slot Manifold System (Biometra, Göttingen, Germany). The membranes were then blocked and incubated with the respective primary antibodies (Table 5.1) overnight at 4°C. The immune-reactive proteins were detected separately with secondary antibodies (Table 5.1) at room temperature. Membranes were reacted with ECF detection system (GE

Healthcare, Freiburg, Germany) and read using the BioRad GelDoc XR (Bio-Rad, Hemel Hempstead, UK). Densities from each band were quantified using Quantity One software (Bio-Rad, Hemel Hempstead, UK).

DNA fragmentation assay

Sperm suspensions were centrifuged and suspended on filtered PBS. Sperm cells DNA fragmentation was assessed with Halotech® Halosperm® G2 Kit (Halotech DNA SL, Madrid, Spain) using the manufacturer's protocol.

NMR spectroscopy

¹H-NMR spectroscopy was performed in culture media to determine lactate and acetate production, glucose consumption and variations of other substrates, such as pyruvate or acetate, during the incubation of spermatozoa in the different experimental conditions, as routinely performed by our team (Alves et al., 2011). Sodium fumarate (final concentration of 2 mM) was used as an internal reference (6.50 ppm) to quantify metabolites in solution (multiplet, δ , ppm); lactate (doublet, 1.33); acetate (single, 1.91); pyruvate (singlet, 2.36); H1- α glucose (doublet,

Table 5.1: List of Antibodies used in this study

Antibody	Source	Molecular Weight (kDa)	Dilution	Vendor	Catalog #
4-HNE	Goat	---	1:5000	EMD Millipore, Temecula, CA, USA	AB5605
DNP	Rabbit	---	1:5000	Sigma-Aldrich, Roedermark, Germany	D9656
Anti-Phospho-tyrosine	Mouse		1:5000	Merck Milipore, Darmstadt, Germany	05-321
Mouse	Goat	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A3562
Rabbit	Goat	---	1:5000	Sigma-Aldrich, Roedermark, Germany	A3687
Goat	Donkey	---	1:5000	Santa Cruz Biotechnology, Heidelberg, Germany	SC-2020

DNP- Dinitrophenyl; 4-HNE- 4-Hydroxynonenal

5.22). Spectra analysis was performed offline. The relative areas of ^1H -NMR resonances were quantified using the curve-fitting routine using the NutsProTM NMR spectral analysis program (Acorn, NMR Inc., Fremont, CA, USA).

Statistical analysis

Statistical differences between experimental groups were assessed by 2-way ANOVA uncorrected Fisher's LSD. All data are shown as mean $\pm$ SEM ($n = 15$ for each condition). Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered significant.

Results

Short-term preservation in a medium supplemented with MHY 1485 increased sperm metabolic activity without inducing oxidative damages

The metabolic activity of sperm is essential for the fertility of males. Alteration in sperm capability to utilize glucose or other substrates to produce ATP will compromise its quality and thus, the fertility potential of the individual (Dias et al., 2014; Lin et al., 2009). Enhancement of mTOR during preservation increased sperm cells metabolic activity when compared with those spermatozoa preserved in a commercial storage medium. Although there was no difference concerning the glucose consumption between the different groups (Fig. 5.1A), after 120 min of sperm preservation in a medium with the mTOR enhancer MHY 1485 there was an increased production of lactate to 0.039 ± 0.005 pmol/cell, when compared with the production of this metabolite by spermatozoa preserved in the commercial medium (0.032 ± 0.004 pmol/cell) (Fig. 5.1B). Increased metabolic activity is associated with increased production of ROS. It is well known that human spermatozoon is very susceptible to oxidative stress, resulting in the carbonylation of proteins and lipid peroxidation (Aitken and Baker, 2006).

Interestingly, our results show that activation and inhibition of mTOR did not induce an increase of oxidative damages. The levels of protein carbonylation detected were 1.03 ± 0.05 -fold variation to control (Fig. 5.2A), while the lipid peroxidation levels were 1.03 ± 0.06 -fold variation to control (Fig. 5.2B).

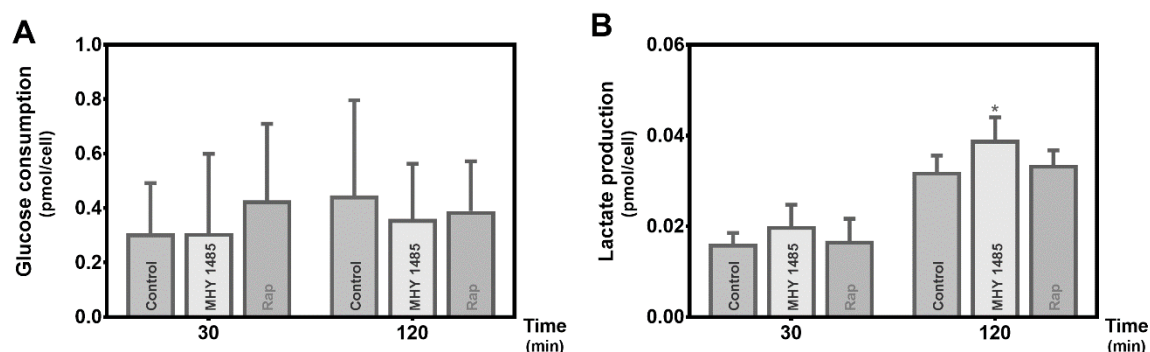


Figure 5.1 – Human spermatozoa metabolism during storage for 120 minutes at 37°C. Spermatozoa were stored in Sperm Preparation Medium (ORIGIO, Måløv, Denmark) not supplemented (Control) or supplemented with an mTOR activator (MHY 1485). Spermatozoa were also incubated with an mTOR inhibitor (Rapamycin; Rap) as a negative control. Sperm glucose consumption (Panel A) and lactate production (Panel B) were assessed by ^1H -Nuclear Magnetic Resonance. Results are presented as mean $\pm$ standard error of the mean (n=12); Significant results (P < 0.05) are indicated as *-vs. Control.

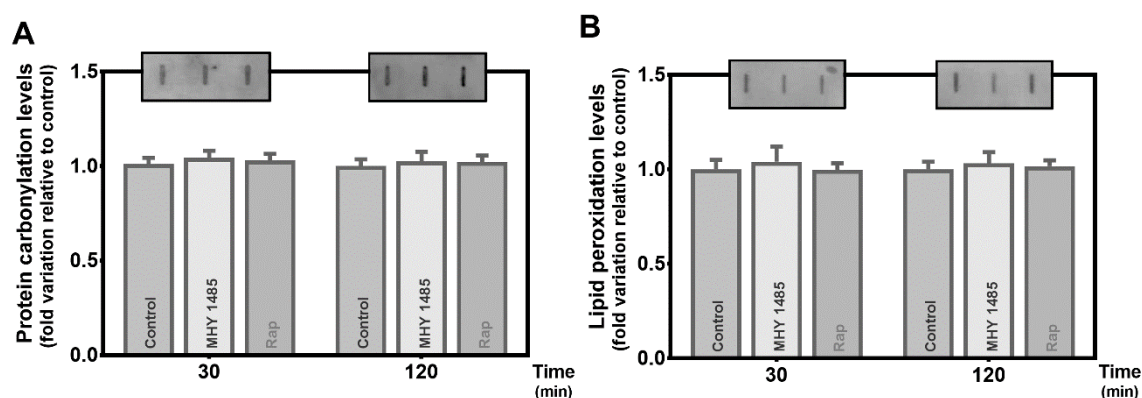


Figure 5.2 – Human spermatozoa oxidative damage to proteins (carbonylation) (Panel A) and lipids (peroxidation) (Panel B) after storage for 120 minutes at 37°C. Spermatozoa were stored in Sperm Preparation Medium (ORIGIO, Måløv, Denmark) not supplemented (Control) or supplemented with an mTOR activator (MHY 1485). Spermatozoa were also incubated with an mTOR inhibitor (Rapamycin; Rap) as a negative control. Protein carbonylation was assessed by immunoblotting a specific @DNP antibody (SigmaAldrich, St. Louis, MO, USA). Lipid peroxidation was assessed by immunoblotting a specific @HNE antibody (Merck Millipore, Temecula, USA). Results are presented as mean $\pm$ standard error of the mean (n=12); Significant results (P < 0.05) are indicated as *-vs. Control.

Short-term preservation in a medium supplemented with MHY 1485 improves sperm viability and motility

Sperm quality was evaluated after 120 minutes preservation in a commercial medium or in a commercial medium supplemented with mTOR inhibitor or enhancer. Our results show that, after that period, the number of motile spermatozoa increases to 74 ± 3 % in spermatozoa preserved in the medium supplemented with mTOR enhancer when compared with the control group, which presented 65 ± 4 % of motile spermatozoa after short-term storage in a non-supplemented medium (Fig 5.3A and B). To further characterize sperm quality we evaluated the percentage of viable spermatozoa. Our results show that, after 30 minutes preservation in a medium supplemented with mTOR enhancer MHY 1485, the percentage of viable spermatozoa is higher (68 ± 3 %), when compared with preservation in a commercial medium, which presented 60 ± 3 % of viable spermatozoa. The results were even more significant after 120 minutes preservation of spermatozoa in the medium supplemented with mTOR enhancer since the percentage of viable spermatozoa was strikingly higher (70 ± 2 %), when compared with 60 ± 3 % after preservation in a commercial medium and 59 ± 3 % after preservation in medium supplemented with rapamycin, the mTOR inhibitor (Fig. 5.3C and D).

Short-term sperm preservation in a medium supplemented with MHY 1485 decreases DNA fragmentation and maintains spermatozoa binding capacity

Spermatozoa DNA integrity after preservation is essential for further use in ART. Thus, we evaluated DNA fragmentation in spermatozoa preserved in a commercial medium and in a medium supplemented with mTOR inhibitor and enhancer. Our results show that the percentage of spermatozoa with fragmented DNA is lower (40 ± 2 %) after preservation during 120 minutes in a medium supplemented with MHY 1485, when compared with spermatozoa preserved in

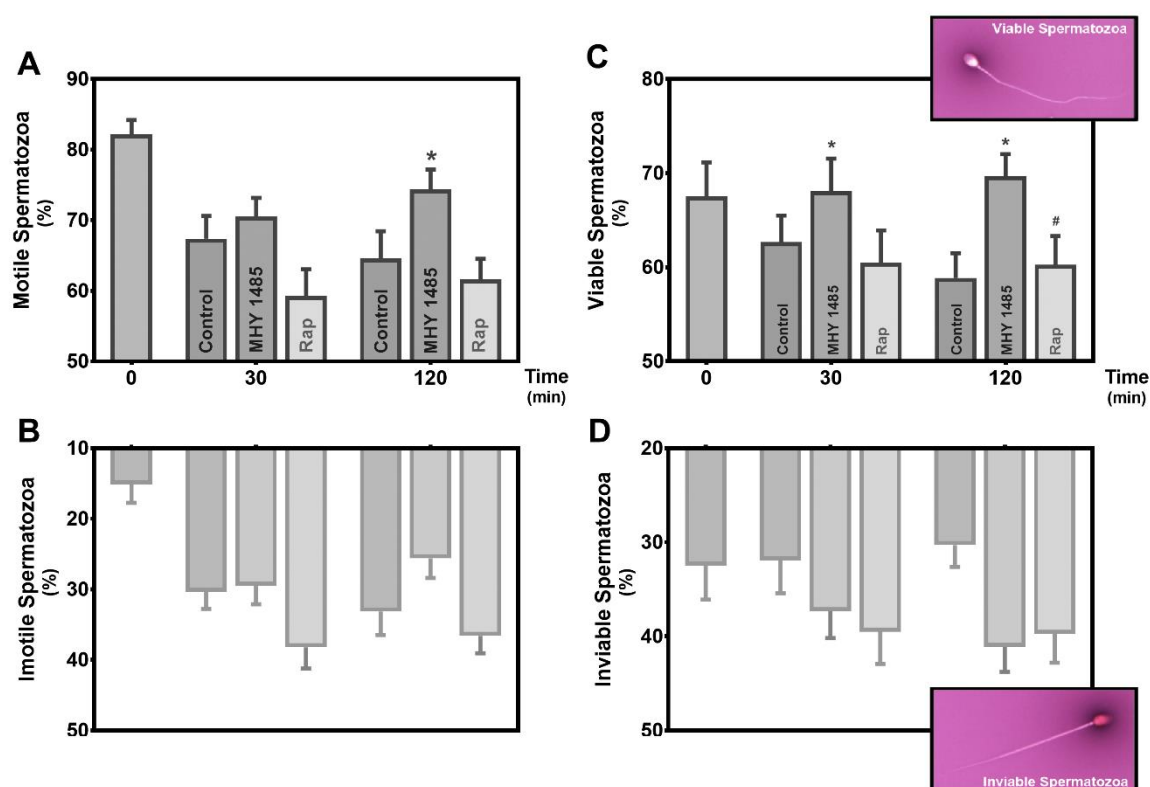


Figure 5.3 – Human spermatozoa motility (Panels A and B) and viability (Panels C and D) after storage during 120 minutes at 37°C. Spermatozoa were stored in Sperm Preparation Medium (ORIGIO, Måløv, Denmark) not supplemented (Control) or supplemented with an mTOR activator (MHY 1485). Spermatozoa were also incubated with an mTOR inhibitor (Rapamycin; Rap) as a negative control. Viability was assessed by the eosin-nigrosin staining method. Results are presented as mean $\pm$ standard error of the mean (n=12); Significant results ($P < 0.05$) are indicated as *-vs. Control.

a commercial medium (control group), which presented 51 ± 2 % of spermatozoa with fragmented DNA. Interestingly, inhibition of mTOR during preservation did not result in any alteration in the percentage of spermatozoa DNA fragmentation, when compared with the levels detected in spermatozoa maintained in the control group (Fig. 5.4A and B). After testing DNA integrity, we evaluated if sperm capacitation, as it is a preparatory step for acrosome reaction, was altered by the supplementation of the commercial sperm preservation medium with mTOR inhibitor or enhancer. One of the key processes known to occur for sperm capacitation is protein tyrosine phosphorylation. Our results show that the phosphotyrosine levels in spermatozoa preserved in a control commercial medium was similar as those of spermatozoa preserved in medium supplemented with both mTOR inhibitor and activator (Fig. 5.4C).

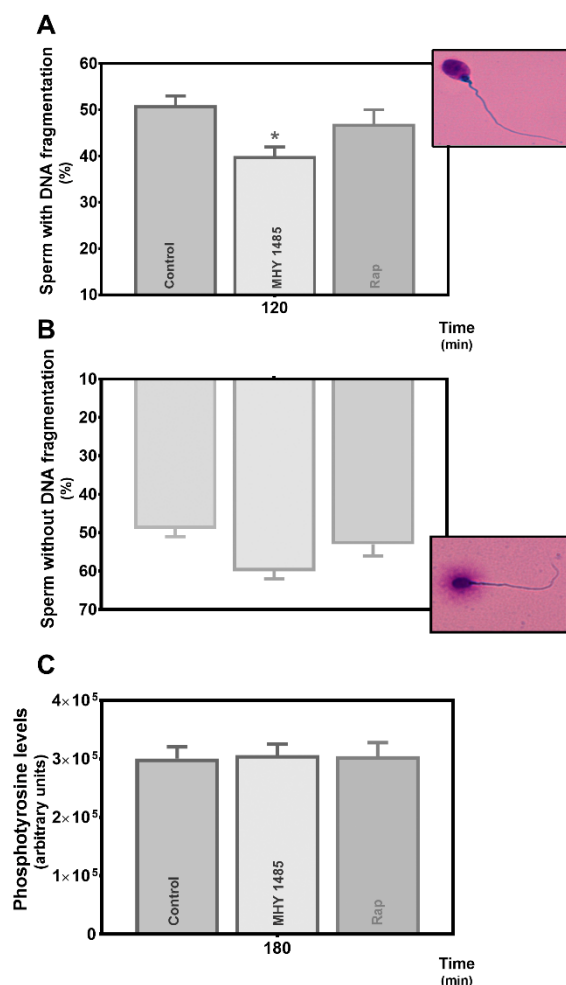


Figure 5.4 – Human spermatozoa DNA fragmentation (Panels A and B) and sperm capacitation (Panel C) after storage for 120 minutes at 37 °C. Spermatozoa were stored in Sperm Preparation Medium (ORIGIO, Måløv, Denmark) not supplemented (Control) or supplemented with an mTOR activator (MHY 1485). Spermatozoa were also incubated with an mTOR inhibitor (Rapamycin; Rap) as a negative control. DNA fragmentation was assessed using a Halosperm G2 kit (HALOTECH, Madrid, Spain). Sperm capacitation was assessed by evaluating sperm phosphotyrosine levels after 180 minutes' incubation in Sperm Capacitation Medium (LifeGlobal Europe, Brussels, Belgium) following the 120 minutes in treatment medium. Results are presented as mean $\pm$ standard error of the mean (n=12); Significant results ($P < 0.05$) are indicated as *-vs. Control.

Discussion

More than 15% of the couples are infertile and seek for assisted reproductive technologies (ART). For the use of those technologies, sperm collection is mandatory. However, after collection and during preservation, sperm quality rapidly declines, which hampers the success of ART. Although there are several

options of commercial medium specifically developed for sperm preservation, they are still far from ideal. Thus, supplementation of commercial media with compounds that may allow preservation of sperm quality with better results would be essential to improve ART success. Herein we report that sperm preservation in a medium supplemented with MHY 1485, an mTOR enhancer, increases sperm metabolic activity without inducing oxidative damages. Notably, the supplementation of preservation media with MHY 1485 also improved sperm viability and motility, while decreasing DNA fragmentation and maintaining spermatozoa binding capacity. Our data suggest that mTOR is not only involved in sperm quality, but also that the use of an enhancer of this protein in preservation media improves spermatozoa quality.

Mature spermatozoa are very differentiated cells that mostly use glucose and fructose to sustain their high energetic demands (Bucci et al., 2011). Indeed, their main goal consists in fertilize the oocyte and thus, join male haploid DNA to female DNA. All those processes require energy being that some functions, such as motility, are highly sensitive to substrates availability and external metabolic conditions. The main metabolic pathways involved in energy production are anaerobic glycolysis and oxidative phosphorylation, being that both are associated with production of reactive oxygen species (ROS). Sperm cells are highly susceptible to ROS. Not only their membrane is rich in polyunsaturated fatty acids (PUFA), and thus are readily attacked by ROS (Aitken et al., 2012), but high ROS levels cause an unbalance on OS resulting in apoptosis (Aitken and Koppers, 2011). Therefore, it is crucial to sustain a high metabolic activity without changing the “physiological levels” of ROS. Several signaling pathways have been associated with sperm metabolism and quality (Freitas et al., 2017). The mTOR signaling is known to regulate several crucial cellular events, including energy supply and oxidative stress (Zhao et al., 2017), but its role on male reproduction remains fairly unknown. The first evidence for a possible role for mTOR on male fertility was described when male patients treated with the mTOR inhibitor sirolimus presented reversible infertility and altered sperm quality (Deutsch et al., 2007). Later, it was demonstrated that mTOR mediates several physiological functions in testes, particularly in germ and testicular cells (Mok et al., 2014; Mok et al., 2013; Xu et al., 2016). Our team has been focused in

understanding how mTOR can control spermatogenesis and sperm physiology. Our first results were attained using human Sertoli cells, which are the somatic testicular cells responsible for the nutritional support of spermatogenesis. We showed that mTOR controls glucose consumption and redox balance in human Sertoli cells (Jesus et al., 2016), exerting a high level of specialization in the determination of a successful spermatogenesis. Herein, we show that short-term (up to 2 hours) preservation of spermatozoa at 37°C is accompanied by a maintenance of the metabolic activity. Interestingly, inhibition of mTOR signaling by rapamycin did not induce any alteration in spermatozoa metabolic activity. However, when the medium was supplemented with the mTOR enhancer MHY 1485, there was an increased metabolic activity since we detected a higher lactate production, although a similar consumption of glucose was observed. Pyruvate and lactate are considered the most important sources of energy to sustain key sperm functions, including motility (Darr et al., 2016). However, increased metabolic activity is often linked with increased production of ROS and oxidative stress (O'Flaherty, 2015). Protein carbonyl content is commonly used as a marker for protein oxidation in spermatozoa (Oliveira et al., 2015), as carbonyl group formation results from ROS attack and is directly associated with protein damage induced by OS (West, 2000). In addition, lipid membranes present in spermatozoa can also be oxidized and thus we measured lipid peroxidation. Notably, our data shows that supplementation of sperm preservation medium with mTOR enhancer MHY 1485 during 2 hours at 37°C increases the production of lactate without increasing any of the oxidative stress markers studied. These results illustrate that MHY 1485 serves as a metabolic enhancer without inducing oxidative damages in proteins and membrane lipids. As expected, this enhanced metabolic activity was followed by an increase in the percentage of motile spermatozoa. Indeed, after 2 hours preservation in a commercial medium there was a decrease in the percentage of motile spermatozoa however, when the medium was supplemented with mTOR enhancer the percentage of motile spermatozoa was significantly higher than in those preserved in a non-supplemented commercial medium. In addition, the percentage of viable spermatozoa was also higher at the end of 30 minutes and 120 minutes in those samples preserved in a medium supplemented with mTOR enhancer than in non-supplemented commercial medium. These results clearly

illustrate that mTOR activation enhances sperm metabolic activity, which is reflected in a higher percentage of viable and motile spermatozoa.

Adequate DNA integrity maintenance during preservation is crucial to guarantee that there is an effective and safe transmission of paternal information to the embryo. The presence of DNA breaks in spermatozoa may have a severe negative outcome, not only for the developmental stages of the embryo, but also after birth during childhood and adult life (Lewis et al., 2013). It is crucial to maintain or if possible increase sperm DNA integrity during preservation for ART. Our results show that supplementation of a commercial medium with the mTOR enhancer MHY 1485 decreases the percentage of sperm with DNA fragmentation, when compared with sperm preserved in the non-supplemented commercial medium. Indeed, mTOR signaling is closely associated to the DNA damage response and cellular protection mechanisms. Recently, some authors even suggested that the interaction between genotoxicity and DNA damage may be mediated by mTOR (Ma et al., 2018). Our data shows that sperm preserved in a medium supplemented with mTOR activator presents a significant decrease in the percentage of DNA fragmentation when compared with the percentage detected in sperm preserved in the non-supplemented commercial medium. Nevertheless, it is also crucial that during and after sperm preservation it retains the ability to gain fertilization competence, which is dependent of a phenomenon known as capacitation. One of the most important events that occur is tyrosine phosphorylation. Thus, we evaluated the protein levels of phosphotyrosine. Our results show that even after 180 minutes of sperm preservation in a medium supplemented with the mTOR enhancer MHY 1485 there is no alteration in phosphotyrosine levels when compared with the preservation of spermatozoa in a non-supplemented commercial medium.

The role for mTOR in male reproductive tract has been overlooked though it integrates signals that derive from intra- and extracellular cues, such as toxicants, amino acids, energy, oxygen, DNA damage and many others. On the other hand, spermatozoa employ an extremely complex and sophisticated network of signaling pathways that control the key events before and during fertilization. Overall, our results show that the addition of the mTOR enhancer MHY 1485 to

a commercial sperm preservation medium improves the percentage of viable and motile spermatozoa and decreases sperm DNA fragmentation. This was due to a stimulation of spermatozoa metabolic activity that did not increased oxidative damages nor altered its capacitation ability. Based on our findings we may affirm that enhancement of mTOR during preservation improves sperm quality, which may be crucial to improve the outcomes of ART or prolong the time for storage without spermatozoa losing their most relevant qualities. Further research will be needed but this work opens new insights on the relevance of mTOR to sperm physiology.

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Chapter 6

Concluding Remarks

6. Concluding remarks

Infertility has been affecting millions of couples worldwide. The causes for infertility of a couple may be due to problems only in the man, only in the woman or a result from alterations in both individuals. It has been highlighted that infertility affects about 7% of all men and 40 - 50% of infertility cases are due to the male factor (Brugh and Lipshultz, 2004; Lotti and Maggi, 2015). Nearly one third of the cases results from male factor alone (Hamilton and Ventura, 2006; Lutz, 2006). Over the last few decades, a decline in male reproductive health has been observed in European men, with studies identifying a high prevalence of low sperm counts in young men (<40 years) (Fernandez et al., 2012; Jorgensen et al., 2001; Jorgensen et al., 2002; Nordkap et al., 2012).

SCs have long been known for their metabolic plasticity (for review (Oliveira et al., 2015)), presenting a high glycolytic flux in order to ensure the production of lactate required for spermatogenesis (Boussouar and Benahmed, 2004; Rato et al., 2012). An enhanced aerobic glycolysis, or a Warburg-like metabolic behavior, has been established as essential for the metabolic cooperation established between SCs and the developing germ cells (Oliveira et al., 2015). The modulation of metabolic pathways in testicular somatic cells, particularly in SCs, is determined by multiple factors including metabolic substrates availability and the action of hormones (Alves et al., 2012; Galardo et al., 2008; Hall and Mita, 1984; Oliveira et al., 2011; Rato et al., 2012; Riera et al., 2001) and other endogenous or exogenous factors that have a contribution in the progression of the spermatogenic event. In addition, all those events are mediated by specific signaling pathways.

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. In the first study we aimed to evaluate the effect of an mTORC1 inhibition through exposure to rapamycin in hSCs carbohydrate metabolism, metabolite secretion/consumption, oxidative status, and also alterations in mitochondrial complexes expression. Inhibition of the mTORC1/S6K pathway by rapamycin on hSCs induces an increase in

glucose consumption to produce the same amount of lactate and acetate as cells with an active mTORC1. Similarly, in human skeletal muscle, rapamycin administration augmented glucose uptake in the presence of combined hyperaminoacidemia and prandial-like peripheral hyperinsulinemia (Krebs et al., 2007). Under unfavorable conditions, such as insulin deprivation (Alves et al., 2012; Oliveira et al., 2012), hSCs sustain lactate production by adapting their metabolism. In the second study we aimed to evaluate how different carbon sources availability (and at distinct concentrations) in cultured hSCs communicate with mTORC1 signaling. We evaluated the glycolytic profile (particularly lactate production) and mitochondrial complexes expression of hSCs by placing these cells under distinct glycemic conditions or feeding them with an essential and a non-essential amino acid (leucine and glutamine, respectively) and further examined if the metabolic profile for each condition was followed by specific changes in mTORC1 signaling pathway players. Exposure to elevated glucose levels (17.5 mM), which we might correlate with a hyperglycemic status (315 mg/dL), led to a reduction of lactate production, although the consumption of glucose was higher, as compared to the cells cultured with 5.5 mM glucose (100 mg/dL). It has been described that SCs cultured under physiological levels of glucose convert the vast majority of glucose (around 95%) to lactate (Oliveira and Alves, 2015; Robinson and Fritz, 1981). When hSCs were exposed for a longer period (6 hours) to elevated glucose levels (17.5 mM) a transition from a Warburg-like effect (converting most glucose to lactate) to a non-glycolytic mode (decrease in lactate production) was observed. A similar trend has been described when cancer cells were cultured under high glucose levels, in which lactate accumulation seem to virtually block net lactate generation (Xie et al., 2014). Glutamine (through glutaminolysis) contributes with the anabolic carbons for the biosynthesis of distinct metabolic building blocks, and also accounts for lactate production (Zielke et al., 1980), when glucose is insufficient for the metabolic demands of the cells (DeBerardinis and Cheng, 2010). Exposing hSCs to glutamine as the only carbon source was able to cause a dramatic increase in acetate production as soon as after 30 minutes of culture, comparing to hSCs cultured in the absence of glucose, with different concentrations of glucose or with 5 mM leucine. Nevertheless, this was reverted in cells cultured for 6 hours with glutamine where an accumulation of pyruvate in the media of hSCs was

observed. Interestingly, we observed that glutaminolysis correlates with mTORC1 activation in the 30 min time frame of culture and that glutaminolysis promotes a rapid accumulation of acetate, which latter enhances the synthesis and export pyruvate, without altering lactate production. We observed that culturing hSCs with leucine promotes a dramatic reduction on the export of the key endpoints of SCs glucose metabolism (lactate and pyruvate) as compared to those cells cultured with physiological levels of glucose, both at 30 min or after 6 h. These observations, together with the fact that leucine is being consumed, suggest that this amino acid might be promoting an enhancement of oxidative phosphorylation. In fact, this may be the reason for the concurrent increase on oxidation of unsaturated fatty acids. When comparing the results obtained after cultivating hSCs with two distinct amino acids, it seems that glutamine (a non-essential amino acid), and thus stimulation of glutaminolysis, may be more relevant as a metabolic substrate for the testicular metabolic cooperation than leucine (an essential amino acid). This process appears to be mediated by mTOR since the timeframe of our experiments promotes mTOR phosphorylation at Ser²⁴⁴⁸, with the phosphorylated rpS6 levels remaining elevated within the first 30 min, but after 6 hours there is a dephosphorylation of both targets, suggesting transient activation and latter desensitization of this pathway, what was reported previously (Nicklin et al., 2009).

As referred, when male factor is the contributor for the infertility of the couple, the treatment is not usually directed to a specific identified cause, because a clear diagnosis is not established for the observed couple's infertility. Treatment associated with ART is frequently applied in an empirical manner to maximize the chances to conceive, but without an in-depth study on the causes of male infertility. For the use of those technologies, sperm collection is mandatory. However, after collection and during preservation, sperm quality rapidly declines, which hampers the success of ART. In the third study, we hypothesized that supplementation of a commercial sperm preservation medium with an mTOR enhancer (MHY 1485) could improve sperm quality after short-term storage, without changing DNA integrity and their capacitation. To further understand how these effects are mediated, we focused on sperm metabolic activity and oxidative damages during storage. This is the first report focused on a possible role for

mTOR during short-term sperm preservation. Sperm preservation in a medium supplemented with MHY 1485, an mTOR enhancer, increases sperm metabolic activity without inducing oxidative damages. Notably, the supplementation of preservation media with MHY 1485 also improved sperm viability and motility, while decreasing DNA fragmentation and maintaining spermatozoa binding capacity. Our data suggests that mTOR is not only involved in sperm quality, but also that the use of an enhancer of this protein in preservation media improves spermatozoa quality.

In sum, our data show that mTOR signaling pathway is a regulator of male fertility. Our first study revealed that mTOR pathway, particularly by mTORC1 path, alters the glycolytic and oxidative profile of hSCs, suggesting a role on the modulation of the metabolism of these cells. The second study, besides showing the SC metabolism plasticity with different nutrients availability, reveals also a timeframe integration of these environmental cues by mTOR pathway. Both works present some limitations since we only used an *in vitro* approach, with isolated hSCs, and the testicular tissue includes several types of cells, not being possible to entirely expand the conclusions to an *in vivo* situation and extrapolate these results in terms of whole testis environment. The third study clearly show that a stimulation of mTOR pathway on sperm, by addition of the mTOR enhancer MHY 1485 to a commercial sperm preservation medium, improves the percentage of viable and motile spermatozoa and decreases sperm DNA fragmentation. This was due to a stimulation of sperm metabolic activity that did not increased oxidative damages nor altered its capacitation ability. Based on this we may affirm that enhancement of mTOR during preservation improves sperm quality, which may be crucial to improve the outcomes of ART.

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Mammalian target of rapamycin controls glucose consumption and redox balance in human Sertoli cells

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Objective: To study the role of mammalian target of rapamycin (mTOR) in the regulation of human Sertoli cell (hSC) metabolism, mitochondrial activity, and oxidative stress.

Design: Experimental study.

Setting: University research center and private assisted reproductive technology centers.

Patient(s): Six men with anejaculation (psychological, vascular, neurologic) and conserved spermatogenesis.

Intervention(s): Testicular biopsies were used from patients under treatment for recovery of male gametes. Primary hSCs cultures were established from each biopsy and divided into a control group and one treated with rapamycin, the inhibitor of mTOR, for 24 hours.

Main Outcome Measure(s): Cytotoxicity of hSCs to rapamycin was evaluated by sulforhodamine B assay. The glycolytic profile of hSCs was assessed by proton nuclear magnetic resonance and by studying protein expression of key glycolysis-related transporters and enzymes. Expression of mitochondrial complexes and citrate synthase activity were determined. Protein carbonylation, nitration, lipid peroxidation, and sulfhydryl protein group contents were quantified. The mTOR signaling pathway was studied.

Result(s): Rapamycin increased glucose consumption by hSCs, maintaining lactate production. Alanine production by rapamycin-exposed hSCs was affected, resulting in an unbalanced intracellular redox state. Rapamycin-exposed hSCs had decreased expression of mitochondrial complex III and increased lipid peroxidation, whereas other oxidative stress markers were unaltered. Treatment of hSCs with rapamycin down-regulated phospho-mTOR (Ser-2448) levels, illustrating an effective partial inhibition of mTORC1. Protein levels of downstream signaling molecule p-4E-BP1 were not altered, suggesting that during treatment it became rephosphorylated.

Conclusion(s): We show that mTOR regulates the nutritional support of spermatogenesis by hSCs and redox balance in these cells. (Fertil Steril® 2016;105:825–33. ©2016 by American Society for Reproductive Medicine.)

Key Words: mTOR, Sertoli cells, spermatogenesis, testis, rapamycin

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Spermatogenesis is a highly regulated process that takes place in the seminiferous epithelium where Sertoli cells (SCs) directly interact with developing germ cells (1). The somatic SCs form the blood-testis barrier (BTB) and establish an adequate luminal environment within the seminiferous tubules for the process of sperm cells development (2). The SCs are known as "nurse" cells because of their functions in the physical and nutritional support of spermatogenesis

(1, 3, 4). Sertoli cell (SC) metabolism is essential for the normal occurrence of spermatogenesis (5), as developing germ cells are incapable of using glucose for energy metabolism and are dependent on the lactate produced by SCs (6).

The mammalian target of rapamycin (mTOR) is a Ser/Thr protein kinase that plays an essential role on several cellular events such as cell growth and proliferation (7, 8). It is also a key regulator for sensing and integrating various environmental cues, including growth factors and nutrients (9). The mTOR can form two complexes to execute its functions, mTORC1 and mTORC2, by associating with different binding partners (9, 10). mTORC1 is composed of mTOR, regulatory associated protein of mTOR (raptor) and other factors. It mediates the mTOR action by modulating protein synthesis. This complex is sensitive to rapamycin, which acts as an allosteric inhibitor of mTORC1 by dissociating raptor from mTOR (11, 12). The key binding partner of mTORC2 is rictor (rapamycin-insensitive companion of mTOR), which according with its name is insensitive to rapamycin.

Recently new functions have been described for mTOR in the male reproductive system. It was shown that mTOR is involved in BTB restructuring during the epithelial cycle of spermatogenesis (13, 14), illustrating that this molecule may have a preponderant role in the control of male fertility. Hence, we hypothesized that mTOR can play an essential role in the control of SC metabolism and in the nutritional support of spermatogenesis. In addition, as mitochondrial function is also crucial for spermatogenesis and regulates SC metabolism (15), we hypothesized that mTOR signaling could modulate mitochondrial activity in human SCs (hSCs). To test our hypotheses we examined the effect of exposure to rapamycin, the inhibitor of mTORC1, in the glycolytic profile and mitochondrial function of hSCs.

MATERIALS AND METHODS

Chemicals

Tris-Base and NZYColour Protein Maker II were purchased from NZYTech. All other chemicals were purchased from Sigma-Aldrich unless stated otherwise.

Patient Selection, Ethical Issues, and Testicular Biopsies

Patient selection and clinical study were performed at the Center for Reproductive Genetics Professor Alberto Barros (Porto, Portugal) after approval by the local Ethics Committee. The studies followed the Guidelines of the Local, National and European Ethical Committees and the Declaration of Helsinki. Testicular biopsies were obtained from patients seeking fertility treatment, after informed written consent. Only cells left in culture plates after the fertility treatment were used. The hSCs were isolated from testicular biopsies from patients with anejaculation (psychological, vascular, neurologic) and with conserved spermatogenesis.

Sertoli Cells Culture

Testicular biopsies were washed using a routine method (16). The primary cultures of hSCs were obtained using routine methods (17). The resulting pellet was suspended in SC culture medium (Dulbecco's minimum essential medium [DMEM]:-Ham's F-12 1:1, containing 15 mM HEPES, 50 U/mL penicillin, 50 mg/mL streptomycin sulfate, 0.5 mg/mL fungizone, 50 µg/mL gentamicin, and 10% heat-inactivated fetal bovine serum). Cells were plated and incubated at 32°–34°C, 5% CO₂ in air. Cultures were examined and those with contaminants <5% were used. Cultures purity was tested by immunoperoxidase detection of antimüllerian hormone and vimentin-specific markers (18).

Experimental Groups

The hSCs were allowed to grow (90%–95% of confluence). Then, cells were washed and cultured in serum-free medium (supplemental DMEM:F12 1:1 at pH 7.4). The medium was supplemented either with insulin-transferrin-sodium selenite (ITS) (5 µg/mL – 5 µg/mL – 5 ng/mL, respectively; control group), or with ITS plus rapamycin (ITS+R) (100 ng/mL; rapamycin group). Cells were maintained for 24 hours. After treatment, culture media were collected, the cells were detached using a trypsin-ethylenediaminetetraacetic acid (EDTA) solution, and collected. Total protein in each group was quantified using the Pierce BCA Protein assay kit (Pierce Biotechnology). A viability test was performed on the cells of the different experimental groups using the trypan blue exclusion test. Viability averaged 85%–90%, always with values >85%.

Cytotoxicity Assay

The cytotoxicity of hSCs to rapamycin was determined by the colorimetric sulforhodamine B assay (19). In brief, cells were seeded and cultured for 24 hours in ITS or ITS+R medium. After treatment, cells were washed twice in phosphate-buffered saline (PBS) solution and fixed overnight in 1% acetic acid methanol. Cells were then incubated with 0.5% (wt/vol) sulforhodamine B assay in 1% acetic acid for 1 hour at 37°C. The unbound dye was removed by washing with 1% acetic acid solution. Dye bound to cells was extracted with 10 mM Tris solution (pH 10). Optical densities of the media were measured at 540 nm. No cytotoxicity was observed for the concentration of rapamycin used in this work during 24 hours (Supplemental Fig. 1, available online).

Nuclear Magnetic Resonance Spectroscopy

¹H-nuclear magnetic resonance spectroscopy was performed with routine methods used by our team (20). Sodium fumarate (final concentration of 1 mM) was used as an internal reference (6.50 ppm) to quantify metabolites in solution (multiplet, δ, ppm); lactate (doublet, 1.33); alanine (doublet, 1.45); acetate (single, 1.91); pyruvate (singlet, 2.36); H1-α glucose (doublet, 5.22). Spectra analysis was performed offline. The relative areas of ¹H-nuclear magnetic resonances were quantified using the curve-fitting routine with the NutsPro NMR spectral analysis program (Acorn, NMR Inc.).

Western Blot

Western blot was performed using standard methods (21). In brief, protein samples (75–100 μg) were fractionated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were blocked and incubated with primary antibodies (Supplemental Table 1, available online) overnight at 4°C. Mouse anti- β -tubulin was used as the protein-loading control, except for total oxidative phosphorylation (OXPHOS), in which mouse anti- β -actin was used as loading control. The immune-reactive proteins were detected separately with secondary antibodies. Membranes were reacted with the ECF detection system (GE Healthcare) and visualized with the BioRad GelDoc XR (Bio-Rad). The densities from each band were obtained using the Quantity One software (Bio-Rad).

Slot Blot

The levels of carbonyl and nitrotyrosine protein groups and of 4-hydroxynonenal were measured by the slot-blot technique. Protein carbonyl group levels were determined as described previously (15). For determination of nitrotyrosine and 4-hydroxynonenal levels, 5 μg of protein were diluted in PBS and transferred to polyvinylidene difluoride membranes using a Hybri-slot manifold system (Biometra). The membranes were then blocked and incubated with the respective primary antibodies (Supplemental Table 1) overnight at 4°C. The immune-reactive proteins were detected separately with secondary antibodies. Membranes were reacted and visualized as described previously. Densities were quantified using the Quantity One software.

Citrate Synthase Activity

Citrate synthase activity was measured according to Coore and colleagues (22) using a spectrophotometric assay (412 nm), in which the amount of 5,5'-dithio-bis(2-nitrobenzoic acid) that reacted with coenzyme A (released from the reaction of acetyl-CoA with oxaloacetate) was determined by measuring the appearance of 5-thio-2-nitrobenzoic acid (molar extinction coefficient $\epsilon = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$). Results were expressed in nanomoles per minute per microgram of protein.

Sulfhydryl Protein Groups

Sulfhydryl protein groups were spectrophotometrically determined as described by Hu (23). The absorbance of the samples was determined at 412 nm against a blank, after reaction of 5 μL of protein extract in phosphate buffer (pH 7.4) with 1 μL of 5,5'-dithio-bis(2-nitrobenzoic acid) (10 mM) in a medium containing 15 μL of Tris buffer (0.25 M Tris, 20 mM EDTA) and 79 μL of methanol. Sulfhydryl content was calculated measuring the appearance of 5-thio-2-nitrobenzoic acid (using the molar extinction coefficient $\epsilon = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$) and expressed in nanomoles per milligrams of protein.

Statistical Analysis

All data are shown as mean $\pm$ SEM ($n = 6$ for each condition). Statistical differences between experimental groups were assessed by unpaired t test using GraphPad Prism 6 (GraphPad Software). $P < .05$ was considered significant.

RESULTS

Rapamycin Increases Glucose Consumption by hSCs

The SCs metabolize the extracellular glucose and ensure the production of metabolic precursors necessary for developing germ cells (for review, see Alves et al. [24]). After treatment with rapamycin, glucose consumption by hSCs was higher when compared with cells cultured in control conditions (383 ± 19 vs. 219 ± 62 nmol of glucose/ μg of protein) (Fig. 1A). Glucose enters into hSCs through glucose transporters (GLUTs), particularly GLUT1, GLUT2, and GLUT3 (25–28). There were no alterations in the expression levels of GLUT1, GLUT2, or GLUT3, as determined by Western blot, in hSCs exposed to rapamycin (Fig. 1B–1D).

Production of Monocarboxylates by hSCs is Insensitive to Rapamycin Treatment

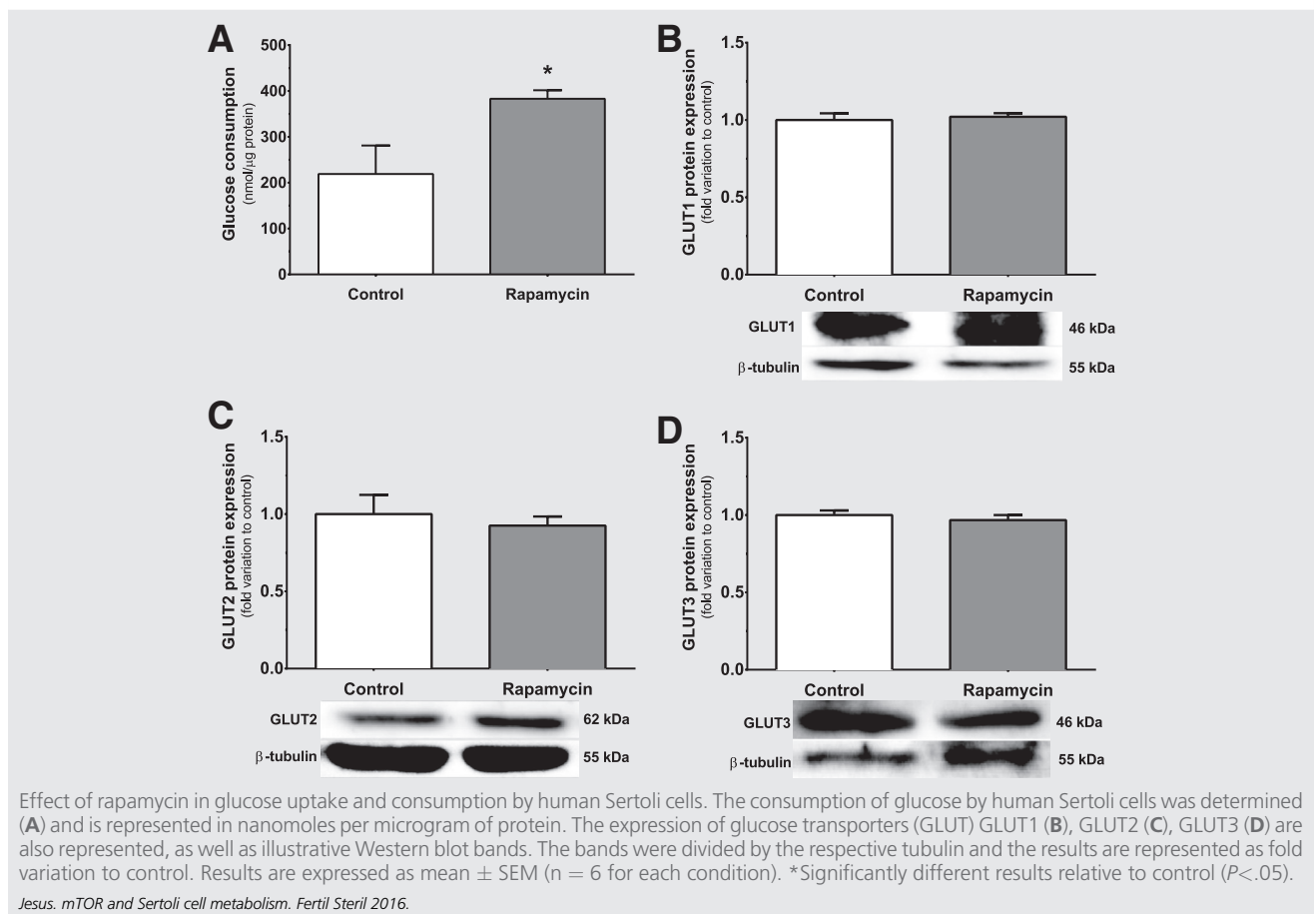
Glucose is metabolized in a sequence of multistep reactions. Phosphofructokinase mediates the first rate-limiting step in glycolysis (29) and plays a crucial regulatory role in the control of SC metabolism (28, 30). Nevertheless, exposure to rapamycin was not able to alter the protein expression of this enzyme, as measured by Western blot (data not shown). Also, the consumption of pyruvate by hSCs was not altered by exposure to rapamycin (Supplemental Fig. 2A, available online).

The pyruvate originating from glycolysis can [1] be converted to alanine by alanine aminotransferase; [2] be converted to lactate by lactate dehydrogenase, with the simultaneous oxidation of reduced nicotinamide adenine dinucleotide (NADH) to oxidized nicotinamide adenine dinucleotide (NAD^+); [3] or be converted to acetyl-CoA, which enters mitochondria to be used in the Krebs cycle, or is converted into acetate. In hSCs, most of the pyruvate taken from the media or derived from glycolysis is converted to lactate. When hSCs were exposed to rapamycin, we did not detect (by Western blot) alterations in lactate dehydrogenase protein levels (data not shown) or in lactate production (Fig. 2A) when compared with cells cultured in control conditions. The production of acetate by hSCs exposed to rapamycin was also not altered when compared with hSCs in control conditions (Supplemental Fig. 2B). However, treatment of hSCs with rapamycin induced a decrease in alanine production when compared with cells in control conditions (1.8 ± 0.8 vs. 4.3 ± 0.9 nmol of alanine/ μg of protein) (Fig. 2B).

Rapamycin Promotes Intracellular Redox Unbalance and Stimulates Lipid Peroxidation in hSCs

Exposure of hSCs to rapamycin augmented the lactate-to-alanine ratio (1.0 ± 0.4), when compared with cells cultured

FIGURE 1



in control conditions (2.6 ± 0.6) (Fig. 2C). The $\text{NAD}^+:\text{NADH}$ ratio reflects the oxidative status of the cell. Our results suggested an unbalanced redox status in rapamycin-exposed hSCs. Reactive oxygen and nitrogen species may oxidize protein side chains, generating the formation of carbonyl group and nitrotyrosine groups (31). Rapamycin-exposed hSCs did not present altered levels of protein carbonylation (dinitrophenylhydrazine reacts with free carbonyl groups on proteins, leading to adduction of dinitrophenyl groups) or nitration when compared with nonexposed cells (Fig. 2D and E). The protein sulfhydryl groups are also susceptible to oxidation (32–35). We observed no differences in total sulfhydryl groups in hSCs exposed to rapamycin compared with cells cultured in control conditions (Fig. 2F). 4-Hydroxynonenal is a byproduct formed during oxidation of unsaturated fatty acids (36). The levels of 4-hydroxynonenal are higher in cultured hSCs exposed to rapamycin than in nonexposed cells (Fig. 3A).

Exposure to Rapamycin Decreases Mitochondrial Complex III Expression in hSCs

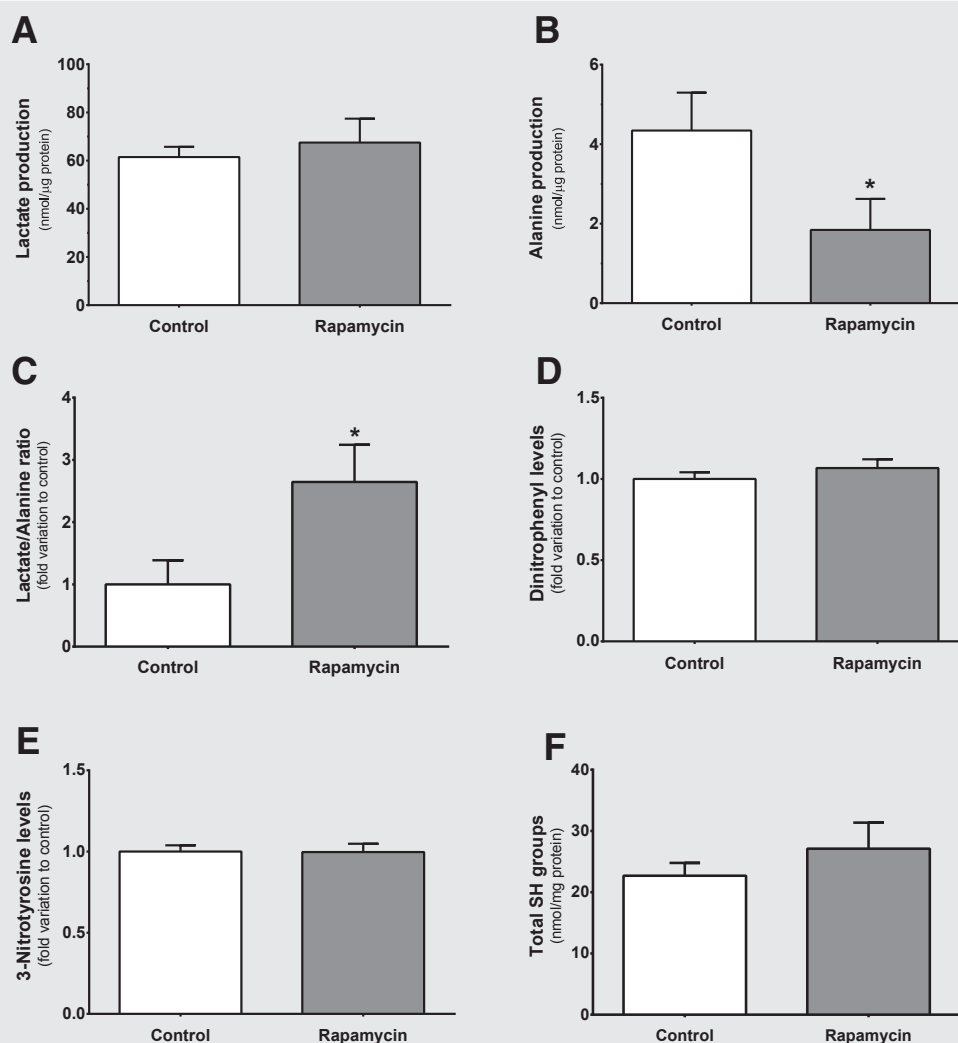
Mitochondria are responsible for adenosine triphosphate (ATP) synthesis by oxidative phosphorylation. The electrons resulting from oxidation of substrates are guided through

redox carriers (mitochondrial complexes I, II, III, and IV) and linked with proton pumping by an F-ATPase (mitochondrial complex V). Rapamycin-exposed hSCs presented no alterations in the expression of the mitochondrial complexes II, IV, and V, compared with nonexposed cells (Fig. 3B 3D, and 3E). However, a decrease was detected (from 1.00 ± 0.17 to 0.50 ± 0.04) in rapamycin-exposed hSCs expression of mitochondrial complex III when compared with nonexposed cells (Fig. 3C). The enzyme citrate synthase stands as a pace-making enzyme for the Krebs cycle (37) and is used as a quantitative marker for intact mitochondria. The activity of citrate synthase was not altered in hSCs exposed to rapamycin when compared with nonexposed cells (Fig. 3F).

Phosphorylation of Mammalian Target of Rapamycin is Inhibited in hSCs Exposed to Rapamycin

Phosphatidylinositol 3-kinase is an upstream regulator of mTORC1, which is phosphorylated and activated with the binding of insulin to its receptors (8). Rapamycin treatment did not alter phosphatidylinositol 3-kinase expression in hSCs (Fig. 4A). PIP_3 is a second messenger, phosphorylated by phosphatidylinositol 3-kinase. Protein kinase B (Akt) is recruited by phosphatidylinositol (3,4,5)-trisphosphate (PIP_3)

FIGURE 2



Effect of rapamycin in intracellular glucose metabolism and in oxidative profile of human Sertoli cells. The production of lactate (A) and alanine (B) by human Sertoli cells was determined. The results are expressed as nanomoles per microgram of protein. The lactate-to-alanine ratio (C) is represented as an indicator of intracellular redox state. Oxidative damages were measured in proteins carbonylation (D) and nitration (E) and by sulfhydryl (SH) groups content (F). Results are expressed as mean $\pm$ SEM ($n = 6$ for each condition). *Significantly different results relative to control ($P < .05$).

Jesus. mTOR and Sertoli cell metabolism. *Fertil Steril* 2016.

and, besides being an upstream regulator of mTORC1, it phosphorylates glycogen synthase kinase 3 (38). The expression of glycogen synthase kinase 3 is not altered in hSCs exposed to rapamycin (Fig. 4B). However, as expected, treatment of hSCs with rapamycin caused a decrease in the expression of phosphorylated mTOR when compared with nonexposed cells (Fig. 4C). The eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) is a downstream signaling molecule of mTORC1 (9, 10). Notably, the expression of phosphorylated 4E-BP1 is not altered in hSCs exposed to rapamycin (Fig. 4D).

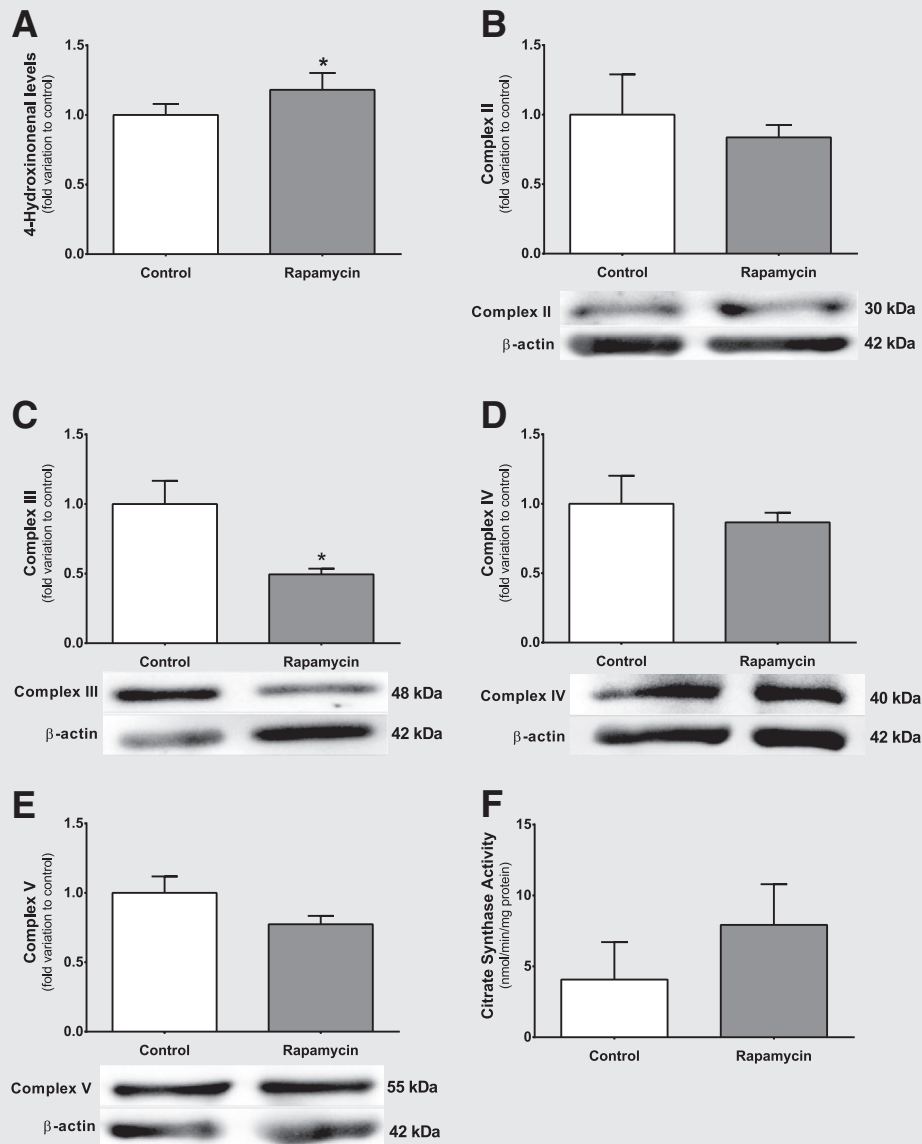
DISCUSSION

Sertoli cells create the BTB and are responsible for the physical and nutritional support of developing germ cells (1, 3,

4). Thus, SC metabolism is an essential event for the normal occurrence of spermatogenesis (5). Compelling evidence showed that mTOR is pivotal in the establishment and reorganization of BTB. However, other functions for mTOR signaling pathway in male reproductive system remain to be unveiled. Thus, we aimed to evaluate the effect of an mTORC1 inhibitor (rapamycin) in the nutritional support of spermatogenesis by hSCs. We determined the effect of rapamycin in hSCs carbohydrate metabolism, metabolite secretion/consumption, oxidative status, the involvement of mTOR signaling pathway, and alterations in the expression of mitochondrial complexes.

Our data show that mTOR is a novel regulator of hSCs glycolytic and oxidative profiles. Cultured hSCs use glucose to produce lactate at high concentrations in a typical

FIGURE 3



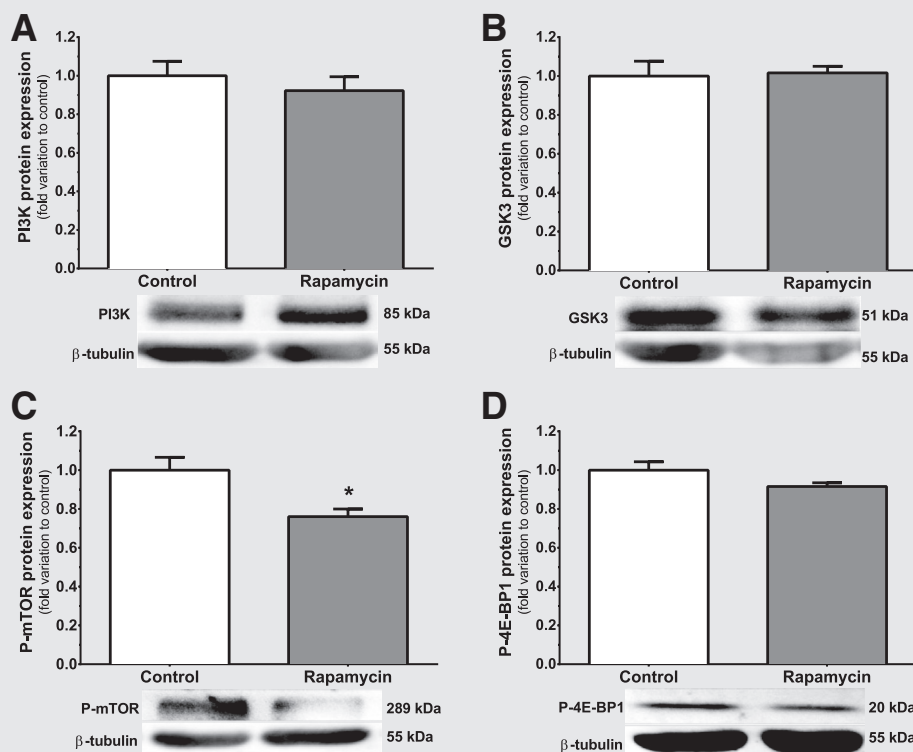
Effect of rapamycin in the oxidative profile, mitochondrial complexes proteins levels and in mitochondrial integrity of human Sertoli cells. Oxidative damages were measured in lipid peroxidation (A). The protein levels of mitochondrial complex II (B), complex III (C), complex IV (D), and complex V (E) are represented, as well as illustrative Western blot bands. The bands were divided by the respective actin and the results are represented in fold variation to control. The presence of/or intact mitochondria was measured by citrate synthase activity (F). The results are expressed as nanomoles of coenzyme A per minute per milligram of protein. Results are expressed as mean $\pm$ SEM ($n = 6$ for each condition). *Significantly different results relative to control ($P < .05$).

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Warburg-like metabolism (5). Interestingly, glucose consumption was increased in rapamycin-treated cells, although lactate, pyruvate, and acetate production remained unchanged. SCs display abnormally elevated glycolytic activity under normal conditions and a high metabolic plasticity to sustain lactate production (39, 40). Rapamycin, the inhibitor of mTORC1 pathway, is a regulator of the glycolytic flux in mammalian cells (41). The mTORC1 pathway controls glucose metabolism and glycolysis through regulation of glucose transporters and rate-limiting glycolytic enzymes

(42, 43). In human skeletal muscle, rapamycin administration augmented glucose uptake in the presence of combined hyperaminoacidemia and prandial-like peripheral hyperinsulinemia (44). Under unfavorable conditions, such as insulin deprivation (21, 39), hSCs sustain lactate production by adapting their metabolism. Inhibition of the mTORC1/ribosomal protein S6 kinase pathway by rapamycin induces an increase in glucose consumption to produce the same amount of lactate and acetate as control cells. This was not followed by alterations in GLUTs,

FIGURE 4



Effect of rapamycin in the protein expression levels of target of rapamycin (TOR) signaling pathway. The protein expression levels of phosphatidylinositol 3-kinase (PI3K) (A); glycogen synthase kinase 3 (GSK3) (B); phosphorylated mammalian TOR (P-mTOR) (C), and phosphorylated eukaryotic translation initiation factor 4E-binding protein 1 (P-4E-BP1) (D) in human Sertoli cells were determined. The bands were divided by the respective tubulin and the results are represented in fold variation to control. Results are expressed as mean $\pm$ SEM ($n = 6$ for each condition). *Significantly different results relative to control ($P < .05$).

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phosphofructokinase, or lactate dehydrogenase protein levels, suggesting that the mTORC1 pathway regulates glucose transporters and rate-limiting glycolytic enzymes activity in hSCs.

The mTOR signaling pathway is also linked to amino acids metabolism (45). Cells cultured in conditions of amino acids deprivation show a rapid decline in the phosphorylation of proteins involved in the mTOR pathway (46). In addition, amino acids activate the ribosomal protein S6 kinase and the downstream signaling of mTOR pathway. Pyruvate produced by hSCs can be either converted to alanine or lactate through reactions coupled with the NAD^+/NADH equilibrium. Because rapamycin-exposed hSCs decrease the production of alanine, a higher lactate-to-alanine ratio was inferred. As so, the cellular NAD^+/NADH ratio should be increased in rapamycin-exposed hSCs, which led us to hypothesize that oxidative stress and/or mitochondrial dysfunction could occur. We analyzed specific markers for oxidative stress such as protein carbonylation, nitration, sulfhydryl groups, and lipid peroxidation levels. Our data shows that rapamycin-exposed SCs present a higher lipid peroxidation. Rapamycin is known to sensitize cells with a high glycolytic activity, namely cancer cells, to oxidative stress (for review, see Nogueira and Hay [47]). Rapamycin hyperactivates Akt through the inhibition of a negative feedback loop induced

by mTORC1 to inhibit Akt activity (48). This has been suggested as a strategy to selectively kill cells treated with rapamycin, particularly cancer cells (49). The hSCs present some remarkable metabolic similarities with cancer cells (for review, see Oliveira et al. [5]) and cellular glucose metabolism is closely related to oxidative stress. Thus, our data suggest that rapamycin treatment sensitizes hSCs to oxidative stress, particularly lipid peroxidation.

Mitochondrial dysfunction is a major contributor for lipid peroxidation (50). In addition, an interplay between mitochondrial function and mTOR signaling has been shown, as the mTOR pathway plays a critical role in oxygen consumption and oxidative capacity in mammalian cells (51). The inhibition of mTOR signaling by rapamycin in Jurkat cells promoted the shift of glucose metabolism from mitochondrial respiration to glycolysis (52). It was also suggested that mTOR activity may be sensitive to cells redox status, which is concomitant with our data concerning lipid peroxidation. The mTOR was also shown to bind to the mitochondrial outer membrane and alter mitochondrial genes expression (52, 53). Our data show that exposure to rapamycin decreases mitochondrial complex III protein levels in hSCs, illustrating that mTOR signaling is pivotal for maintenance of normal bioenergetics. Although hSCs present a clear Warburg-like metabolism,

under detrimental conditions, such as insulin deprivation, mitochondrial-mediated apoptosis is activated (54), thus mitochondrial bioenergetics are also pivotal. This is crucial as SCs are only capable of sustaining a limited number of germ cells (55, 56) and the male reproductive potential is often associated with the number and function of SCs. Further studies will be needed to enlighten the relevance of mTOR in oxidative stress, mitochondrial physiology, and apoptosis in these cells.

The treatment of hSCs with rapamycin for 24 hours led, as expected, to a decrease in mTOR phosphorylation at Ser-2448, illustrating an effective partial inhibition of mTORC1 by rapamycin. Interestingly, there were no differences in phosphatidylinositol 3-kinase, glycogen synthase kinase 3, and phosphorylated 4E-BP1 levels. We aimed to determine the impact of rapamycin on hSCs metabolism and thus, we selected a period of 24 hours to allow changes in metabolic pathways to occur, although not long enough to induce mTORC2 inhibition. The mTORC1 inhibition is reported to stimulate Akt signaling (48), which can up-regulate the mTOR pathway. In addition, rapamycin is not able to completely inhibit the mTORC1 phosphorylation/pathway. The 4E-BP1 is a downstream signaling molecule of mTORC1 (57). However, 4E-BP1 is rephosphorylated and is refractory in exposure to rapamycin during longer periods (58, 59). A 24-hour treatment of hSCs with rapamycin partially inhibits mTOR phosphorylation at Ser-2448, although downstream phosphorylated 4E-BP1 levels remain unchanged, probably due to rephosphorylation.

The mTOR signaling was shown to regulate BTB dynamics (13, 14, 60, 61), but few studies have been performed to unveil other mechanisms by which mTOR can regulate spermatogenesis. Rapamycin inhibits apoptosis, promoted by reactive oxygen species inducers by alleviating mitochondrial dysfunction (62). In addition, rapamycin treatment in mice led to reduced testicular size and arrest of germ cells development, illustrating that mTORC1 is required for mice spermatogonial differentiation in vivo (63). Nevertheless, the effect of rapamycin in isolated testicular cells and in the human male reproductive system remained undisclosed. In the present study we show that inhibition of mTORC1 in hSCs increases glucose consumption and decreases alanine production, promoting an unbalanced redox state. This redox unbalance is reflected in an increased lipid peroxidation that may be associated with decreased mitochondrial complex III expression.

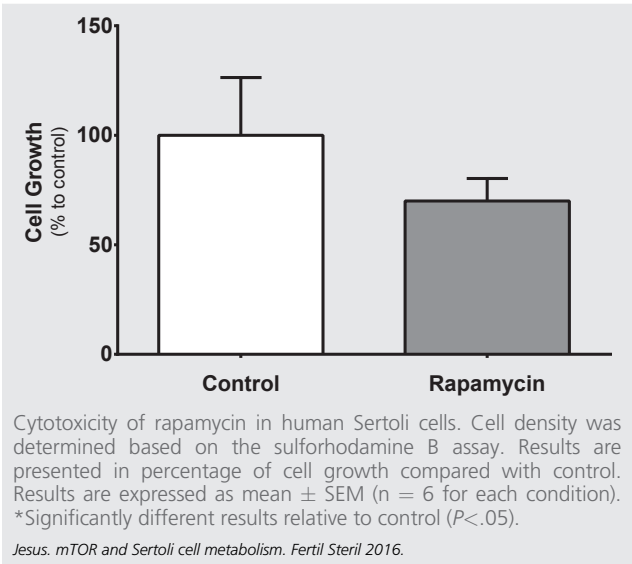
Our data show that mTOR signaling, particularly mTORC1, alters the glycolytic and oxidative profile of hSCs. Our report presents some limitations. The testicular tissue includes several types of cells and this work was done using an in vitro approach, with isolated hSCs. Therefore it was not possible to fully expand the conclusions to an in vivo situation, and extrapolate these results in terms of the whole testis environment, in which the role of mTORC1 remains to be unveiled. Further studies will be needed to disclose the full involvement of mTOR in the control of human male reproductive potential, particularly in individuals suffering from metabolic diseases, aged men, those with low sperm counts, and with other fertility problems.

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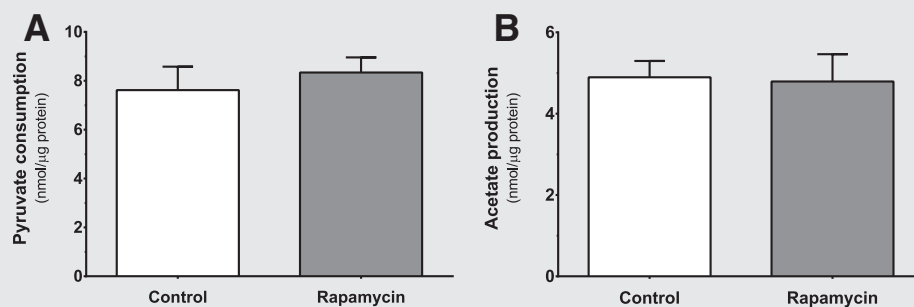
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SUPPLEMENTAL FIGURE 1



SUPPLEMENTAL FIGURE 2



Effect of rapamycin in intracellular glucose metabolism. The production of pyruvate (A) and acetate (B) by human Sertoli cells was determined. The results are expressed as mean $\pm$ SEM ($n = 6$ for each condition) in nanomoles per microgram of protein. *Significantly different results relative to control ($P < .05$).

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SUPPLEMENTAL TABLE 1

List of antibodies used in this study.

Antibody	Source	Molecular weight (kDa)	Dilution	Vendor	Catalog no.
GLUT1	Rabbit	46	1:5,000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-7903
GLUT2	Rabbit	61	1:1,000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-9117
GLUT3	Goat	46	1:500	Santa Cruz Biotechnology, Heidelberg, Germany	sc-7582
PFK-1	Rabbit	85	1:500	Santa Cruz Biotechnology, Heidelberg, Germany	sc-67028
LDH	Rabbit	37	1:5,000	Abcam, Cambridge, MA, USA	ab52488
MCT4	Rabbit	43	1:1,000	Santa Cruz Biotechnology, Heidelberg, Germany	sc-50329
PI3K	Rabbit	85	1:2,500	Milipore, Darmstadt, Germany	Cat.#06-195
GSK3	Mouse	51(alfa)	1:1,000	Milipore, Darmstadt, Germany	Cat.#05-412
P-mTOR	Rabbit	289	1:1,000	Milipore, Darmstadt, Germany	Cat.#09-213
P-4EB-P1	Rabbit	20	1:1,000	Cell Signaling Technology, Beverly, MA, United States	#2855
Total OXPHOS	Mouse	20, 30, 40, 48, 55	1:1,000	Abcam, Cambridge, MA, USA	ab110413
β -Tubulin	Mouse	55	1:5,000	Thermo Fisher Scientific, Rockford, IL, USA	BT7R
β -Actin	Mouse	42	1:5,000	Thermo Fisher Scientific, Rockford, IL, USA	BA3R
Mn SOD	Mouse	43	1:1,000	ENZO Life Sciences, Farmingdale, NY, USA	ALX-804-265-C100
DNP	Rabbit	—	1:5,000	Sigma-Aldrich, Roedermark, Germany	D9656
4-HNE	Goat	—	1:5,000	EMD Millipore, Temecula, CA, USA	AB5605
Nitrotyrosine	Rabbit	—	1:2,500	Cell Signaling Technology, Danvers, MA, USA	9691S
Mouse	Goat	—	1:5,000	Sigma-Aldrich, Roedermark, Germany	A3562
Rabbit	Goat	—	1:5,000	Sigma-Aldrich, Roedermark, Germany	A3687
Goat	Donkey	—	1:5,000	Santa Cruz Biotechnology, Heidelberg, Germany	SC-2020

Note: 4-HNE = 4-hydroxynonenal; DNP = dinitrophenyl; GLUT1 = glucose transporter 1; GLUT2 = glucose transporter 2; GLUT3 = glucose transporter 3; GSK3 = glycogen synthase kinase 3; LDH = lactate dehydrogenase; MCT4 = monocarboxylate transporter 4; Mn SOD = manganese superoxide dismutase; P-4EBP-1 = phosphorylated eukaryotic translation initiation factor 4E-binding protein 1; PFK-1 = phosphofructokinase 1; PI3K = phosphatidylinositol 3-kinase; P-mTOR = phosphorylated mammalian target of rapamycin; OXPHOS = oxidative phosphorylation.

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

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REVIEW ARTICLE

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.

ARTICLE HISTORY

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KEYWORDS

mTOR; Sertoli cells;
spermatogenesis; male
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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

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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 (Laplane & 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 (Laplanche & 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-

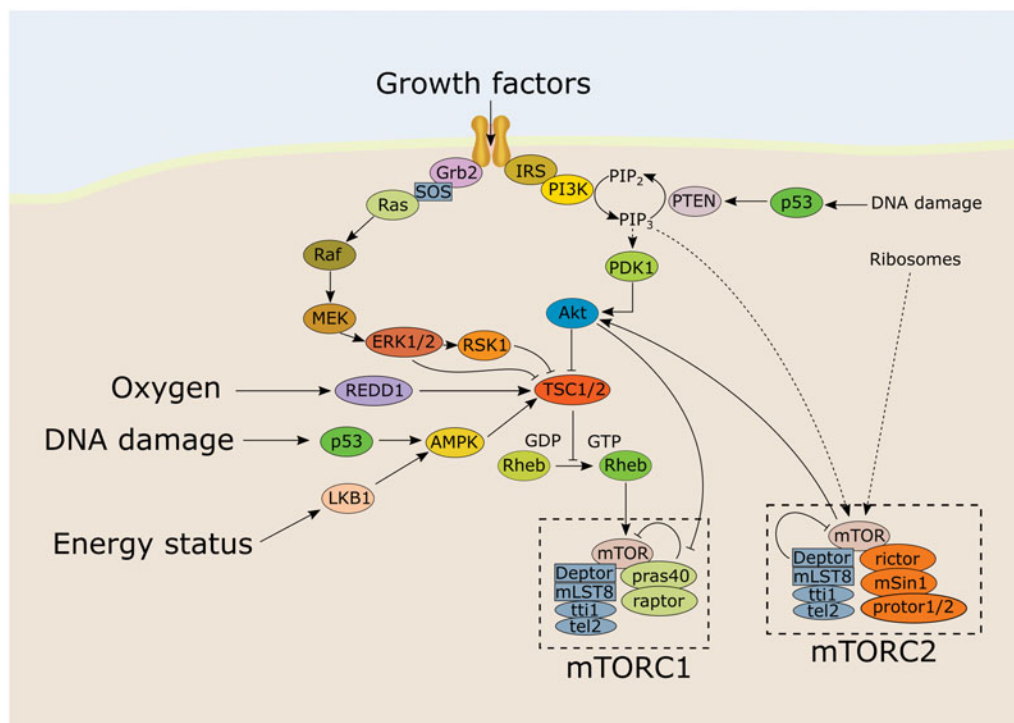


Figure 1. mTOR-signaling pathway: mTORC1 and mTORC2 complexes and the key signaling nodes that regulate mTORC1 and mTORC2. Critical inputs regulating mTORC1 include growth factors, DNA damage, energy status, and oxygen. mTORC2 is activated by growth factors and ribosomes in a poorly understood mechanism. 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).

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 intracellular 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 tuberlin), 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).

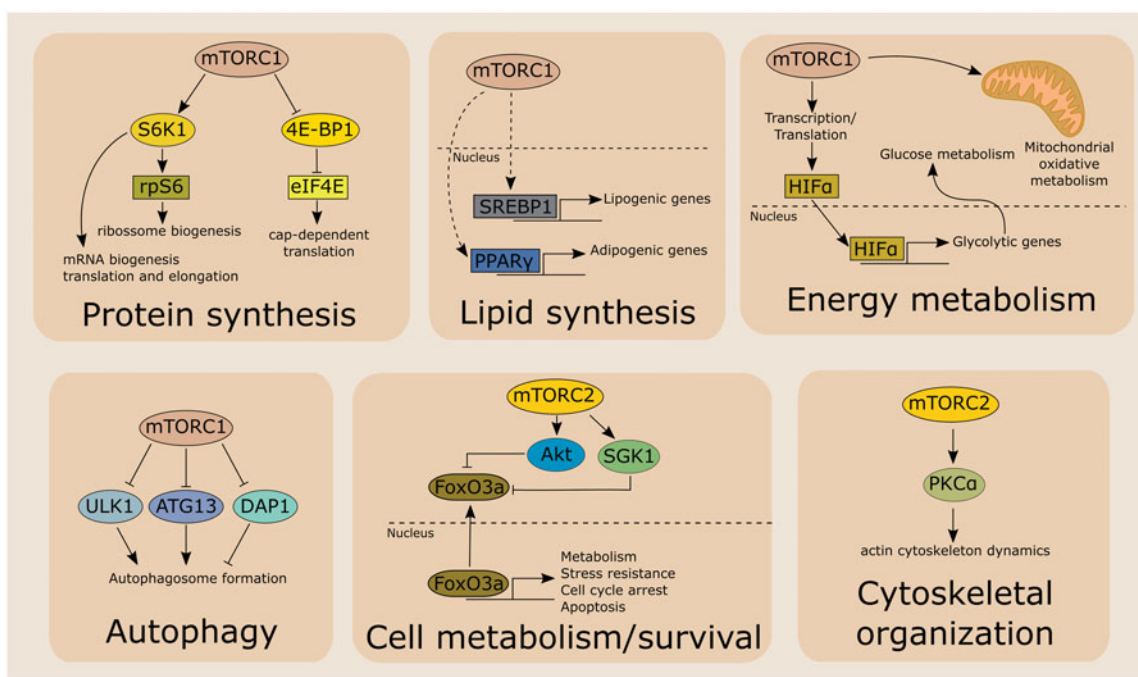


Figure 2. Key outputs of the mTORC1 and mTORC2 pathways. mTORC1 regulates many biological processes through phosphorylation of several proteins, positively regulating anabolic processes, such as protein and lipid synthesis and energy metabolism, and negatively regulating catabolic processes such as autophagy. 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).

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 α 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; Sarbassov *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; Sarbassov *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

Table 1. Clinically orientated studies associating mTOR inhibitors and male reproductive dysfunction.

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 (<i>n</i> = 66); Group 2: CNI (<i>n</i> = 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 (<i>n</i> = 28); Group 2: Sirolimus (<i>n</i> = 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 (<i>n</i> = 15); Group 2: Sirolimus (<i>n</i> = 15); Group 3: Sirolimus + CNI (<i>n</i> = 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 (<i>n</i> = 19); Group 2: no Sirolimus (<i>n</i> = 67); Group 3: intermittent Sirolimus (<i>n</i> = 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.

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

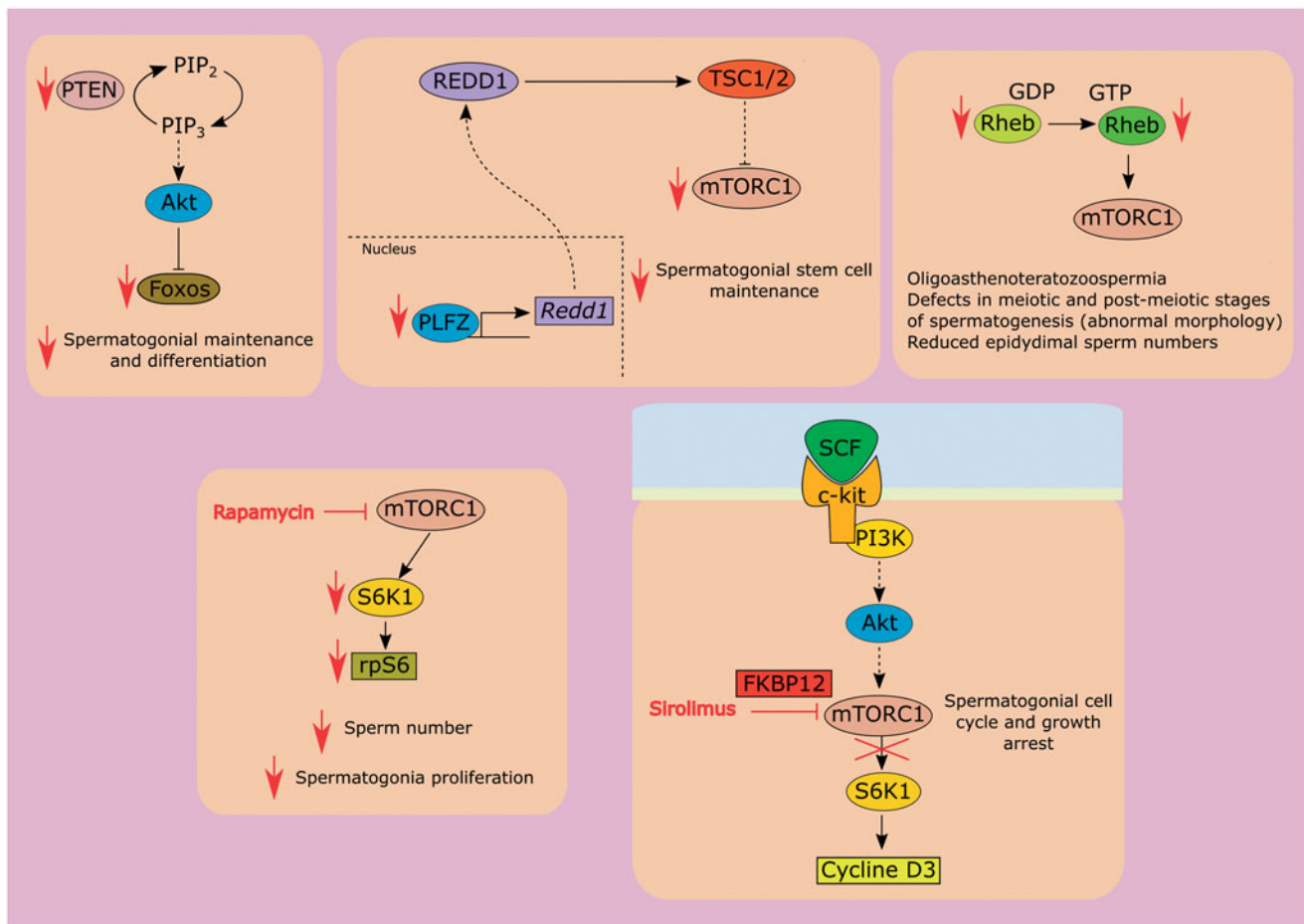


Figure 3. Effects of alterations in mTOR signaling pathway on spermatogenesis. Pten inactivation with the germ line-specific Vasa-Cre (Tg(Ddx4-cre)1Dcas) results in severe defects in spermatogonial maintenance and differentiation, in part through Foxo1. It was identified a role for PLZF-mediated Redd1 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. 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).

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.

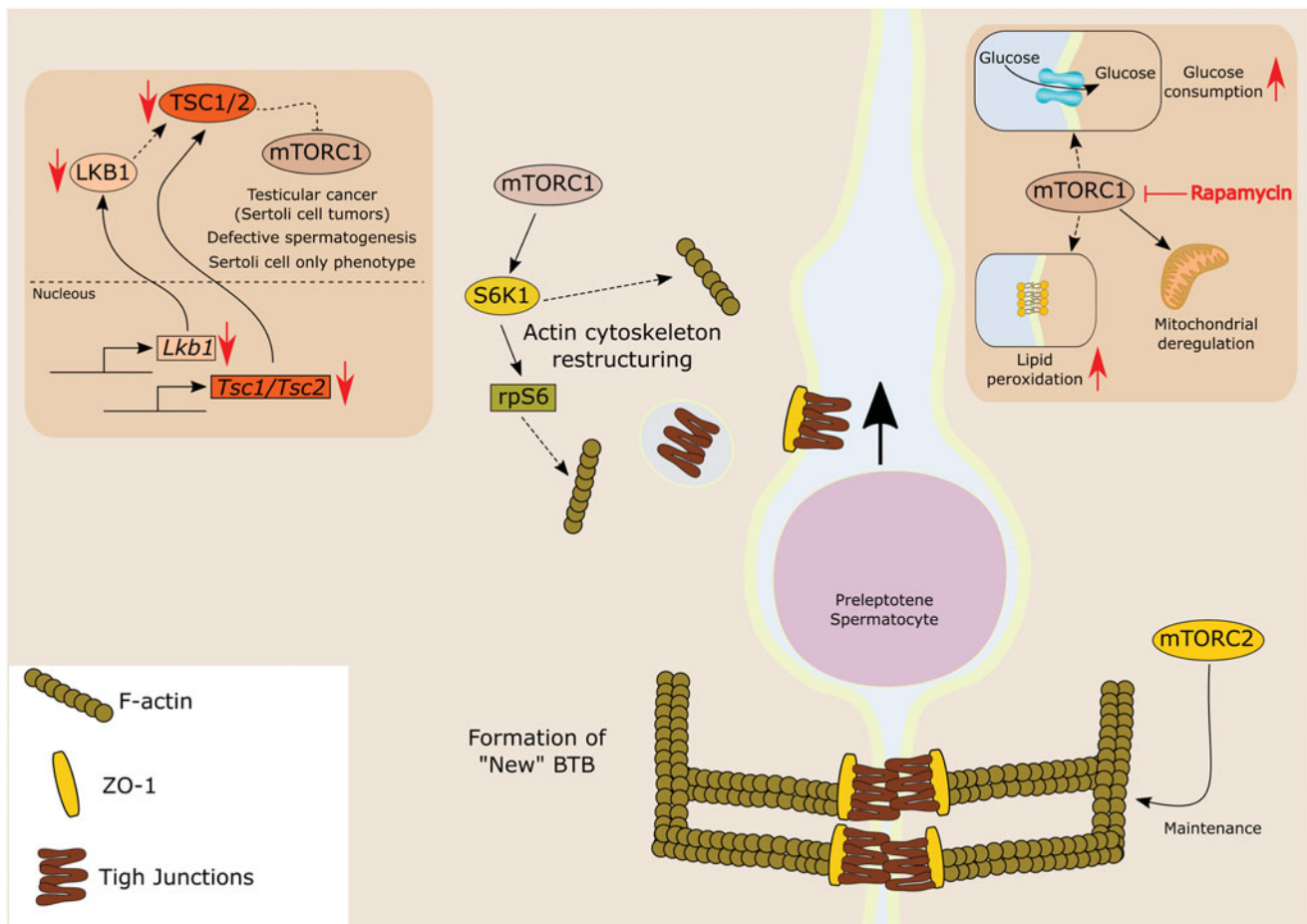


Figure 4. Role of mTOR complexes in Sertoli cells. Effects on male reproduction resulting from alterations on mTOR signaling: loss of *Lkb1* causes severe defects in spermatogenesis including a Sertoli cell only tubule phenotype. The deletion of *Tsc1* and *Tsc2* causes similar defects. Rapamycin-exposed 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).

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 *Lkb1*cko 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 the tightest blood–tissue barriers, due to its constitution composed by coexisting tight junction, basal ectoplasmic specialization, and other adhesion junction 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 (Laplante & 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.

Disclosure statement

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