Molecular Characterization and Functional Analysis of Cashmere Goat Mammalian Target of Rapamycin

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The mammalian target of rapamycin (mTOR) is an evolutionarily conserved protein kinase that belongs to the phosphatidylinositol kinase-related kinase family. We describe our molecular characterization of mTOR and its function (GenBank accession HM114224) in Cashmere goat (*Capra hircus*). The goat *mTOR* complementary DNA is 8617 bp, comprising an open reading frame of 7650 bp—corresponding to a polypeptide of 2549 amino acids—and a 909 bp 3′ untranslated region with a polyA tract and a polyadenylation signal at nucleotides 8575–8580. In a bioinformatics analysis, goat mTOR has typical sites of activity and domains. *mTOR* mRNA was measured in brain, heart, testis, liver, spleen, kidney, and lung by real-time polymerase chain reaction, and the expression of mTOR in fetal fibroblasts was detected by western blot. The viability of fetal fibroblasts was inhibited on treatment with CCI-779, a specific inhibitor of mTOR. Our data supplied evidence that the transcription of *mTOR* was detected in the seven tissues in Cashmere goat, and mTOR protein was translated in fetal fibroblasts. The proliferation of fetal fibroblasts decreases on inhibition of mTOR.

Introduction

THE MAMMALIAN TARGET of rapamycin (mTOR) (molecular weight Mr 289,000), also called FKBP rapamycin-associated protein (FRAP), is an evolutionarily conserved serine/threonine kinase that belongs to the phosphatidylinositol kinase-related kinase (PIKK) family. It controls cell growth, metabolism, and survival in response to nutrients, growth factors, cellular energy, and stress and has pleiotropic effects (Schmelzle and Hall, 2000; Fingar and Blenis, 2004).

mTOR is a downstream target of Akt. PI3K activates many signaling molecules, of which the Akt/mTOR pathway is notable, due its function in inhibiting apoptosis and mediating proliferation (Aoki *et al.*, 2001). mTOR is believed to regulate growth, and governs protein synthesis by phosphorylating downstream substrates, including p70S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (Burnett *et al.*, 1998).

The mTOR signaling pathway has significant physiological and pathological functions in cancers and metabolic diseases (Schmelzle and Hall, 2000; Proud, 2002; Wullschlegel *et al.*, 2006). mTOR is active in diabetes (Fraenkel *et al.*, 2008; Zoncu *et al.*, 2010), obesity (Um *et al.*, 2004; Chakraborty *et al.*, 2010), the nervous system (Li *et al.*,

2010; Neasta *et al.*, 2010), aging (Selman *et al.*, 2009; Kapahi *et al.*, 2010) and in diverse immune cells (Powell and Delgoffe, 2010).

Rapamycin (sirolimus) is a macrolide antibiotic that is produced by *Streptomyces hygroscopicus* and binds FKBP-12 (FK506-binding protein). The rapamycin-FKBP12 complex inhibits mTOR activity. Rapamycin decreases the expression of phospho-mTOR, phospho-S6K, cyclin D1, and VEGF-A (Wu *et al.*, 2010). Rapamycin also interferes with the G₂/M transition in murine one-cell embryos by inhibiting the activity of M-phase promoting factor (MPF). The mTOR signaling pathway regulates the activity value of MPF in murine one-cell embryos (Xu *et al.*, 2009). CCI-779 (temsirolimus), an analog of rapamycin, induces cell cycle arrest in G₁ and apoptosis (Teachey *et al.*, 2006; Gridelli *et al.*, 2008) and has been developed as oral and intravenous formulations for clinical use.

The *mTOR* gene and protein have been extensively examined in human, mouse, and rat but not in goat, due to the lack of basic data in this animal. To study the function and regulation of mTOR in Inner Mongolia Cashmere goat cells, we cloned full-length *mTOR* complementary DNA (cDNA), measured its transcription in various tissues by quantitative real-time polymerase chain reaction (PCR), and investigated its function in goat cell growth.

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Materials and Methods

Animals and tissue collection

Inner Mongolia cashmere goats were bred on a natural diet in Inner Mongolia, China. Brain, heart, testis, liver, spleen, kidney, and lung were collected from five adult male goats after slaughter in a commercial goat slaughter farm in the spring. Tissue samples were flash frozen in liquid nitrogen immediately after harvesting and stored at -80°C .

Cell cultures

Inner Mongolia cashmere goat fetal fibroblasts (GFbs) were cultured in DMEM/F12 (Gibco), supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc.), 100 U/mL penicillin G, and 100 mg/mL streptomycin (Sigma-Aldrich, Inc.), and maintained in a monolayer culture at 37°C in humidified air with 5% CO_2 . Morphology was examined on a light microscope.

Reagents and antibodies

CCI-779 (temsirolimus), a bioavailable derivative of rapamycin, was synthesized by Wyeth Pharmaceuticals Inc. (Philadelphia) and provided by Dr. Naomoto, Okayama University, Japan. CCI-779, a TORISEL injection, 25 mg/mL was supplied with DILUENT for TORISEL, stored at 4°C , and diluted to an appropriate final concentration with culture media before use. The following primary antibodies were used: anti- β -actin (Sigma-Aldrich, Inc.) and anti-mTOR, a mouse serum polyclonal antibody that we raised against the C-terminal peptide of Cashmere goat mTOR kinase.

RNA extraction and cDNA synthesis

Total RNA was isolated using RNazol (RNAiso Plus; TaKaRa Co. Ltd.) from brain, heart, testis, liver, spleen, kidney, lung, and fetal fibroblasts of Inner Mongolia cashmere goat. RNA was reverse transcribed with an oligo (dT)₁₂₋₁₈ primer using the AMV 1st Strand cDNA Synthesis kit (Takara Co. Ltd.) as per the manufacturer's instructions. cDNA from various tissues was subjected to quantitative real-time PCR, and full-length mTOR was cloned using cDNA from fetal fibroblasts. One microgram of total RNA was used for each reaction.

Cloning and sequencing of mTOR

Due to the length of mTOR, the coding sequence of mTOR was divided into three fragments for amplification. The predicted fragment length of the 5' terminal fragment was 2218bp; it was amplified with the following primers: forward: 5' GAACCTCAGGGCAAGATGCTTGG 3', reverse: 5' TGAGCATCTTGCGCAGGAAAGG 3'. The 3' terminal and the middle sections were 2886bp and 2612bp, respectively, primers for 3' terminal fragment were as follows: forward: 5' TGGTTTCTTGCCACATGCTGTCC 3', reverse: 5' CCAGTT ACCAGAAAGGACACCAG 3'; and forward: 5' CCTTCC TGCAGCAAGATGCTCATC 3', reverse: 5' TCGGACAGCA TGTGGCAAGAAACC 3' were primers for the middle section. Primers were designed using the sequence of *homo sapiens* mTOR in GenBank and commercially synthesized. These fragments were amplified for 35 cycles with cDNA as the template at various annealing temperatures (60°C , 55°C ,

59.5°C). The PCR products were cloned into a plasmid and sequenced on an ABI PRISM 377XL DNA Sequencer (Applied Biosystems, Inc.).

3' rapid amplification of cDNA ends

RNA was reverse transcribed with a 3' rapid amplification of cDNA ends (3' RACE) 1st strand cDNA synthesis kit and a 3' RACE adaptor primer per the manufacturer (Takara Co. Ltd.). The 3' untranslated region (3' UTR) fragment was amplified with cDNA as the template for 35 cycles at the appropriate annealing temperature (50.5°C) with the following primers: forward: 5' CTGGATGTCCCAACA CAAGTC 3', reverse: 5' CTGATCTAGAGGTACCGGATCC 3'. 3' RACE generated a single fragment of 1070 bp.

Quantitative real-time PCR analysis of mTOR expression in various tissues

mTOR mRNA levels were measured by quantitative real-time PCR. mTOR cDNA was amplified with cDNA as the template by real-time PCR using the following primers: forward: 5' CCCATTCCACCAGTTTCCAGG 3', reverse: 5' CAGCGAGTTCTTGCTATTCC 3'; β -actin was amplified with forward: 5' CCACTGGCATTGTCATGGACTC 3', reverse: 5' TTCCTTGATGTACGGACGATTT 3'.

Quantitative real-time PCR were performed on a Bio-Rad Chromo 4 PCR System using SYBR[®] Premix Ex Taq[™] (Perfect Real Time) (TaKaRa Co. Ltd.). One microliter of cDNA was amplified in a 25- μL mixture containing 10 μM forward primer (0.5 μL), 10 μM reverse primer (0.5 μL), 2XSYBR[®] Premix Ex Taq[™] (12.5 μL), and nuclease-free water (10.5 μL). The program comprised an initial denaturation step at 95°C for 5 min; followed by 40 cycles at 95°C for 15 s, 54°C for 30 s, and 72°C for 30 s; 72°C for 10 min; and a final melting curve. Three technical replicas were run. Delta CT (ΔCT) values were calculated to determine tissue-specific expression. The PCR results were analyzed by one-way analysis of variance to compare expression between tissues.

Western blot analysis of expression of mTOR in Inner Mongolia Cashmere goat fetal fibroblasts

Cashmere GFbs were used to seed 6-well tissue culture plates at 3×10^5 cells per well and incubated. Subconfluent cells were harvested with trypsin and washed with cold phosphate buffered saline, and cells were lysed in a buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 100 g/L glycerol, 50 g/L Triton X-100, 2 g/L Na_2VO_4 , and 4 g/L EDTA; 10 μL PMSF (0.1 M) and 10 μL ALT (10 g/L) were added per 1 mL lysis buffer immediately before use. The cell lysates were put on ice for 15 min and centrifugated at 15,000 rpm at 4°C for 20 min, and the supernatant was transferred into a new tube. The concentrations of the lysates were measured by the Bio-Rad protein determination method (Bio-Rad Laboratories).

Equal amounts (40 μg) of protein were electrophoresed in 12% or 8% (w/v) sodium dodecylsulfate polyacrylamide gels. Proteins were transferred to Hybond-polyvinylidene difluoride membranes (Amersham) and incubated with the primary antibodies overnight at 4°C and horseradish peroxidase-conjugated sheep (polyclonal) anti-mouse IgG (GE Healthcare UK Limited) at room temperature for 1 h. Signals were detected by chemiluminescence (Amersham).

MTT assay and IC_{50} calculation

Exponentially growing cells were seeded in 96-well plates at 6×10^3 cells per well 24 h before drug treatment. Then, cells were incubated with CCI-779 at various concentrations (0.037 μ M, 0.11 μ M, 0.33 μ M, 1.0 μ M, 3.0 μ M, 9.0 μ M, 18.0 μ M, and 27.0 μ M) for 48 h. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (20 μ L) (MTT, 5g/L; Sigma-Aldrich, Inc.) was added to each well in fresh medium and incubated for 4 h at 37°C. The formazan product was dissolved in 100 μ L dimethyl sulfoxide per well and incubated for 10 min at 37°C. The MTT absorbance value was detected at 490/630 nm on a spectrophotometer set (Bio-TEK ELx800; Bio-tek instruments, Inc.). IC_{50} values were calculated using the Logit model.

Bioinformatics analysis of mTOR

Theoretical molecular weights of the deduced polypeptides and isoelectric points were predicted by calculating the isoelectric point (<http://isoelectric.ovh.org>). Based on the amino-acid sequence of mTOR, the active sites and domains were predicted using Psite (www.softberry.com) and InterProScan (www.ebi.ac.uk), respectively. Three-dimensional models of certain regions were generated using SWISS-MODEL Workspace (Guex and Peitsch, 1997; Schwede *et al.*, 2003; Arnold *et al.*, 2006) (<http://swissmodel.expasy.org>). In addition, the location of mTOR in cells was predicted using PSORT (<http://psort.nibb.ac.jp>).

Statistical analysis

Descriptive statistics were generated for all quantitative data, expressed as mean $\pm$ SD. Each assay was performed in triplicate.

Results

Cloning and sequence analysis of Cashmere goat mTOR

The mTOR cDNA (GenBank accession HM114224) from Inner Mongolia cashmere goat comprises an open reading frame (ORF) of 7650 bp and a 952-bp residue of the 3' UTR, which contains a polyadenylation signal at nucleotides 8575–8580 (AATAAA), 24 bp upstream of the polyA tract. The full cDNA sequence is 92% identical to homo sapiens mTOR and 92%, 92%, 91%, 89%, and 89% identical to that of *pan troglodytes*, *equus caballus*, *canis lupus familiaris*, *rattus norvegicus*, and *mus musculus*, respectively. The amino acid sequence of mTOR is 99% identical to that of *homo sapiens* and 98% to others.

Primary and secondary structure of Cashmere goat mTOR

The deduced primary structure of Inner Mongolia Cashmere goat mTOR consists of 2549 amino acids. There are 8 N-glycosylation sites, 5 cAMP- and cGMP-dependent protein kinase phosphorylation sites, 21 protein kinase C phosphorylation sites, 3 tyrosine kinase phosphorylation sites, 3 endoplasmic reticulum targeting sequences (Fig. 1A), an FAT domain (residues 1382–1982), an FKBP12 rapamycin-binding domain (FRB, residues 2019–2112), a protein kinase-like domain (residues 2086–2480), and an FATC domain (residues 2517–2549) (Fig. 1B).

The predicted molecular weight of unmodified mTOR is M_r 288.6 kDa, and the estimated isoelectric point (pI) is 7.24. With regard to basic residues, mTOR contains 5.1% lysine, 6% arginine, 3.26% histidine, and has 5.02% aspartate and 6.51% glutamate for acidic residues. mTOR was predicted to be an integral membrane protein using PSORT (<http://psort.nibb.ac.jp>). There are three dominating regions in goat mTOR, as calculated by the SWISS-MODEL workspace—a region from 370 to 1187, an FRB domain, and an FATC domain (Fig. 2).

Tissue distribution of the Cashmere goat mTOR mRNA

By quantitative real-time PCR, mTOR mRNA was found to be more abundant in brain, testis, kidney, and spleen compared with liver, heart, and lung tissue from Cashmere goat (Fig. 3A).

Expression of mTOR in Cashmere goat fetal fibroblasts

Total protein from Cashmere GFbs was extracted and assayed by western blot. mTOR was expressed in GFbs (Fig. 3B).

CCI-779 inhibits Cashmere goat fetal fibroblast proliferation

We measured the IC_{50} value of CCI-779 to examine its cytostatic effects on GFbs and optimize its concentration for further experiments. GFbs were treated with various concentrations of CCI-779 (0.037 μ M–27 μ M) for 48 h, and their susceptibility was measured by MTT assay. The IC_{50} of CCI-779 against GFbs was 10.88 μ M (Fig. 4), and their proliferation was inhibited.

Discussion

By understanding how signaling molecules regulate mTOR function, we can gain insights into mechanisms of cell growth, proliferation, and apoptosis. mTOR governs important functions in peripheral organs (Catania *et al.*, 2010)—its activation is sufficient to stimulate specific metabolic pathways, such as glycolysis, *de novo* lipid biosynthesis (Duvel *et al.*, 2010), cholesterol trafficking, and fasting-induced ketogenesis (Sengupta *et al.*, 2010; Xu *et al.*, 2010).

The aim of our study was to describe mTOR in Inner Mongolia Cashmere goat. The ORF of goat mTOR cDNA encodes the same number of amino acids as those of *rattus norvegicus*, *mus musculus*, and *homo sapiens* (2549), and its sequence is 99% identical to that of the human ortholog and 98% to others.

mTOR is an evolutionarily conserved, nutrient-sensing protein kinase that regulates growth and metabolism in all eukaryotic cells (Wullschlegel *et al.*, 2006). mTOR was predicted to be an integral membrane protein, and mTOR lies on the surface of lysosomes (Sancak *et al.*, 2010). There are three predicted endoplasmic reticulum-targeting sequences in mTOR, but it is unknown whether these sequences are related to the location of mTOR or which one takes effect. Nevertheless, mTOR is active in the cytoplasm and nucleus (Kantidakis *et al.*, 2010; Wei and Zheng, 2009), regulating transcription. However, how mTOR executes its various functions is unknown.

The 3' UTR of goat mTOR is much longer than those of five other cDNAs with intact 3' UTRs of goat genes in

FIG. 1. The predicted amino-acid sequence and domains of mTOR. (A) Alignment of amino-acid sequences of *mus musculus* (BC112904.1), *rattus norvegicus* (NM_019906.1), *homo sapiens* (NM_004958.2), and *equus caballus* (XM_001492351.1). Predicted N-glycosylation sites (Asn-Xaa-Ser/Thr) are boxed, and cAMP- and cGMP-dependent protein kinase phosphorylation sites (Arg/Lys-Xaa-Ser/Thr) are shaded gray. Protein kinase C phosphorylation sites (Ser/Thr-Xaa-Lys/Arg) are marked by double wavy lines. Tyrosine kinase phosphorylation sites are underlined, and endoplasmic reticulum-targeting sequences are marked by both pane and gray shading. All sites were determined using Psite (www.softberry.com). (B) Domains in mTOR, predicted with SMART Software (<http://smart.embl.de/>). mTOR, the mammalian target of rapamycin.

GenBank: *S6*, *GPx4*, *K26*, *LDH-A*, and *S6K1*. In transiently transfected Chinese hamster ovary cells with a firefly luciferase reporter, the length of the 3' UTR regulates the translational efficiency and stability of mRNA (Tanguay and Gallie, 1996). On average, genes that have more miRNA sites have longer 3' UTRs; proteins with 3' UTRs that have more target sites are predominantly active during development, whereas with fewer target sites in the 3' UTR execute basic functions (Stark *et al.*, 2005), suggesting that mTOR is an important regulator of cellular growth.

As an evolutionarily conserved protein kinase that belongs to the PIKK family, mTOR has a C-terminal protein kinase-like domain that is common to other members. Immediately upstream of the catalytic domain lies the FRB domain, to which FKBP12-rapamycin binds to mTOR to inhibit its activity. In addition, FAT is another ubiquitous domain of the PIKK family, located upstream of the FRB domain. The C-terminus contains a FATC domain that regulates the activity of mTOR (Peterson *et al.*, 2000; Takahashi *et al.*, 2000). In this study, we described the basic data about

FIG. 2. Three-dimensional model of mTOR regions. (A) The model of the N-terminal region, residues 370 to 1187. (B) FRB domain, residues 2019 to 2112. (C) FATC domain, residues 2519 to 2549. Color images available online at www.liebertonline.com/dna

FIG. 3. Quantitative real-time PCR analysis of *mTOR* mRNA expression in various tissues and Western blot analysis of *mTOR* expression in Cashmere goat fetal fibroblasts. **(A)** *mTOR* mRNA levels in brain, heart, testis, liver, spleen, kidney, and lung from Cashmere goat by quantitative real-time PCR. **(B)** *mTOR* expression in GFbs; β -actin is the positive control. PCR, polymerase chain reaction; GFbs, goat fetal fibroblasts.

goat *mTOR*, and the predicted domains of goat *mTOR* were as same as those in former research.

S6Ks are well-characterized downstream molecules that are regulated by *mTOR* (Chung *et al.*, 1992; Hay and Sonenberg, 2004). S6K is suppressed by the *mTOR* inhibitor RAD001 in SEG-1 esophageal cancer cells, (Wang *et al.*, 2010), and S6K regulates the lifespan (Blagosklonny and Hall, 2009). Akt is an upstream regulator of *mTOR*, and S6Ks negatively regulate insulin signaling through insulin receptor substrate 1, impairing PI3-kinase/Akt signaling downstream of the insulin receptor (Shah and Hunter, 2006).

The *mTOR* signaling pathway has been extensively investigated in humans and some model animals, and *mTOR* has recently been demonstrated to regulate protein synthesis in pigs and bovines (Burgos *et al.*, 2010; Murgas Torrazza *et al.*, 2010; Suryawan *et al.*, 2010; Toerien *et al.*, 2010). Lack of the fundamental information about genes and their expression as well as regulation has become a bottleneck for the molecular studies in these important livestock. Our study provides an original work about *mTOR* of Cashmere goat, and the results may be useful to the researchers of goat and sheep.

In this study, we examined the function of *mTOR* in GFbs, demonstrating that it is important for their growth. We infer that *mTOR* regulates the proliferation of GFbs in addition to

FIG. 4. Inhibition curve of CCI-779 on GFbs growth. GFbs were treated with various concentrations of CCI-779 (0.037 μ M to 27 μ M) for 48 h and subjected to MTT assay.

human esophageal cancer cells SEG-1, but further study is needed to generate more data on how the *mTOR* signaling pathway is regulated in Inner Mongolia Cashmere goat cells.

Conclusions

We cloned *mTOR* from Inner Mongolia Cashmere goat fetal fibroblasts, which includes an ORF of 7650 bp and a 3' UTR of 909 residues. *mTOR* is expressed at higher levels in testis, spleen, kidney, and brain compared with heart, liver, and lung. The treatment of CCI-779 decreases the viability of GFbs, and the IC_{50} of CCI-779 against GFbs was 10.88 μ M. *mTOR* plays a key role in growth regulation of Inner Mongolia Cashmere goat fetal fibroblasts.

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Disclosure Statement

No competing financial interests exist.

References

- Aoki, M., Blazek, E., and Vogt, P.K. (2001). A role of the kinase *mTOR* in cellular transformation induced by the oncoproteins P3k and Akt. *Proc Natl Acad Sci U S A* **98**, 136.
- Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. (2006). The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* **22**, 195.
- Blagosklonny, M.V., and Hall, M.N. (2009). Growth and aging: a common molecular mechanism. *Aging* **1**, 357.
- Burgos, S., Dai, M., and Cant, J. (2010). Nutrient availability and lactogenic hormones regulate mammary protein synthesis through the mammalian target of rapamycin signaling pathway. *J Dairy Sci* **93**, 153–161.
- Burnett, P.E., Barrow, R.K., Cohen, N.A., Snyder, S.H., and Sabatini, D.M. (1998). RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc Natl Acad Sci U S A* **95**, 1432.
- Catania, C., Binder, E., and Cota, D. (2010). *mTORC1* signaling in energy balance and metabolic disease. *Int J Obes* **35**, 751–761.
- Chakraborty, A., Koldobskiy, M.A., Bello, N.T., Maxwell, M., Potter, J.J., Juluri, K.R., Maag, D., Kim, S., Huang, A.S., and Dailey, M.J. (2010). Inositol Pyrophosphates Inhibit Akt Signaling, Thereby Regulating Insulin Sensitivity and Weight Gain. *Cell* **143**, 897–910.
- Chung, J., Kuo, C.J., Crabtree, G.R., and Blenis, J. (1992). Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell* **69**, 1227–1236.
- Duvel, K., Yecies, J.L., Menon, S., Raman, P., Lipovsky, A.I., Souza, A.L., Triantafellow, E., Ma, Q., Gorski, R., and Cleaver, S. (2010). Activation of a metabolic gene regulatory network downstream of *mTOR* complex 1. *Mol Cell* **39**, 171–183.
- Fingar, D.C., and Blenis, J. (2004). Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene* **23**, 3151–3171.
- Fraenkel, M., Ketzinel-Gilad, M., Ariav, Y., Pappo, O., Karaca, M., Castel, J., Berthault, M.F., Magnan, C., Cerasi, E., and

- Kaiser, N. (2008). mTOR inhibition by rapamycin prevents β -cell adaptation to hyperglycemia and exacerbates the metabolic state in type 2 diabetes. *Diabetes* **57**, 945.
- Gridelli, C., Maione, P., and Rossi, A. (2008). The potential role of mTOR inhibitors in non-small cell lung cancer. *Oncologist* **13**, 139.
- Guex, N., and Peitsch, M.C. (1997). SWISS-Model and the Swiss-Pdb Viewer: an environment for comparative protein modeling. *Electrophoresis* **18**, 2714–2723.
- Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. *Genes Dev* **18**, 1926.
- Kantidakis, T., Ramsbottom, B.A., Birch, J.L., Dowding, S.N., and White, R.J. (2010). mTOR associates with TFIIC, is found at tRNA and 5S rRNA genes, and targets their repressor Maf1. *Proc Natl Acad Sci* **107**, 11823.
- Kapahi, P., Chen, D., Rogers, A.N., Katewa, S.D., Li, P.W.L., Thomas, E.L., and Kockel, L. (2010). With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. *Cell Metab* **11**, 453–465.
- Li, N., Lee, B., Liu, R.J., Banasr, M., Dwyer, J.M., Iwata, M., Li, X.Y., Aghajanian, G., and Duman, R.S. (2010). mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science* **329**, 959.
- Murgas Torrazza, R., Suryawan, A., Gazzaneo, M.C., Orellana, R.A., Frank, J.W., Nguyen, H.V., Fiorotto, M.L., El-Kadi, S., and Davis, T.A. (2010). Leucine supplementation of a low-protein meal increases skeletal muscle and visceral tissue protein synthesis in neonatal pigs by stimulating mTOR-dependent translation initiation. *J Nutr* **140**, 2145.
- Neasta, J., Ben Hamida, S., Yowell, Q., Carnicella, S., and Ron, D. (2010). Role for mammalian target of rapamycin complex 1 signaling in neuroadaptations underlying alcohol-related disorders. *Proc Natl Acad Sci* **107**, 20093.
- Peterson, R.T., Beal, P.A., Comb, M.J., and Schreiber, S.L. (2000). FKBP12-rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions. *J Biol Chem* **275**, 7416.
- Powell, J.D., and Delgoffe, G.M. (2010). The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. *Immunity* **33**, 301–311.
- Proud, C.G. (2002). Regulation of mammalian translation factors by nutrients. *Eur J Biochem* **269**, 5338–5349.
- Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A.L., Nada, S., and Sabatini, D.M. (2010). Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell* **141**, 290–303.
- Schmelzle, T., and Hall, M.N. (2000). TOR, a central controller of cell growth. *Cell* **103**, 253–262.
- Schwede, T., Kopp, J., Guex, N., and Peitsch, M.C. (2003). SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Res* **31**, 3381.
- Selman, C., Tullet, J., Wieser, D., Irvine, E., Lingard, S.J., Choudhury, A.I., Claret, M., Al-Qassab, H., Carmignac, D., and Ramadan, F. (2009). Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. *Science* **326**, 140.
- Sengupta, S., Peterson, T.R., Laplante, M., Oh, S., and Sabatini, D.M. (2010). mTORC1 controls fasting-induced ketogenesis and its modulation by ageing. *Nature* **468**, 1100–1104.
- Shah, O.J., and Hunter, T. (2006). Turnover of the active fraction of IRS1 involves raptor-mTOR and S6K1-dependent serine phosphorylation in cell culture models of tuberous sclerosis. *Mol Cell Biol* **26**, 6425.
- Stark, A., Brennecke, J., Bushati, N., Russell, R.B., and Cohen, S.M. (2005). Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3'UTR evolution. *Cell* **123**, 1133–1146.
- Suryawan, A., Orellana, R., Fiorotto, M., and Davis, T. (2010). Leucine acts as a nutrient signal to stimulate protein synthesis in neonatal pigs. *J Anim Sci* **89**, 2004–2016.
- Takahashi, T., Hara, K., Inoue, H., Kawa, Y., Tokunaga, C., Hidayat, S., Yoshino, K., Kuroda, Y., and Yonezawa, K. (2000). Carboxyl-terminal region conserved among phosphoinositide-related kinases is indispensable for mTOR function *in vivo* and *in vitro*. *Genes Cells* **5**, 765–775.
- Tanguay, R.L., and Gallie, D.R. (1996). Translational efficiency is regulated by the length of the 3'untranslated region. *Mol Cell Biol* **16**, 146.
- Teachey, D.T., Obzut, D.A., Cooperman, J., Fang, J., Carroll, M., Choi, J.K., Houghton, P.J., Brown, V.I., and Grupp, S.A. (2006). The mTOR inhibitor CCI-779 induces apoptosis and inhibits growth in preclinical models of primary adult human ALL. *Blood* **107**, 1149.
- Toerien, C.A., Trout, D.R., and Cant, J.P. (2010). Nutritional stimulation of milk protein yield of cows is associated with changes in phosphorylation of mammary eukaryotic initiation factor 2 and ribosomal S6 kinase 1. *J Nutr* **140**, 285.
- Um, S.H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P.R., Kozma, S.C., and Auwerx, J. (2004). Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* **431**, 200–205.
- Wang, Z.H.I.G., Fukazawa, T., Nishikawa, T., Watanabe, N., Sakurama, K., Motoki, T., Hatakeyama, S., Omori, O., Ohara, T., and Tanabe, S. (2010). RAD001 offers a therapeutic intervention through inhibition of mTOR as a potential strategy for esophageal cancer. *Oncol Rep* **23**, 1167–1172.
- Wei, Y., and Zheng, X.F. (2009). TORC1 association with rDNA chromatin as a mechanism to co-regulate Pol I and Pol III. *Cell Cycle* **8**, 3802–3803.
- Wu, M.J., Chang, C.H., Chiu, Y.T., Wen, M.C., Shu, K.H., Li, J.R., Chiu, K.Y., and Chen, Y.T. (2010). Rictor-dependent AKT activation and inhibition of urothelial carcinoma by rapamycin. *Urol Oncol* [Epub ahead of print]; doi: 10.1016/j.urolonc.2009.11.009
- Wullschlegel, S., Loewith, R., and Hall, M.N. (2006). TOR signaling in growth and metabolism. *Cell* **124**, 471–484.
- Xu, J., Dang, Y., Ren, Y.R., and Liu, J.O. (2010). Cholesterol trafficking is required for mTOR activation in endothelial cells. *Proc Natl Acad Sci* **107**, 4764.
- Xu, X.Y., Zhang, Z., Su, W.H., Zhang, Y., Yu, Y.Q., Li, Y.X., Zong, Z.H., and Yu, B.Z. (2009). Characterization of p70 S6 kinase 1 in early development of mouse embryos. *Dev Dyn* **238**, 3025–3034.
- Zoncu, R., Efeyan, A., and Sabatini, D.M. (2010). mTOR: from growth signal integration to cancer, diabetes and ageing. *Nature Rev Mol Cell Biol* **12**, 21–35.

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