mTOR Signaling in Lymphangiomyomatosis

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Abstract

The protein mammalian target of rapamycin (mTOR) plays a central role in cell growth and proliferation. Excessive mTOR activity is a prominent feature of many neoplasms and hamartoma syndromes, including lymphangiomyomatosis (LAM), a destructive lung disease that causes progressive respiratory failure in women. Although pharmacological inhibitors of mTOR should directly target the pathogenesis of these disorders, their clinical efficacy has been suboptimal. Recent scientific findings reviewed here have greatly improved our understanding of mTOR signaling mechanisms, provided new insights into the control of cell growth and proliferation, and facilitated the development of new therapeutic approaches in LAM, as well as other neoplastic disorders that exhibit excessive mTOR activity.

Introduction

MAMMALIAN TARGET OF RAPAMYCIN (mTOR) is a highly-conserved serine-threonine kinase that plays a central role in the regulation of cell growth and proliferation. In all eukaryotic cells, TOR senses environmental stress or cellular metabolism, and then executes the appropriate anabolic or catabolic cellular responses. Conditions such as hypoxia, low ATP levels, amino acid withdrawal, or glucose deprivation inhibit mTOR activity. The resulting catabolic responses include cell cycle arrest, reduced protein synthesis, atrophy, autophagy, and attenuated mitochondrial respiration. In higher eukaryotes, mTOR can also integrate signals from growth factor-activated signaling pathways.¹ The prototypical signaling sequence begins with ligation of the insulin-like growth factor-1 (IGF-1) receptor, the recruitment of insulin receptor substrate-1 (IRS-1), and the activation of phosphatidylinositol-3 kinase (PI3K). Elevated phosphatidylinositol 3-phosphate (PI3P) levels leads to the repression of tuberin, the protein encoded by the tuberous sclerosis complex-2 (TSC2) gene. mTOR can then be activated by the small G-protein Ras homologue enriched in brain (Rheb), which is normally suppressed by TSC2. Therefore, loss of TSC2 or gain of PI3K activity leads to excessive Rheb and mTOR activity, which contribute to the pathogenesis of LAM,²⁻⁴ as well as many neoplastic tumors.⁵⁻⁷ Subsequent anabolic responses include the synthesis of oncogenic and angiogenic proteins, ribosomal biogenesis, cell growth, and cell proliferation. Here,

we discuss the mechanisms by which abnormalities in mTOR signaling might contribute to the pathogenesis of LAM, and other disorders of abnormal cell proliferation.

The Molecular Organization of mTOR

Initially reported in 1994,⁸⁻¹⁰ mTOR is a ~240-kDa protein composed of structural domains that permit multiple protein-protein interactions, and the assembly of macromolecular signaling complexes that control anabolic functions¹¹ (Fig. 1). A C-terminal PI3K-like domain contains the serine/threonine kinase active site. The heterogeneity of its target sequences suggests that the mTOR kinase domain directly phosphorylates certain effector substrates, but may control the phosphorylation of others via intermediary kinases or phosphatases.¹² Of the few known post-translational modification sites in mTOR, all involve serine/threonine phosphorylation.^{13,14} The auto-phosphorylation of serine 2481 (S2481) correlates with mTOR kinase activity, but not necessarily the ability to activate downstream effectors.¹⁵ Although it inhibits mTOR signaling, rapamycin does not necessarily reduce mTOR autokinase activity. Novel mTOR inhibitors are currently undergoing characterization.¹⁶⁻¹⁸ The phosphorylation of S2448 also correlates with mTOR kinase activity, as well as with mitogen or nutrient levels.^{19,20} Phosphorylation of S1261 in cells exposed to insulin is required for the activation of mTOR, its autophosphorylation at S2481, and its regulation of cell size.²¹

mTOR also contains amino acid residues that mediate its subcellular localization. mTOR nuclear trafficking requires two leucine residues in its HEAT repeat region (Fig. 1); constitutive nuclear localization of mTOR is observed in several tumor cell lines.^{22,23} A distinct set of residues in the HEAT repeat region determines mTOR localization to endoplasmic reticulum (ER) and/or Golgi membranes.²⁴ The ER may provide a physical platform for mTOR metabolic sensing functions, control of the ER stress response,²⁵ and coordination of mTOR signaling to the translational and transcriptional machinery.^{26,27} mTOR can also localize to mitochondrial membranes, although the molecular mechanism is unknown.²⁸ mTOR mitochondrial localization was associated with an effect on ATP production and oxygen consumption.²⁹ Furthermore, mTOR directly sensed changes in cell redox state, possibly via highly-conserved cysteine residues in its FATc domain.^{30,31}

The Core mTOR Complexes

From yeast to mammals, TOR nucleates two distinct macromolecular complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2)^{32,33} (Fig. 2). mTORC1 is defined by the presence of 'regulatory associated partner of mTOR' (raptor). Raptor physically links mTOR to its effectors (e.g., 4E-BP1, p70 S6 kinase (S6K)) by binding their conserved 'target of rapamycin signaling' (TOS) motifs.^{34–36} mTORC1 enhances protein synthesis and ribosomal biogenesis, whereas it suppresses catabolic responses such as autophagy and the induction of cell cycle inhibitor genes.

mTORC2 is defined by the presence of 'rapamycin insensitive companion of mTOR' (rictor), and regulates cell proliferation, polarity, and cytokinesis by phosphorylating the hydrophobic and/or turn motifs on a subset of 'cAMP-dependent protein kinase/protein kinase G/protein kinase C' (AGC kinases; e.g., protein kinase C α , Akt) kinases.³⁷ Other known mTORC2-specific proteins include mammalian SAPK-interacting protein 1 (mSIN1), and 'protein observed with rictor-1' (PROTOR1). Rictor and mSIN1 determine mTORC2 substrate specificity, and are required for the phosphorylation of Akt or PKC α .^{38–41} PROTOR1, also known as PRR5, appears to regulate platelet-derived growth factor (PDGF) signaling and cellular apoptosis.^{42–44} Both mTOR complexes contain mLST8 (originally termed G β L), which is

essential for the function of mTORC2, and modulates mTORC1 activity in nutrient-sensitive manner.⁴⁵

Regulated mTORC-Protein Interactions

The small GTPase Rheb is an essential binding partner and activator of mTOR. GTP-bound Rheb stimulates mTORC1 activity. The GDP-bound (inactive) state is favored by the C-terminal GAP activity of tuberin (TSC2), but does not appear to affect the interaction between Rheb and mTOR.^{46–49} TSC2 is therefore an endogenous suppressor of mTORC1. In contrast, TSC2 appears to positively regulate mTORC2 independent of its GAP activity or Rheb.^{50,51}

Additional mTOR binding partners act as 'signal input receivers' for the mTOR complexes. The mTORC1 protein 'proline-rich Akt/PKB Substrate 40' (PRAS40) contains a TOS (i.e., raptor-binding) motif, and appears to be a *bone fide* mTORC1 substrate.^{52,53} However, PRAS40 also constitutively binds and inhibits mTORC1, dissociating from the complex in cells exposed to insulin or nutrients.^{43,54,55} The integral ER membrane protein FKBP38 constitutively inhibits mTORC1 activity by blocking the interaction between mTOR and Rheb.^{56–58} FKBP38 dissociates from Rheb in cells exposed to mitogens, and thus facilitates the activation of mTOR. Phospholipase D (PLD), a mitogen-activated Rheb- and mTOR-binding protein, catalyzes the synthesis of phosphatidic acid, which directly activates mTOR via binding to the FRB domain.^{59–61} The rapamycin/FKBP12 complex might inhibit mTOR in part by competing for the phosphatidic acid-binding site in the FRB domain.⁶² Ras-related GTP binding (Rag) GTPases were also recently identified as mTOR-associated proteins.⁶³ Rag heterodimers targeted mTOR to cytoplasmic vesicular structures in response to amino acid repletion; their possible effects on mitogen-dependent mTOR signaling have not been defined.

mTOR interacts with, and regulates, the protein phosphatase 2A catalytic subunit (PP2Ac) via its associated adaptor subunit α 4. In yeast, TORC1 controls the nuclear localization of transcription factors by associating with, and likely phosphorylating, the α 4 homologue Tap42.^{64,65} α 4, or Tap42, can be negative^{64,66,67} or positive^{68–70} regulators of PP2Ac, which in turn can dephosphorylate multiple targets, including AGC kinases.⁷¹ Some propose that mitogen-stimulated phosphorylation of S6K at its T389 residue results from the inhibition of

FIG. 1. Structural domains and motifs in mTOR. mTOR consists of 2549 amino acids organized in several structural domains. The N-terminal portion of mTOR contains multiple HEAT (Huntington, Elongation factor 3, A subunit of type 2A protein phosphatase (PP2A), and TOR). The FAT (FRAP, Ataxia telangiectasia mutated (ATM), Transformation/transcription domain associated protein (TRRAP)) and FAT c-terminal (FATc) domains flank the kinase domain, are required for kinase activity, and appear to cooperatively bind certain mTOR-interacting proteins.³⁷ Highly conserved, and homologous to that in ATM and ATR, the c-terminal kinase domain (PI3_PI4_kinase) contains the serine/threonine kinase active site. The FKBP12-rapamycin-binding (FRB) domain binds the rapamycin/FKBP12 complex or phosphatidic acid.^{143,144}

FIG. 2. Composition of the mTOR core complexes. mTOR complexes integrate mitogenic and metabolic signals, and control anabolic or catabolic cellular functions as appropriate. mTORC1, as defined by the presence of raptor, is shown to the *left* of mTORC2, which is defined by its association with rictor. Other adapter proteins that coordinate the functions of mTOR complexes are also shown.

PP2Ac activity by mTOR.⁶⁶ Genetic studies indicate a role for $\alpha 4$ in certain functions attributed to mTORC2, such as cytokinesis and cell spreading.^{72,73}

Signaling Inputs to the mTOR Complexes

Mitogen-activated and metabolic sensing pathways converge upon the core mTOR signaling components at multiple levels (Fig. 3). The best studied is that involving PI3K, phosphatidylinositol-dependent kinase-1 (PDK1), and Akt. Ligation of growth factor receptors permits the recruitment and activation of IRS-1 and PI3K. Phosphorylated phosphatidylinositols (PI3Ps) bind PDK1, which then phosphorylates Akt in its activation loop (T308). Akt phosphorylates TSC2, suppresses its GAP activity, and relieves the inhibitory effect of TSC2 on Rheb and mTORC1 activity.^{74,75} Commonly mutated in neoplastic disorders, the protein phosphatase and tensin homologue (PTEN) is a PI3P phosphatase; its loss results in elevated PDK1, Akt, and mTOR activity. PDK1 can also directly phosphorylate the activation loop of other AGC kinases that undergo phosphorylation by mTOR at their hydrophobic motifs (e.g., PKC α , PKC δ , S6K).^{38,39,76–78} Therefore, the PI3K/PDK1 pathway can activate AGC kinases independently of TSC2 or Rheb. The concurrent phosphorylation of AGC kinases by PDK1 and mTOR may be required for their maximal activation or stability.³⁷ In agreement, the ability of rapamycin to block tumor progression or apoptosis in mice varied according to PI3P levels or Akt activity, suggesting that the net cellular growth and proliferation of neoplastic cells depends on the relative activities of the PI3K and mTOR signaling pathways.^{79–82}

Whereas Akt enhances mTORC1 activity, its S473 residue is also a substrate for mTORC2.⁸³ The biological significance of Akt as both a substrate of mTORC2 and a kinase for mTOR signaling intermediates remains unclear. Akt appears to act as a signal transducer of growth factor signaling in the control of mTORC1 and cell growth, while simultaneously mediating mTORC2 suppression of apoptosis and enhancement of pro-

liferation. Future studies investigating the intracellular regulators of mTORC2, as well as the subcellular localization and phosphorylation kinetics of the respective mTOR complexes may shed light on their relative contributions to the balance between cell proliferation and apoptosis.

mTORC1 simultaneously controls a negative feedback mechanism that dampens growth factor signaling. The mTORC1 effector S6K phosphorylates and inhibits IRS-1, thereby limiting the activation of mTOR in TSC2-deficient tumors^{84,85} (Fig. 3). This may in part explain the relatively benign nature of TSC and LAM hamartomas, as well as the limited clinical efficacy of rapamycin.⁸⁶ That is, by inhibiting mTORC1, rapamycin relieves the S6K-dependent inhibition of IRS-1/PI3K/Akt signaling, and limits its therapeutic effect on tumor growth.

In addition to the PI3K pathway, receptor tyrosine kinases activate Ras, an oncogenic protein that promotes the transformation and growth of neoplasms. Ras activates intermediates, including p90 ribosomal S6 kinase (Rsk), the mitogen-activated protein kinase Erk, and phospholipase D, all of which can modulate mTOR signaling (Fig. 3). TSC2 and raptor contain Erk- or Rsk-phosphorylated residues.^{87–90} Like its negative feedback effect on the PI3K/Akt pathway, S6K also attenuated Erk activity, and Erk was 'de-repressed' in cells exposed to rapamycin.⁹¹

Other inputs to the TSC2/Rheb/mTOR signaling axis include metabolic sensing pathways, which are commonly dysregulated in cancer or hamartoma syndromes.^{6,92} The energy (ATP)-sensing protein AMP-dependent protein kinase (AMPK) can control TSC2 or raptor via direct phosphorylation.^{93–95} Its upstream kinase LKB1 is lost or mutated in a variety of cancers.⁵ Phosphorylation of TSC2 by AMPK permits its phosphorylation by GSK3, an intermediate in the Wnt signaling pathway.^{94,96} Thus, Wnt, an important controller of differentiation and proliferation, can activate mTORC1, cell growth, and tumor progression independent of its known effect on the β -catenin-mediated Wnt transcriptional program.⁹⁷

FIG. 3. Coordination of mTORC1 and mTORC2 signaling activities. mTOR signaling can be organized into concentric spheres consisting from center to periphery of the mTORCs (*center*), their regulatory interaction partners, proteins that indirectly modify mTORC activity or mediate mTORC functions, and the mTORC-related physiological stimuli or functions. Proteins that modify mTORC activity are *above* the horizontal line, whereas those which mediate mTORC functions are *below*. Although multiple feedback and crosstalk mechanisms occur, the directionality of mTOR signaling is depicted in the *center*, with the mTOR complexes acting as signal integrators. Color images available online at www.liebertonline.com/ltb.

mTORC Signaling Targets in LAM and Neoplasia

Translation and cell growth

Excessive cell growth and proliferation require a robust protein synthesis machinery.^{98,99} In response to mitogens, S6K and 4E-BP1 associate with mTORC1 via their respective TOS motifs. Phosphorylation of 4E-BP1 at multiple sites inhibits its interaction with the mRNA CAP-binding protein eIF4E, and permits the initiation of translation.¹⁰⁰ This mechanism recognizes a subset of mRNAs, many of which are involved in cell growth, proliferation, and angiogenesis (e.g., ribosomal proteins, cyclin D1, HIF-1 α). eIF4E was sufficient to transform cells,¹⁰¹ and overexpression of 4E-BP1 partially reversed v-src-induced transformation.¹⁰² S6K phosphorylates components of the translational machinery, and promotes ribosomal biogenesis via the transcriptional induction of ribosomal RNA and genes encoding ribosomal proteins.

Apart from S6K and Akt, mTORCs can associate with and phosphorylate two other AGC kinases that modify cell proliferation and apoptosis: serum/glucocorticoid kinase-1 (SGK1) and protein kinase C δ .^{78,103,104} Genetic or biochemical studies in *C. elegans* and mammalian cell lines indicate phosphorylation and regulation of SGK by TORC2 and not mTORC1.^{105–107} In a cell line overexpressing mTOR, however, SGK was phosphorylated by mTORC1, and subsequently blocked cell cycle arrest.¹⁰⁸ In contrast to the mTORC2 substrate PKC α , PKC δ is phosphorylated by mTORC1, phosphorylates 4E-BP1, and controls the initiation of translation.¹⁰⁴ Interestingly, PKC δ is a kinase for the transcription factor STAT1 kinase, and was detected in a rapamycin-sensitive macromolecular complex with mTOR and STAT1.^{109–111}

Gene transcription

Neoplasms exhibit transcriptomic programs that determine oncogenic potential and pathogenesis.^{112,113} Emerging data indicate that mammalian or yeast TOR can localize to the nucleus, bind DNA regulatory elements, and modify the transcription of nutrient and stress response genes.^{114–116} mTOR can physically associate with transcription factors that control cell proliferation, apoptosis, ribosomal biogenesis, and angiogenesis. In response to growth factors, mTORC1 can associate with and phosphorylate STAT3, an oncogenic transcription factor that is increased in LAM, and a variety of cancers.^{117–119} Conversely, inactivation of mTOR or its associated phosphatase complex, promoted its association with STAT1, enhanced STAT1 nuclear content, and amplified the transcription of STAT1-dependent anti-proliferative and pro-apoptotic genes.¹⁰⁹ The balance between STAT1 and STAT3 is dysregulated in LAM, and is thought to control the fate of neoplastic cells.¹²⁰ mTOR also activates NF- κ B, a pro-proliferative transcription factor implicated in the pathogenesis of cancer.¹²¹ Others demonstrated a physical association between mTOR and IKK α , the IKK kinase required for NF- κ B translocation to the nucleus.¹²² Loss of TSC2 inhibited NF- κ B-dependent responses to apoptosis inducing agents.¹²³

The metabolic switch from oxidative phosphorylation to glycolysis is a prominent feature of tumor biology.⁹² mTOR physically bound the promoters of mitochondrial biogenesis genes, and acted as a transcriptional co-activator via physical interactions with the transcription factors PGC-1 α and YY1.¹²⁴

mTORC1 also interacted with hypoxia inducible factor-1 α (HIF-1 α), a transcription factor that, in cells exposed to hypoxia, controls a gene transcription program of angiogenesis, proliferation, and metabolic switch to anaerobic metabolism.¹²⁵ A TOS motif in HIF-1 α mediated its interaction with raptor and enhanced transcriptional activity.¹²⁶ mTORC1 also promoted the synthesis of HIF-1 α protein.¹²⁷ Moreover, in a negative feedback mechanism, the hypoxia-induced protein 'regulated in development and DNA damage responses-1' (REDD1) bound TSC2, thereby suppressing mTOR activity.¹²⁸ The loss of REDD1 or TSC2 likely prevents hypoxic inhibition of cell growth and proliferation, an important tumor survival mechanism. Neoplastic disorders with elevated PI3K and mTOR activity often exhibit elevated HIF-1 α levels, dysregulated angiogenesis, and therapeutic sensitivity to mTOR inhibitors.

Other emerging mTOR targets in LAM

Recent studies revealed an important role for autophagy in oncogenesis and tumor survival.¹²⁹ mTORC1 interacts with, phosphorylates, and inhibits the activity of Ulk1, a protein that initiates autophagy.^{130,131} Whereas mTOR inhibitors block cell growth and proliferation, they may limit their own efficacy by promoting autophagy and reducing apoptosis in established tumors.

TSC2-deficient cells clearly exhibit abnormalities in cytoskeletal function consistent with their ability to invade and metastasize.^{132,133} mTORC2 directly interacted with and regulated Prex1, a Rac1 guanine nucleotide exchange factor that controls cytoskeletal rearrangement.¹³⁴

Although this discussion has been restricted to direct mTOR signaling interactions (Fig. 3), certain indirect effects of mTOR on biological processes relevant to LAM are worthy of mention. TSC2-deficient cells exhibit an activated ER stress responses, consistent with their requirement for protein synthesis and cell growth.²⁵ The ER stress-activated transcription factor ATF6 rendered dormant cancer cells resistant to rapamycin-induced apoptosis and tumor regression.¹³⁵ ER stress-activated proteins (e.g., PERK, DAPK, eIF2 α , ATF6) may represent potential targets for the sensitization of LAM and neoplastic cells to apoptotic stimuli.

Consistent with the restriction of LAM to women, estrogen promotes the proliferation and metastasis of TSC2-deficient tumors *in vivo*, or TSC2-deficient cells derived from renal angiomyolipomas, in mTOR-dependent fashion.^{136–139} Separate studies suggested that prolactin stimulates mTOR, and modifies STAT1 activity in mTOR-dependent fashion.^{140,141} Further studies are required to better understand how mTORCs interact with androgen or estrogen signaling pathways.

Although the cell of origin in LAM is unknown, the expression of both neuroendocrine and smooth muscle cell lineage markers in LAM cells suggests abnormalities in cellular differentiation. In TSC1-deficient mice, mTOR controlled hematopoietic stem cell quiescence, renewal, and lineage development. Further studies investigating the role of mTOR in the biology of LAM or cancer stem cells will permit a better understanding on the origins of LAM.

Conclusions

Since the identification of the core mitogen-activated mTOR pathway (i.e., PI3K/Akt/TSC2/mTOR), a large

number of additional mTOR-interacting proteins and signaling pathways have been identified. It has become clear that neoplastic cells express oncogenes, or lack tumor suppressors, that can render them resistant to the antiproliferative effects of rapamycin. Moreover, the normal feedback mechanisms that limit cell proliferation can be blocked by rapamycin. Finally, TSC2 and Rheb participate in signaling mechanisms other than the mTOR pathway that contribute to the cytoskeletal,¹³² hormonal,¹³⁷ and proliferative¹⁴² characteristics of neoplastic cells. Further characterization of the mTOR complexes and their signal integration functions will lead to therapeutic approaches for LAM and neoplasia that simultaneously target multiple receptors and signaling intermediates.

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Disclosures

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Abbreviations

AGC = cAMP-dependent protein kinase/
 protein kinase G/protein kinase C
 ER = endoplasmic reticulum
 HEAT = Huntington, Elongation factor 3, A subunit of
 type 2A protein phosphatase (PP2A), and TOR
 LAM = lymphangioleiomyomatosis
 mTOR = mammalian target of rapamycin
 mTORC = mTOR complex
 PI3K = phosphatidylinositol-3 kinase
 PI3P = phosphatidylinositol-3 phosphate
 PKC = protein kinase C
 PP2A = protein phosphatase 2A
 Rheb = Ras homologue enriched in brain
 S6K = p70 S6 kinase
 TOS = target of rapamycin signaling
 TSC = tuberous sclerosis complex
 PDK1 = phosphatidylinositol-dependent kinase-1