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(Article begins on next page)
MicroRNA-21 is Induced by Rapamycin in a Model of Tuberous Sclerosis (TSC) and Lymphangioleiomyomatosis (LAM)

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Abstract

Lymphangioleiomyomatosis (LAM), a multisystem disease of women, is manifest by the proliferation of smooth muscle-like cells in the lung resulting in cystic lung destruction. Women with LAM can also develop renal angiomyolipomas. LAM is caused by mutations in the tuberous sclerosis complex genes (TSC1 or TSC2), resulting in hyperactive mammalian Target of Rapamycin (mTOR) signaling. The mTOR inhibitor, Rapamycin, stabilizes lung function in LAM and decreases the volume of renal angiomyolipomas, but lung function declines and angiomyolipomas regrow when treatment is discontinued, suggesting that factors induced by mTORC1 inhibition may promote the survival of TSC2-deficient cells. Whether microRNA (miRNA, miR) signaling is involved in the response of LAM to mTORC1 inhibition is unknown. We identified Rapamycin-dependent miRNA in LAM patient angiomyolipoma-derived cells using two separate screens. First, we assayed 132 miRNA of known significance to tumor biology. Using a cut-off of \textgreater 1.5-fold change, 48 microRNA were Rapamycin-induced, while 4 miRs were downregulated. In a second screen encompassing 946 miRNA, 18 miRs were upregulated by Rapamycin, while eight were downregulated. Dysregulation of miRs 29b, 21, 24, 221, 106a and 199a were common to both platforms and were classified as candidate “RapamirRs.” Validation by qRT-PCR confirmed that these microRNA were increased. miR-21, a survival miR, was the most significantly increased by mTOR-inhibition (p<0.01). The regulation of miR-21 by Rapamycin is cell type independent. mTOR inhibition promotes the processing of the miR-21 transcript (pri-miR-21) to a premature form (pre-miR-21). In conclusion, our findings demonstrate that Rapamycin upregulates multiple miRs, including pro-survival miRs, in TSC2-deficient patient-derived cells. The induction of miRs may contribute to the response of LAM and TSC patients to Rapamycin therapy.

Introduction

Lymphangioleiomyomatosis (LAM) is a devastating multisystem disease that almost exclusively affects women and can result in end-stage lung disease. LAM is characterized by the diffuse proliferation of smooth muscle-like cells (LAM cells) that express melanocyte lineage proteins. In the lungs, LAM cells can lead to small airway obstruction, blockage of lymphatic vessels leading to the formation of chyloous pleural effusions, and cystic parenchymal destruction which is believed to be due to the release of matrix metalloproteinases and other catabolic enzymes [1,2]. LAM occurs in two forms: in association with germline mutations in the tuberous sclerosis complex (TSC) genes, and in women who do not have tuberous sclerosis (sporadic LAM). The majority of women with TSC-LAM and about 40% of women with sporadic LAM have renal angiomyolipomas, which are benign tumors consisting of smooth muscle, fat and dysplastic vasculature. Angiomyolipoma cells have many similarities to LAM cells and may arise from a common progenitor cell [3]. LAM cells from women with sporadic LAM can carry somatic mutations in the TSC2 gene, which is a tumor suppressor gene that regulates the mammalian Target of Rapamycin (mTOR) [4,5,6,7,8].

mTOR is a kinase that integrates cellular and environmental cues, including growth factor activity and glucose levels, to regulate cell growth and proliferation. mTOR exists in two distinct complexes: mTOR complex 1 (mTORC1), which includes Raptor, and mTOR complex 2, which includes Rictor [9,10]. Recent clinical trials of allosteric mTORC1-inhibitors such as Sirolimus (Rapamycin) in women with LAM have been promising in that they confer a partial reduction in angiomyolipoma volume and stabilization of lung function [11,12]. However, upon cessation of therapy lung function decline resumes and angiomyolipomas regrow, suggesting that allosteric mTORC1 inhibitors exert a cytostatic but not cytotoxic effect.

MicroRNA (miRNA or miRs) are small RNA molecules that regulate gene expression, primarily by affecting transcript stability. Many miRNA have their own promoter regions and undergo transcription in a tightly regulated manner. Mature miRNA form
miRs. The expression of hundreds of mRNA in a cell-type dependent fashion toward a previously unknown miR-regulated signaling network associated with activation of mTORC1, including the majority of miRNAs dysregulated with Rapamycin therapy for TSC and LAM patients. Rapamycin induces miR-21 expression in multiple TSC2-deficient cell types and miR-21 expression may be TSC2-independent.

To identify whether miR-21 is regulated by Rapamycin and/or TSC2 in other cell lines, tuberin (TSC2) was stably downregulated in C3H-10T1/2 mouse pre-pericyte fibroblasts with a lentiviral shRNA vector (Figure 4A). Cells were cultured in DMEM containing 10% FBS and treated with Rapamycin (20 nM) or vehicle control for 24 hours. Rapamycin increased miR-21 levels approximately 2-fold in both tuberin-deficient and control shRNA cells (Figure 4B), but unexpectedly no decrease in miR-21 was observed in the cells with tuberin downregulation despite the increased phosphorylation of ribosomal protein S6, a downstream target of mTORC1. Next, we utilized Tsc2-null mouse embryonic fibroblasts and EL3 T cells (Tsc2-null cells from an Eker rat uterine leiomyoma), which are established cellular models of TSC [26,27,28] compared to HEK293 and A549 cells, which express TSC2. Cells were cultured in DMEM containing 10% FBS and treated with Rapamycin (20 nM, 24 h) with or without vehicle control. The human cells were normalized to RNU44, murine cells to snoRNA4202, and rat cells to U87. miR-21 was induced >1.5-fold (p<0.05, n = 3) by Rapamycin in each of these cell lines (Figure 4C). These results further confirm that miR-21 is induced by Rapamycin in TSC2-deficient cell lines.
by Rapamycin in different cell lineages and species and suggest that the regulation of miR-21 may be TSC2-independent.

**Rapamycin regulates miR-21 levels independently of AKT signaling**

TSC2-null cells display feedback inhibition to the PI3K/AKT signaling pathway via hyperactivation of mTORC1 [29,30]. When TSC2-null cells are treated with Rapamycin this feedback inhibition is released and AKT is phosphorylated and activated. To determine whether Rapamycin regulates miR-21 expression via an AKT-dependent mechanism, we treated 621-101 cells with Rapamycin and the AKT inhibitor MK2206 (Selleckchem, Catalog No. S1078) (Figure 5). In Figure 5A (lanes 1–2), western blot analysis was performed to confirm that p-AKT levels are

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### Table 1. Rapamycin-regulated miRNA in LAM patient-derived cells identified by the Signosis Array (Fold Change >1.5).

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold Change (Rapa 24 h/ DMSO)</th>
<th>miRNA</th>
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### Table 2. Rapamycin-regulated miRNA in LAM patient-derived cells identified by the Exiqon Array (Fold Change >1.5, normalized to RNU44).

<table>
<thead>
<tr>
<th>microRNA</th>
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<th>microRNA</th>
<th>Expression Change (Rapamycin/DMSO)</th>
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<td>miR-513a-5p</td>
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Rapamycin potentiates the post-transcriptional processing of pri-miR-21

MicroRNA biogenesis is a tightly regulated process, with key enzyme complexes participating in transcription and processing at three major junctions (Figure 6A). Post-transcriptional processing is a key mechanism to controlling miRNA levels [31]. The regulation of miR-21, in particular, has been well-characterized, with Davis and colleagues demonstrating that TGF-beta and Smad signaling induces a Drosha-mediated post-transcriptional processing of pri-miR-21 to induce miR-21 expression [32]. To determine whether Rapamycin influences the processing of pri-miR-21, we treated 621-101 cells, cultured in DMEM containing 10% FBS, with Rapamycin (20 nM) or DMSO for 24 hours and assayed the expression of pri-miR-21, pre-miR-21 and miR-21 using qRT-PCR. Rapamycin significantly induced pre-miR-21 and miR-21 at 24-hours, but did not affect expression of pri-miR-21 (Figure 6B). This result suggests that Rapamycin potentiates the DROSHA-mediated processing of pri-miR-21.

Discussion

The landmark Multicenter International LAM Efficacy of Sirolimus (MILES) Trial, a randomized, placebo controlled trial in 89 women with sporadic or TSC-associated LAM, demonstrated that Rapamycin (an allosteric mTOR inhibitor) stabilized lung function during one year of therapy; discontinuation of therapy resulted in a rate of lung function decline similar to untreated patients [12]. These findings are consistent with results from an earlier phase I/II trial of patients with angiomyolipomas, some of who also had sporadic or TSC-associated LAM, in which Rapamycin promoted a decrease in the volume of renal angiomyolipomas by almost 50%, with regrowth to approximately
the original size after treatment was stopped [11]. These studies suggest that temporarily slows disease progression, but does not eradicate the TSC2-deficient LAM and angiomyolipoma cells. Therapeutic targeting of factors that promote the survival of LAM cells during Rapamycin therapy could lead to more robust and/or durable responses.

While miRNA have been studied in many human diseases, whether miRNA contribute to the therapeutic response to

Figure 2. Exiqon miRNA microarray confirms 8 Rapamycin-dependent miRNA. 621-101 cells were treated with Rapamycin 20 nM or DMSO for 24 hours. Total RNA was isolated and applied to the Exiqon platform, which assays 946 human miRNA. A) Heat map of miRNA dysregulated by Rapamycin >1.5-fold, log2 scale. RNA from three biologic replicates per condition was pooled; each miRNA was assayed in quadruplet on the array. B) miRNA dysregulated by Rapamycin >1.5-fold (normalized to RNU44). Highlighted miRNA (except miR-31 and 210) are common to both the Exiqon and Signosis platforms. miR-21 is circled.

doi:10.1371/journal.pone.0060014.g002
Rapamycin in LAM and TSC is unknown. To address this, we performed two screens to determine whether Rapamycin impacts miRNA levels in LAM patient angiomyolipoma-derived cells. The first screen assayed 132 miRNA of known importance to cancer signaling pathways, while the second was a more comprehensive screen that assayed all 946 known miRNA listed in miRBase Version 15.0. The screens revealed a complex and previously unrecognized network of Rapamycin-regulated miRNA, which we termed “RapamiRs”. Using qRT-PCR to confirm our results, we demonstrate that miR-21 is significantly induced by Rapamycin, whereas miR-210 is repressed. We further analyzed Rapamycin’s effect on miR-21, demonstrating that miR-21 is induced in several different TSC2-deficient cell lines. Utilizing mouse pre-pericyte fibroblasts with stable downregulation of tuberin, we confirmed that the dysregulated expression of miR-21 is mTOR-dependent, but tuberin independent. Finally, we revealed that the induction of miR-21 expression by Rapamycin is mediated by post-transcriptional processing of the primary miR-21 transcript, as opposed to increased transcription. These studies reveal a novel regulatory network that is upregulated by Rapamycin.

We hypothesize that the upregulation of miR-21 by Rapamycin in LAM patient-derived cells impacts the therapeutic response to Rapamycin. First, miR-21 is a known “oncomiR,” inhibiting multiple tumor suppressor genes, including phosphatase and tensin homolog (PTEN), programmed cell death 4 (PDCD4) and sprouty 2 (SPRY2) to promote growth, differentiation and proliferation [33,34,35,36,37]. These pro-survival effects of miR-21 may partially explain why there is a resumption of disease upon treatment discontinuation in LAM patients treated with Rapamycin. Interestingly SPRY2 has previously been shown to regulate mTORC1 signal transduction and vascularization of the lung [38]. Second, miR-21 is a known regulator of smooth muscle morphology, promoting a de-differentiated state marked by growth and migration, which is essential for angiogenesis [39,40]. Krymskaya and colleagues have previously demonstrated a role for regulators of smooth muscle function in the pathogenesis of LAM, identifying that RhoA is activated in TSC2-deficient cells [41,42]. Smooth muscle-like LAM cells can be identified in vivo in two different morphologic states, a highly proliferative “spindle shape” and a more static “epithelioid” state [43]; the pathways that regulate the phenotypic switch of LAM cells is unknown.

![Figure 3. qRT-PCR confirmation of Rapamycin-dependent miRNA in TSC2-deficient cells.](image-url)

TSC2−/− cells were treated with Rapamycin 20 nM or DMSO for 24 hr and miRNA expression was assessed by qRT-PCR. A) miRNA expression is similar in 621-101 cells using RNU44 (left panel) or RNU48 (right panel) for normalization. B) miRNA expression in 621-101 cells normalized to RNU44. Highlighted results are significant using a Bonferroni correction.
doi:10.1371/journal.pone.0060014.g003
Third, it is possible that miR-21 induces a pro-inflammatory state that promotes the survival and metastasis of TSC2-deficient LAM cells. miR-21 is regulated by inflammatory mediators and in turn acts to promote inflammation. For example, interleukin-6 is a potent modulator of miR-21, acting via STAT3 signaling [44]. Additionally, mTORC1 has been shown to activate STAT3 signaling in mice and humans making this an intriguing link [45,46]. Moreover, miR-21 inhibits PTEN and PDCD4 to repress NF-κB signaling and IL-10, an anti-inflammatory interleukin [47].

A number of groups have identified a link between innate immunity pathways and TSC-mTOR signaling. Weichhart and colleagues have shown that TSC2-deficient cells exhibit an anti-inflammatory state via the induction of IL-10, whereas Rapamycin treatment promotes a pro-inflammatory condition by suppressing NF-κB signaling and IL-10, an anti-inflammatory interleukin [47].

![Figure 4. miR-21 is mTOR-dependent and may be TSC2-independent. A) Stable downregulation of tuberin in C3H-10T1/2 pre-pericytes results in increased phosphorylation of ribosomal protein S6, as expected. Treatment with Rapamycin (20 nM, 24 h) inhibits phosphorylation of S6. B) Downregulation of TSC2 in C3H-10T1/2 cells does not affect miR-21 expression. Inhibition of mTORC1 with Rapamycin induces ~2-fold increase in miR-21 expression in both control shRNA and TSC2 shRNA cells. Bars represent the mean of two biologic replicates +/- SD. * p<0.05. C) LAM patient-derived cells (621-101), TSC2-null rat uterine leiomyoma-derived cells (ELT3), TSC2-null mouse embryonic fibroblasts (MEFs), HEK293 and lung adenocarcinoma (A549) cells were treated with Rapamycin 20 nM vs Control for 24 h. Relative MiR-21 expression was determined by qRT-PCR. Human cells were normalized to RNU44, mouse cells to snora202 and rat cells to U87, which are all small nucleolar RNA molecules. For all charts, bars represent the mean of three biologic replicates +/- standard error. * p<0.05. ** p<0.01. doi:10.1371/journal.pone.0060014.g004]
IL-10 and inducing TNF-alpha, IL-6, and IL-12p40 [48]. Moss and colleagues have also contributed to the recognition of inflammatory processes underlying the pathogenesis of LAM, demonstrating that chemokines and chemokine receptors, especially CCL-2/MCP-1, are dysregulated in LAM cells and in bronchoalveolar lavage fluid obtained from LAM patients [49].

Therefore, the induction of miR-21 by Rapamycin may induce a proliferative smooth muscle morphology and contribute to a pro-inflammatory milieu.

Our identification of Rapamycin-induced miRNA reveals a novel and complex signaling network downstream of mTOR with potential therapeutic implications for women with LAM and...
patients with TSC receiving Rapamycin therapy. For example, if miR-21 induction by Rapamycin proves to be a strong pro-survival stimulus in TSC2-deficient cells, then suppression of miR-21 in conjunction with Rapamycin could represent an effective therapeutic strategy for TSC and LAM. Manipulation of miRNA expression is currently being studied as a therapy for malignancies and cardiomyopathies, with promising results in multiple pre-clinical models of disease [50,51]; ‘antagomiRs’ that...
suppress miRNA expression are now being tested in clinical trials. Rapamycin may also be useful as serum biomarkers of response to Rapamycin. Finally, our findings may be relevant to other diseases in which Rapamycin and its analogs are currently being used therapeutically, including cancer, for which there are currently more than 100 active cancer clinical trials using mTOR inhibitors.

Methods

Cell culture and Tsc2-downregulation

621-101 (The Rothberg Institute), Tsc2-null mouse embryonic fibroblasts (provided by Dr. David Kwiatkowski, HEK293, C3H10T1/2 cells (American Type Culture Collection) and ELT3 cells (provided by Dr. Cheryl Walker) were maintained in DMEM supplemented with 10% FBS (Sigma), Penicillin (50 units/mL) and Streptomycin (50 mg/mL); media for ELT3 cells also contained G418 (0.5 mg/mL). Lentiviral particles expressing shTSC2 were generated by transfecting HEK293T cells with plasmid DNA expressing shTSC2 (or empty vector) and lentiviral packaging particles (VSVG, PLP1, PLP2). Supernatant containing virions was applied to C3H10T1/2 cells (passage 7) for infection. C3H10T1/2 with lentiviral transfection were maintained in DMEM supplemented with 10% FBS (Sigma) with Puromycin (for selection).

Immunoblot analyses

Lysates were mixed with Laemmli Sample buffer and boiled for 10 minutes. 30 μg of sample were resolved in a 4–12% acrylamide gel and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blotted overnight with rabbit anti-phospho-S235/236 S6 ribosomal protein (Cell Signal-Hercules, CA). The membranes were blotted overnight with rabbit anti-phospho-S235/236 S6 ribosomal protein (Cell Signal-Hercules, CA). The membranes were blotted overnight with rabbit anti-phospho-S235/236 S6 ribosomal protein (Cell Signal-Hercules, CA). The membranes were blotted overnight with rabbit anti-phospho-S235/236 S6 ribosomal protein (Cell Signal-Hercules, CA).

MicroRNA Screens

621-101 cells were grown in DMEM supplemented with 10% FBS until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO). Cells were washed twice with phosphate buffered saline (PBS) until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO). Cells were washed twice with phosphate buffered saline (PBS) until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO). Cells were washed twice with phosphate buffered saline (PBS) until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO). Cells were washed twice with phosphate buffered saline (PBS) until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO). Cells were washed twice with phosphate buffered saline (PBS) until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO). Cells were washed twice with phosphate buffered saline (PBS) until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO). Cells were washed twice with phosphate buffered saline (PBS) until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO). Cells were washed twice with phosphate buffered saline (PBS) until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO). Cells were washed twice with phosphate buffered saline (PBS) until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO). Cells were washed twice with phosphate buffered saline (PBS) until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO). Cells were washed twice with phosphate buffered saline (PBS) until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO). Cells were washed twice with phosphate buffered saline (PBS) until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO). Cells were washed twice with phosphate buffered saline (PBS) until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO). Cells were washed twice with phosphate buffered saline (PBS) until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO). Cells were washed twice with phosphate buffered saline (PBS) until 60% confluent. Cells were then treated for 24 hours with Rapamycin (20 nM) or equal volume Dimethylsulfoxide (DMSO).

Acknowledgments

We are grateful to Augustine Choi, Mark Perrella, David Kwiatkowski and Stephen Chan for their critical comments. We thank David Kwiatkowski for providing TSC2-null MEFs and Cheryl Walker for providing ELT3 cells.

Author Contributions

Conceived and designed the experiments: AJT DAM NAN CP EPH. Performed the experiments: AJT DAM FM NAN. Analyzed the data: AJT DAM NAN CP EPH. Contributed reagents/materials/analysis tools: AJT DAM NAN FJ CP EP. Wrote the paper: AJT DAM.


MicroRNA in TSC and LAM