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Rapamycin-insensitive up-regulation of *MMP2* and other genes in TSC2-deficient LAM-like cells

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At a Glance Commentary

Scientific Knowledge on the Subject: Increased MMP-2 expression in LAM-like cells is TSC1/TSC2-dependent but rapamycin insensitive.

What This Study Adds to the Field: TSC1/TSC2 deficiency can lead to abnormalities that are mTOR independent.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

Abstract

Rationale:

Increased matrix metalloproteinase (MMP) activity has been implicated in the pathogenesis of lymphangioleiomyomatosis (LAM).

Objectives:

To investigate how TSC1 or TSC2 deficiency alters MMP expression and regulation.

Methods:

We studied immortalized cells that lack TSC2 derived from an angiomyolipoma (AML) of a LAM patient, and a TSC2 addback derivative; and murine embryonic fibroblast cells that lack Tsc1 or Tsc2 and respective controls. Global gene expression analysis was carried out in the AML and derivative cell lines. MMP levels in the conditioned media from these cells were analyzed by zymography and ELISA.

Measurements and Main Results:

We found increased MMP-2 expression in cells lacking TSC1/TSC2 compared to their respective controls by zymography. MMP-2 overproduction by these cells was not affected by rapamycin treatment. Gene expression analysis confirmed increased MMP-2 gene expression that was not affected by rapamycin.

Furthermore, multiple other genes were found to be over-expressed in rapamycin-treated TSC2-deficient cells compared to TSC2+ cells.

Conclusions:

We conclude that TSC1/TSC2 deficiency leads to MMP-2 overproduction that is rapamycin insensitive, and that several genes exhibit similar patterns suggesting TSC1/TSC2 dependent but mTOR independent pathways may be involved in the pathogenesis of LAM.

Abstract Word Count: 191.

Key Words: Interstitial Collagenase, Neoplasms, Sirolimus.

Introduction:

Pulmonary lymphangioleiomyomatosis (LAM) is a progressive interstitial lung disease that has no proven effective treatment. Clinically the disease can present with insidious onset of dyspnea, or sudden onset of dypsnea and/or chest pain due to spontaneous pneumothorax (1). Pathologically, LAM is characterized by both destruction of lung parenchyma with development of thin-walled cysts, and occurrence of numerous deposits of distinctive spindle-shaped and epithelioid cells, often in clusters termed LAM nodules(2). LAM occurs in about 30% of adult women with the genetic disorder tuberous sclerosis complex (TSC), but is more commonly seen in women with no history of TSC, so-called sporadic LAM(1). In both TSC–associated and sporadic LAM, kidney involvement by a related neoplasm, angiomyolipoma (AML), occurs frequently (1, 2).

Biallelic inactivating mutations in *TSC2* are common in both TSC-associated and sporadic LAM (3, 4). In addition, kidney AML and LAM cells from sporadic LAM patients have the same mutation in *TSC2* (in each individual patient), suggesting that both lesions are derived from the same cell lineage and may spread from one site to another through a metastatic mechanism(3). These genetic data are consistent with pathologic studies demonstrating that there is widespread involvement of the lymphatic system, both within the thorax and infra-diaphragmatic, by proliferating cells of appearance highly similar to those seen in LAM nodules(2, 5).

The TSC1 and TSC2 proteins (also called hamartin and tuberin, respectively) form a complex that has a critical function in regulating the state of activation of the mTOR kinase through Rheb-GTP (2, 6, 7). Thus, phospho-S6 (downstream of mTOR complex 1, or mTORC1 activation) is elevated in the AML and LAM cells of TSC patients. This observation, with support from positive results in mouse and rat models of TSC, has led to clinical trials testing the efficacy of rapamycin, an mTORC1 inhibitor, in LAM (1, 8, 9).

Matrix metalloproteinases (MMPs) are a group of enzymes able to degrade components of the extracellular matrix (ECM). MMPs have been implicated in the pathogenesis of chronic lung diseases (10) as well as tumor growth and metastasis (11). Recent studies have provided evidence that MMPs, especially MMP-2, are increased in expression in LAM nodules by immunohistochemistry analyses (12-15). Furthermore, MMP-14 (membrane type 1 matrix metalloproteinase or MT-1-MMP), an important activator of MMP-2, also appears to be expressed at high levels in LAM lesions (15-17), while TIMP-3 (tissue inhibitor of metalloproteinase 3), an inhibitor of MMP-2, appears expressed at reduced levels in LAM lesions (15). The observation that TIMP-3-null mice develop emphysema with pulmonary parenchymal destruction provides evidence of the importance of this enzymatic system in lung disease (18). In addition, blockade of MMP activity with doxycycline was associated with improvement in respiratory function in a woman with LAM in a case report (19).

The above circumstantial evidence suggests that MMPs may contribute to ECM degradation and development of cystic lesions, an important aspect of LAM pathology that likely contributes significantly to respiratory dysfunction. However, the full range of MMP expression abnormalities in LAM has not been studied in detail. Here we use a recently developed AML cell line from a patient with LAM to explore the relationship between TSC1/TSC2 expression and MMP expression.

Methods:

Cell Culture

Cell culture media and supplements were from GIBCO, Frederick, MD. An immortalized TSC2-deficient human cell line derived from the AML of a LAM patient(20), and its corresponding TSC2-rescued control cell line has been described previously (21). These cells were cultured in DMEM/F12 supplemented with 10%FBS, 0.2 μM hydrocortisone, 0.1 nM triiodothyronine, 0.01μU/ml vasopressin, 1.6μM FeSO4, cholesterol, ITS, 100ng/ml EGF, 100μg/ml zeomycin, and 1% penicillin-streptomycin-amphotericin B (PSA). Immortalized *Tsc1*$^{-/-}$ or *Tsc2*$^{-/-}$ murine embryonic fibroblast (MEF) cell lines with their perspective controls have been described previously (22, 23). MEFs are maintained in DMEM, supplemented with 10% FBS and 1% PSA.

All experiments were performed in triplicate. For biochemical analyses and conditioned media analysis, cells were seeded at $2.5x10^5$ cells/ml in 6-well plates in their normal growth media for 24hr. Media was replaced by serum-free media (Alpha-MEM with 1% PSA) with rapamycin (20nM) or vehicle. 24 hours later, cell-free conditioned media was collected, and cell lysate was prepared with RIPA (Boston Bioproducts, Boston, MA) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN) and a phosphatase inhibitor (Thermo Scientific, Waltham, MA).

Long-term rapamycin treatment was performed in 10mm culture dishes. Vehicle or rapamycin-containing media was replaced daily for 5 days. Cells were then detached and seeded at $2.5x10^5$ cells/ml into 6-well plates as above.

RNA Interference Studies

Small-interfering RNA (siRNA) constructs were purchased from Ambion (Austin, TX), and used as instructed by the manufacturer. Briefly, 30-100nM Silencer siRNA constructs against Rheb (s12021), mTOR (s603) or nonsense negative control were incubated in Opti-MEM (Invitrogen, Carlsbad, CA) with NeoFX transfection agent. The mixture was then plated into 6-well plates and overlaid with $3x10^5$ cells/well in Opti-MEM for 24hr. Media was replaced with 2ml/well of serum-free media and incubated for another 24hr before collection of cell-free media and cell lysate as described above.

Western blotting

Protein samples were analyzed by SDS-PAGE using 4-12% NuPAGE Gel (Invitrogen), and transferred to a nitrocellulose membrane. Immunoblotting was performed by standard methods using HRP-conjugated secondary antibodies, and chemiluminescence using Supersignal West Pico Chemiluminescent substrate (Thermo Scientific) and exposure to film. Anti-pS6 (S240/244), mTOR, Rheb antibodies were purchased from Cell Signaling, Danvers, MA.

MMP-2 assay using zymography

Gelatinase activity in conditioned media was assessed by zymography as previously described (24). Serum-free conditioned media, normalized to cell number, was mixed with non-reducing sample buffer (Boston Bioproducts), and loaded onto a 10% polyacrylamide gel containing 0.1% gelatin (Bio-Rad, Hercules, CA). After electrophoresis, the gels were soaked in 2.5% Triton X-100 with gentle shaking for 30 min at ambient temperature with one change of solution. The gels were then rinsed and incubated overnight at 37°C in developing buffer (Boston Bioproducts). Gels were then stained for 15 to 30 min in 0.5% Coomassie Blue R-250 in acetic acid, isopropyl alcohol, and water (1:3:6); destained in acetic acid, ethanol, and water (1:3:6), and photographed. MMPs were identified by localized clearing of the gel. MMP-2 and MMP-9 were identified by molecular weight, and confirmed as previously reported(24).

ELISA

A Quantikine Human MMP-2 ELISA kit from R&D Systems, Minneapolis, MN was used as directed.

Gene Expression Analysis

Gene expression levels were measured using the CodeLink Human Whole Genome microarray with 53,423 30-mer oligonucleoide probes. In the Microarray Quality Control (MAQC) studies, the measurements of gene expression changes on these microarrays exhibited a high level of concordance with other microarray platforms and with quantitative RT-PCR (25). Total RNA was extracted from cells using the Qiagen RNeasy kit (Qiagen Inc., Valencia, CA). The quality of the total RNA was assessed by analysis on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The ratio of the 28S/18S bands was required to be ≥ 2.0. 0.75 µg of total RNA was reverse transcribed into cDNA, and the product used in an in vitro transcription (IVT) reaction to generate complementary RNA (cRNA) labeled with biotin for hybridization to arrays following the CodeLink labeling protocol (Applied Microarrays, Inc., Tempe, AZ). Briefly, RNA was first reverse transcribed using a T7-Oligo (dT) promoter primer, followed by second-strand cDNA synthesis using RNase H. Double-stranded cDNA was purified and used as a template in the IVT using T7 RNA Polymerase and a biotinylated nucleotide analog/ribonucleotide mix for to generate biotin-labeled cRNA.

For hybridization to the CodeLink arrays 10 µg of labeled cRNA was fragmented, denatured at 90℃ for 5 min, and then hybridized at 37℃ for 20 hours. After washing, the arrays were incubated with streptavidin-phycoerythrin and

biotinylated anti-streptavidin antibodies, and the arrays scanned on a GenePix 4000B scanner (Axon Instrument, Sunnyvale, CA).

Global Gene Analysis

Gene expression data were obtained in triplicate on three groups of cells: TSC2- cells treated with rapamycin (group A), vehicle treated TSC2- cells (group B), and TSC2+ cells (group C). The gene expression levels on each array were normalized to a median expression level of 1.0 using the CodeLink software. To focus on genes expressed well above background, we excluded genes from consideration if no sample had an expression level of at least 2. Coefficient of variations (CV), inter-group p-values (Student's t-test), and gene ratios of two-group comparisons were calculated using Microsoft Excel, and then imported to Microsoft Access for further analysis. To identify differentially expressed genes we first required that the a within-group CV <0.2 to exclude highly variable genes and then a p-value < 0.05 for the Student's t-test for the average expression between any pair of groups.

To identify statistically significant gene expression changes for the 23 MMP and 4 TIMP gene probes on the array, we imposed the Bonferroni multiple testing correction on the t-test for statistically significant changes on the expression of these families of genes corresponding to p-values of 0.002 and 0.0125 respectively.

To identify additional genes whose expression is increased due to loss of TSC2 but independent of mTORC1 activation, we selected genes that met the following

criteria: 1. Expression ratio of Group A/Group C > 2 with P < 0.05. 2. Expression ratio of Group B/Group C > 2 with P < 0.05. 3. Group A and Group B expression not significantly different (p > 0.05). We also identified a subset of these genes in which expression levels were increased > 5-fold in the TSC2-deficient cells with or without rapamycin treatment (Groups A, B) in comparison to TSC2+ cells (Group C).

Statistics

The two-sided Student's t-test was used to compare expression between pairs of groups. The simple Bonferroni correction was used to correct for multiple comparisons in gene expression data for the MMPs and TIMPs.

The gene expression levels in all three groups were compared in a pairwise fashion. The TSC2-deficient cells with or without rapamycin treatment (Groups A, B) were compared with each other to identify rapamycin dependent genes and both groups A and B were compared with the TSC2+ cells (Group C) to identify TSC2 related genes.

Results:

TSC-deficient cell lines secrete more MMP-2 than control cell lines.

We used an immortalized cell line derived from an AML of a patient with LAM (20) to examine production of MMPs in a relevant cell type. Conditioned media from this line gave clear bands on zymogram gels in the range of 60-85kDa,

corresponding to pro-MMP-2 (higher molecular weight) and active MMP-2 (lower molecular weight), which were clearly increased in comparison to media from the addback TSC2 cell line derived from this AML line (Figure 1A). MMP-9, the other highly gelatinolytic MMP, has a higher molecular weight (~92KD), and that region of the gel showed only a faint, non-reproducible signal. Similar findings were made in the analysis of murine embryo fibroblast (MEF) cell lines engineered to be null for either Tsc1 or Tsc2 (19, 20) (Figure 1). All three of the TSC2 or Tsc1 or Tsc2 deficient cell lines showed increased levels of pS6 (S240/244) compared to controls, as expected.

MMP-2 secretion by TSC1/TSC2 deficient cells is mTORC1 independent.

Since activation of mTORC1 is one of the signaling hallmarks of cells lacking TSC1 or TSC2, we examined whether MMP-2 secretion was dependent upon mTORC1 activity. Cells were treated with rapamycin during serum-free media incubation and collection of conditioned media for zymography. Rapamycin treatment at 20nM for 24 hours had no effect on MMP-2 activity in the media, while it clearly blocked mTORC1 activity, as assessed by reduction in pS6 levels (Figure 2A). Long term rapamycin treatment for seven days similarly had no effect on MMP-2 activity (Online Supplemental Figure E1).

To confirm that MMP-2 was present in the conditioned media at higher levels in cells lacking TSC1/TSC2, and to quantify this increase, an ELISA assay for MMP-2 was performed. MMP-2 levels were approximately 4.5-fold higher in the

TSC2 null AML cell line, in comparison to the addback TSC2 expressing line, and were not affected by rapamycin treatment for 24 hours (Figure 2B).

Increased MMP-2 secretion is mTOR and Rheb-independent.

To confirm that MMP-2 secretion was independent of mTOR in these cells, we examined the effects of knockdown of mTOR or Rheb by siRNA. By densitometry estimates on the immunoblots, we achieved about 90% knockdown of mTOR protein with 40% lower pS6, and about 70% knockdown of Rheb protein with 70% lower pS6 in the siRNA experiments. siRNA knockdown of either mTOR or Rheb appeared to have no effect on MMP-2 secretion by the TSC2 null AML cell line, while each treatment clearly reduced pS6 levels (Figure 3A). Indeed, MMP-2 ELISA analysis showed that mTOR inhibition had no significant effect on secreted MMP-2 levels, while Rheb inhibition actually increased MMP-2 levels to a small but significant extent (Figure 3B). These data suggest that increased MMP-2 in TSC2-deficient cells is mTOR independent.

MMP-2 mRNA levels are increased in TSC2-deficient cells.

To examine the mechanism of increased MMP-2 secretion by the TSC2 null AML cell line, we performed gene expression analysis using CodeLink microarrays. To assess the potential effects of treatment with rapamycin on gene expression, three sets of RNA samples were studied: A: the TSC2 null AML cell line treated with rapamycin for 24hr, B: the TSC2 null AML cell line treated with vehicle, and C: the vehicle-treated TSC2 addback AML cell line. All cells were maintained in

serum-free conditions for 24hr before RNA isolation. Probes for 23 MMPs were present on the microarray. Even after Bonferroni the multiple testing correction the difference in expression levels of MMP-2 between samples B and C (neither treated with rapamycin) was statistically significant with an average increase of 2.34 between the TSC2 null AML cell line and the TSC2 expressing addback cell line (Table 1), p=0.0003. The mRNA levels of MMP-15, also known as MT-2-MMP and a reported activator of MMP-2 (26), were also increased in the TSC2 null AML cell line with a ratio of 3.44, p=0.0004. Rapamycin treatment had no significant effect on MMP-2 levels in the TSC2 null line (Table 1), consistent with our zymography and ELISA results. Multiple other expression differences were seen among the MMPs, but these were not significant after correction for multiple testing. In particular, comparing TSC2- cells to TSC2+ cells, MMP-1 and MMP-3 mRNA levels were reduced (ratios of 0.4883 and 0.6667, with p values of 0.0029 and 0.0063, respectively), while MMP-13, MMP-17, and MMP-25 were increased (ratios of 4.53, 5.05, and 1.42 with p values of 0.0030, 0.0035, 0.0031, respectively). Among these, only MMP-13 mRNA levels were reduced by rapamycin (Table 1).

TIMP expression analysis.

Four TIMPs were analyzed in the gene expression analysis (Table 2). Comparing TSC2- to TSC2+ cells, the *TIMP1*, *TIMP2*, and *TIMP3* showed no statistically significant changes. Interestingly, only the *TIMP4* mRNA expression was significantly different. Although it has not been previously implicated in LAM,

TIMP-4 was increased 3.71 fold in the TSC2- AML line, $p<0.0001$. Rapamycin treatment had no significant effect.

Global gene analysis to identify other genes whose expression was also up-regulated in a TSC2-dependent but rapamycin–insensitive manner.

The full gene expression data set was also analyzed to identify genes that appear to increase along with *MMP2* and *MMP15*. 271 genes were identified for which expression levels were increased two-fold or greater in the TSC2 null cell line compared to the addback cell line, independent of rapamycin treatment (Table 3). 22 genes were identified for which expression levels were increased five-fold or greater in the TSC2 null cell line compared to the addback cell line, independent of rapamycin treatment (Table 4). In these cases the Bonferroni correction for the testing of the 50,000 gene probes on the array would require a p-value < 0.000001 for statistical significance at the 0.05 level. Because of the small N=3 for each group, only two genes (*CCL7* and *SLC16A6*) met this strict requirement, Nevertheless, many genes exhibited large increases from 10- to 65-fold.

Since all of the CodeLink microarray data is publically available in the Gene Expression Omnibus (GEO) database at the NCBI, this data set can be examined in more detail for up and down regulated genes in future work.

Discussion:

The lack of reliable cell lines to serve as an in vitro model system to study aspects of the pathogenesis of LAM has been a major limitation. Here we use a recently derived TSC2 null AML cell line to explore the regulation of expression of MMPs. This cell line was derived from an AML resected from a patient with sporadic (non-TSC-associated LAM), and has been shown to have biallelic inactivation of TSC2 (R611Q on one allele, and loss of the second TSC2 allele) (20). The identical mutations were identified in the LAM cells of this patient, consistent with the 'benign metastasis' model of LAM development, with migration of cells from some initial site to both the renal and lung lesions in this patient (3, 27).

We have shown that this TSC2 null AML cell line secretes higher levels of MMP-2 than its TSC2 addback derivative, and that rapamycin inhibition of mTORC1 does not affect the increased MMP-2 secretion. In addition, knockdown of either mTOR or Rheb has no major effect on MMP-2 secretion by this line. Similar observations were made on MMP-2 secretion by pairs of MEF lines that are null for either Tsc1 or Tsc2. In aggregate these data strongly suggest that MMP-2 production is increased in TSC2 null LAM cells at least partly through a transcriptional mechanism. These data lend support to previous immunohistochemistry observations on the expression of MMPs in LAM lesions, to the speculation that MMPs may play a role in the pathogenesis of pulmonary LAM, and to the finding that MMPs are elevated in the urine of patients with LAM (2, 12-15, 19). As with other chronic lung diseases and cancers, the ability of MMP-2 to degrade ECM may directly contribute to lung cyst formation in

pulmonary LAM. However, it is important to note that how MMP-2 plays a role in the pathogenesis of LAM remains unclear, and thus the therapeutic potential for MMP-2 inhibition is also uncertain.

Another possible role for MMPs in LAM pathogenesis is through stimulation of angiogenesis and lymphangiogenesis. MMPs have been shown to enhance VEGF expression and regulate angiogenesis, and TIMPs are known to suppress the angiogenesis phenotype at a number of stages of the angiogenesis program (28-32). The up-regulation of MMP in LAM may contribute to lymphangiogenesis through the expression of VEGF-C, VEGF-D and the corresponding receptors (5, 33). The observations that VEGF-D is expressed by LAM lesions and that increased circulating VEGF-D levels may be found in LAM patients suggest the importance of pro-lymphangiogenesis factors in development of LAM(34, 35).

We found that the increased expression of MMP-2 by the AML/LAM cell line appeared to be independent of mTORC1, as assessed by the lack of effect of rapamycin treatment, as well as siRNA knockdown of mTOR and Rheb. Although most biochemical and signaling effects in cells lacking TSC1 or TSC2 are thought to occur through activation of mTORC1, there is previous evidence that several abnormalities in TSC2-deficient cells are independent of mTORC1 activation. B-Raf kinase activity is reduced in TSC2-deficient cells due to Rheb-GTP, but independent of mTORC1 (36, 37). In addition, Tsc2-null MEFs exhibit reduced Akt kinase activation partially due to impaired mTORC2 activity (38). Last, Tsc1 or Tsc2-null MEFs have a higher percentage of cilium-containing cells comparing to the respective controls, and rapamycin treatment has no effect on

this observation (39). However, none of these observations seems to be connected to increased expression of MMP-2 seen in this TSC2 null cell line, and indeed the lack of effect of Rheb knockdown on MMP-2 levels argues against this being due to Rheb.

Much enthusiasm has been generated by the prospect that inhibitors of mTOR may represent ideal therapeutic agents in the treatment of LAM, as an mTOR pathway disease. Initial clinical trials have indicated that rapamycin may have some benefit in LAM/TSC-related diseases, but further investigation is required (8, 9). The existence of pathways aberrantly activated in TSC2 null cells that do not respond to rapamycin treatment suggest that rapamycin treatment alone may be inadequate for disease control. Treatments aimed at inhibiting MMP thus may have potential benefit in LAM. Clinically available agents such as doxycycline and HMG-CoA reductase inhibitors, statins, have been shown to reduce MMP production and pathologic proliferation in vascular smooth muscle cells (19, 40-43), and may have benefit for clinical LAM in combination with rapamycin. Statins may be particularly attractive, since they also have some ability to reduce Rheb-GTP and Rho-GTP levels in cells lacking TSC1/TSC2 (44).

In addition to MMPs, gene expression analysis identified several genes whose expression is increased TSC2- cells compared to TSC2+ controls. The expression of these genes also did not appear to be affected significantly by rapamycin treatment. Although these differences in gene expression had extremely low p values ($p = 10^{-3} – 10^{-6}$, Table 4), it is possible that some of these have occurred by chance given the large number of comparisons being made.

Nonetheless, they suggest that there are additional, mTORC1-independent effects of in TSC1/TSC2 loss in this AML/LAM cell line. *WISP2*, *WNT9A,* and *SOX4* are increased in expression in the AML/LAM cell line, suggesting that there may be aberration of Wnt signaling in these cells, consistent with a previous observation of increased β-catenin levels within LAM lesion by IHC (45). Wnt activation has been reported to induce MMP-2 expression (46), suggesting a possible link between Wnt and MMP in LAM. Further investigation into their potential role in the pathogenesis of LAM is required.

In conclusion, we report that MMP-2 expression is significantly increased in TSC2-deficient cells derived from a LAM-associated AML, and is not affected by rapamycin treatment. In addition, several other genes show expression changes that fit a similar pattern, suggesting the existence of mTORC1-independent pathway events in LAM-like cells lacking TSC2. Finally, our observations raise the possibility that rapamycin treatment of LAM may have limited clinical effectiveness.

Table 1. Gene expression analysis of MMP genes in three different groups of AML/LAM cell lines. Group A, TSC2- cells treated with rapamycin; B, TSC2- cells treated with vehicle; C, TSC2+ treated with vehicle. Gene expression ratios and P values for comparisons of pairs of the three groups are presented. Comparing TSC2- (group B) to TSC2+ (group C), we found that *MMP2* and *MMP15* expression was significantly increased. Rapamycin treatment had no significant effect on any MMP expression (comparing groups A, B). The Bonferroni correction was used to conservatively correct for multiple comparisons, so that a P value of <0.002 was considered significant.

MMP Name	Ratio A/B	Ratio A/C	Ratio B/C	P (A-B)	P (A-C)	P (B-C)
MMP1	1.15	0.56	0.49	0.3125	0.0045	0.0029
MMP2	1.14	2.66	2.34	0.3971	0.0094	**0.0003**
MMP3	1.27	0.85	0.67	0.0262	0.0266	0.0063
MMP7	0.82	0.80	0.98	0.1011	0.1359	0.7752
MMP8	1.93	1.34	0.69	0.3522	0.6231	0.4585
MMP9	1.12	1.38	1.23	0.2796	0.0414	0.0161
MMP10	1.05	1.09	1.04	0.3922	0.1230	0.1999
MMP11	1.57	1.69	1.08	0.0254	0.0336	0.7037
MMP12	2.89	5.13	1.77	0.0374	0.0275	0.4306
MMP13	0.20	0.91	4.53	0.0183	0.9253	0.0030
MMP14	1.12	1.34	1.19	0.1749	0.0143	0.0846
MMP15	0.85	2.91	3.44	0.4740	0.0510	**0.0004**
MMP16	0.97	1.10	1.13	0.9109	0.8767	0.8245
MMP17	1.14	5.75	5.05	0.4888	0.0032	0.0035
MMP19	1.44	1.67	1.16	0.0030	0.0010	0.1317
MMP20	1.07	1.06	0.99	0.3777	0.4013	0.9130
MMP21	1.17	1.05	0.89	0.7837	0.9037	0.8257
MMP23	0.64	1.08	1.67	0.3521	0.9012	0.3332
MMP24	1.28	1.86	1.45	0.6926	0.4654	0.4756
MMP24	1.02	1.01	0.99	0.7416	0.8682	0.8282
MMP25	0.95	1.35	1.42	0.0982	0.0091	0.0031
MMP26	0.85	0.95	1.11	0.0638	0.3624	0.0802
MMP27	3.04	1.48	0.49	0.1176	0.3704	0.0759
MMP28	1.82	0.84	0.46	0.1045	0.4865	0.0879

Table 2. Gene expression analysis of TIMP genes in three different groups of AML/LAM cell lines. Group A, TSC2- cells treated with rapamycin; B, TSC2- cells treated with vehicle; C, TSC2+ treated with vehicle. Gene expression ratios and P values for comparisons of pairs of the three groups are presented. Comparing TSC2- (group B) to TSC2+ (group C), we found that *TIMP4* was expressed at a significantly higher level, and rapamycin treatment had little effect on this difference. The Bonferroni correction was used to conservatively correct for multiple comparisons, so that a P value of 0.0125 was considered significant.

TIMP Name	RatioA/B	RatioA/C	RatioB/C	P (A-B)	P (A-C)	P (B-C)
TIMP1	1.29	1.59	1.24	0.1696	0.0299	0.1257
TIMP2	1.22	1.03	0.85	0.0259	0.7392	0.1575
TIMP3	1.05	1.25	1.19	0.4789	0.5966	0.6807
TIMP4	0.89	3.30	3.71	0.2524	0.0016	**<0.0001**

Table 3. Selection criteria for candidate genes that are up-regulated and insensitive to rapamycin treatment in the absence of TSC2. Group A, TSC2- cells treated with rapamycin; B, TSC2- cells treated with vehicle; C, TSC2+ treated with vehicle. P values and expression ratio of intergroup comparisons used as selection criteria and the number of candidate genes identified are presented.

Parameter	P value A vs. B	P value A vs. C	P value B vs. C	Ratio of A/B	Ratio of A/C	Ratio of B/C	#Unique Gene
First selection	>0.05	<0.05	<0.05		>2	>2	271
Second Selection	>0.05	<0.05	<0.05		>5	>5	22

Table 4. Highly overexpressed genes that are TSC2-dependent rapamycin-insensitive in the AML/LAM cell lines. Global gene expression analysis was performed as described in Methods and Table 3. Genes listed here were: 1) expressed at ≥ 5-fold increase in TSC2-null cells with (Group A) or without (Group B) rapamycin treatment as compared to TSC2+ cells (Group C); 2) not differentially expressed between Groups A and B (P>0.05). Gene expression ratios and P values of intergroup comparisons are presented.

Gene	Description	Ratio A/B	Ratio A/C	Ratio B/C	P(A-B)	P (A-C)	P (B-C)
ARL7	ADP-ribosylation factor-like 7	1.12	6.03	5.35	0.32	0.000141	0.000936
CCL7	Chemokine (C-C motif) ligand 7	0.98	63.93	65.06	0.67	0.000015	<0.000001
CKLFSF8	Chemokine-like factor super family 8	1.3	12.43	9.49	0.07	0.000079	0.001112
CNIH3	Cornichon homolog 3	0.92	26.29	28.56	0.22	0.000004	0.000042
COL1A2	Collagen, type I, alpha 2	1.1	8.57	7.76	0.34	0.000285	0.000065
COLEC12	Collectin sub-family member 12	1.17	9.82	8.37	0.14	0.00008	0.000224
CYB5R2	Cytochrome b5 reductase b5R2	1.08	20.29	18.72	0.2	0.000001	0.000052
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	1.05	57.32	54.28	0.55	0.000011	0.000224
EPHB2	EPH receptor B2	0.89	5.41	6.05	0.1	0.000109	0.000004
G1P3	Interferon, alpha-inducible protein	1.15	6.06	5.25	0.1	0.000068	0.000135
GJA1	Gap junction protein, alpha 1	0.96	13.07	13.48	0.62	0.000053	0.000008
HGF	Hepatocyte growth factor	1.09	6.47	5.93	0.44	0.000231	0.000479
INPP5F	Inositol polyphosphate-5-phosphatase F	1.07	9.32	8.68	0.38	0.00001	0.000206
MAGEH1	Melanoma antigen, family H, 1	1.16	5.96	5.1	0.19	0.000386	0.000246
MT1L	Metallothionein 1L	1.13	6.49	5.73	0.14	0.000092	0.000053
PKIB	Protein kinase (cAMP-dependent, catalytic) inhibitor beta	0.94	5.47	5.77	0.48	0.000151	0.000033
PYCARD	PYD and CARD domain containing	1.06	5.76	5.4	0.42	0.000186	0.000034
SLC16A6	Solute carrier family 16 (monocarboxylic acid transporters), member 6	1.14	9.08	7.94	0.22	0.000549	<0.000001
SOX4	SRY (sex determining region Y)-box 4	0.87	13.22	15.15	0.16	0.00018	0.000029
WISP2	WNT1 inducible signaling pathway protein 2	0.94	7.07	7.51	0.5	0.000006	0.000354
WNT9A	Wingless-type MMTV integration site family, member 9A	0.89	9.59	10.75	0.15	0.000024	0.000056
ZNF537	Zinc finger protein 537	1.23	6.73	5.44	0.08	0.000415	0.00006

Figure Legends

Figure 1. Secretion of MMP-2 by TSC1 or TSC2-deficient cells. Representative zymography (Zym: MMP-2) of conditioned media and Western Blots (WB: pS6, tubulin) of cell lysates are shown. First two lanes, a TSC2-deficient human AML cell and addback control line; last four lanes, Tsc2-null or Tsc1-null MEF cells and respective controls. In TSC1/TSC2-deficient cells, pS6 (S240/244) levels are increased, reflecting mTORC1 activation.

Figure 2. Lack of effect of rapamycin treatment on MMP-2 secretion by TSC1/TSC2-deficient cells.

A. Representative zymography (Zym: MMP-2) of conditioned media and Western Blots (WB: pS6, tubulin) of lysates from TSC2-deficient human AML/LAM cells and Tsc1- or Tsc2-null MEF cells with and without rapamycin treatment for 24 hours are shown. Rapamycin effectively suppressed pS6 (S240/244), but had no effect on MMP-2 secretion by the TSC1/TSC2-deficient cells.

B. ELISA quantification of MMP-2 in the conditioned media of human AML/LAM cells. Averages and standard deviations of triplicate experiments are presented here. Rapamycin-treated TSC2-nulls secreted same amount of MMP-2 as untreated TSC2-nulls cells ($P=0.54$), while TSC2+ addbacks secreted much lower MMP-2 ($P<0.001$).

Figure 3. Lack of effect of mTOR or Rheb knockdown on MMP-2 secretion by the TSC2-deficient AML cell line.

A. Representative zymography (Zym: MMP-2) of conditioned media and Western Blots (WB: mTOR, Rheb, pS6, tubulin) of lysates from TSC2-deficient human AML cells. RNA interference with siRNA targeting mTOR (left panel) or Rheb (right panel) successfully reduced expression of the target proteins and reduced pS6 (S240/244) levels, but had no effect on MMP-2 levels in the media.

B. ELISA quantification of MMP-2 levels in conditioned media of TSC2- AML cells treated with siRNA targeting mTOR, or Rheb. Averages and standard deviations of triplicate experiments are shown. Compared to control cells, knockdown of mTOR did not affect MMP-2 secretion ($P=0.48$) while knockdown of Rheb caused a minor increase in MMP-2 production ($P<0.01$).

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Figure 1.

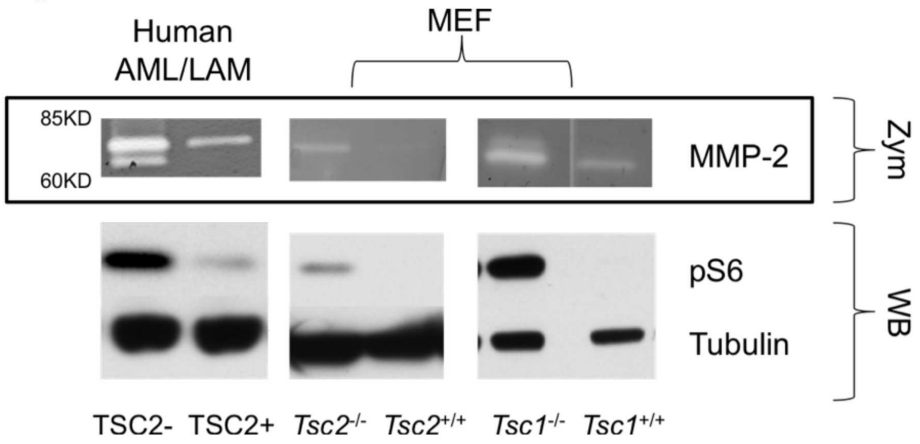

Figure 1. Secretion of MMP-2 by TSC1 or TSC2-deficient cells.
Representative zymography (Zym: MMP-2) of conditioned media and Western Blots (WB: pS6, tubulin) of cell lysates are shown. First two lanes, a TSC2-deficient human AML cell and addback control line; last four lanes, Tsc2-null or Tsc1-null MEF cells and respective controls. In TSC1/TSC2-deficient cells, pS6 (S240/244) levels are increased, reflecting mTORC1 activation.

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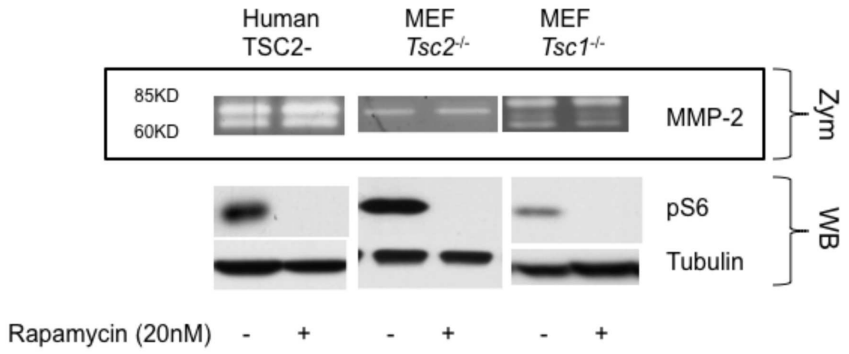
Figure 2.

A.



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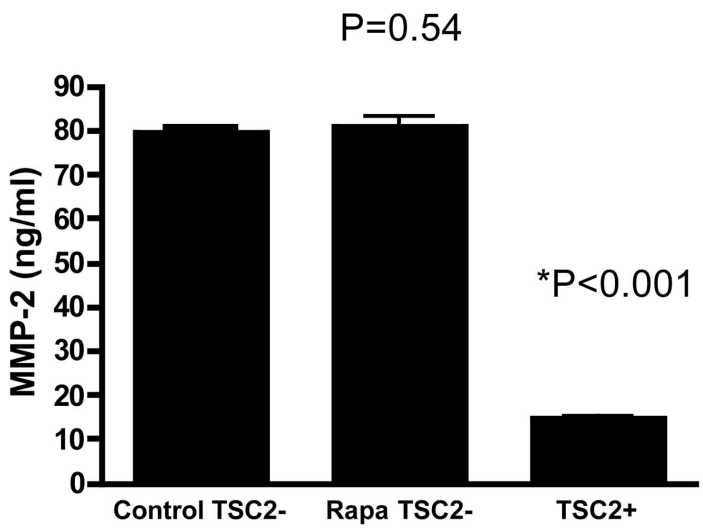

Figure 2. Lack of effect of rapamycin treatment on MMP-2 secretion by TSC1/TSC2-deficient cells. A. Representative zymography (Zym: MMP-2) of conditioned media and Western Blots (WB: pS6, tubulin) of lysates from TSC2-deficient human AML/LAM cells and Tsc1- or Tsc2-null MEF cells with and without rapamycin treatment for 24 hours are shown. Rapamycin effectively suppressed pS6 (S240/244), but had no effect on MMP-2 secretion by the TSC1/TSC2-deficient cells.
B. ELISA quantification of MMP-2 in the conditioned media of human AML/LAM cells. Averages and standard deviations of triplicate experiments are presented here. Rapamycin-treated TSC2-nulls secreted same amount of MMP-2 as untreated TSC2-nulls cells (P=0.54), while TSC2+ addbacks secreted much lower MMP-2 (P<0.001).

177x212mm (600 x 600 DPI)

Figure 3.

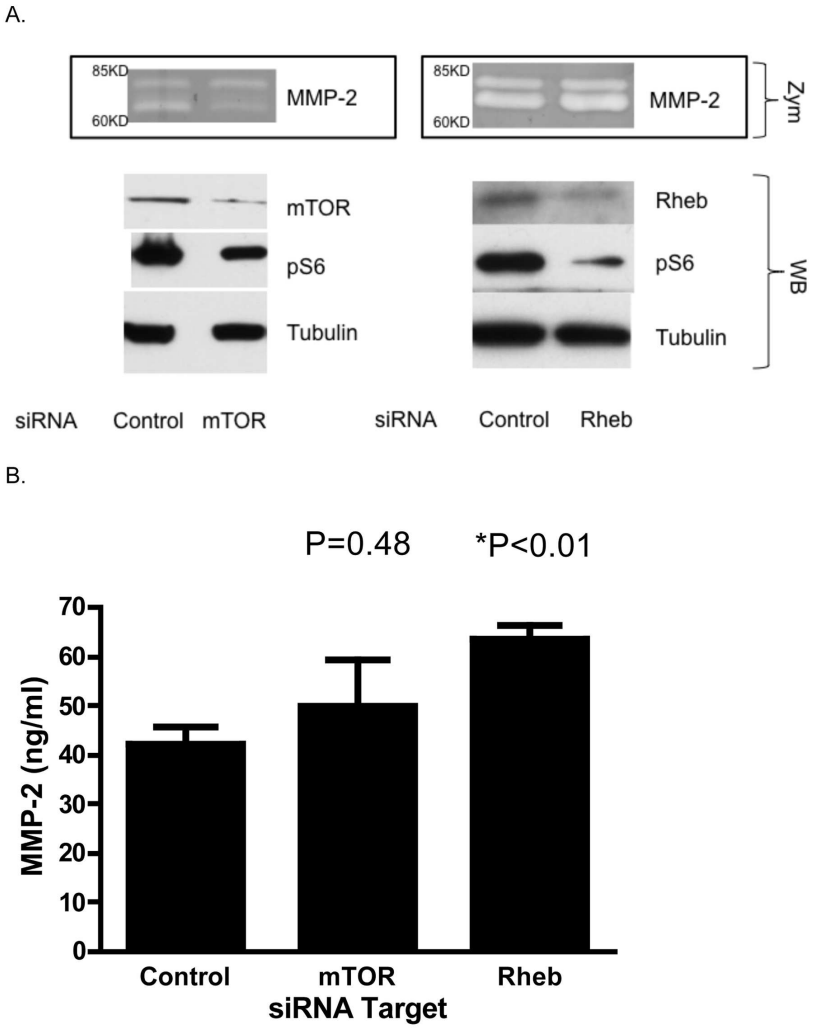

Figure 3. Lack of effect of mTOR or Rheb knockdown on MMP-2 secretion by the TSC2-deficient AML cell line.

A. Representative zymography (Zym: MMP-2) of conditioned media and Western Blots (WB: mTOR, Rheb, pS6, tubulin) of lysates from TSC2-deficient human AML cells. RNA interference with siRNA targeting mTOR (left panel) or Rheb (right panel) successfully reduced expression of the target proteins and reduced pS6 (S240/244) levels, but had no effect on MMP-2 levels in the media.

B. ELISA quantification of MMP-2 levels in conditioned media of TSC2- AML cells treated with siRNA targeting mTOR, or Rheb. Averages and standard deviations of triplicate experiments are shown. Compared to control cells, knockdown of mTOR did not affect MMP-2 secretion (P=0.48) while knockdown of Rheb caused a minor increase in MMP-2 production (P<0.01).

177x225mm (600 x 600 DPI)

Online Data Supplement

Rapamycin-insensitive up-regulation of *MMP2* and other genes in TSC2-deficient LAM-like cells

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6. Harvard Medical School, Boston, MA
7. Department of Biological Sciences, Virginia Tech, Blacksburg, VA

Supplemental Figure E1. Lack of effect of long term rapamycin treatment on MMP-2 production by TSC1/TSC2-deficient cells. Representative zymography (Zym: MMP-2) of conditioned media and Western Blots (WB: pS6, tubulin) of lysates from TSC2-deficient human AML/LAM cells and Tsc1- or Tsc2-null MEF cells with and without rapamycin treatment for seven days are shown. Long term rapamycin treatment effectively suppressed pS6 (S240/244), but had no effect on MMP-2 secretion by these cells.