Rapamycin-Resistant Poly (ADP-Ribose) Polymerase-1 Overexpression Is a Potential Therapeutic Target in Lymphangioleiomyomatosis

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Abstract

Lymphangioleiomyomatosis (LAM) is a female-predominant cystic lung disease that can lead to respiratory failure. LAM cells typically have inactivating tuberous sclerosis complex 2 (TSC2) mutations and mammalian target of rapamycin (mTOR) complex (mTORC) 1 activation. Clinical response to the mTORC1 inhibitors has been limited, prompting a search for additional therapy for LAM. In this study, we investigated the impact of TSC2 on the expression of poly (ADP-ribose) polymerase (PARP)-1 that initiates the DNA repair pathway, and tested the efficacy of PARP1 inhibitors in the survival of TSC2-deficient (TSC2⁻) cells. We analyzed publicly available expression arrays of TSC2⁻ cells and validated the findings using real-time RT-PCR, immunoblotting, and immunohistochemistry. We examined the impact of rapamycin and Torin 1 on PARP1 expression. We also tested the effect of PARP1 inhibitors, 8-hydroxy-2-methylquinazoline-4-one and 3,4-dihydro-5[4-(1-piperindinyl) butoxy]-1(2H)-isoquinoline, on the survival of TSC2⁻ cells. We identified the up-regulation of *PARP1* in TSC2⁻ cells relative to cells in which wild-type TSC2 has been reintroduced (TSC2-addback [TSC2⁺] cells). The transcript levels of *PARP1* in TSC2⁻ cells were not affected by rapamycin. PARP1 levels were increased in TSC2⁻ cells, xenograft tumors of rat-derived TSC2⁻ cells, renal cystadenomas from *Tsc2*^{+/-} mice, and human LAM nodules. RNA interference of mTOR failed to reduce PARP1 levels. Proliferation

and survival of TSC2⁻ cells was reduced in response to PARP1 inhibitor treatment, more so than TSC2⁺ cells. TSC2⁻ cells exhibit higher levels of PARP1 relative to TSC2⁺ cells in an mTOR-insensitive manner. PARP1 inhibitors selectively suppress the growth and induce apoptosis of TSC2⁻ cells from patients with LAM. Targeting PARP1 may be beneficial in the treatment of LAM and other neoplasm with mTORC1 activation.

Keywords: tuberous sclerosis complex; mammalian target of rapamycin; rapamycin; lymphangioleiomyomatosis; poly (ADP-ribose) polymerases

Clinical Relevance

Tuberous sclerosis complex (TSC) 2-deficient cells exhibit higher levels of poly (ADP-ribose) polymerase (PARP)-1 relative to TSC2-addback cells in a mammalian target of rapamycin (mTOR)-insensitive manner. PARP1 inhibitors selectively suppress the growth and induce apoptosis of cells derived from TSC2-deficient patients with lymphangioleiomyomatosis (LAM). Targeting PARP1 may be beneficial in the treatment of LAM and other neoplasm with mTOR complex 1 activation.

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Lymphangioleiomyomatosis (LAM) is a female-predominant, progressive cystic lung disease. LAM is characterized by proliferation of abnormal smooth muscle-like cells in nodules in the lung parenchyma and other sites, and by cystic changes with lung tissue destruction (1–4). LAM occurs in 30–40% of women with tuberous sclerosis complex (TSC) (5, 6). LAM can also occur as a sporadic disease in women who do not have generalized features of TSC. TSC is due to germline mutations in either *TSC1* or *TSC2*, and similar inactivating somatic mutations are found in LAM cells from patients with sporadic LAM (7, 8).

About 60% of women with the sporadic form of LAM also have renal angiomyolipomas. The presence of *TSC2* mutations in LAM cells and renal angiomyolipoma cells from women with sporadic LAM, but not in normal tissues, has led to the model that LAM cells spread to the lungs via a metastatic mechanism. Genetic analyses of recurrent LAM after lung transplantation also support the metastatic model for LAM pathogenesis (7, 9). The only treatment for end-stage LAM and respiratory failure is lung transplantation, with its intrinsic limitations. Furthermore, LAM can recur in the transplanted lungs (9, 10).

The protein products of the *TSC1* and *TSC2* genes, hamartin and tuberin, respectively, interact with Tre2-Bub2-Cdc16 (TBC) 1 domain family, member 7 (TBC1D7) (11) to form a complex (12) that regulates the state of activation of the mammalian target of rapamycin (mTOR) complex (mTORC) 1 (13). Mutation and loss of either gene leads to mTORC1 activation, which has been observed in LAM-derived cells and LAM nodules (4, 14–17). However, we previously reported that *TSC2*-deficient (*TSC2*[−]) cells derived from a LAM patient have multiple expression characteristics that are independent of mTORC1 activation (18). Therefore, we hypothesize that some of these proteins may contribute significantly to LAM pathogenesis and represent potential therapeutic targets in LAM/TSC-related diseases.

Poly (ADP-ribose) (PAR) polymerase (PARP)-1, the founding and most studied member of PARP family proteins, is a large, abundant nuclear protein activated by DNA damage, which plays a role in chromosomal repair and stability (19, 20).

PARP inhibitors have gained increasing popularity as possible chemotherapeutic agents. Studies have documented that breast cancer, early onset-deficient and phosphatase and tensin homolog-deficient tumor cells have particular sensitivity to the cytotoxic effect of PARP inhibitors (21–24), suggesting that PARP may be a promising therapeutic target in many forms of cancer.

Here, we report that PARP1 expression is up-regulated in *TSC2*[−] cells from patients with LAM *in vitro*, and this is insensitive to treatment with either rapamycin (mTORC1 inhibitor) or Torin1 (mTORC1 and mTORC2 dual inhibitor). *In vivo*, PARP1 expression is abundant in *TSC2*[−] xenograft tumors, in spontaneously arising renal tumors in *Tsc2* heterozygous mice, and in human angiomyolipomas and in LAM nodules. PARP1 inhibitors suppress the growth and induce apoptosis of *TSC2*[−] cells from patients with LAM. Based on these preclinical studies, we propose that the PARP1 inhibitors may have therapeutic benefit for women with LAM.

Materials and Methods

Detailed descriptions of the MATERIALS AND METHODS are available in the online supplement.

DNA Damage Assessment

H2A.X is required for checkpoint-mediated DNA repair after double-stranded DNA breaks, which results in rapid phosphorylation of H2A.X (Ser139). Cells were fixed in 4% paraformaldehyde for 15 minutes. Immunofluorescent staining with phospho- γ -H2A.X was performed. Nuclear staining of phospho- γ -H2A.X is the readout of DNA damage.

Gene Expression Analysis

Reanalysis of previously published expression array data (GSE16944 [18]; GSE10072 [25, 26]; GSE19804 [25, 26]; and GSE12027 [27]) was performed. Transcript levels of PARPs were compared between *TSC2*[−] and *TSC2*-addback (*TSC2*⁺) cells, or rapamycin- and vehicle-treated *TSC2*[−] cells. Expression levels of *PARP1* were compared among HMB45-positive LAM cells collected by laser-capture microdissection from nodular structures in the lungs of 14 LAM cases (LAM) (27), 107 lung adenocarcinomas from the

Environment and Genetics in Lung cancer Etiology study in Italy (25, 26), and 60 lung carcinomas (lung tumor) and their paired adjacent normal lung tissue (NL) collected from nonsmoking females in Taiwan (25, 26).

Quantitative RT-PCR was performed using One-Step qRT-PCR Kits in the Real-Time PCR System from Applied Biosystems (Life Technologies, Grand Island, NY) and normalized to β -actin.

Cell Culture and Reagents

Mouse expression arrays of *Tsc2*^{−/−} *p53*^{−/−} and *Tsc2*^{+/+} *p53*^{−/−} murine embryonic fibroblasts (MEFs) (28) were preformed (29, 30). An immortalized *TSC2*[−] human cell line derived from angiomyolipoma of a patient with LAM (31), and its corresponding *TSC2*-rescued control cell line (*TSC2*⁺ cells) has been described previously (18). In brief, patient-derived cells were transfected with pcDNA3.1zeo-hTSC2 or its corresponding empty vector, pcDNA3.1zeo (32). Eker rat uterine leiomyoma-derived *TSC2*[−] (ELT) 3 cells were developed by Howe and colleagues (33, 34). ELT3 cells were transduced with a retroviral plasmid, pMSCVneo-hTSC2, or its corresponding empty vector, pMSCVneo (35).

PARP Inhibitors

8-Hydroxy-2-methylquinazoline-4-one (NU1025), 3,4-dihydro-5[4-(1-piperindinyl)butoxy]-1(2H)-isoquinoline (DPQ), and Olaparib were purchased from EMD Chemicals (Billerica, MA).

Cell Viability Assay

Cell numbers were quantified using CyQuant (Invitrogen, Carlsbad, CA). Values are expressed as mean ($\pm$ SEM; $n = 8$ /group. * $P < 0.05$; Student's *t* test).

Cell Death Assay

Cells were treated with PARP1 inhibitors for 24 hours, hydrogen peroxide (H₂O₂) was added for 1 hour, cells were harvested and subjected to Annexin V:FITC staining (BD, San Jose, CA), and flow cytometry analysis was performed.

Animal Studies

Tsc2^{+/−} C57Bl6 mice (9–10 mo old) (36) were treated with three doses of 3 mg/kg rapamycin or vehicle. For the xenograft tumor study, 2×10^6 ELT3-V3 (*TSC2*-null) or ELT3-T3 (*TSC2*-re-expressing) cells were inoculated bilaterally into the

posterior back region of female intact CB17-SCID mice (Taconic, Hudson, NY), as previously described (37, 38). Tissues were homogenized in RIPA buffer supplemented with protease and phosphatase inhibitors.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded 10- μ m sections using antibodies against PARP1 (Cell Signaling, Danvers, MA).

Western Blotting

Protein samples were analyzed by SDS-PAGE using 4–12% NuPAGE Gel (Life Technologies). Immunoblotting was performed by standard methods using horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. All antibodies were purchased from Cell Signaling.

RNA Interference

Small interfering RNA (siRNA) Silencer siRNA constructs against Raptor (Rap; s33216) 5'-CCATCGGTGCAAAATTACA-3', mTOR (s603) 5'-CATTCGCATTTCAGTCCAT A-3', or nonsense negative control were purchased from Ambion (Life Technologies). Silencer siRNA constructs (30 nM) were used for transfection.

Statistical Analyses

Statistical analyses were performed using Student's *t* test when comparing two groups. Results are presented as means ($\pm$ SD).

Results

DNA Damage Repair Mechanism Is Dysfunctional in Cells Lacking TSC2

It has been reported that the cellular levels of reactive oxygen species (ROS) were significantly higher in TSC2-null rat uterine-derived ELT3 cells compared with TSC2⁺ cells (39). Here, we found that the levels of ROS were 36% higher in TSC2⁻ patient-derived cells relative to TSC2⁺ cells ($P < 0.01$; Figure 1A), consistent with the published data (39). Considering ROS as a main contributor of oxidative DNA damage (40, 41), we next examined whether TSC2 regulates ROS-triggered DNA damage using phospho- γ H2A.X as the readout. Immunofluorescent staining of

Figure 1. Tuberin attenuates reactive oxygen species (ROS) production and promotes DNA damage. Patient-derived tuberous sclerosis complex (TSC) 2-deficient (TSC2⁻) and TSC2-addback (TSC2⁺) cells were treated with vehicle, 20 nM rapamycin, or 0.5 mM hydrogen peroxide (H₂O₂). (A) Cellular levels of ROS in TSC2-null cells and TSC2⁺ cells were measured. (B) Representative immunofluorescent staining images of phospho- γ H2A.X in patient-derived cells. Scale bars, 30 μ m. ** $P < 0.01$, Student's *t* test.

phospho- γ H2A.X was more prominent in TSC2-re-expressing cells relative to TSC2⁻ cells (Figure 1B). Notably, rapamycin treatment did not affect phospho- γ H2A.X

staining in TSC2⁻ cells (Figure 1B). Together, these data indicate a dysfunctional DNA damage repair mechanism in cells lacking TSC2.

Figure 2. TSC2 negatively regulates the expression of poly (ADP-ribose) (PAR) polymerase (PARP)-1 *in vitro*. (A) Global gene expression assay was performed using RNA isolated from patient-associated angiomyolipoma-derived TSC2⁻ cells, TSC2⁺ cells, and TSC2⁻ cells treated with rapamycin for 24 hours (TSC2-R). The *heatmap* shows differentially expressed PARP genes. (B) Transcript levels of PARP family members were compared between TSC2⁻ and TSC2⁺ cells, or rapamycin- and vehicle-treated TSC2⁻ cells (Rapa TSC2⁻). (C) Comparison of the differentially expressed PARP genes in four independent expression arrays, including one data set of murine embryonic fibroblasts (MEFs) from patients with lymphangioleiomyomatosis (LAM) and three Tsc2^{-/-} and Tsc2^{+/+} MEFs (MEF-1, -2, and -3). (D) Comparison of the differentially expressed PARP family member in four independent expression arrays. ***P* < 0.01, **P* < 0.05, Student's *t* test. N/S, no significance.

Identification of PARP1

Up-Regulation in TSC2⁻ Cells

PARP family members play a critical role in repair of single-stranded DNA breaks (42, 43). We hypothesized that TSC2 regulates the function of PARPs in cells under oxidative stress. To determine whether PARP1 expression is altered in cells lacking TSC2, we performed expression array analysis using our previously published data sets (18). Gene set enrichment analysis showed that the transcript levels of 10 PARP family members, including *PARP1*, *PARP8*, *PARP9*, *PARP10*, *PARP11*, *PARP12*, and *TIPARP*, were significantly higher in TSC2⁻ cells from patients with LAM compared with TSC2⁺ cells (Figure 2A). Interestingly, rapamycin treatment did not affect the expression of PARPs overall, and in some cases appeared to increase expression of some PARP isoforms (*PARP1*, *PARP9*, *PARP12*, and *PARP14*) (Figures 2A and 2B). Next, we performed quantitative real-time PCR to validate our array findings. The transcript levels of *PARP1*, *PARP9*, *PARP12*, and *TIPARP* were higher in TSC2⁻ patient-derived cells relative to TSC2⁺-re-expressing cells (see Figure E1 in the online supplement).

To determine which PARP member(s) is critical in the context of TSC2 deficiency, we analyzed three additional, publicly available expression array data sets. The transcript levels of 12 PARP members were altered in *Tsc2*^{-/-} MEFs compared with *Tsc2*^{+/+} MEFs from data sets GSE21755, GSE27982 (29), and GSE28021 (29), respectively. Notably, the cross-comparison of differently expressed PARPs identified *PARP1* as the commonly altered gene in TSC2⁻ cells relative to TSC2-expressing cells from all expression array data sets (Figures 2C and 2D).

To examine the impact of PARP inhibitors on the transcript levels of PARPs, we treated TSC2⁻ patient-derived cells with PARP inhibitors for 24 hours. DPQ is a potent inhibitor of PARP1 (half maximal inhibitory concentration [IC₅₀] = 40 nM) (44), although much less potent against PARP2 (45). NU1025 is a potent inhibitor of PARP1 (IC₅₀ = 400 nM) (46, 47). Olaparib (AZD2281) is a selective inhibitor of PARP1/2 (IC₅₀ = 1–5 nM) (48). Quantitative real-time PCR analysis showed that the transcript levels of *PARP1*, *PARP9*, *PARP12*,

or *TIPARP* were not significantly altered by PARP inhibitor treatment (Figure E2).

TSC2 Negatively Regulates PARP1 Expression in an mTOR-Independent Manner *In Vitro*

To define the molecular mechanisms responsible for PARP1 overexpression, we

reanalyzed our previous expression array of TSC2⁻ cells from patients with LAM (18) and found that *PARP1* expression was increased by 2.3-fold in TSC2⁻ cells from patients with LAM compared with TSC2⁺ cells ($P < 0.05$; Figure 3A). Interestingly, rapamycin treatment did not affect the level of PARP1 (Figure 3A). To validate the

Figure 3. TSC2 negatively regulates the expression of PARP1 *in vitro*. (A) The relative level of PARP1 in the microarray of patient-associated angiomyolipoma-derived cells compared with cells treated with rapamycin or TSC2 addback cells. (B) The transcript levels of PARP1 in cells derived from patients with LAM treated with rapamycin or vehicle. (C and D) TSC2⁻ or TSC2⁺ cells (LAM patient-derived or rat-derived cells) were treated with 20 nM rapamycin for 24 hours. Immunoblots of PARP1, phospho-S6, and S6 are shown. (E) Cells were treated with small interfering RNAs (siRNAs) to various mammalian target of rapamycin (mTOR) components, and lysates were subjected to immunoblotting. Knockdown of either mTOR or raptor (Rap) reduced phospho-S6 (S240/244) levels compared with cells treated with nonsense control (NC) siRNA. (F) TSC2⁻ patient-derived cells were treated with vehicle (Con), rapamycin (Rapa; 20 nM), or Torin1 (0.05, 0.25, 0.5, and 1 μM) for 4 hours. Immunoblots of PARP1 and phospho-S6 are shown. Duplicate lanes were loaded. Rapamycin (rapa) at 20 nM suppressed mTOR complex (mTORC) 1, as reflected by lower phospho-S6 levels, whereas PARP inhibitors, 3,4-dihydro-5[4-(1-piperidinyl)butoxy]-1(2H)-isoquinoline (DPQ) and 8-hydroxy-2-methylquinazoline-4-one (NU1025), had no effect on phospho-S6 levels. ** $P < 0.01$, Student's *t* test.

findings of the expression array, we first performed real-time PCR analysis. TSC2⁻ cells from patients with LAM exhibited a twofold increase of PARP1 ($P < 0.01$; Figure 3B). Importantly, rapamycin treatment did not alter the levels of PARP1, consistent with the changes identified in the expression array study (18) (Figure 3B). Torin 1 is a potent ATP-competitive mTORC1 and mTORC2 inhibitor. We treated cells with Torin 1 for 24 hours. Interestingly, Torin 1 treatment did not affect PARP1 expression (Figure 3B). Our data suggest that up-regulation of PARP1 expression is independent of mTOR.

We next assessed the protein levels of PARP1 using Western blot analysis. PARP1 protein levels were higher in TSC2⁻ cells from patients with LAM compared with TSC2⁺ cells (Figure 3C). Rapamycin treatment suppressed phosphorylation of the ribosomal protein p70S6 (S6), but did not affect PARP1 levels (Figure 3C). Similar observations were made in rat uterine leiomyoma-derived ELT3 cells (33, 34) expressing empty vector (TSC2⁻) relative to TSC2-re-expressing cells (TSC2⁺; Figure 3D). Collectively, our data show that TSC2 negatively regulates the expression of PARP1 in a rapamycin-insensitive manner.

Rapamycin only suppresses part of mTORC1 activity, and has little effect on mTORC2 (49, 50). Therefore, we tested whether PARP1 overexpression in TSC2⁻ cells may be due to other mTOR-associated pathways. mTOR or Rap (a component of mTORC1) was depleted by siRNA, resulting in significant suppression of phospho-S6 compared with nontargeting siRNA control (nonsense negative control; Figure 3E). However, PARP1 remained highly expressed in TSC2⁻ cells under mTOR or Rap siRNA knockdown (Figure 3E). To examine in greater detail whether Torin 1 treatment affects PARP1 protein levels, we performed a dose-response study of Torin 1 and found that Torin 1 from 0.25 nM to 1 μ M did not affect PARP1 protein levels in TSC2⁻ cells from patients with LAM (Figure 3F). Our data suggest that PARP1 overexpression is independent of mTORC1 or other mTOR components in cells lacking TSC2.

TSC2 Negatively Regulates PARP1 Expression *In Vivo*

To determine whether TSC2 regulates PARP1 expression *in vivo*, we first used

xenograft tumors from mice inoculated with TSC2⁻ ELT3-V3 (TSC2⁻) cells and TSC2-re-expressing ELT3-T3 (TSC2⁺) cells. Immunoblot analysis showed that TSC2 was detected in ELT3 (TSC2⁺) xenograft tumors, but not in ELT3 (TSC2⁻) tumors (Figure 4A, *left panel*). PARP1 protein levels were markedly higher in TSC2⁻ xenograft tumors with increased phospho-S6 compared with TSC2⁺ tumors (Figure 4A). We next examined spontaneously arising renal cystadenomas, commonly observed in *Tsc2*^{+/-} transgenic mice (36). Tumors exhibited abundant accumulation of PARP1 in the nucleus of tumor cells (Figure 4B). Adjacent normal kidney tubule cells were moderately positive for PARP1, but glomeruli were

negative for PARP1 in *Tsc2*^{+/-} mice and in *Tsc2*^{+/+} mice (Figure 4B).

To confirm these observations and to test the role of mTORC1 in PARP1 overexpression *in vivo*, *Tsc2*^{+/-} mice were subjected to short-term treatment with rapamycin (6 mg/kg every 2 d for 1 wk) or vehicle control before harvesting renal tumors for immunoblotting. Renal tumors from a *Tsc2*^{+/-} mouse treated with vehicle control exhibited high phospho-S6, as expected, reflecting mTORC1 activation compared with adjacent normal kidney tissue. Importantly, PARP1 expression is also noticeably higher in tumor relative to adjacent normal kidney (Figure 4C). Renal tumors from a *Tsc2*^{+/-} mouse treated with rapamycin exhibited suppressed

Figure 4. TSC2 negatively regulates the expression of PARP1 *in vivo*. (A) Immunoblots of PARP1 and phospho-S6 in xenograft tumors from TSC2⁻ rat uterine-derived cells (TSC2⁻) and TSC2⁺ cells. (B) *Tsc2*^{+/-} mice were treated with rapamycin (6 mg/kg/d, intraperitoneal) or vehicle control for 1 week. Representative microscopic images of immunohistochemistry of PARP1 on renal tumor sections are shown. Kidney cystadenomas stained heavily (dark brown) for PARP1, especially within the nuclei (arrows). Normal kidney tubules were also noted; tumors stained lightly for PARP1, whereas glomeruli stained negative. (C) Immunoblots of PARP1 and phospho-S6 in kidney cystadenomas (T) and adjacent normal kidney (N) from *Tsc2*^{+/-} mice. Tubulin was used as a loading control.

phospho-S6, confirming effective mTORC1 inhibition. In contrast, the levels of PARP1 expression remained high in two renal tumors compared with adjacent normal kidney tissues (Figure 4C). These data support the model that TSC2 deficiency leads to rapamycin-insensitive up-regulation of PARP1.

PARP1 Overexpression Is Evident in Pulmonary LAM Nodules

We then examined whether our *in vitro* and *in vivo* findings were relevant to human LAM. Accordingly, we analyzed publicly available expression array data sets from LAM cells collected by laser-capture microdissection from LAM nodules (Gene Expression Omnibus [GEO] data set GSE12027) (27), lung cancer/tumor (GEO data sets GSE10072 and GSE19804) (25, 26), and control lungs (GEO data sets GSE10072 and GSE19804) (25, 26). LAM nodule cells expressed higher levels of PARP1 transcript compared with control lungs (NL; $P < 0.01$; Figure 5A). Furthermore, immunoblotting analysis showed that phospho-S6–positive LAM lungs exhibited elevated levels of PARP1 protein relative to control lungs (NL; Figure 5B). Moreover, immunohistochemistry showed that nuclear accumulation of PARP1 is abundant in smooth muscle actin–positive LAM nodules (Figure 5C). Furthermore, we performed dual immunostaining of smooth muscle α -actin and PARP1 in LAM lung tissues. Smooth muscle α -actin–positive cells exhibited prominent nuclear localization of PARP1, indicating that PARP1 is indeed up-regulated in LAM cells, but not in pulmonary artery smooth muscle cells (Figure 5D).

Pharmacologic Inhibition of PARP1 Selectively Suppresses the Growth of TSC2[−] Cells

Having shown that mTORC1 inhibition has no effect on PARP1 expression, we asked whether PARP1 suppression could affect mTORC1 activation in TSC2[−] cells. As expected, rapamycin treatment suppressed phosphorylation of S6 (S240/244) and increased phosphorylation of Akt (S473; Figure 6A), consistent with previous reports of a negative-feedback inhibition between mTORC1 and Akt (28, 51, 52). Interestingly, DPQ and NU1025, two PARP inhibitors, did not affect phosphorylation of S6 at high doses (1 μ M or 10 μ M, respectively; Figure 6A). Importantly, NU1025 at 10 μ M modestly

Figure 5. PARP1 overexpression is evident in pulmonary LAM. (A) The public expression array data of LAM lung cells, lung tumors, and NLs were analyzed. The transcript levels of PARP1 are shown. (B) Immunoblots show the protein levels of PARP1 and phospho-S6 in LAM lung and NL tissues. (C) Immunohistochemical staining of smooth muscle actin (SMA) and PARP1 in pulmonary LAM nodules (LAM-1 and LAM-2). (D) LAM lung tissue sections stained for smooth muscle α -actin and PARP1. $^{**}P < 0.01$, Student's *t* test. DAPI, 4',6-diamidino-2-phenylindole; HE, hematoxylin and eosin stain; NL, normal lung.

increased phosphorylation of Akt (S473), suggesting that inhibition of PARP1 has no impact on mTORC1 activation in TSC2[−] cells, although possible interactions between Akt and PARP1 may warrant future studies.

To assess the efficacy of PARP inhibitors on PARP1 expression, we treated patient-derived cells with DPQ (1 and 10 μ M) and NU1025 (1 and 10 μ M) for 24 hours. Immunoblot analysis with PARP1 showed that PARP inhibitors did

Figure 6. Inhibition of PARP1 suppresses the growth of TSC2⁻ cells. (A) Immunoblots of PARP1, phospho-Akt, and phospho-S6 in TSC2⁻ patient-derived cells treated with DPQ or NU1025. (B) Immunoblots of PARP1, PAR, and β-actin in TSC2⁻ patient-derived cells. (C) Cell viability of patient-derived cells including TSC2⁻ and TSC2-addback cells treated with PARP1 inhibitors (1–30 μM DPQ; 1–30 μM Olaparib) with or without combination with rapamycin (20 nM). Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (D) Cell proliferation of patient-derived cells, including TSC2⁻ and TSC2-addback cells treated with PARP1 inhibitors (1–30 μM DPQ; 1–30 μM Olaparib) with or without combination with rapamycin (20 nM). Cell proliferation was measured using crystal violet assay. ***P* < 0.01, **P* < 0.05, Student's *t* test.

not affect the protein levels of PARP1 (Figure 6A), suggesting that the mechanism of action of PARP inhibitors is unlikely the suppression of PARP1 expression.

PARP1 activity is to catalyze the formation of PAR. To examine whether PARP1 inhibitors affect the activity of PARP1, we treated TSC2⁻ patient-derived cells with DPQ, NU1025, or Olaparib, and then performed immunoblot analysis for PAR. Treatment with 10 μM DPQ, 10 μM NU1025, or 1 μM and 10 μM Olaparib markedly decreased the levels of PAR compared with the control (Figure 6B), indicating that PARP

inhibitors suppress the activity rather than the expression of PARP1, consistent with other findings (53).

We then tested the possible dose-dependent cytotoxic effect of PARP1 inhibitors, DPQ (1–30 μM) and Olaparib (1–30 μM), in TSC2⁻ patient-derived cells and their TSC2-re-expressing counterparts, and performed side-by-side comparison of the drug effects in cell viability. DPQ or Olaparib (30 μM) selectively decreased cell viability by 15–40% (*P* < 0.01; Figure 6C) and proliferation (Figure 6D) of TSC2⁻ cells. Importantly, TSC2⁺ cells were resistant to Olaparib and DPQ treatment

(Figures 6C and 6D). We noted that rapamycin treatment up to 24 hours had no appreciable cytotoxic effect on cell growth (Figures 6C and 6D).

To determine whether mTORC1 and PARP1 pathways intersect, we treated TSC2⁻ cells from patients with LAM with rapamycin plus PARP1 inhibitors, DPQ or Olaparib, and then measured cell viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, cell proliferation using crystal violet staining, and cell death using propidium iodide exclusion assay. PARP1 inhibition marginally decreased cell viability

Figure 7. Inhibition of PARP1 promotes apoptosis of TSC2⁻ cells. (A) Cell death of patient-derived cells, including TSC2⁻ and TSC2⁺ cells treated with PARP1 inhibitors (1–30 μ M DPQ; 1–30 μ M Olaparib) with or without combination with rapamycin (20 nM). Cell death was measure using propidium iodide (PI) exclusion assay. (B) Cell death of patient-derived cells, including TSC2⁻ and TSC2-addback cells treated with PARP1 inhibitors (1–30 μ M DPQ; 1–30 μ M Olaparib) with or without combination with H₂O₂ (0.5 mM). Cell death was measure using PI exclusion assay. (C) Patient-derived

(Figure 6C) and cell proliferation (Figure 6D). Interestingly, combination treatment of rapamycin and PARP1 inhibitor did not further decrease cell viability (Figure 6C) or cell proliferation (Figure 6D) compared with PARP1 inhibitor alone. These data indicate that there is no synergistic effect between rapamycin and PARP1 inhibitors in the growth of TSC2⁻ cells from patients with LAM.

Pharmacologic Inhibition of PARP1 Promotes Apoptosis of TSC2⁻ Cells

It has been reported that PARP1 plays a role in repair of oxidative stress-induced DNA damage and cell survival (54). To examine whether PARP1 inhibitors affect cell survival, TSC2⁻ and TSC2-re-expressing patient-derived cells were treated with 10 and 30 μ M DPQ or 30 μ M Olaparib. Both drugs selectively killed TSC2⁻ cells without affecting the survival of TSC2-re-expressing cells (Figure 7A). Next, we treated TSC2⁻ patient-derived cells and TSC2-re-expressing cells with 1–30 μ M DPQ or Olaparib in the presence of H₂O₂. H₂O₂ treatment alone induced apoptosis to the same extent in both TSC2⁻ and TSC2-re-expressing cells (Figure 7B). Interestingly, in the presence of H₂O₂, 3–30 μ M DPQ or Olaparib significantly induced the death of TSC2⁻ cells by roughly 50% ($P < 0.01$; Figure 7B). Notably, PARP inhibitors plus rapamycin treatment did not further induce cell death, indicating no synergistic effect between rapamycin and PARP1 inhibitors in the survival in TSC2⁻ cells from patients with LAM.

To quantify the levels of cell death triggered by H₂O₂ exposure in TSC2⁻ patient-derived cells, we performed a cytometric Annexin V and propidium iodide assay. TSC2⁻ cells treated with DPQ exhibited 33% higher population of apoptotic cells compared with control (67.3 versus 50.7%; Figure 7C). In addition, TSC2⁻ cells treated with NU1025 showed 35% higher population of apoptotic cells compared with control (68.3% versus

50.7%; Figure 7C). Together, these data indicate that inhibition of PARP1 suppresses the growth and promotes cell death of cells derived from patients with LAM under oxidative stress.

Discussion

PARP1 is activated by DNA damage and plays a role in chromosomal repair and stability (19, 20). Recently, PARP inhibitors have regained attention as possible chemotherapeutic agents in human cancers (55). We report here, for the first time, that TSC2⁻ cells demonstrate increased PARP1 expression *in vitro* and *in vivo*, and that the increase is rapamycin insensitive and mTOR independent. Furthermore, TSC2⁻ cells exhibited higher cytotoxicity to PARP inhibition compared with TSC2⁺ cells.

Our data suggest that PARP1 up-regulation is likely a direct consequence of TSC2 loss, because, in several human or animal-derived TSC2⁻ cells/tissues, PARP1 up-regulation can be documented. PARP1 expression can only be suppressed by TSC2 reconstitution, and is not affected by mTOR inhibition from rapamycin or Torin 1 treatments, or mTOR or Rap siRNA interference. Therefore, TSC2 deficiency upstream of mTOR pathway is the culprit leading to PARP1 up-regulation (Figure 7D).

Several studies have documented possible mTORC1-independent effects in cells lacking TSC1/TSC2. B-Raf kinase activity is reduced in TSC2⁻ cells due to Rheb-GTP activation, which interacts with B-Raf and inhibits its activity in an mTORC1-independent manner (56, 57). Tsc2-null MEFs exhibit reduced Akt kinase activation partially due to impaired mTORC2 activity (16). Tsc1- or Tsc2-null MEFs have a higher percentage of cilium-containing cells compared with the respective controls, and rapamycin treatment has no effect on this observation (17). We previously found that matrix metalloproteinase overexpression in TSC2⁻

cells is resistant to rapamycin suppression of mTORC1 (18). Recently, we found that TSC2 negatively regulates cyclooxygenase-2 expression and prostaglandin production in a rapamycin-insensitive, but mTORC2-dependent, manner (38). The current report adds to the accumulating evidence that mTORC1-independent, aberrantly regulated pathways contribute to TSC-related diseases, and that rapamycin alone may be insufficient as a clinical therapy.

In this study, we found that the levels of ROS were higher in TSC2⁻ patient-derived cells relative to TSC2⁺ cells, consistent with the previous findings in Tsc2-null rat-derived ELT3 cells (39). We also found that the molecular marker of DNA damage, γ H2A.X, was more prominent in TSC2-re-expressing cells relative to TSC2⁻ cells, indicative of a dysfunctional DNA damage repair mechanism in cells lacking TSC2. ROS is a main contributor of oxidative DNA damage (40, 41). PARP family members play a critical role in repair of single-stranded DNA breaks (42, 43). Our findings of PARP1 up-regulation and efficacy of PARP inhibitor in limiting the survival of cells from patients with LAM indicate a therapeutic potential of targeting PARP1 as a curative treatment for LAM.

Compared with TSC2⁺ cells, TSC2⁻ cells exhibited higher sensitivity to the cytotoxic effect of PARP1 inhibition. Rapamycin did not enhance, and actually could antagonize, this effect. Therefore, our data suggest that unsuppressed mTOR activity from loss of TSC2 may not be critical for the survival of TSC2⁻ cells, and that PARP1 up-regulation is important for the viability of TSC2⁻ cells with mTOR hyperactivation. Our data may also have implications in oncologic therapy. We speculate that PARP1 inhibitors may be particularly effective in cancer types exhibiting increased mTOR activity; thus, the status of mTOR activity may serve as a predictive biomarker for PARP1 inhibitors.

Figure 7. (Continued). TSC2⁻ and TSC2⁺ cells were treated with NU1025 (10 μ M) or DPQ (10 μ M) for 24 hours, and then treated with H₂O₂ (0.5 mM) for 1 hour. Cell death was analyzed for PI and Annexin V staining by flow cytometry. Percentage of PI-positive, Annexin V-positive, or double-positive cells was summarized. ** $P < 0.01$, * $P < 0.05$, Student's *t* test. (D) Model demonstrating the role of PARP1 in LAM pathogenesis. Loss of TSC2 results in hyperactivation of mTOR, increased ROS production, and uncontrolled cell proliferation. Rapamycin inhibits mTOR activity and suppresses cell proliferation. ROS causes DNA damage in TSC2⁻ cells, leading to PARP1 expression. PARP1 repairs DNA damage and enhances cell survival under oxidative stress. Inhibition of PARP1 leads to death of cells derived from patients with LAM, indicating that this approach has therapeutic potential.

The Multicenter International LAM Efficacy of Sirolimus Trial demonstrated that the mTORC1 inhibitor, Sirolimus, stabilizes lung function and improves quality of life in women with LAM. However, upon drug withdrawal, lung function decline resumed (58), indicating that mTORC1 inhibitor has a cytostatic, but not cytotoxic, effect in LAM cells. We report here promising preclinical results using PARP1 inhibitors that specifically suppress the growth and survival

of TSC2[−] cells from patients with LAM. In conclusion, we report that PARP1 expression is elevated in TSC2[−] patient-derived cells compared with TSC2[−]-re-expressing cells, and that PARP inhibitors selectively suppress the proliferation of TSC2[−] cells. Our data add to a growing list of U.S. Food and Drug Administration–approved agents with potential efficacy in LAM, including simvastatin (59), chloroquine (60, 61), doxycycline (62), and aspirin (38). We

anticipate that PARP1 inhibitors may provide a novel opportunity for the therapy of LAM. ■

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