

PTEN inhibitor VO-OHpic suppresses TSC2^{-/-} MEFs proliferation by excessively inhibiting autophagy via the PTEN/PRAS40 pathway

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Abstract. Tuberous sclerosis complex (TSC) is a relatively rare autosomal dominant disease which involves multiple organs, including the brain, kidney, lung, skin and heart. Renal angiomyolipomas (RAML) are the main causes of mortality in patients with TSC. The preferred treatment for RAML is the use of mTOR inhibitors, but the efficacy of these are not satisfactory. Therefore, an alternative treatment is urgently required. Autophagy levels decline in TSC associated cortical tubers, and the inhibition of autophagy in animal or cell models of TSC may suppress tumor development and cell proliferation. PTEN is a protein tyrosine phosphatase and can inhibit the activation of Akt. In the present study, it was indicated that the PTEN inhibitor, hydroxyl(oxo)vanadium 3-hydroxypiridine-2-carboxylic acid (VO-OHpic), suppressed proliferation and growth of TSC2^{-/-} murine embryonic fibroblasts (MEFs) by further inhibiting autophagy of cells. The expression levels of human microtubule-associated protein 1 light chain 3-I (LC3-I) and LC3-II, which are autophagy associated proteins, were demonstrated to decline following VO-OHpic treatment. The expression levels of phosphorylated proline-rich Akt substrate 40 kDa (PRAS40) also decreased in TSC2^{-/-} MEFs treated with VO-OHpic. The PTEN inhibitor may inhibit the proliferation of TSC2^{-/-} MEFs through the PTEN-PRAS40 pathway by excessively inhibiting autophagy, without the dependence of the Ras homolog, mTORC1 binding/mTOR pathway. PTEN may be a potential therapeutic target for the treatment of TSC. Further *in vivo* studies are required to confirm these results.

Introduction

Tuberous sclerosis complex (TSC) is a relatively rare autosomal dominant disorder, which can involve multiple organs, including brain, kidney, lung, skin and heart (1). The typical manifestations of TSC in the kidneys include angiomyolipomas (AMLs), multiple renal cysts and renal carcinomas (2). Renal AML (RAML) is present in ~70-90% of patients with TSC, and it is the leading cause of mortality in adult patients with the disorder (3). The recommended first-line therapy for TSC-RAML is the use of mTOR inhibitors. However, ~30-40% patients using mTOR inhibitors do not achieve clinical remission, which is defined as a RAML volume reduction $\geq 50\%$ and severe adverse reactions may also occur in some patients (4,5). Despite these issues, there are currently no other treatment options for RAML (5). The critical role of autophagy in the formation and development of tumors has become increasingly recognized (6). Autophagy influences the rearrangement of the plasma membrane, leading to the sequestration of proteins and organelles intended for delivery into lysosomes for degradation (7). Autophagy is a highly conserved catabolic process of cellular proteins and organelles in which they are sequestered into lysosomes for degradation to maintain cellular homeostasis (8). There are three types of autophagy, including microautophagy, macroautophagy and chaperone-mediated autophagy (7), in which macroautophagy is among the most researched. During macroautophagy, the membrane expands to form phagophores, which expand and bend continuously. The membrane then wraps around the cargo to form complete autophagosomes, which is then delivered into the lysosomes for degradation where the materials are then reused (7). Human microtubule-associated protein 1 light chain 3 (LC3) is an important protein for autophagosome formation (7). LC3 is normally spliced further into LC3-I and LC3-II, which is mainly localized in the cytoplasm and on the autophagosomal double membrane, respectively (9). The levels of LC3-I and II can therefore be used to reflect the degree of autophagy. Miyahara *et al* (9) previously demonstrated in the cerebral cortical tubers of patients with TSC that the levels of LC3-I and LC3-II were lower compared with those in normal cerebral tissues. Autophagy may promote or

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Key words: tuberous sclerosis complex, tuberous sclerosis complex 2^{-/-} murine embryonic fibroblasts, autophagy, PTEN, hydroxyl(oxo)vanadium 3-hydroxypiridine-2-carboxylic acid

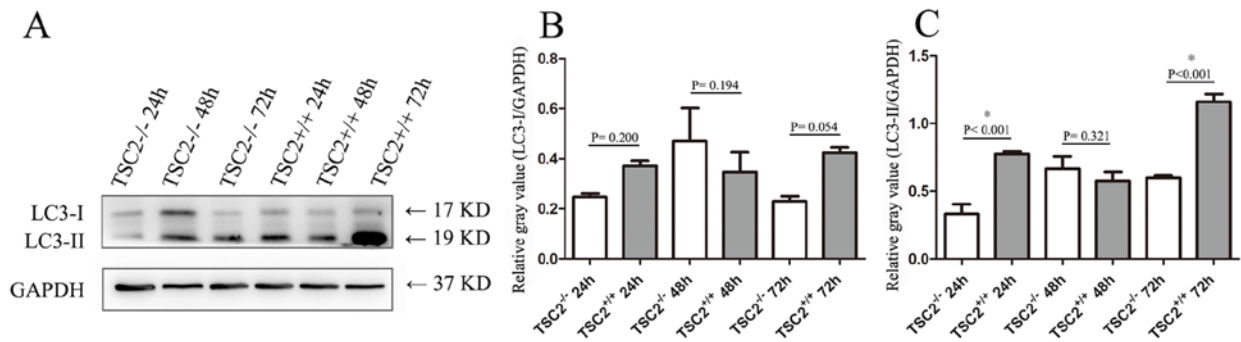


Figure 1. The autophagy levels in TSC2^{-/-} and TSC2^{+/+} MEFs. (A) Western blot analysis of LC3-I and LC3-II in TSC2^{-/-} and TSC2^{+/+} MEFs. (B) Average relative gray values of LC3-I at the 24, 48 and 72 h. (C) Average relative gray values of LC3-II at the 24, 48 and 72 h. The stated time point represents the time point at which the MEFs were harvested. *P<0.001. MEF, murine embryonic fibroblasts; TSC, tuberous sclerosis complex; LC3, human microtubule-associated protein 1 light chain 3.

inhibit tumorigenesis, depending on the cellular context (6). Autophagic dysfunction can lead to a variety of human pathologies, including cancer, metabolic diseases and degenerative conditions (10). In Myc-induced lymphomas, chloroquine may inhibit autophagy and the fusion of autophagosomes and lysosomes, resulting in the suppression of lymphoma development (11). Haploinsufficiency of the autophagy gene, Beclin 1, promotes tumor formation in the TSC2^{-/-} mouse model (6) and allelic loss of Beclin 1 may be associated with breast, ovarian and prostate cancers (12).

mTOR also participates in the regulation of autophagy by inhibiting the formation of the autophagosome (13). Ras homolog-enriched in brain (Rheb) is a principal downstream target of the TSC1/TSC2 complex and an upstream regulator of mTOR. When TSC1 or TSC2 mutates, Rheb can be abnormally activated and promote mTOR activation to downregulate autophagy (13). PTEN is an upstream regulator of Akt that regulates the TSC1/TSC2 complex (14,15), but has also been revealed to potentially regulate autophagy via a different signaling pathway independent of TSC1/TSC2 (15). The current study indicated that the PTEN inhibitor, hydroxyl(oxo)vanadium 3-hydroxypyridine-2-carboxylic acid (VO-OHpic), could suppress the proliferation of TSC2^{-/-} murine embryonic fibroblasts (MEFs) by excessively inhibiting autophagy via the PTEN/proline-rich Akt substrate 40 kDa (PRAS40) pathway.

Materials and methods

Cell culture. The TSC2^{-/-} and TSC2^{+/+} MEF cell lines, as aforementioned (16), were donated by Professor Hongbing Zhang, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). The cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere. The TSC2^{-/-} MEFs were used as two groups: i) With VO-OHpic treatment at 1 μM as previously described (17); or ii) without VO-OHpic treatment. All cultured TSC2^{-/-} and TSC2^{+/+} MEFs were harvested at 24, 48 and 72 h, respectively, for further analysis.

Cell proliferation analysis. Cell proliferation assays were conducted using a Cell Counting kit-8 (CCK-8; Beijing

Solarbio Science & Technology Co., Ltd.; cat. no CA1210) according to the manufacturer's protocol. The harvested cells (5,000 cells/well) were seeded in 96-well plates at 100 μl per well and cultured in growth medium at 37°C and 5% CO₂. A total of 10 μl of CCK-8 were added to each well at 24, 48 and 72 h. Subsequently, the cells were incubated at 37°C for 3 h and the optical density was measured at 450 nm.

Western blot analysis. The cells were stored at -80°C, then proteins were subsequently extracted with protein lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.; cat. no. R0020). The protein concentration was standardized using a BCA protein assay kit (Beijing Solarbio Science & Technology Co. Ltd.; cat. no. PC0020). A total of 30 μg protein extracts from the various groups (with or without VO-OHpic treatment, at 24, 48 or 72 h, respectively) were loaded into a 15% SDS-PAGE gel and transferred onto PVDF membranes. The membranes were blocked with 5% skimmed milk in Tris-buffered saline with Tween 20 (0.01 M Tris-HCl, pH 7.6; 0.15 M NaCl; 0.05% Tween 20) for 2 h at room temperature. The membranes were incubated with rabbit anti-LC3 antibodies Abs (Sigma-Aldrich; Merck KGaA; cat. no. L8918; 1:2,000), rabbit anti-phosphorylated (p)-PTEN Abs (Abcam; cat. no. ab31392; 1:1,000), rabbit anti-PTEN Abs (Abcam; cat. no. ab32199; 1:1,000), rabbit anti-p-PRAS40 Abs (Abcam; cat. no. ab134084; 1:1,000) or rabbit anti-PRAS40 Ab (Abcam; cat. no. ab151719; 1:1,000) overnight at 4°C. GAPDH antibodies were used as an internal control (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.; cat. no. TA-08; 1:1,000). The bound primary Ab was detected with horseradish peroxidase-linked goat anti-rabbit immunoglobulin G (IgG; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.; cat. no. ZB-2301; 1:10,000). The secondary incubation was conducted at room temperature for 1 h. Subsequently, the antigen-antibody complexes were detected using ECL (Tanon Science & Technology Co. Ltd.). Gray values were calculated using Image J 1.50i software (National Institutes of Health).

Statistical analysis. All of the data were analyzed using SPSS version 19.0.0 (IBM Corp.) and GraphPad Prism version 6.0 (GraphPad Software, Inc.). The results are presented as the mean ± SEM. Differences between groups were analyzed

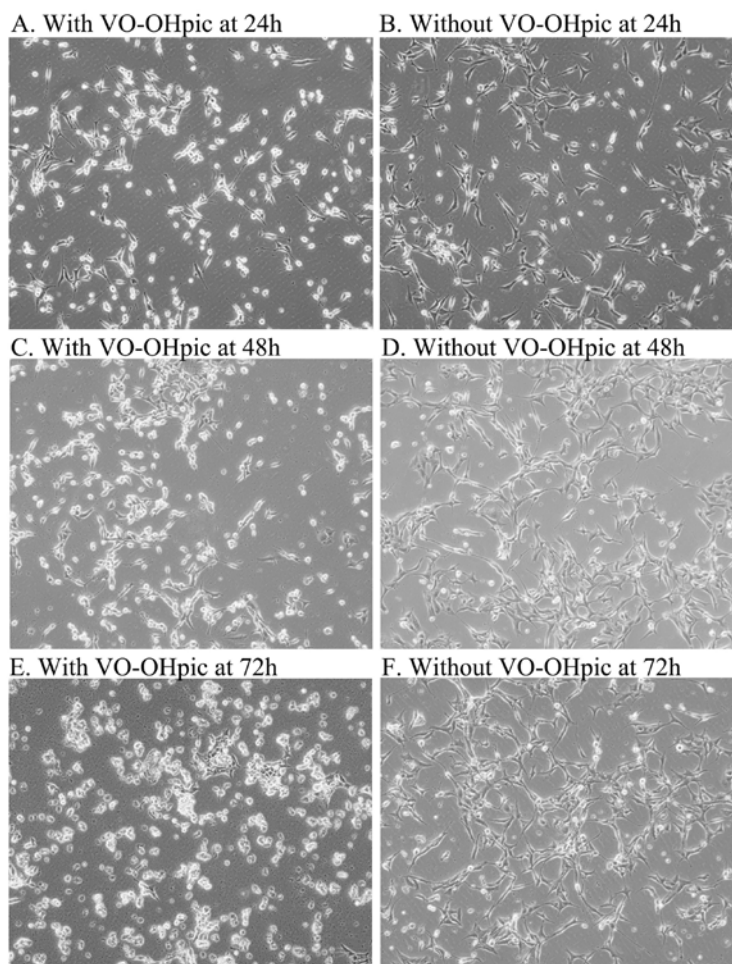


Figure 2. Different morphological features of $TSC2^{-/-}$ MEFs with or without VO-OHpic treatment at 24, 48 and 72 h. (A) $TSC2^{-/-}$ MEFs with VO-OHpic ($1 \mu M$) treatment at 24 h. (B) $TSC2^{-/-}$ MEFs without VO-OHpic treatment at 24 h. (C) $TSC2^{-/-}$ MEFs with VO-OHpic ($1 \mu M$) treatment at 48 h. (D) $TSC2^{-/-}$ MEFs without VO-OHpic treatment at 48 h. (E) $TSC2^{-/-}$ MEFs with VO-OHpic ($1 \mu M$) treatment at 72 h. (F) $TSC2^{-/-}$ MEFs without VO-OHpic treatment at 72 h. The application of VO-OHpic appeared to lead to a reduction in the density of the cells and in the cell volume at each time point. The MEFs which had not received VO-OHpic treatment presented with larger cell bodies and nuclei; more obvious processes; and the cells appeared denser. magnification, x200. MEF, murine embryonic fibroblasts; TSC, tuberous sclerosis complex; VO-OHpic, hydroxyl(oxo)vanadium 3-hydroxypyridine-2-carboxylic acid.

using two-way ANOVAs followed by post-hoc Tukey's tests. $P < 0.05$ was considered to indicate a statistically significant difference. All experiments were repeated at least three times.

Results

To confirm the autophagic conditions in $TSC2^{-/-}$ MEFs, the expression levels of LC3 were analyzed. $TSC2^{-/-}$ and $TSC2^{+/+}$ MEFs were harvested at 24, 48 and 72 h, and western blot analysis was performed (Fig. 1). Overall, LC3 expression in $TSC2^{-/-}$ MEFs was reduced compared with that in $TSC2^{+/+}$ MEFs at 24 and 72 h. Two-way ANOVA analysis indicated that neither $TSC2^{+/+}/TSC2^{-/-}$ nor the time point of collection, had a significant effect on LC3-I expression levels ($P = 0.245$ and $P = 0.291$, respectively; data not shown). Considering the effect of $TSC2^{+/+}/TSC2^{-/-}$, no statistically significant differences were found (All $P > 0.05$; Fig. 1B). By contrast, both $TSC2^{+/+}/TSC2^{-/-}$ and the collection time had a significant effect on LC3-II expression (both $P < 0.001$). When considering the effect of $TSC2^{+/+}/TSC2^{-/-}$, except for the cells at 48 h, LC3-II was expressed at a significantly lower level in $TSC2^{-/-}$ MEFs (24 h, $P < 0.001$; 72 h, $P < 0.001$; Fig. 1C).

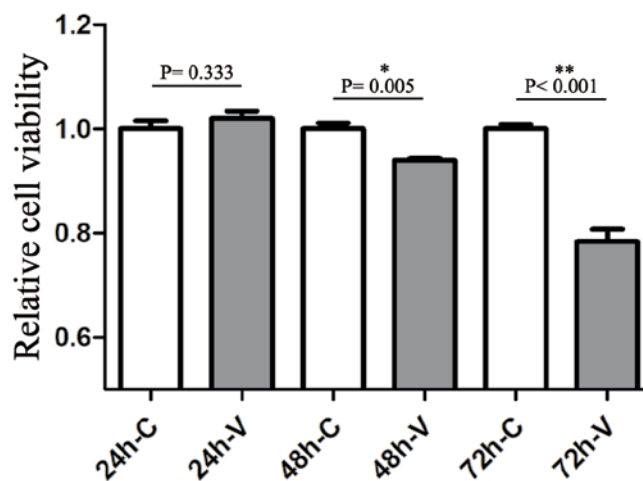


Figure 3. Quantitative analysis of the proliferation of $TSC2^{-/-}$ MEFs with or without VO-OHpic treatment. The results of the Cell Counting kit-8 assay revealed a downwards trend in the relative cell viability of $TSC2^{-/-}$ MEFs treated with VO-OHpic compared with $TSC2^{-/-}$ MEFs without VO-OHpic treatment over time. $^*P < 0.01$ and $^{**}P < 0.001$. C, control; MEF, murine embryonic fibroblasts; TSC, tuberous sclerosis complex; V, with VO-OHpic treatment; VO-OHpic, hydroxyl(oxo)vanadium 3-hydroxypyridine-2-carboxylic acid.

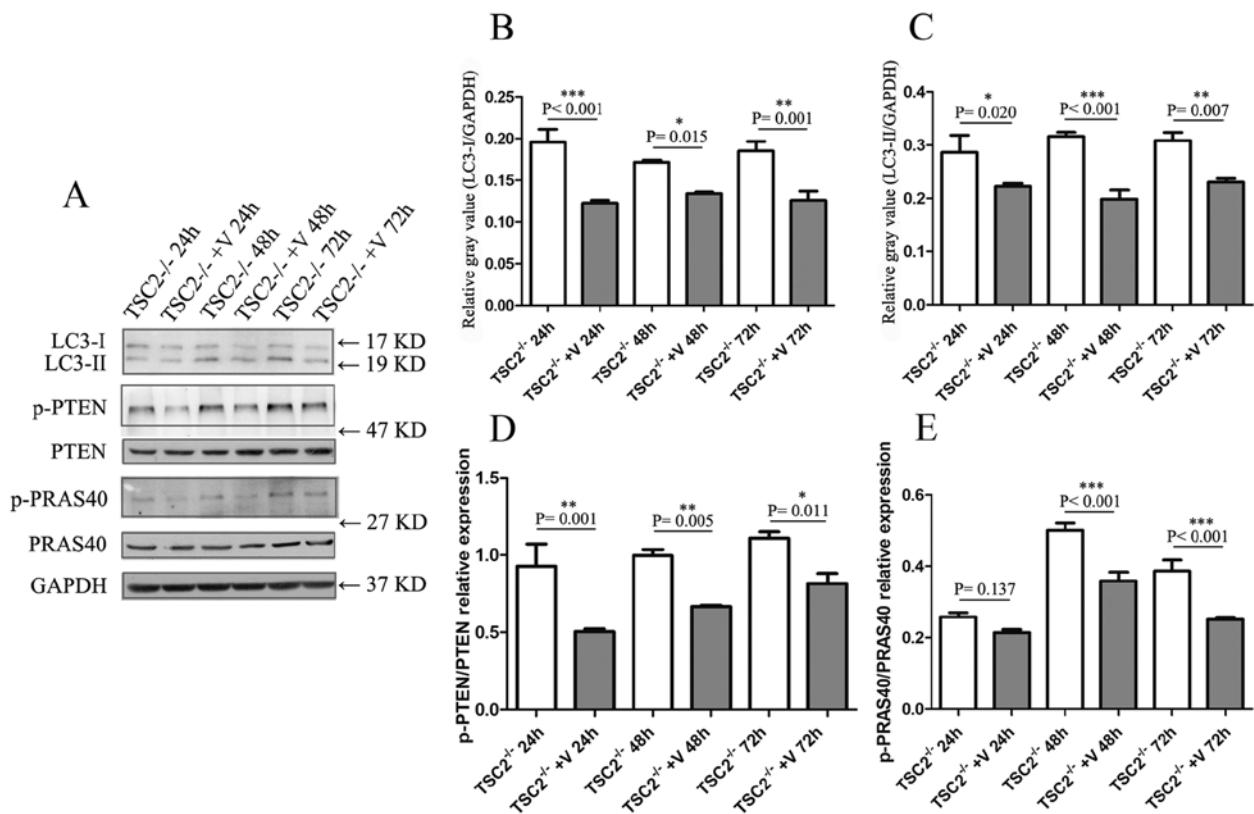


Figure 4. The autophagy levels and related molecular pathways assessed in TSC2^{-/-} MEFs that received VO-OHPic treatment. (A) Western blot assays of LC3, p-PTEN and p-PRAS40 in TSC2^{-/-} MEFs, with or without VO-OHPic treatment. Quantitative analysis of (B) LC3-I and (C) LC3-II expression levels in TSC2^{-/-} MEFs, with or without VO-OHPic treatment. Quantitative analysis of (D) p-PTEN/PTEN and (E) p-PRAS40/PRAS40 relative expression levels in TSC2^{-/-} MEFs, with or without VO-OHPic treatment. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. VO-OHPic, hydroxyl(oxo)vanadium 3-hydroxypyridine-2-carboxylic acid; +V, VO-OHPic treatment; MEF, murine embryonic fibroblasts; p, phosphorylated; TSC, tuberous sclerosis complex; LC3, human microtubule-associated protein 1 light chain 3; PRAS40, proline-rich Akt substrate 40 kDa.

Subsequently, a PTEN inhibitor, VO-OHPic, was added to TSC2^{-/-} MEFs and the effect on cell proliferation and growth was observed. At various time points, the cells that underwent VO-OHPic treatment demonstrated a reduced volume and a decrease in their density (Fig. 2). The MEFs displayed larger cellular bodies and nuclei, with more obvious processes, and the cells grew at a greater density when VO-OHPic was not applied. VO-OHPic treatment led to atrophy of the cell bodies and nuclei of MEFs and a reduction in the size and number of processes. CCK-8 assays were also performed for quantitative analysis. A two-way ANOVA revealed that both the intervention and the time points of collection had a significant effect on the extent of cell viability (both, $P < 0.001$). When considering the effect of the intervention, VO-OHPic led to a reduction in cell viability (with vs. without VO-OHPic treatment: 24 h, $P = 0.333$; 48 h, $P = 0.005$; 72 h, $P < 0.001$; Fig. 3).

To further analyze the effects of the PTEN inhibitor on autophagy of TSC2^{-/-} MEFs, the LC3 levels of cells treated with or without VO-OHPic, were examined (Fig. 4). Two-way ANOVA analysis showed that the intervention had a significant effect on LC3-I expression levels (overall, $P < 0.001$; intervention vs. control: 24 h, $P < 0.001$; 48 h, $P = 0.015$; and 72 h, $P = 0.001$); however, this did not change with time ($P = 0.800$). Similar results were indicated for LC3-II expression levels (overall intervention, $P < 0.001$; overall time, $P = 0.644$; intervention vs. control: 24 h, $P = 0.020$; 48 h, $P < 0.001$; and 72 h, $P = 0.007$).

The potential pathways which allowed for this change were subsequently investigated. p-PTEN and PTEN levels were examined in TSC2^{-/-} MEFs under various conditions (with or without VO-OHPic treatment at 24, 48 or 72 h). The relative expression ratios of p-PTEN/PTEN were then calculated. Two-way ANOVA analysis revealed that both interventions and time points had a significant effect on the p-PTEN/PTEN expression ratio (interventions, $P < 0.001$; time points, $P = 0.013$). When considering the effect of the PTEN inhibitor intervention, the relative expression ratio of p-PTEN/PTEN declined in TSC2^{-/-} MEFs which were treated with VO-OHPic (Fig. 4), reflecting the decrease in the activity of the cells (intervention vs. control: 24 h, $P = 0.001$; 48 h, $P = 0.005$; and 72 h, $P = 0.011$; Fig. 4). The activity of PRAS40, which is a downstream regulator of PTEN, was also affected by treatment with VO-OHPic. Both interventions and time point measured had a significant effect on p-PRAS40/PRAS40 expression ratio, as indicated by two-way ANOVA analysis (both, $P < 0.001$). When considering the effect of the intervention, the relative expression ratio of p-PRAS40/PRAS40 decreased in TSC2^{-/-} MEFs treated with VO-OHPic (intervention vs. control: 24 h, $P = 0.137$; 48 and 72 h, $P < 0.001$; Fig. 4).

Discussion

TSC-associated RAML is a life-threatening disease in adult patients (3). Recently, mTOR inhibitors such as everolimus are

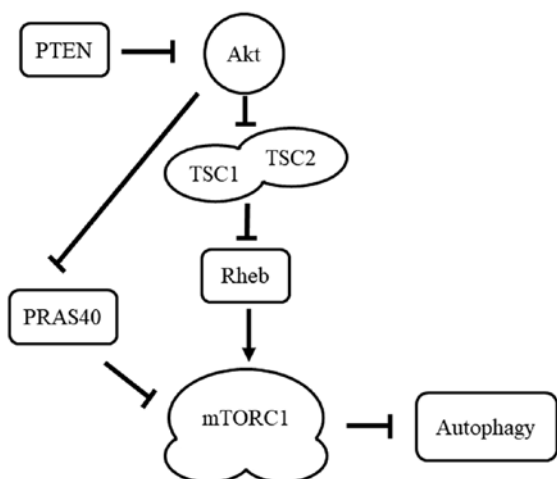


Figure 5. PTEN may regulate autophagy by allowing for a bypass of PTEN/PRAS40 signaling in TSC2^{-/-} cells and influence cell proliferation. PTEN could suppress the activation of mTOR and promote autophagy not only by inhibiting Akt/Rheb/mTOR pathway, but also by activating the PTEN/PRAS40 pathway.

the preferred treatment option for TSC-RAML (4). However, in addition to severe adverse reactions associated with these treatment options, ≤40% patients don't respond effectively to mTOR inhibitors (4,5). Therefore, finding an alternative therapeutic strategy for TSC-RAML is of urgent importance. The present study revealed that PTEN inhibitors can suppress TSC2^{-/-} MEFs proliferation by inhibition of autophagy through the PTEN/PRAS40/mTOR pathway, bypassing the PTEN/Akt/Rheb/mTOR pathway. These observations provide a research basis for further feasibility study of PTEN inhibitor in TSC treatment.

mTORC1 is a complex, which contains mTOR, and the Rheb-mTORC1 pathway activates the translation of mRNA (18). The excessive activation of mTORC1 has been revealed to participate in a number of different diseases, including lung cancer, diabetes, neurodegeneration and age-related diseases (19). Overactive mTORC1 can perturb ribosome synthesis and reduce the accuracy of translation, therefore leading to the production and accumulation of abnormal proteins (19). Hamartin and tuberlin negatively regulate mTORC1 and GAP negatively regulates Rheb (18). In a study on patients with TSC and in TSC animal models, the overactive mTORC1 pathway was revealed to be inhibited using rapamycin (20,21). Rapamycin and its homologs are used in the clinical treatment for TSC associated RAML, subependymal giant cell astrocytoma and lymphangioliomyomatosis (4,6,22-24). However, rapamycin is not a cytotoxic agent, but a cytostatic agent; thus, cannot be used to eliminate RAML. The tumors will recover following the withdrawal of treatment; therefore, alternative medical strategies are required (4,5).

In a previous study investigating TSC and autophagy, Parkhitko *et al* (6) reported that chloroquine, which may function by preventing the fusion of autophagosomes and lysosomes, could prevent the proliferation of TSC2^{-/-} MEFs. It was also demonstrated that in TSC2^{-/-} xenograft tumors or renal tumors from TSC2^{+/-} mice, rapamycin combined with chloroquine presented a stronger inhibitory effect than either single drug. Beclin 1 knockdown has also been demonstrated

to inhibit the development of the tumors (6). It is hypothesized that there is a balance between the inhibition and activation of autophagy in TSC tumors (6). The inhibition of autophagy leads to the accumulation of P62/SQSTM1 in TSC2^{-/-} MEFs, which initiates the development of TSC tumors (6). Autophagy cannot be induced adequately with numerous metabolic defects existing in TSC2^{-/-} MEFs; therefore, further suppression of autophagy may exhibit an inhibitory effect on tumor development. Therefore, the development of TSC tumors may also depend on autophagy.

PTEN is a highly conserved dual-function protein tyrosine phosphatase, which can dephosphorylate both inositol lipids and proteins. PTEN can dephosphorylate the lipid second messengers phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate to produce phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate, respectively, leading to the inhibition of Akt activation (14). VO-OHpic is a specific and reversible small molecule inhibitor of PTEN (14). PRAS40 is a component of the mTORC1 complex and is also a downstream target of Akt (25). However, p70 ribosome S6 kinase and 4E-binding protein 1, substrates of mTORC1, can combine with mTORC1, thereby competing with PRAS40 (26). The inhibition of PRAS40 increases the frequency of interactions between mTORC1 and its substrates, and suppresses autophagy by upregulating mTOR-p70S6K, resulting in further mTORC1 activation and autophagy inhibition (26).

In a study by Nardella *et al* (15) on prostate cancer, PTEN deficiencies promoted prostate tumor formation in Rheb overexpressing mice. The aforementioned study indicated that PTEN not only regulates autophagy through the Akt/Rheb/mTOR pathway, but also through the Akt/PRAS40 pathway (Fig. 5). In the present study, the effect of PTEN inhibitors on autophagy in TSC2^{-/-} MEFs was investigated. It was revealed that the PTEN inhibitor, VO-OHpic, could suppress autophagy, cell proliferation and cell growth in TSC2^{-/-} MEFs. The expression of p-PTEN and p-PRAS40 also declined, revealing that VO-OHpic may regulate autophagy via the PTEN-PRAS40 pathway. The results are based on *in vitro* experiments, and further *in vivo* studies are required to validate these results. The further downstream proteins also require investigation.

In conclusion, the PTEN inhibitor, VO-OHpic, may suppress the proliferation of TSC2^{-/-} MEFs, potentially through the excessive inhibition of autophagy via the PTEN/PRAS40 pathway, and may be a potential therapeutic target for the treatment of TSC-RAML.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

YZ and HL conceived and designed the experiments. WW, XW and HG performed the cellular experiments. YC provided and performed the cell culture. WW produced the manuscript. WW and YZ conducted data analysis. All the authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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