Induction by rapamycin and proliferation-promoting activity of Hspb1 in a *Tsc2*-deficient cell line

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Abstract. Tuberous sclerosis complex (TSC) is an intractable inherited disease caused by a germline mutation in either the TSC complex subunit 1 (*TSC1*) or *TSC2* tumor suppressor genes. Recent progress in the treatment of TSC with rapamycin has provided benefits to patients with TSC. However, the complete elimination of tumors is difficult to achieve as regrowth often occurs after a drug is suspended; thus, more efficient medication and novel therapeutic targets are required. To overcome tumor remnants in the treatment of TSC, the present study investigated rapamycin-responsive signaling pathways in *Tsc2*-deficient tumor cells, focusing on heat shock protein-related pathways. The expression levels of heat shock protein family B (small) member 1 (Hspb1; also known as HSP25/27) were increased by rapamycin treatment. The phosphorylation of Hspb1 was also increased. The knockdown of *Hspb1* suppressed cell proliferation in the absence of rapamycin, and the overexpression of Hspb1 enhanced cell proliferation both in the presence and absence of rapamycin. Pathways associated with Hspb1 may present target candidates for treatment of TSC.

Introduction

Tuberous sclerosis complex (TSC) is a hereditary intractable disease characterized by various neoplastic lesions and psychoneurotic symptoms. Currently, mTOR inhibitors (rapamycin and its derivatives) are used to treat TSC due to the fact that loss of the causative gene (*TSC1* or *TSC2*) (1,2) results in the increased activity of mTOR kinase complex 1 (mTORC1) in the pathological process of TSC (3-8). While therapeutic benefits have been obtained to some extent in the reduction of neoplastic lesions (9,10), the lack of complete tumor eradication (9), the side effects associated with long-term drug administration (such as interstitial pneumonia, severe stomatitis and immunosuppression) (11), and the sporadic occurrence of unresponsive cases has led to the urgent need to develop new therapeutic options.

Numerous studies have attempted to identify new drug targets for TSC in order to improve treatment. For example, rapamycin has been shown to induce feedback activation of pro-oncogenic kinases upstream of mTORC1, such as Akt and mitogen-activated protein kinase (MAPK), in TSC gene-deficient tumor cells (12-14). These kinases promote cell proliferation in rapamycin-treated TSC gene-deficient tumor cells (15). Studies have shown that endoplasmic (ER) stress is involved in the mechanism underlying negative feedback regulation by hyper-activated mTORC1 (16). Autophagy is activated by rapamycin independent of such feedback regulation and might support the proliferation of TSC gene-deficient tumor cells by providing free amino acids (17,18). These rapamycin-responsive pathways are thought to be candidate for drug targets. The single intervention of these pathways, along with certain rapamycin combination treatments for TSC gene-deficient tumors have been tested using cellular and animal models (15,18-20). While some of these interventions were effective, none have been applied in practical scenarios.

Many reports in the literature have addressed the dysregulation of mTORC1-independent signaling pathways in TSC gene deficiency. Because the protein complex of TSC1/TSC2 gene products (hamartin/tuberin) directly regulates the GTP-binding protein Rheb as a GTPase-activating protein (GAP), researchers have attempted to identify new pathways downstream of Rheb (21,22). For example, the contribution of PAK2 has already been demonstrated (23). In terms of neuropsychiatric disorders, previous research identified that the GDP-binding form of Rheb downregulates the expression levels of syntenin in an mTORC1-independent manner and helps to maintain the appropriate formation of the spine (24,25). The contribution of syntenin-related pathways in TSC-related tumorigenesis has yet to be reported. There are many other reports relating to mTORC1- and Rheb-independent pathways, although these findings have yet to be applied clinically.

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In addition, heat shock protein (HSP)-related pathways have been implicated in the development and progression of cancers (26). The expression levels of HSP, especially HSP70, HSP90, and Hspb1 (HSP25/27), have been identified as markers of the stage and prognosis of various cancers (26,27). These HSPs are thought to represent therapeutic targets for the treatment of cancer (26). Research has identified an interaction between hamartin and HSP70; this interaction was found to regulate apoptosis in TSC (28). Other research has shown that harmatin acts as a chaperone for HSP90 (29). However, the significance of these functions in the pathogenesis of TSC has yet to be fully elucidated. Furthermore, the relationship between Hspb1 and TSC-related tumorigenesis and therapy has yet to be defined.

In an attempt to identify key pathways for the development of new drugs, we analyzed rapamycin-induced changes in *Tsc2*-deficient tumor cells from an animal model of TSC (30-32). In this study, we focused on the pathways related to Hspb1 and found identified their role in the proliferation of *Tsc2*-deficient cells under rapamycin treatment.

Materials and methods

Cell culture. A *Tsc2*-deficient kidney tumor cell line, MKOC1-277 (MKOC) was established in our laboratory from the *Tsc2* knockout mouse and, together with its derivatives, had been used in various researches (30-35). MKOC cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin. Rapamycin (Sigma-Aldrich, Darmstadt, Germany) treatment was performed with dimethylsulfoxide (DMSO) as a vehicle. HeLa, COS7 and Plat-E cells (COSMO BIO, Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 1 μg/ml of puromycin, 100 U/ml of penicillin and 100 μg/ml of streptomycin. In the case of Plat-E cells, 10 μg/ml of blasticidin was also added. All cell cultures were performed at 37˚C with 5% CO_2. SB203580 was purchased from Sigma.

Cell proliferation assays. Cell proliferation was assessed with the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay system (Roche, Basel, Switzerland) in accordance with the manufacturer's guidelines using 96-well plates. To 200 μl of culture medium, XTT reagent (50 μl) was added to each well and incubated for 4 h. Measurement of the absorbance at 450 nm (reference wavelength of 650 nm) was performed with a microplate reader (Benchmark Plus-microplate Spectrophotometer; Bio-Rad, Hercules, United States).

Western blot analysis. Cell lysates were prepared in Laemmli's sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determination by DC Protein Assays (Bio-Rad). Equal amounts of proteins were separated by SDS-PAGE using standard methodology and proteins were transferred to nylon membranes (Millipore, Burlington, United States). For blocking and antibody reactions, we used 1% skimmed milk/Tris buffered saline supplemented with 0.05% of Tween 20 (TBST). Anti-mouse or anti-rabbit Envision HRP-conjugate (DAKO, K4001 or K4003, respectively) was used as secondary antibodies. SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, United States) or Immobilon Forte (Millipore) was used for the development of signals. A Bio-Rad ChemiDoc MP imaging system was used for the detection and analysis of signals. We used several different primary antibodies: anti-HSP27 (#sc13132), -pHSP27(S82) (#sc166693), -Hsp70 (#sc24, #sc59569), and Hsp90 (#sc13119); these antibodies were purchased from Santa Cruz Biotechnology (Dallas, United States). We also used anti-pS6(S235/S236) (#2211), -S6 (#2217), -tuberin (#3612), and -ß-actin (#4970) antibodies from Cell Signaling Technology (Danvers, United States) and anti-α-tubulin (B-5-1-2, #T5168) from Sigma.

RNA interference (RNAi). Transfection of siRNA was performed with Lipofectamine RNAi MAX (Thermo Fisher Scientific) according to the manufacturer's protocol. Cells were treated with 10 nM siRNA for the *Hspb1* gene (5'-CGGAGG AGCUCACAGUGAATT-3' and 5'-UUCACUGUGAGCUCC UCCGGA-3') (36) or control RNA (Thermo Fisher Scientific, #4390843) for 48-72 h. To analyze cell proliferation, rapamycin treatment was started one day after siRNA transfection to avoid severe toxicity.

Plasmid construction. The full-length cDNA of mouse *Hspb1* was amplified using primers mHsp27-F, 5'-AAG AATTCCCAGTGCTTCTAGATCCTCA-3' (forward), and mHsp27-R, 5'-AACTCGAGGCAATGGCTATGGGAG ATAG-3' (reverse). The amplified fragment was digested with *Eco*RI and *Xho*I and subcloned into the pBabe-puro vector (pBabe-puro was a gift from Hartmut Land & Jay Morgenstern & Bob Weinberg (Addgene plasmid #1764 ; http://n2t.net/addgene:1764 ; RRID:Addgene_1764)) (37). The vector had been modified to contain the *Xho*I recognition sequence by introducing synthetic oligonucleotides to the cloning site. The resulting Hspb1 expression vector was designated as pBabe-mHSP27. To generate cDNA for the Ser86-to-Asp86 (S86D) Hspb1 mutant, PCRs were carried out with specific primers: mHsp27-F and H27ASPR1, 5'-ACC CCGCTGTCGAGCTGT-3' (reverse), H27ASPF1, 5'-ACA GCTCGACAGCGGGGT-3' (forward), and mHsp27-R, using pBabe-mHSP27 as a template. Then, the two amplified fragments were mixed and a second PCR was performed using mHsp27-F and mHsp27-R to amplify the full-length cDNA. Similarly, cDNA for the Ser86-to-Ala86 (S86A) Hspb1 mutant (pBabe-mHsp27-S86A) was amplified using the following primers: mHsp27F, H27ALAR1, 5'-ACCCCG CTTGCGAGCTGT-3' (reverse), H27ALAF1, 5'-ACAGCTC GCAAGCGGGGT-3' (forward), and mHsp27R. The resulting full length mutant cDNA fragments were cloned into the pBabe-puro vector to generate pBabe-mHSP27-S86D and -S86A. The accuracy of the cDNA sequence was checked by the dideoxy-sequencing method using an ABI 310 sequencer (Thermo Fisher Scientific).

Establishment of stable cell lines. Plat-E cells (pre-cultured without puromycin or blasticidin) were transfected with the vector for wild-type, S86A and S86D Hspb1, or empty vector using FuGENE6 transfection reagent (Promega, Wisconsin,

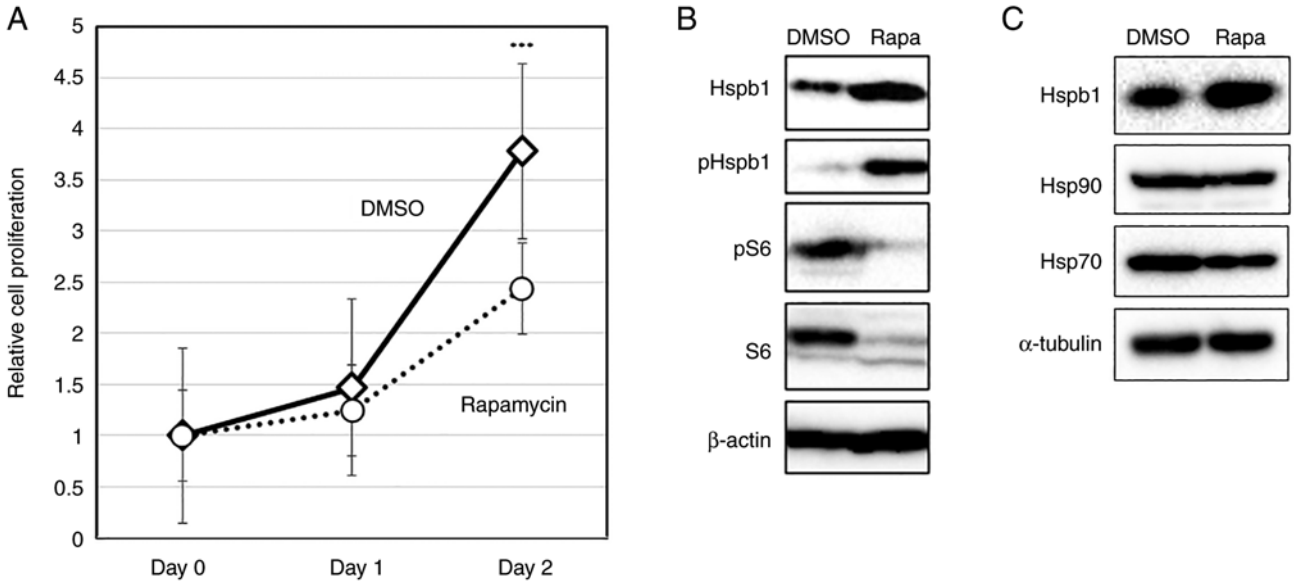

Figure 1. Analysis of Hspb1 expression in TSC complex subunit 2-deficient kidney tumor cells (MKOC). (A) MKOC cells were treated with 20 nM Rapa or vehicle only (DMSO) for 48 h. Cell proliferation was then analyzed by XTT assays. Data are representative of three independent experiments. Each experiment was performed with three independent culture wells. The data are presented as the mean + SD. ***P<0.001 compared with Rapa. (B) MKOC cells were treated with 20 nM Rapa or vehicle only (DMSO) for 24 h. Total cell lysates were analyzed by western blotting with the indicated antibodies (Hspb1, pHspb1, pS6, S6 and βactin). (C) MKOC cells were treated with 20 nM Rapa or vehicle only (DMSO) for 24 h. Total cell lysates were analyzed by western blotting with the indicated antibodies (Hspb1, Hsp90, Hsp70 and α-tubulin). Hsp90, heat shock protein 90; Hsp70, heat shock protein 70; Hspb1, heat shock protein family B (small) member 1; MKOC, MKOC1-277; p, phosphorylated; Rapa, rapamycin.

United States) according to the manufacturer's protocol. Next, 48 h after transfection, we collected the culture media which was filtered through a 0.45-μm filter. Then, we added polybrene to a final concentration of 8 μg/ml. MKOC cells were infected with supernatant and the selection of stably transduced cells with 0.6 μg/ml of puromycin was started 48 h after infection. Established cell lines were maintained in the presence of puromycin in the medium.

Statistical analysis. Statistical analyses were performed with Microsoft Excel software (Microsoft, Redmond, United States), and Statistical analysis program for pharmaceutical data (Osaka university, Osaka, Japan; http://www.gen-info.osaka-u.ac.jp/MEPHAS/tukey-e.html). The significance of difference was examined by unpaired Student's t-test for two groups comparison, and one-way ANOVA followed by Tukey's post hoc test for multiple groups comparison. A p value less than 0.05 was considered statistically significant.

Results

Hspb1 expression was upregulated by rapamycin treatment in Tsc2-deficient tumor cells. To analyze the levels of Hspb1 protein, we treated MKOC cells with rapamycin (20 nM) for 24 and 48 h and then performed western blotting. Rapamycin suppressed the proliferation of *Tsc2*-deficient cells by approximately 35% (P<0.001) (Fig. 1A). In this condition, we found that the amount of Hspb1 protein was increased by 24 h rapamycin treatment (Fig. 1B). The phosphorylation of Hspb1 on Ser86 (corresponding to Ser82 in human HSPB1), the main phosphorylation site that regulates oligomerization, was also upregulated, concomitant with an increase in the total amount of protein (Fig. 1B). Furthermore, rapamycin treatment did not result in the increased expression of HSP70 and Hsp90 (Fig. 1C), thus revealing that each HSP responded differently to rapamycin. To ascertain whether these phenomena occurred generally in cultured cells, we investigated the effects of rapamycin on HSP expression in routine HeLa and COS7 cells. No induction of Hspb1 expression was observed in either HeLa or COS7 cells (Fig. 2). These results indicated that the

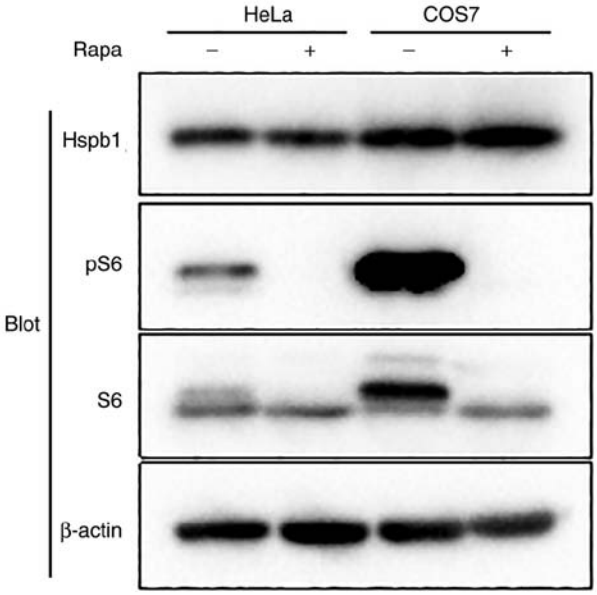

Figure 2. Confirmation of Hspb1 expression in HeLa and COS7 cells. Cells were treated with 20 nM Rapa (+) or vehicle only (-) for 24 h. Total cell lysates were analyzed by western blotting with the indicated antibodies. Hspb1, heat shock protein family B (small) member 1; p, phosphorylated; Rapa, rapamycin.

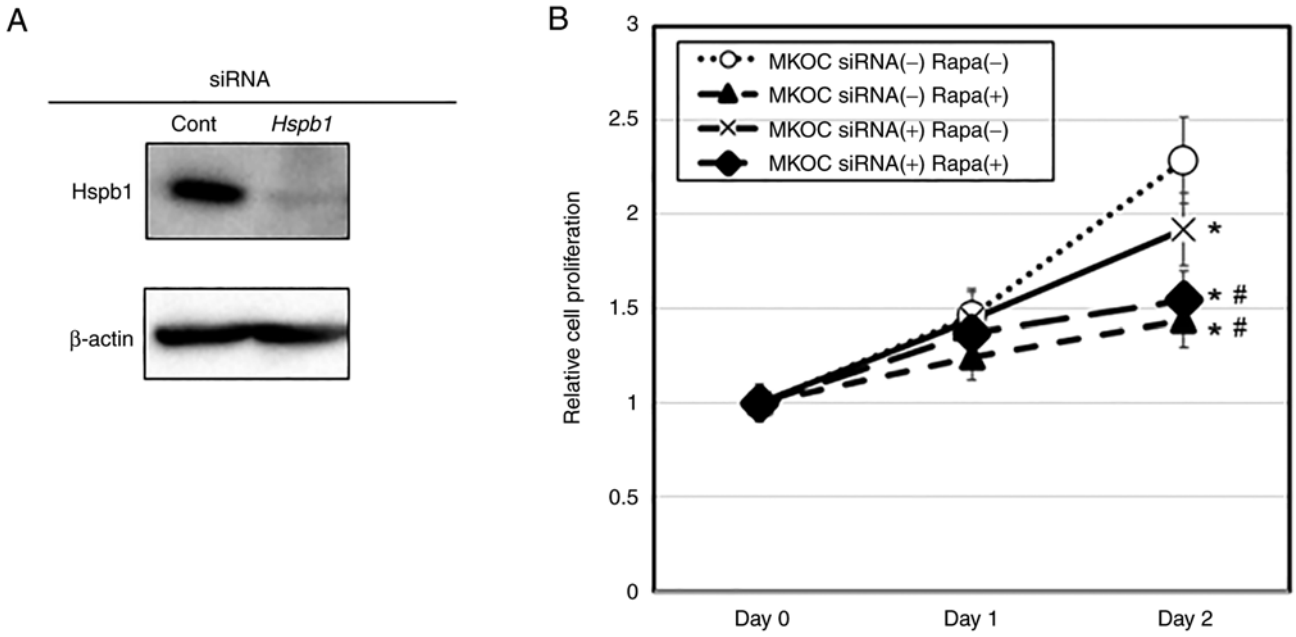

Figure 3. Analysis of MKOC cells transfected with *Hspb1* siRNA. (A) MKOC cells were treated with *Hspb1* siRNA for 48 h. Total cell lysates were analyzed by western blotting with the indicated antibodies. (B) MKOC cells were treated with siRNA (+) or siRNA (-) for 24 h. Subsequently, each group of cells was treated with 20 nM Rapa or vehicle only for 48 h. The start point of the drug treatment was defined as Day 0. Cell proliferation was analyzed by XTT assays. Data are representative of three independent experiments. Each experiment was performed with three independent culture wells. The data are presented as the mean + SD. *P<0.05 compared with siRNA (-) and Rapa (-); #P<0.05 compared with siRNA (+) and Rapa (-). Cont, control; Hspb1, heat shock protein family B (small) member 1; MKOC, MKOC1-277; siRNA, small interfering RNA; siRNA (+), *Hspb1* siRNA; siRNA (-), control RNA; Rapa, rapamycin; Rapa (-), vehicle only.

suppression of mTORC1 by rapamycin causes the upregulation of Hspb1 expression in *Tsc2*-deficient cells.

SiRNA for Hspb1 suppressed cell proliferation. To investigate whether Hspb1 exerted a positive or negative role in cell proliferation under rapamycin treatment, we used RNAi to suppress the expression of Hspb1. The efficacy of siRNA for the *Hspb1* gene was confirmed by western blotting analysis (Fig. 3A). *Hspb1* siRNA treatment significantly inhibited the proliferation of MKOC cells under normal culture condition (P<0.05) (Fig. 3B). Under the rapamycin treatment, however, the extent of inhibition was similar when compared between control RNA and *Hspb1* siRNA conditions (P<0.05 between DMSO and rapamycin without siRNAs; no significance between control and *Hspb1* siRNA under the rapamycin-treated condition) (Fig. 3B), thus suggesting that rapamycin-induced growth regulation may involve the suppression of Hspb1-related pathways.

The forced expression of Hspb1 enhanced proliferation under rapamycin treatment. Next, we analyzed the effects of the forced expression of Hspb1 on cell proliferation. We established MKOC cells stably expressing Hspb1 and control cells (Fig. 4A). When cultured without rapamycin for 48 h, the proliferation of HSP27-expressing cells was promoted when compared with control cells (P<0.05) (Fig. 4B). Under the action of rapamycin, there was also proliferation promoting activity in the wild-type Hspb1 (P<0.05) (Fig. 4B). These results suggested that the function of Hspb1 promoted proliferation in *Tsc2*-deficient cells even in the presence of rapamycin.

We also assayed the effect of Ser86 phosphorylation in Hspb1 upon cell proliferation by the forced expression of a phosphorylation-mimetic S86D mutant (D-mutant) and resistant S86A mutant (A-mutant) Hspb1 in MKOC cells (Fig. 4A). Western blotting revealed a reduction in pHspb1 antibody binding in D-mutant and A-mutant cases, thus suggesting that site-directed mutagenesis had been successfully accomplished. In the proliferation assays, both with and without rapamycin treatment, there was a reduction of proliferation-promoting activity in the A-mutant case (P<0.05 vs. wild-type case both with and without rapamycin) when compared with the wild-type case, thus suggesting that phosphorylation on S86 might be a positive regulator of proliferation-promoting activity in Hspb1 (Fig. 4A and B). However, there was no obvious difference in cell proliferation when compared between the A- and D-mutant (Fig. 4A and B). From these observations, it was difficult to conclude that Ser86 phosphorylation on Hspb1 affects cell proliferation.

Discussion

In this study, we focused on the stress-response pathways involving Hspb1 that have not been explored in the pathogenesis and treatment of TSC. We confirmed that Hsp27 was not increased by rapamycin treatment in other cells, a phenomenon that may be related to *Tsc2* mutation, thus, we decided to further analyze Hsp27. We found that the Hspb1 induced by rapamycin promoted cell proliferation in *Tsc2*-deficient tumor cells in culture.

There was no significant difference in proliferation inhibition at 24 h, but there was a difference in Hspb1 expression.

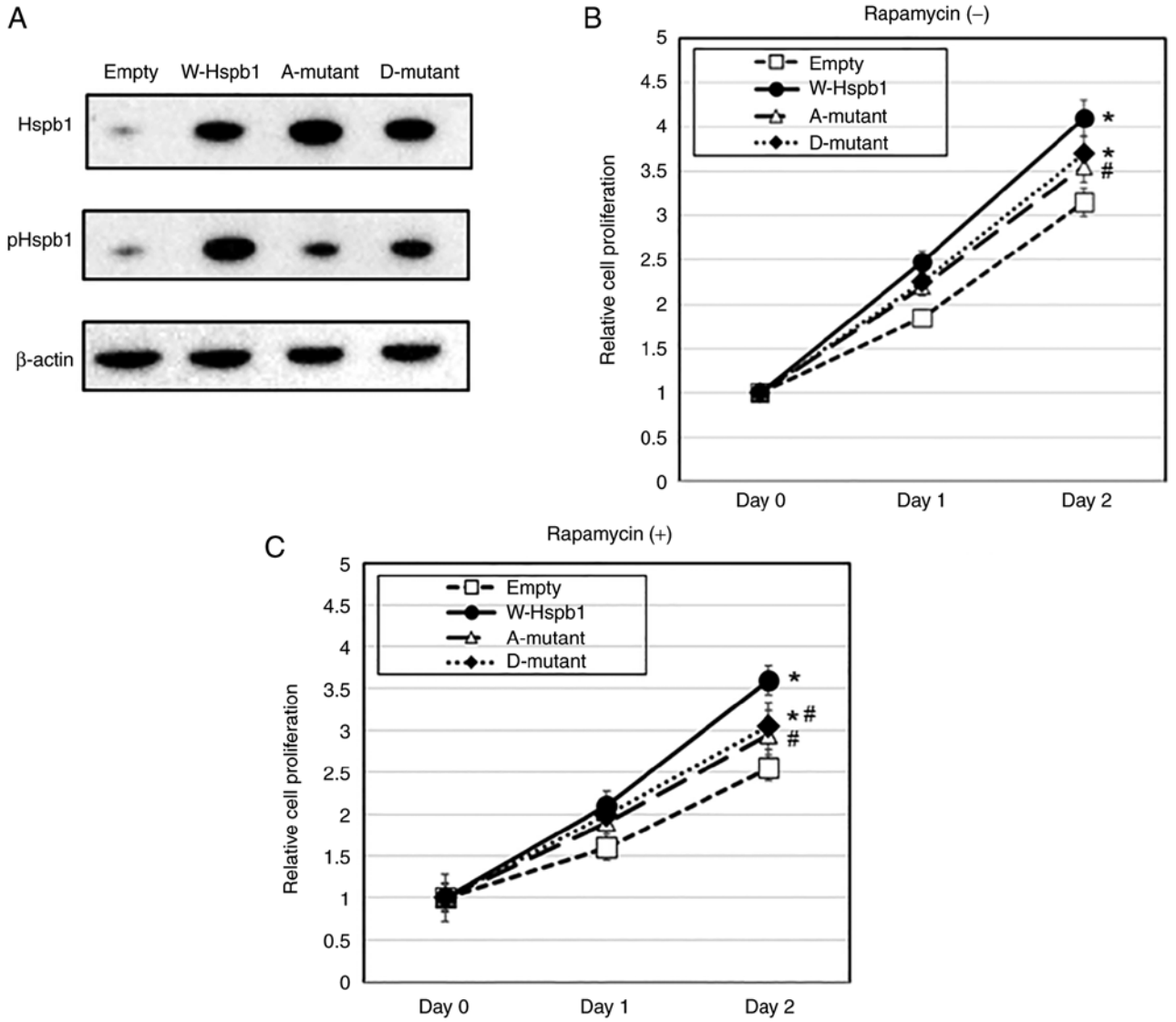

Figure 4. Analysis of MKOC1-277 cells stably expressing Hspb1 and its mutants. (A) Total cell lysates were analyzed by western blotting with the indicated antibodies. Cells were treated with (B) vehicle only or (C) 20 nM rapamycin for 48 h. Cell proliferation was analyzed by XTT assays. Data are representative of three independent experiments. Each experiment was performed with three independent culture wells. The data are presented as the mean + SD. *P<0.05 compared with empty vector; #P<0.05 compared with wild-type Hspb1. Empty, control empty vector; A-mutant, phosphorylation-resistant S86A-mutant; D-mutant, phosphorylation-mimetic S86D-mutant; Hspb1, heat shock protein family B (small) member 1; p, phosphorylated; W-Hspb1, wild-type Hspb1 overexpression.

Although the mechanism is unknown at present, we hypothesize that Hspb1 expression fluctuations occur quickly in response to rapamycin the network of proliferation inhibitory mechanisms (Fig. S1), and then actual cell proliferation suppression appears as a phenotype.

Previous studies have shown that the resistance to anti-cancer drugs in many cancers is associated with higher expression levels of Hspb1 (26). The findings of the present study suggest that Hspb1 might be responsible, at least in part, for tumor remnants then TSC is treated with rapamycin (9). In siRNA experiments, no effect of suppression of *Hspb1* was observed in addition to the action of rapamycin, but in forced expression experiments, a proliferation-promoting effect of increased Hspb1 expression appeared under the rapamycin treatment condition. In such a condition, it is presumed that the proliferation-promoting pathway that can be supported by Hspb1 expression was sufficiently suppressed, and that further suppression of *Hspb1* by siRNA did not show any effect. In the case of forced expression experiments, it is presumed that the suppression of proliferation by rapamycin could be partly recovered by increased amount of Hspb1. The regulatory mechanisms of proliferation exerted by Hspb1 under rapamycin treatment remain unclear. Nonetheless, the combined treatment of TSC gene-deficient tumors with a drug targeting Hspb1-related pathway might be more effective when compared with the single use of rapamycin. It might be possible that the effect of Hspb1 suppression will appear at the concentration of rapamycin-related drugs acting during treatment.

In this study, we revealed the functional importance of Hspb1 in the proliferation of *Tsc2*-deficient cells. The mechanistic effect of Hspb1 has been implicated in the regulation of ferroptosis, a mechanism of cell death that is associated with iron metabolism (38,39). In *Hspb1*-suppressed cells, it is

possible that a proliferation suppressive mechanism related to ferroptosis might be induced.

In the present study, we also observed elevated levels of phosphorylation. However, the forced expression of phosphorylation-mimicking and non-phosphorylated mutants both showed slight attenuation of the proliferation-promoting effect. At present, the significance of phosphorylation remains unclear but should be investigated in the future.

A recent study found a possibility that Hspb1-related pathway might be a chemotherapeutic target of TSC-related neuropsychiatric disorders (40). To the best of our knowledge, the present study is the first to report the function of Hspb1 in the proliferation of TSC-related tumors. Further experiments are needed to determine whether other types of TSC gene-deficient cells, especially human-origin cells, show identical phenotypes.

In conclusion, we identified Hspb1 as a key molecule in the proliferation-supporting mechanism that occurs under rapamycin treatment. Intervention of the Hspb1-related pathway may increase the efficacy of rapamycin in the treatment of TSC. It is now necessary to perform *in vitro* as well as *in vivo* studies with animal models to clarify the importance of Hspb1 in the pathogenesis and treatment of TSC.

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Availability of data and materials

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

Authors' contributions

TKi, OH and TKo designed the study. TKi, KN, TT, YS and TKo performed the experiments. TKi and TKo prepared the figures. TKi and TKo prepared the manuscript. TKi and TKo confirm the authenticity of all the raw data. All authors have read and approved the final 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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