



# Synergistic effects of Rapamycin and Fluorouracil to treat a gastric tumor in a PTEN conditional deletion mouse model

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## Abstract

The tumor suppressor gene phosphatase and tensin homolog (PTEN) in PI3K/Akt/mTOR pathway is essential in inhibiting tumor growth and metastasis. However, whether the mutation of *PTEN* gene could induce tumorigenesis and impact the treatment of gastric cancer is still unclear. The purpose of the study was to investigate the combined treatment of gastric tumorigenesis using Rapamycin and Fluorouracil (5-Fu) through interfering with the Akt/mTOR pathway in a mouse model with *PTEN* conditional deletion. Three groups of mice were exposed for 5 days to Rapamycin and 5-Fu separately and together. The gene expression of the Akt/mTOR pathway, the protein expression of caspase-3 and p-Akt, p-S6K and p-4EBP1, and the pathological changes in stomachs were analyzed. Our study demonstrates that the conditional *PTEN* deletion in the cells of glandular stomach induces hyperplastic gastric tumors in mice. The combined Rapamycin administration with 5-Fu resulted in better outcomes than their separate administration for the treatment of gastric cancer by inhibiting the mTOR signal pathway. Our study indicates that Rapamycin has a synergistic interaction with chemotherapeutic 5-Fu, and demonstrates a potential therapeutic combination treatment on glandular stomach tumor with PTEN functional absence or aberrantly activated Akt/mTOR pathway. It provides important insights into the inhibition of the Akt/mTOR pathway in gastric cancer clinical therapy.

**Keywords** *PTEN* · Gastric tumorigenesis · Rapamycin · Akt/mTOR · 5-Fluorouracil

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## Introduction

Gastric cancer (GC) is the fifth most common malignancy and remains the third cause of death worldwide [1]. Established clinical treatments and interventions (e.g., surgical resection) are not fully successful in treating GC [2]. Although adjuvant therapeutic strategies, such as chemotherapy and radiotherapy greatly improve the prognosis for GC patients, the 5-year overall survival rate is still relatively low (< 30% worldwide) [3, 4]. There is an urgent need to find new therapeutic strategies to cure GC.

Tumorigenesis involves a series of genetic events that disrupt or alter signaling networks controlling cell proliferation and survival. Multiple cell signaling pathways, including G-protein-coupled receptors signaling to MAPK/Erk, p53 pathway, NF- $\kappa$ B, and caspase-3 pathway, as well as phosphoinositide 3-kinase (PI3K)/Akt/ Mammalian Target of Rapamycin (mTOR) pathway, are involved in the tumorigenesis [5–17]. The PI3K/Akt pathway can be considered as a central integrator of a tangled web of

signaling networks with direct and indirect effects on each other [18]. PI3K/Akt activation can produce diverse changes in cell physiology and inhibits apoptosis, indicating that the PI3K/Akt pathway mutation may have broad consequences on tumorigenesis and treatment responses [17, 18].

The mTOR protein is a 289-kDa serine-threonine kinase that belongs to the PI3K-related kinase family and is conserved throughout evolution. Studies show that the mTOR signaling pathway is activated during various cellular processes (e.g., tumor formation and angiogenesis, insulin resistance, adipogenesis, and T-lymphocyte activation) and is deregulated in humans who have diseases such as cancer and type 2 diabetes [7, 19, 20]. Though PI3K/Akt/mTOR pathway plays an important role in the development of human gastric tumorigenesis [21], whether a mutation in a specific component of the PI3K/Akt pathway as a single factor could produce the same effects on tumor physiology or induce tumorigenesis is still unclear.

The PI3K/Akt pathway has a natural inhibitor called Phosphatase and tensin homolog deleted on chromosome ten (PTEN) whose function is to limit proliferation in cells, helping to prevent cancer [22]. One well-known impact of PTEN loss on cell metabolism is increased protein synthesis through modulation of two important components of the protein synthesis machinery, eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and p70S6 kinase, two downstream effects of raptor-mTOR (mTORC1) [23]. PTEN works by dephosphorylating PIP3 to PIP2 which limits Akt's ability to bind to the membrane, decreasing its activity. *PTEN* gene can be found mutated frequently both very early in tumorigenesis (as in hereditary cancer syndromes) and also much later in advanced cancers [24]. Previous studies have demonstrated that the specific inactivation of the *PTEN* alleles in mouse gastric epithelium induced an aberrant Akt-p53-miR365-cyclin D1/cdc25A pathway, which ultimately initiated spontaneous gastric tumor [25–28]. Considering the important role of the Akt/mTOR pathway [29–31], it is necessary to investigate the status of this pathway in the glandular stomach during the process of tumorigenesis initiated by *PTEN* alleles conditional deletion in mice gastric epithelia.

Moreover, fluorouracil (5-Fu) is widely used as an important first-line anti-cancer drug in clinical use, while the drug resistance of 5-Fu to patients with gastric cancer has become a serious therapeutic problem [32]. The mTOR pathway has long been identified as the target of the antibiotic Rapamycin [33]. Previous studies have shown that the PI3K/Akt/mTOR pathway plays an important role in mediating drug resistance in many kinds of human cancers [34–37]. We hypothesized that specific inhibition of the Akt/mTOR pathway or combination with 5-Fu could become an effective therapeutic strategy for gastric cancer therapy.

To test our hypothesis, we first developed a novel mouse model with *PTEN* conditional deletion to find whether *PTEN* gene deletion can induce Akt/mTOR pathway activation directly leading to gastric tumor genesis. We then use the immune impresser, Rapamycin combined with 5-Fu to improve the tumor treatment with the advantages of chemosensitivity, lower metastasis, and lower proliferation (Fig. 1). Our study provides important insights into the inhibition of the Akt/mTOR pathway in gastric cancer clinical therapy.

## Results

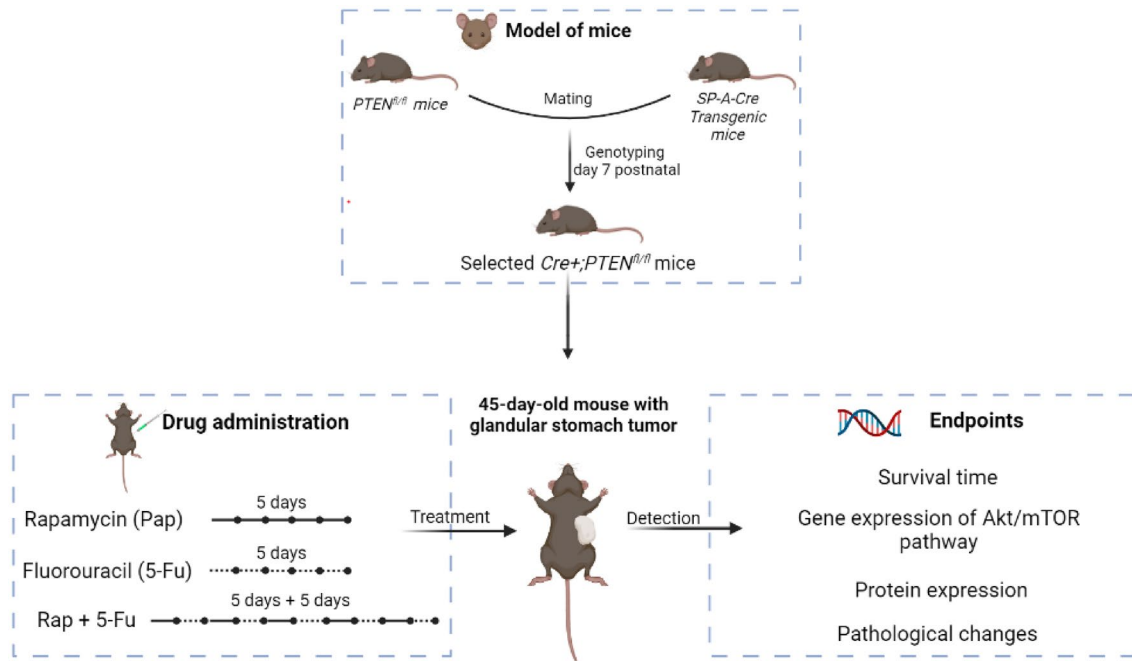
### Genotyping for the hyperplastic gastric tumors induced by *PTEN* conditional deletion

The *Cre*<sup>+</sup>;*PTEN*<sup>fl/fl</sup> mice with glandular stomach cells' specific *PTEN* alleles deletion developed hyperplastic glandular stomach tumor in an early age (before postnatal 45 days) (Fig.S1). Tumorigenesis in glandular stomach resulted in a progressive decrease in the survival of the *SP-A-Cre*<sup>+</sup>;*PTEN*<sup>fl/fl</sup> mice, with a median survival time of (58 ± 1.9) days (*n* = 15) (Fig. S1). The tumor is mainly characterized by irregular proliferation of the glandular stomach. Gender did not introduce any differences in the prevalence of tumorigenesis (data not shown). No obvious differences in phenotypes among mice with different gene types upon birth, while the bodyweight of the *Cre*<sup>+</sup>;*PTEN*<sup>fl/fl</sup> mice at the 45th postnatal day was lower than the *Cre*<sup>-</sup>;*PTEN*<sup>fl/fl</sup> mice with the same gender and same age (Fig. S1).

### Akt/mTOR pathway activation in the hyperplastic gastric tumors induced by *PTEN* conditional deletion

RT-PCR results for the glandular stomach exhibited the activation of the Akt/mTOR pathway at gene level as indicated in Fig. 2B and Fig. S2. The direct activators of mTOR and its upstream regulator gene, *Akt* were found to be significantly increased in the *Cre*<sup>+</sup>;*PTEN*<sup>fl/fl</sup> mice (*p* < 0.05) as compared to the littermate control *Cre*<sup>-</sup>;*PTEN*<sup>fl/fl</sup>, although 4E-binding protein (4EBP1) and p70S6 kinase (S6K), another two downstream effectors, did not display a significant change (Fig. 2B).

The phosphorylated level of p-Akt (p-Akt, Ser473), p-S6K (Thr389), and p-4EBP1 (Thr37/46) in the glandular stomach of the *S-PA-Cre*<sup>+</sup>;*PTEN*<sup>fl/fl</sup> mice are shown in Fig. 2C, D (Fig. S3). At the protein level, p-Akt and p-4EBP1 showed a significant increase (*p* < 0.05) in the *Cre*<sup>+</sup>;*PTEN*<sup>fl/fl</sup> mice compare to the *Cre*<sup>-</sup>;*PTEN*<sup>fl/fl</sup> mice as indicated in Fig. 2C, D, while S6K showed no statistically significant increase (*P* > 0.05). The results indicate that the activation of the Akt/mTOR pathway happens at the protein level.



**Fig. 1** Schema of the protocol for the model of mice, drug administration and endpoints of detection

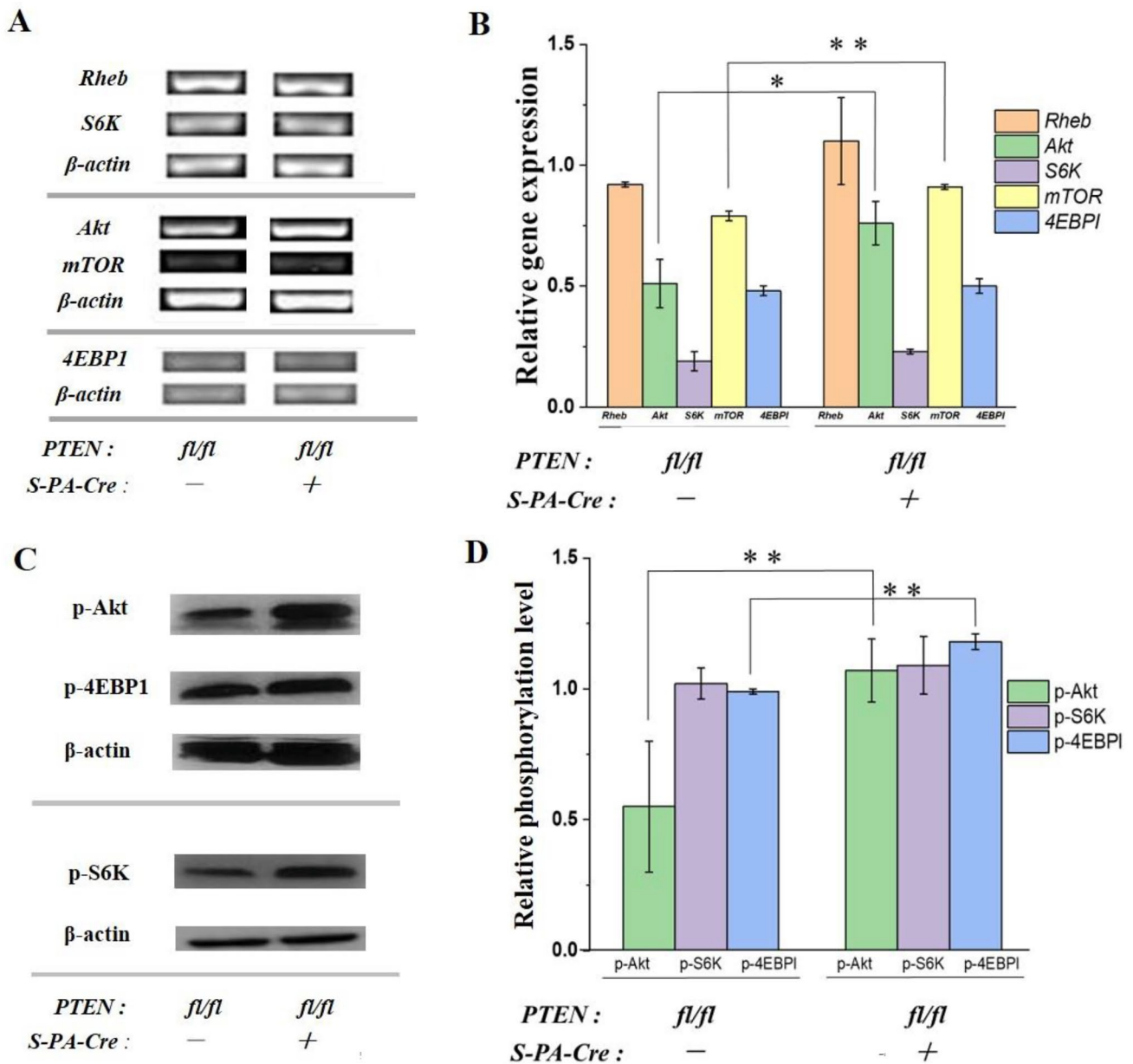
### Reversal of *PTEN* conditional deletion induced glandular stomach tumorigenesis and the attenuation of aberrant Akt/mTOR pathway activation after Rapamycin administration

We hypothesized that the pharmacological inhibition of mTOR has anti-tumor effects, especially for tumors accompanied by *PTEN* functional mutation or Akt signaling aberrant activation. We found that administration of Rapamycin alone can rescue the hyperplastic and metaplastic changes in Akt/mTOR pathway (Fig. 3), with a moderately prolonged survival time (the median survival time of mouse with gastric cancer increased significantly as compared to the control group [(72 ± 5.3) days versus (59 ± 3) days]) (Fig. 3B). Histological analysis showed that Rapamycin treatment inhibited the proliferation of the glandular stomach tumor cells, promoted normal differentiation, as well as induced increased cell apoptosis in epithelium mucosae (morphologically distinct cells were counted as apoptotic cells) (Fig. 3A). Moreover, a significant reduction of the inflammatory cell infiltration in the gastric glandular stomachs of the Rapamycin-treated *SP-A-Cre<sup>+</sup>;PTEN<sup>fl/fl</sup>* mice (Fig. 3A—b versus A—c) was observed. Our results show that intraperitoneal Rapamycin administration alone led to modest tumor regression and significantly amended the damaged stomachs of the mice with gastric epithelial specific *PTEN* alleles deletion when compared to the controls (Fig. 3A—a/b versus A—c/d).

The immunohistochemistry staining with Ki67 and 4EBP1 showed that administration of Rapamycin alone significantly reduced proliferation of the gastric epithelial cells and attenuated invasion (Fig. S4—c/d versus a/b), which suggests that the effect of Rapamycin on gastric cancer can be directly or indirectly related to the inhibition of cell proliferation. To determine the inhibition of Rapamycin on mTOR, proteins expression related to the primary glandular stomach tumor including p-Akt, p-S6K, and p-4EBP1 were detected by western blot (Fig. 3C, D) (Fig. S5). As expected, Rapamycin administration rescued the aberrant overexpression of p-S6K and p-4EBP1 in the stomach of *SP-A-Cre<sup>+</sup>;PTEN<sup>fl/fl</sup>* mice. Notably, the level of p-Akt was also reduced significantly, which indicated the down-regulation of its activity.

### Reversal of the glandular stomach tumorigenesis and attenuation of aberrant activation of the Akt/mTOR pathway by combined Rapamycin and 5-Fu administration

Administration of 5-Fu alone did not change the malignant phenotype of the gastric cancer mice in the Akt/mTOR pathway (Fig. 4). The aberrant level of the p-Akt, p-S6K, and p-4EBP1 are also the same with and without administration of 5-Fu (Fig. 4C, D) (Fig. S6). This finding suggested that *PTEN* conditional deletion induced the abnormal activated Akt/mTOR pathway cannot be rescued by 5-Fu



**Fig. 2** Gastric epithelial specific *PTEN* alleles deletion induced aberrant constitutional activation of Akt/mTOR pathway in glandular stomach. **A, B** The expression of Akt/mTOR pathway relative genes, including *Akt*, *Rheb*, *mTOR*, *S6K*, and *4EBP1* in glandular stomach of *Cre*<sup>+</sup>;*PTEN*<sup>fl/fl</sup> mice and littermates control *Cre*<sup>-</sup>;*PTEN*<sup>fl/fl</sup> mice, was detected by semi-quantitative RT-PCR, densitometry was quantified using the Image J software. **C, D** The protein levels of p-Akt (Ser473), p-S6K (Thr389), and p-4EBP1 (Thr37/46) in glandular

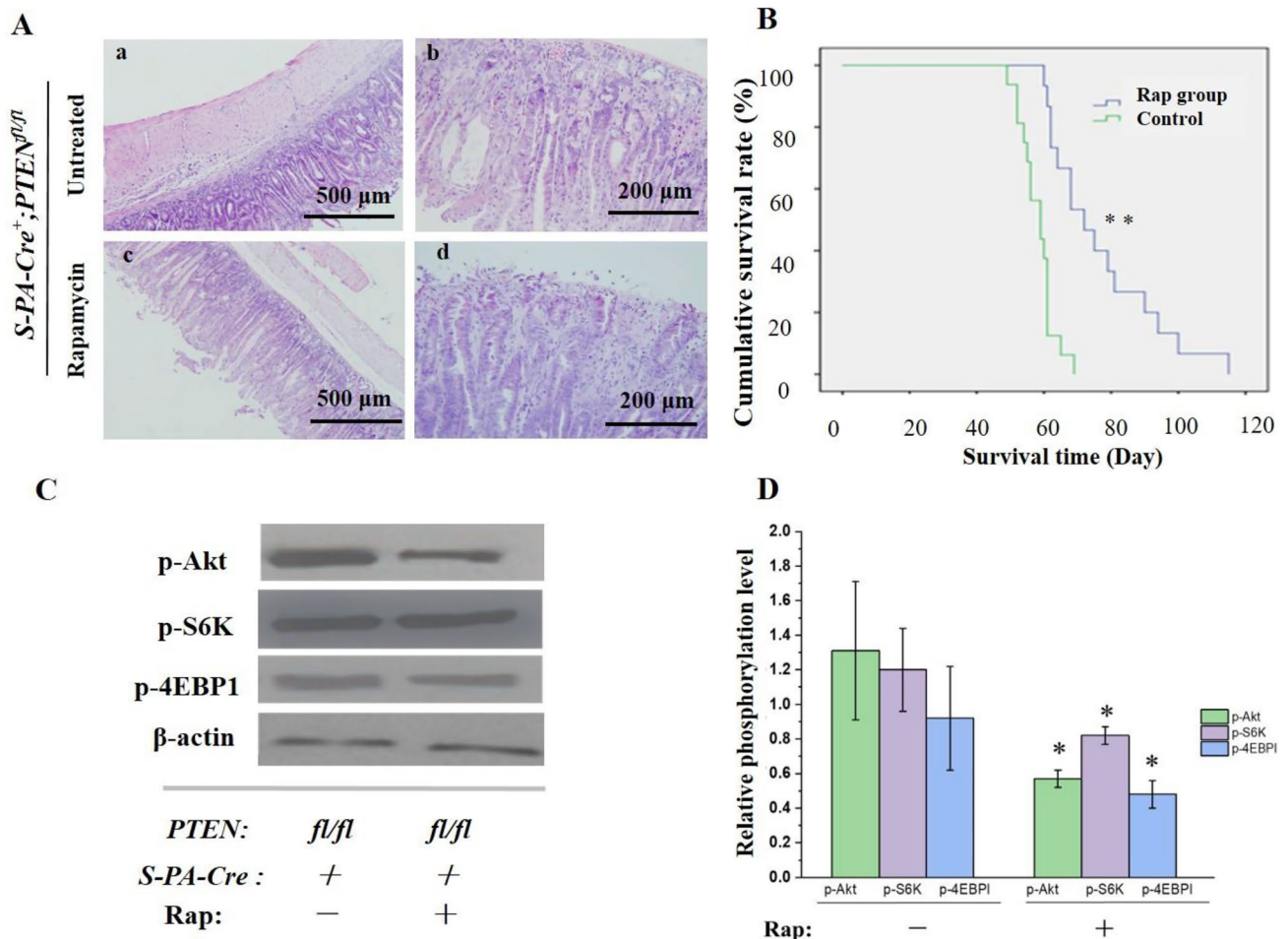
stomach of *Cre*<sup>+</sup>;*PTEN*<sup>fl/fl</sup> mice and littermates control *Cre*<sup>-</sup>;*PTEN*<sup>fl/fl</sup> mice were assayed by western blot. All tests were done when mice were at 45-day-old. The grouping of gene blots was cropped from different parts of the same gel (Fig. S2). The grouping of protein blots was cropped from different gels (Fig. S3). Data were shown as the mean  $\pm$  SD of at least three replicates. \**P* < 0.05, \*\**P* < 0.01, versus wild type *Cre*<sup>-</sup>;*PTEN*<sup>fl/fl</sup> mice

administration. Besides, the median survival time ( $61 \pm 2.0$ ) days by 5-Fu administration was the same as the control group with vehicle ( $60 \pm 2.6$ ) days (Fig. 4B). However, there was a significant increase in the activated caspase-3 (Fig. 3C, D), which likely suggests increased cell apoptosis. These results suggest the drug failure of 5-Fu may be

responsible for the ineffective inhibition of the Akt/mTOR pathway activated by the *PTEN* deficiency.

The administration of Rapamycin combined with 5-Fu led to modest tumor regression and significantly slowed down the damages to stomachs in *SP-A-Cre*<sup>+</sup>;*PTEN*<sup>fl/fl</sup> mice as compared to the control (Fig. 5). The combined administration



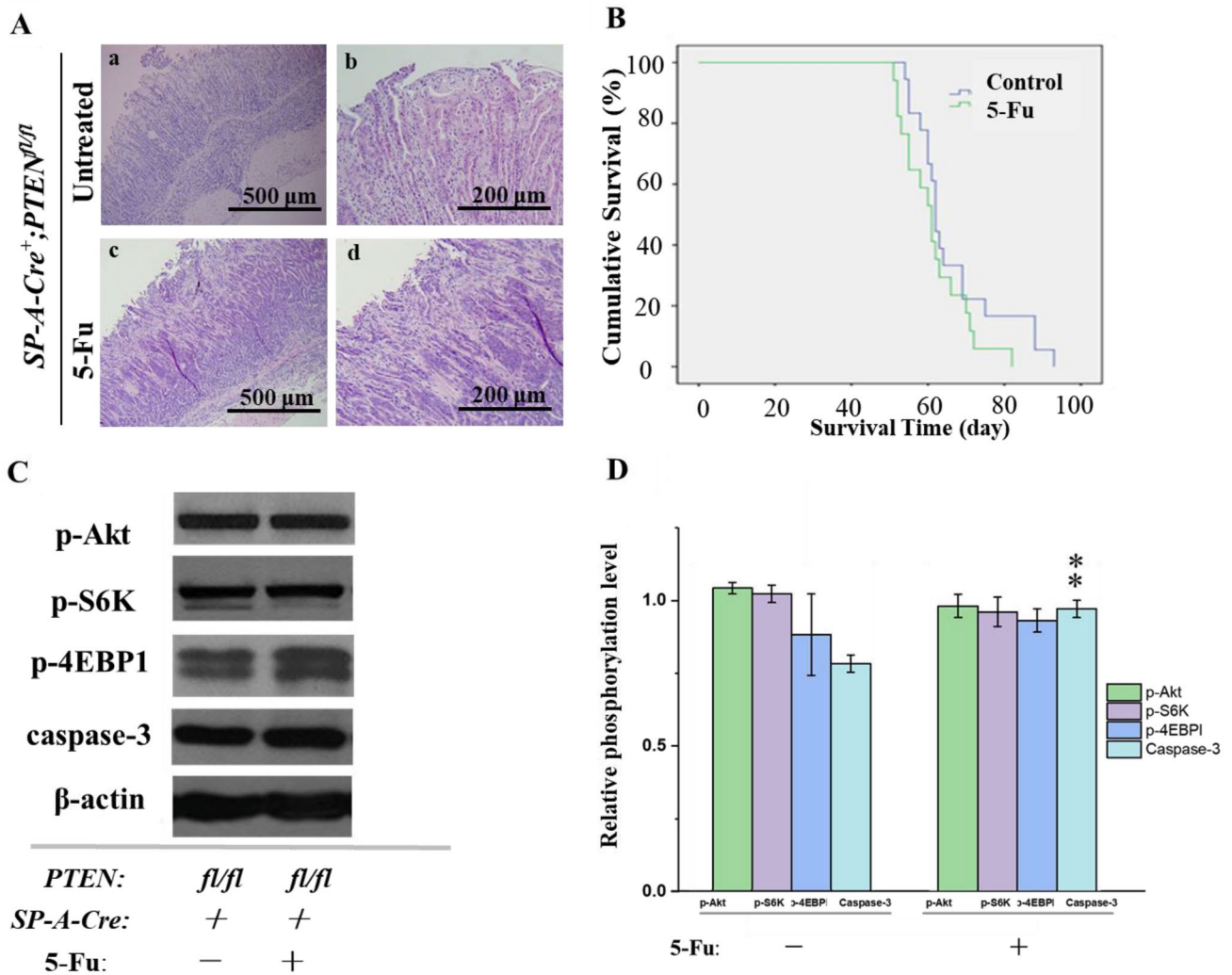


**Fig. 3** Rapamycin reduced the malignancy degree and inhibited proliferation of the gastric epithelial cells, attenuated the activation of Akt/mTOR pathway, and prolonged survival time of the *SP-A-Cre<sup>+</sup>;PTEN<sup>fl/fl</sup>* mice with gastric glandular carcinoma induced by conditional *PTEN* absence. **A** The pathological changes of stomachs for control (c, d) and after 5 days' Rapamycin treatment (a, b). The mice were dissected and the stomachs were collected for histology and immunohistochemistry analysis. Rapamycin alone treatment promoted normal differentiation and apoptosis of the epithelium, and lastly, led to modest tumor regression and significantly improved the damaged stomach (c versus a). d showed higher magnification of

apoptotic cells in the epithelium mucosa. **B** The survival rate of mice was analyzed by the Kaplan–Meier survival analysis approach to generate a Kaplan–Meier survival plot using SPSS17.0. The median survival time of Rapamycin untreated and treated group was  $(59 \pm 3)$  days ( $n = 16$ ) and  $(72 \pm 5.3)$  days ( $n = 15$ ), respectively ( $P < 0.01$ ).  $*P < 0.05$ ,  $**P < 0.01$ , versus the littermate control *Cre<sup>-</sup>;PTEN<sup>fl/fl</sup>* mice. **C**, **D** Protein level of p-Akt, p-S6K, and p-4EBP1 of Rapamycin-treated group and the untreated group was detected by western blot. The grouping of protein blots was cropped from different gels (Fig. S5).  $*P < 0.05$ , Rapamycin treatment group versus untreated group

attenuated the malignance characteristics (such as the poorly differentiated and over-proliferation phenotype and tumor invasion states) of the glandular stomach tumor significantly and almost restored the abnormal histopathological changes of gastric mucosa after 5 days of treatment (Fig. 5A). The levels of p-Akt and p-4EBP1 are significantly decreased after the combined administration (Fig. 5C, D) (Fig. S7). Our results suggest that combined administration effectively inhibited the aberrant activation of the Akt/mTOR pathway in the glandular stomach of *SP-A-Cre<sup>+</sup>;PTEN<sup>fl/fl</sup>* mice. Besides, in comparison with the control group and with the groups where 5-Fu or Rapamycin were administered alone, the median survival

of *SPA-Cre<sup>+</sup>;PTEN<sup>fl/fl</sup>* mice treated with a combination of Rapamycin and 5-Fu increased significantly [ $(79 \pm 5.3)$  days, Figs. 3B, 4B, 5B]. No significant increase of the activated caspase-3 was observed, which suggested that cell apoptosis was likely restored compared with 5-Fu alone administration (Fig. 5C, D).



**Fig. 4** The therapeutic effect of 5-Fu was attenuated in *SP-A-Cre<sup>+</sup>;PTEN<sup>fl/fl</sup>* mice with gastric glandular carcinoma induced by conditional *PTEN* absence. **A** 5-Fu alone administration did not change the phenotype of the glandular stomach tumor by histological analysis (c and d versus a and b). **B** The median survival time of 5-Fu untreated and treated group was  $(60 \pm 2.6)$  days ( $n=12$ ) and  $(61 \pm 2.0)$  days ( $n=17$ ), respectively ( $P>0.05$ ). The results showed

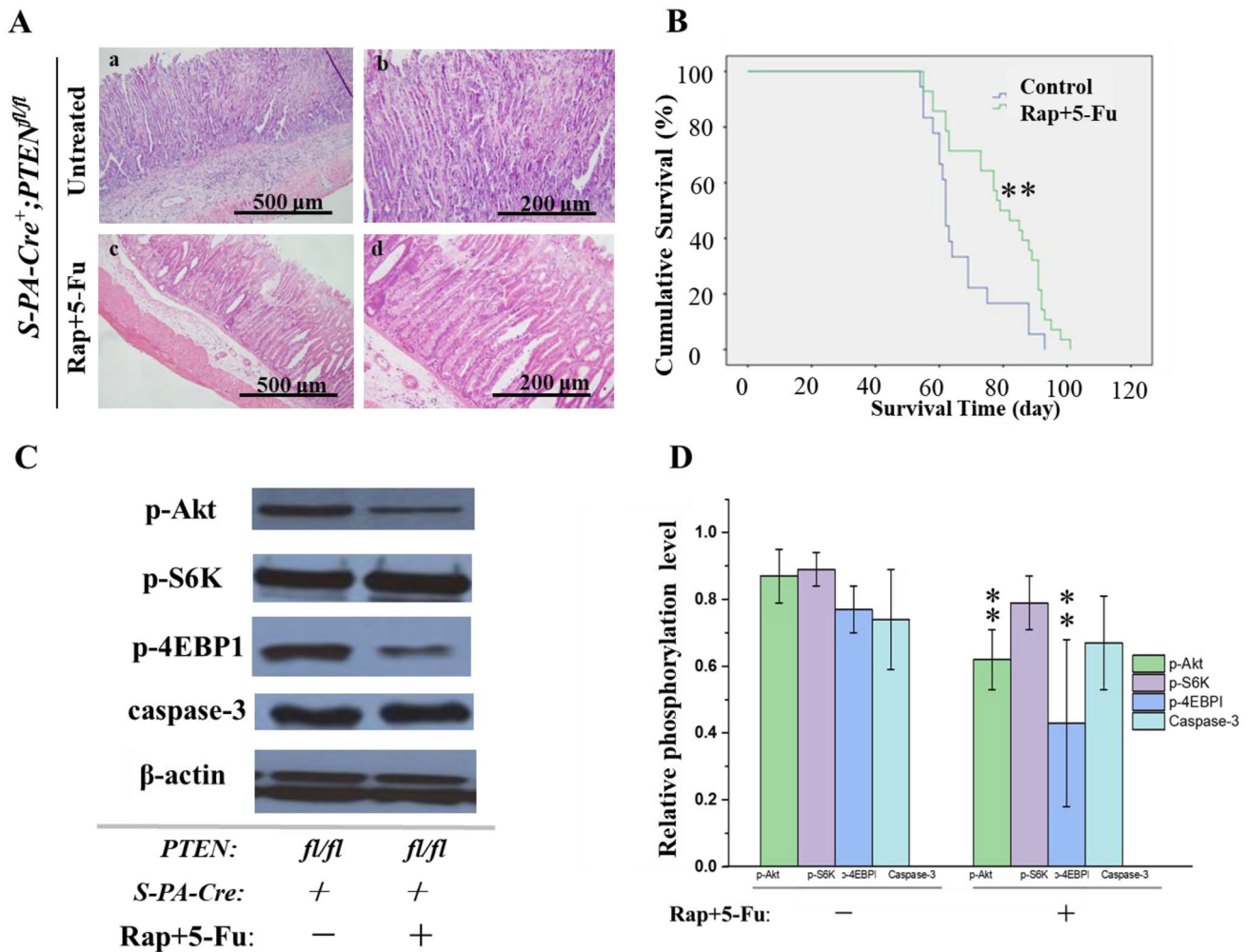
no significant difference. **C, D** The protein level of p-Akt, p-S6K, p-4EBP1, and caspase-3 of the 5-Fu treated and the untreated group were detected by western blot. The results showed 5-Fu alone treatment did not change the aberrant Akt/mTOR pathway activation except for a significant increasing of caspase-3. The grouping of protein blots was cropped from different gels (Fig. S6).  $**P<0.01$ , 5-Fu treatment group versus untreated group

## Discussion

Our study developed a novel gastric tumorigenesis model induced by conditional *PTEN* alleles deletion. The model shows that the proliferation of glandular stomach's cells was increased, accompanying by poorly differentiated and impaired gastric epithelium mucosae. These results indicate that conditional deletion of *PTEN* alleles in the glandular stomach as a single genetic factor could lead to aberrant activation of the Akt/mTOR pathway in the glandular stomach of the *SP-A-Cre<sup>+</sup>;PTEN<sup>fl/fl</sup>* mice. We also found macrophages in the process of the glandular

stomach tumorigenesis, which suggests the relevance of the inflammation to gastric cancer development.

Protein phosphorylation is a reversible post-translational modification of proteins. Phosphorylation alters the structural conformation of a protein, causing it to become activated, deactivated, or modifying its function [38]. In the gastric tumorigenesis model, Akt was activated significantly at the gene and phosphorylated protein level, which provides additional evidence that an excessively activated Akt has broader consequences for tumorigenesis. This result is consistent with the clinical studies which have shown that regulation of Akt expression and phosphorylation in an appropriate range is important for maintaining normal cell



**Fig. 5** Combined treatment with Rapamycin and 5-Fu showed significant improvement on the malignancy of the glandular gastric cancer in *SP-A-Cre<sup>+</sup>;PTEN<sup>fl/fl</sup>* mice. **A** Combined administration significantly alleviated the malignant phenotype of the glandular stomach tumor by histological analysis (c and d versus a and b). Scale bar in a and c was 500  $\mu\text{m}$ , and in b and d was 200  $\mu\text{m}$ . **B** The median survival time of the combined untreated group and treated group was (62  $\pm$  0.7) days ( $n=18$ ) and (79  $\pm$  5.3) days ( $n=28$ ) respectively

( $P<0.01$ ). That of the combined treated group was significantly longer. **C**, **D** Protein levels of p-Akt, p-S6K, p-4EBP1, and caspase-3 in the gastric tumor of combined treated and the untreated group were detected by western blot. The results showed combined administration significantly decreased the expression of p-Akt and p-4EBP1 in *SP-A-Cre<sup>+</sup>;PTEN<sup>fl/fl</sup>* mice. The grouping of protein blots was cropped from different gels (Fig. S7). \*\* $P<0.01$ , drug combination treatment group versus the vehicle control group

growth, preventing tumorigenesis and tumor drug resistance to conventional chemotherapies [18, 39].

As a key downstream substrate of p-Akt, the mTOR activation stimulates protein synthesis and promotes cell proliferation in different organs and systems [19, 29, 30, 40]. Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4EBP1) is a member of a family of translation repressor proteins, and a well-known substrate of the PI3K/Akt signaling pathway. Phosphorylation of 4EBP1 causes its release from eIF4E to allow cap-dependent translation to proceed. Wendel and his colleagues described that eIF4E showed a clear potent oncogene and phenotypes consistent with an anti-apoptotic gene by effecting mRNA 5'cap [41].

P-S6K is an enzyme (specifically, a protein kinase) that in humans is encoded by the *RPS6KB1* gene. It is a serine/threonine kinase that acts downstream of PIP3 and phosphoinositide-dependent kinase-1 in the PI3 kinase pathway. Studies have shown that even though p-S6K phosphorylation is important for glucose homeostasis and controlling cell size, it is not essential to the regulation of 5' TOP mRNAs [29, 30]. Although both of p-S6K and p-4EBP1 play critical roles in translational regulation, in our study p-S6K displayed only a slight increase in the contrast to the significant increase of p-4EBP1 in the *SP-A-Cre<sup>+</sup>;PTEN<sup>fl/fl</sup>* mice. This is consistent with our RT-PCR results [25]. The functional difference between the two genes' expression may account

for such a discrepancy between transcriptional and post-translational modification of the two downstream effectors of mTOR.

Our study shows that administration of Rapamycin alone moderately reduced the proliferation of epithelial cells and promoted transformation to the normal differentiation and gastric epithelium structure. The observed decrease in the levels of p-S6K and p-4EBP1 demonstrates the ability of Rapamycin to inhibit mTOR activity *in vivo*. Notably, administration of Rapamycin reduced the level of p-Akt significantly, which suggested the down-regulation of its activity. The inhibition of mTORC1 caused by Rapamycin likely lead to a reduction of the total TOR, and then impacts the amount or the activity of rictor-mTOR (TORC2), whereas the TORC2 complex directly phosphorylates Akt on the Ser 473 site *in vivo* and facilitates its phosphorylation of Thr 308 site by phosphoinositide-dependent kinase-1 [42, 43].

The administration of 5-Fu alone in the mice model with *PTEN* conditional deletion did neither present any improvement on the malignancy of the glandular stomach tumorigenesis nor prolong the survival time for the mice with gastric tumorigenesis with *PTEN* deficiency. Nevertheless, combined administration of Rapamycin and 5-Fu showed a significant therapeutic effect on the gastric tumorigenesis, effectively suppressed tumor growth and prolonged survival time, as well as showed a modest synergistic therapeutic effect on the mice with gastric tumorigenesis. This finding indicates that inhibition of the Akt/mTOR pathway could enhance the efficacy of chemotherapy and/or combat tumor resistance to 5-Fu in gastric tumorigenesis and thus constitutes an attractive target for cancer drug development [44].

Our results further indicate that tumor harboring activating mutations in the Akt/mTOR pathway or the loss of *PTEN* may be particularly sensitive to PI3K/Akt/mTOR pathway inhibitors. This finding provides new opportunities for developing personalized anti-tumor treatments. Also, previous studies found that human cancer results from multiple known oncogenic mutations. These concomitant oncogenic genetic changes might affect the tumors' response to targeted inhibitors. Our finding demonstrates that the combined administration of Rapamycin and 5-Fu may become a useful treatment method to enhance the targeted inhibitors.

Clinical studies had shown that the loss or diminished expression of *PTEN* in malignant tissues of the stomach may be a major general event in the progression of GC. For example, Li et al. [45] reported that a reduction or loss of *PTEN* expression was detected in over half of GC cases (58.7%, 67/114). Zhu et al. [46], found that the percentage of *PTEN* expression in GC samples (48%, 77/159) was significantly lower compared with that in adjacent normal tissue (75%, 113/151). Yusufu et al. [47] also found that the *PTEN* expression in GC (34%) and metastatic lymph nodes (24.4%) was downregulated compared with in normal tissues

(92.5%). Moreover, the infection of *Helicobacter pylori* (*H. pylori*) may induce GC which was relevant with *PTEN*. For instance, CagA of *H. pylori* can reduce the expression of *PTEN* by enhancing its promoter methylation [48, 49].

Concerning current clinical trials investigating mTOR inhibitors for cancer therapy, our study presented here highlights the possibility of Rapamycin being used in the *PTEN* mutation context for gastric cancer therapy. However, to promote the transformation to practical application, further studies are needed.

## Methods

### Mice generation and genotyping

Mice homozygous for *PTEN* conditional deletion on their glandular stomach was used as the gastric tumorigenesis model. Animal experiments were approved by the Animal Experiment Committee of the Institute of Disease Control and Prevention, Academy of Military Medical Sciences (China) by the guidelines of the Institutional Animal Care and Use Committee (IACUC2009ZX09501-2012). Mice were produced using the *Cre-Loxp* method. 129/SvJ;C57BL/6 mixed background mice was used for constructing the conditional *PTEN* deletion mouse [25]. Briefly, mice carrying conditional floxed *PTEN* (*PTEN<sup>fl/fl</sup>*) alleles were bred with *SP-A-Cre* transgenic mice that express the Cre recombinase in gastric epithelium to produce *Cre<sup>+</sup>;PTEN<sup>fl/fl</sup>* (*PTEN* conditional deletion) mice. The mice were housed in a 12 h light/dark cycle and temperature-controlled SPF environment. Mice identified with homozygous *PTEN* alleles absence (*Cre<sup>+</sup>;PTEN<sup>fl/fl</sup>*) were used as the test system, while the heterozygous *PTEN* allele absence (*Cre<sup>+</sup>;PTEN<sup>fl/-</sup>*) and those *PTEN* floxed mice (*Cre<sup>-</sup>;PTEN<sup>fl/fl</sup>*) were used for the offspring breeding. The offspring were genotyped by PCR analysis.

### Experimental setup and drug administration

The mice (45-day-old) were treated with Rapamycin, 5-Fu, and Rapamycin and 5-Fu combination for 5 days, respectively. Rapamycin (*LC laboratories*) was initially dissolved in 100% ethanol, stored at  $-20^{\circ}\text{C}$ , and further diluted in an aqueous solution of 5.2% Tween-80 and 5.2% PEG-400 (final ethanol concentration 2%) immediately before use in the experiment. Five-Fu (*Sigma*) was dissolved in double distilled water to make the exposure concentrations of 2.5 mg/mL and 2.0 mg/mL, separately. For Rapamycin alone treatment ( $n = 15$ ), Rapamycin (4 mg/kg) was administrated intraperitoneally for 5 days, while the vehicle was given as control ( $n = 16$ ). For 5-Fu alone treatment, 5-Fu (25 mg/kg) was administrated intraperitoneally for 5 days ( $n = 17$ ), while



the placebo was given as control ( $n = 12$ ). For the combination treatment, Rapamycin (4 mg/kg) was given on the first day, followed by the 5-Fu (20 mg/kg) on the second day intraperitoneally for 10 days (five times for each drug, respectively). The survival of mice was recorded every day.

### RNA extraction and RT-PCR detection

Genomic DNA was isolated from tail tips of treated and control mice by standard procedures [25]. The genotyping was determined by PCR [25]. Total RNA was isolated from the glandular stomach of the  $Cre^{-};PTEN^{fl/fl}$  and  $Cre^{+};PTEN^{fl/fl}$  mice using Trizol reagent (Invitrogen, #15,596,026). The first-strand cDNA was synthesized from total RNA using the cDNA synthesis kit (Takara, #6110) according to the manufacturer's instructions, and RT-PCR was performed to evaluate the relative expression of target genes. The results were analyzed by Image J and were normalized to  $\beta$ -actin. The specific primers in Table 1 were used.

### Histology and immunohistochemistry

To analyze the pathological changes of stomachs after 5 days' drug treatment, the mice were dissected and the stomachs (treatment and control) were collected for histology and immunohistochemistry analysis. The collected stomachs were fixed in 10% neutral formalin for 24 h followed by a series of graded alcohol and xylene to dehydrate them, and embedded with paraffin for section. The stomachs were cut into 3  $\mu$ m thick sections. For immunohistochemistry, formalin-fixed paraffin-embedded sections were deparaffinized in xylene and rehydrated through graded alcohol to distilled water. The sections were subjected to antigen retrieval by boiling in a microwave for 5 min in 10 mM/L sodium citrate buffer (pH 6.0) and cooled on the benchtop for about 40 min, and then exposed to 3% hydrogen peroxide

for 20 min at room temperature to block endogenous peroxidase activity. Then the sections were incubated in a blocking solution for 30 min at room temperature, incubated with Ki67 antibody (*IMGENEX*, #NB500-170) overnight at 4 °C, washed with PBS three times. After that, sections were incubated with biotinylated secondary antibody for 30 min and washed by PBS three times, and then sections were incubated with AB enzyme reagent according to the manufacturer's instruction (*Santa Cruz*, #sc2019). After washing three times, the sections were treated with peroxidase substrate until the desired stain intensity was developed. Followed by 5 min washing with distilled water, sections were counterstained with Gill's formulation hematoxylin for 10 s and immediately washed with distilled water. Then they were dehydrated by alcohol and xylene, wiped to remove off excess xylenes, added 1–2 drops of permanent mounting medium, and cover with a glass coverslip.

### Protein lyses and western blot

The stomach tissue was collected from the treated mouse and control. The collected tissues were lysed with modified RIPA buffer (*Beyotime*, R0010) and phosphatase inhibitor on ice for 5 min with gentle grinding and sonication for 8 s to shear DNA and reduce sample viscosity, then the lysates were centrifuged at 11,800 rcf for 20 min at 4 °C. The protein content of the supernatant was quantified using the BCA assay (*Beyotime*, #P0010S). Protein samples (60  $\mu$ g) were loaded onto SDS-PAGE gel for electrophoresis, then the protein was transferred to PVDF membrane, which was blocked with 5% nonfat dry milk in TBST buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20) and then incubated with primary antibody overnight at 4 °C. The membrane was washed three times with TBST buffer and incubated with secondary antibody for 1 h. Then the membrane was washed three times and detected using the enhanced chemiluminescence detection system (*Santa Cruz*, #sc2048). The phosphorylated level of Akt, S6K, and 4EBP1 and the protein level of p-Akt (Ser473), p-S6K (Thr389), and p-4EBP1 (Thr37/46) in the glandular stomach of the SPA- $Cre^{+};PTEN^{fl/fl}$  mice were quantified by *Image J 1.42* (NIH). Each experiment was repeated three times. The following antibodies were used for western blot: mouse monoclonal anti- $\beta$ -actin, rabbit polyclonal anti-phosphorylated-p70S6K (Thr389) (sc-47778 and sc-11759, Santa Cruz), rabbit monoclonal anti-phosphorylated-Akt (Ser473) (4060, Cell Signaling Technology), rabbit monoclonal anti-phosphorylated-4EBP1 (Thr37/46) (2855, Cell Signaling Technology), and rabbit polyclonal anti-caspase-3 (9662, Cell Signaling Technology). The secondary antibodies are HRP AffiniPure Goat Anti-Rabbit IgG (H + L) (ZSGB-BIO, #ZB-2301) and HRP-labeled Goat Anti-Mouse IgG (H + L) (ZSGB-BIO, #ZB-2305). The dilution of the first antibodies

**Table 1** The primer names and sequences of target genes

Name	Sequences
$\beta$ -actin	Forward 5'-AGAGG GAAATCGTGCG TGAC-3'
	Reverse 5'-CAAGAAGGA AGGCTG GAAAA-3'
Akt	Forward 5'-CCGCTACTATGCCATGAAGATC-3'
	Reverse 5'-CACAGC CCGAAG TCC GTTA-3'
Rheb	Forward 5'-AC CAAGATGCCTCAGTCCA-3'
	Reverse 5'-TCCAAGATT CTGCCAAAAGC-3'
mTOR	Forward 5'-GCACAG GTCCTG GAG CCCAC-3'
	Reverse 5'-ATGCCCCAGCAGCGAGAG GA-3'
S6K	Forward 5'-ATGATG TCCGCCA GTATGTT-3'
	Reverse 5'-TGTTCT TCTTAG TCGTTGC T-3'
4EBP1	Forward 5'-GG GACTACAGCACCA CTC-3'
	Reverse 5'-TGGTGC TGAAGA GCGTGCTGGGC-3'

used in immunohistochemistry and Western blot was 1:800 or 1:1000. The dilution of the second antibody is 1:2500. The diluted solution included 5% buffer solution of 1× TBS, TBS (Tris Buffered Saline, pH 7.2).

## Statistical analysis

The survival rate of mice was analyzed by the Kaplan–Meier survival analysis approach to generate a Kaplan–Meier survival plot Using SPSS17.0. The data on gene expression of Akt/mTOR pathway, and protein expression of caspase-3 and p-Akt, p-S6K, and p-4EBP1, were analyzed using a two-tailed student's *t* test or one-way ANOVA followed by Fisher's least significant difference test by SPSS 17.0. Levene's test was used to check the homogeneity of variances. The results were presented as means ± SD, and *P* < 0.05 was considered significant.

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**Author contributions** C-hZ and CP designed experiments, analyzed the data, and wrote the manuscript. C-hZ and L-IC conducted experiments. WC supported results interpretation and reviewed the manuscript. L-sZ, Z-mZ, LJ, and T-fZ cultured mice and analyzed samples. CP submitted the manuscript. S-qP, J-bG, and CP provided financial support and supervision.

## Declarations

**Conflict of interest** The authors declare that there are no conflicts of interest.

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