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ORIGINAL ARTICLE

# L-Glutamine enhances enterocyte growth via activation of the mTOR signaling pathway independently of AMPK

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**Abstract** Neonates (including human infants) require L-glutamine (Gln) for optimal intestinal health. This study tested the hypothesis that Gln enhances enterocyte growth via both mammalian target of rapamycin (mTOR) and AMP-activated kinase (AMPK) signaling pathways. Intestinal porcine epithelial cells (IPEC-1) were cultured for 3 days in Gln-free Dulbecco's modified Eagle medium containing 0 or 2 mM Gln. To determine the role of mTOR and AMPK on cell growth, additional experiments were conducted where medium contained 2 mM Gln and 10 nM rapamycin (Rap, an inhibitor of mTOR) or 1 μM compound C (an inhibitor of AMPK). IPEC-1 cell growth increased with increasing concentrations of Gln from 0 to 2 mM. Compared with 0 mM Gln, 2 mM Gln increased ($P < 0.05$) the amounts of phosphorylated 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase (p70S6 kinase) proteins but did not affect abundances of total or phosphorylated AMPK protein. Gln also increased mRNA levels for Bcl-2, mTOR, p70S6 kinase, 4E-BP1, COX7C, ASCT2, ODC, SGLT-1, CFTR, $Na^+/K^+$-ATPase, HSP70, and ZO-1. Similarly, cells cultured with Rap and Gln exhibited higher ($P < 0.05$) abundances of phosphorylated 4E-BP1 and p70S6 kinase proteins than the Rap-only group, whereas abundances of phosphorylated mTOR and 4E-BP1 proteins were increased when AMPK was inhibited by compound C. Conversely, the amount of phosphorylated AMPK increased when mTOR was inhibited by Rap, suggesting a negative cross-talk between mTOR and AMPK. Collectively, these results indicate that Gln stimulates enterocyte growth by activating the mTOR signaling pathway independently of AMPK.

**Keywords** L-Glutamine · IPEC-1 · mTOR · AMPK

**Abbreviations**

| | |
|---|---|
| AMPK | Adenosine 5′-monophosphate (AMP)-activated protein kinase |
| ASCT2 | $Na^+$-neutral AA exchanger 2 |
| Bcl-2 | B-cell lymphoma 2 |
| Cc | Compound C |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| COX7C | Cytochrome *c* oxidase subunit I |
| HSP70 | Heat shock protein 70 |
| MAPK6 | Mitogen-activated protein kinase 6 |
| mTOR | Mammalian target of rapamycin |
| ODC | Ornithine decarboxylase |
| pBD-1 | Porcine β-defense 1 |
| Rap | Rapamycin |
| SGLT-1 | Sodium/glucose co-transporter-1 |
| Sirt 1 | Sirtuin 1 |
| 4E-BP1 | 4E-binding protein-1 |
| ZO-1 | Zonula occludens-1 |

## Introduction

L-Glutamine (Gln), a conditionally essential amino acid (Wu et al. 2011), plays critical roles in maintaining

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intestinal function and integrity (Wang et al. 2014a), enhancing the absorption of nutrients (Wu et al. 1996) and immune function (Yoo et al. 1997), regulating intestinal gene expression (Wang et al. 2008), stimulating cell proliferation (Kim et al. 2013) and protein turnover (Xi et al. 2012), and inhibiting cell autophagy (Zhu et al. 2014). Of particular interest, Gln is a major energy substrate for intestinal cells (Wu 1998; Wu et al. 2011) and can increase cellular adenosine triphosphate (ATP) levels (Barnabé and Butler 2000; Ahmad et al. 2001). Furthermore, Gln can enhance cytochrome *c* oxidase (COX) activity and mitochondrial function (Groening et al. 2011). Therefore, Gln plays an important role in cellular energy metabolism, particularly in the small-intestinal mucosa (Wu 2014). Because AMP-activated protein kinase (AMPK) is an energy sensor of cellular energy status (Liu et al. 2012), relationships may exist between Gln and AMPK signaling (Wu 2010).

AMPK is mainly recognized as a critical regulator of energy metabolism and activated when the cellular energy level is low (Jobgen et al. 2006). Once activated, AMPK phosphorylates and inactivates a number of metabolic enzymes involved in ATP-consuming cellular events, including fatty acid and protein synthesis, and also activates ATP-generating metabolic pathways, including the uptake and oxidation of glucose, fatty acids, and amino acids (Motoshima et al. 2006). Of particular note, activated AMPK may inhibit the mammalian target of rapamycin (mTOR) signaling, a cellular protein synthesis regulator. Previous study showed that AMPK and mTOR may represent two antagonistic forces governing muscle adaptation to nutrition, starvation, and growth stimulation (Mounier et al. 2011). Recent evidence clearly indicates that Gln stimulates cellular protein synthesis through activating downstream targets (4E-binding protein-1 and 70-kDa ribosomal S6 kinase-1) of mTOR in IPEC-1 cells (Xi et al. 2012). Therefore, it seems that both mTOR and AMPK signaling pathways may play critical roles on mediating effects of Gln on intestinal protein synthesis and cell growth. However, it is not clear whether the mTOR signaling pathway can be activated directly by Gln, or indirectly through the inhibition of AMPK. Moreover, the cross-talk between mTOR and AMPK signaling pathways is also poorly understood. In view of the foregoing, the present study tested the hypothesis that Gln enhances enterocyte growth via both mTOR and AMP signaling pathways.

## Materials and methods

### Reagents

L-Glutamine, rapamycin (Rap, a mTOR inhibitor), and Compound C (Cc, a AMPK inhibitor) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's F12 Ham medium (DMEM-F12), fetal bovine serum (FBS), and antibiotics were purchased from invitrogen (Grand Island, NY, USA). Epidermal growth factor and selenium were obtained from BD Biosciences (Bedford, MA, USA). Plastic culture plates were manufactured by Corning Inc. (Corning, NY, USA). Unless indicated, all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

### Cell culture

IPEC-1 cells were incubated with DMEM-F12 according to previous studies (Haynes et al. 2009; Tan et al. 2010; Zhu et al. 2014). Briefly, cells were grown in serial passage in uncoated plastic culture flasks (75 $cm^2$) in DMEM-F12 containing 17.5 mM D-glucose, 2 mM Gln, 0.7 mM arginine, 15 mM HEPES (pH = 7.4), 5 %FBS, epidermal growth factor (5 μg/L), insulin (5 μg/mL), transferrin (5 μg/mL), selenium (5 ng/mL), penicillin (50 μg/mL), streptomycin (4 μg/mL) and 0.25 μg/mL amphotericin B (Fungizone®). Medium was changed everyday. All cell cultures were carried out at 37 °C in a 5 % $CO_2$ incubator. At confluence, cells were passaged using trypsinization.

### Determination of cell growth

At confluence, IPEC-1 cells were trypsinized, washed thoroughly with DMEM-F12, and then seeded in 24-well cell culture plates with approximately $4 \times 10^4$ cells per well and maintained at 37 °C in a 5 % $CO_2$ incubator. After overnight culture, the cells were starved for 6 h in 1 mL of Gln-free Dulbecco's modified Eagle medium (DMEM). The cells were then cultured in 1 mL of Gln-free DMEM containing 5 % FBS, 5 mM D-glucose, 0.1 mM sodium pyruvate and 0, 0.2, 0.5, 1, 2 or 5 mM Gln (Tan et al. 2010; Xi et al. 2012). The physiological concentrations (μM) of other amino acids in DMEM medium were as follows: L-alanine 350; L-arginine, HCl, 100; L-asparagine, 50; L-aspartic acid, 20; L-cystine 2HCl, 75; L-glutamic acid, 75; glycine, 250; L-histidine HCl $H_2O$, 100; L-isoleucine, 150; L-leucine, 200; L-lysine·HCl, 200; L-methionine, 75; L-phenylalanine, 100; L-proline, 200; L-serine, 200; L-threonine, 200; L-tryptophan, 75; L-tyrosine 2Na $2H_2O$, 100; L-valine, 250; and taurine, 100 (Xi et al. 2012). The media were changed every day. Cell growth was determined by counting the number of cells in each well of the plates using automated cell counter (TC20™, Bio-Rad Laboratories, Inc., CA, USA). The optimum time and Gln dose for cell growth were determined on the basis of cell number after 0, 1, 2, 3 and 4 days incubation.

To determine the effect of Rap or Cc on the cell growth, IPEC-1 cells incubated with DMEM (containing 0.5 mM Gln) were treated for 3 days with (1) Rap (0, 10, 20, 40, 80, or 180 nM) (Bauchart-Thevret et al. 2010); or (2) Cc

(0, 0.25, 0.5, 1, or 2 μM) (Li et al. 2013). Cell growth was determined by counting the number of cells using an automated cell counter and the approximate 50 % inhibition of cell growth was determined.

To test the effects of Gln and Rap on cell growth, IPEC-1 cells were treated for 3 days (72 h) with: (1) Gln (0 mM) + Rap (0 nM); (2) Gln (2 mM) + Rap (0 nM); (3) Gln (0 mM) + Rap (10 nM); or (4) Gln (2 mM) + Rap (10 nM). Cell growth was determined by counting cell number with an automated cell counter. Additionally, gene expression, abundances of mTOR and AMPK signaling molecules were also measured.

On the other hand, to test the effects of Gln and Cc on cell growth, IPEC-1 cells were treated for 3 days (72 h) with: (1) Gln (0 mM) + Cc (0 μM); (2) Gln (2 mM) + Rap (0 μM); (3) Gln (0 mM) + Rap (1 μM); or (4) Gln (2 mM) + Rap (1 μM). Cell growth was determined by counting cell number with an automated cell counter. Additionally, gene expression, abundances of mTOR and AMPK signaling molecules were measured.

## Real-time PCR (RT-PCR) analysis of gene expression

After 3 days of incubation, total cell RNA was extracted and purified using TRIzol Reagent protocol (Invitrogen, Carlsbad, CA, USA). Total RNA was quantified using the NanoDrop® ND-2000 UV–VIS spectrophotometer (Thermo Scientific, Wilmington, DE, USA) at an OD of 260 nm, and its purity was assessed by determining the OD260/OD280 ratio. All of the samples had an OD260/OD280 ratio above 1.8, corresponding to 90–100 % pure nucleic acids. Meanwhile, RNA integrity in each sample was determined using 1 % denatured agarose gel electrophoresis. RNA was used for RT-PCR analysis when it had a 28S/18S rRNA ratio ≥1.8 (Hou et al. 2013). Total RNA was reverse transcribed using a PrimeScript® RT reagent kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instruction. cDNA was synthesized and stored at −20 °C until use.

To amplify cDNA fragments, the primer pairs (Table 1) were used for RT-PCR. To minimize amplification of potentially contaminating genomic DNA, the primers were designed to span introns and intron–exon boundaries. The RT-PCR was performed using the SYBR® Premix Ex Taq™ (Takara, Dalian, China) on an Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA, USA). The total volume of PCR reaction system was 50 μL. Briefly, the reaction mixture contained 0.2 μM of each primer, 25 μL of SYBR® Premix Ex Taq™ (2×) and 4 μL of cDNA in a 50-μL reaction volume. All PCRs

**Table 1** Sequences of the primers used for quantitative PCR analysis

| Genes | Forward | Reverse |
|---|---|---|
| mTOR | TTGTTGCCCCCTATTGTGAAG | CCTTTCGAGATGGCAATGGA |
| p70S6 kinase | GGAAACAAGTGGAATAGAGCAGATG | TTGGAAGTGGTGCAGAAGCTT |
| 4E-BP1 | CCGGAAGTTCCTAATGGAGTGT | GGTTCTGGCTGGCATCTGT |
| AMPKα2 | CGACGTGGAGCTGTACTGCTT | CATAGGTCAGGCAGAACTTGC |
| Sirt1 | CTGGAACAGGTTGCAGGAAT | CCTAGGACATCGAGGAACCA |
| UCP2 | AGGGTCCCCGAGCCTTCT | CAGCTGCTCATAGGTGACAAACA |
| COX7A1 | AAGAGGAGGACGCAGGATGA | CAGCCACTCGGTTCTCCAA |
| COX7C | CCGTAGGAGCCACTATGAGGAG | GCAGCAAATCCAGATCCAAAG |
| CFTR | CCGGGCACCATTAAAGAAAAC | GCCATCAATTTACAGACACAGC |
| SGLT-1 | CCCAAATCAGAGCATTCCATTCA | AAGTATGGTGTGGTGGCCGGTT |
| $Na^+/K^+$-ATPase | TCGATAATCTCTGCTTCGTTGG | ATGGCTTTGGCTGTGATGG |
| HSP70 | GACGGAAGCACAGGAAGGA | GAAGACAGGGTGCGTTTGG |
| pBD-1 | ACCGCCTCCTCCTTGTATTC | CACAGGTGCCGATCTGTTTC |
| Claudin-1 | GGTGCCCTACTTTGCTGCTC | CCCACACGGTTTTGTCCTTT |
| ZO-1 | AGGCGATGTTGTATTGAAGATAAATG | TTTTTGCATCCGTCAATGACA |
| Bcl-2 | TGAATCAGAAGCGGAAACCC | GCTCTAGGTGGTCATTCAGGTAAG |
| Bax | TTTCTGACGGCAACTTCAACTG | AGCCACAAAGATGGTCACTGTCT |
| Cyclin D1 | GCAGAAGTGCGAGGAGGAGGTCTT | CGGATGGAGTTGTCGGTGTAGATGC |
| ODC | GGCGATTGGATGCTCTTTGA | GGCCCCGACATCACATAGTAG |
| ASCT2 | GCCAGCAAGATTGTGGAGAT | GAGCTGGATGAGGTTCCAAA |
| MAPK6 | CAAGCAGCTCGAGTCCATAGG | AGAAGGTGTTAATGTGGCCTGTATT |
| RPL19 | AACTCCCGTCAGCAGATCC | AGTACCCTTCCGCTTACCG |
| HMBS | AGGATGGGCAACTCTACCTG | GATGGTGGCCTGCATAGTCT |
| HPRT1 | AACCTTGCTTTCCTTGGTCA | TCAAGGGCATAGCCTACCAC |

were performed in triplicate on a 96-well RT-PCR plate (Applied Biosystems) under the following conditions (two-step amplification): 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s. A subsequent melting curve (95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s) with continuous fluorescence measurements and final cooling to room temperature was processed. The specificity of the RT-PCR reactions was assessed by analyzing the melting curves of the products and size verification of the amplicons (Meurens et al. 2009). Each biological sample was run in triplicate. The delta delta cycle threshold ($C_T$) method was used to analyze the relative expression of the target gene (Fu et al. 2010). To ensure the sensitivity and accuracy of the results obtained by RT-PCR, data were normalized geometric averaging of three internal reference genes: ribosomal protein L19 (RPL19), hydroxymethylbilane synthase (HMBS), and hypoxanthine phosphoribosyltransferase 1 (HPRT1) (Nygard et al. 2007; Bruel et al. 2010).

### Western blot analysis of proteins abundance

After a 3-day period of incubation, cells were obtained for analysis of protein (Li et al. 2008) and Western blotting (Tan et al. 2010; Yao et al. 2012). Briefly, cells in 6-well culture plates were gently washed with Dulbecco's phosphate-buffered saline and viable cells were counted using an automated cell counter. Cells were collected and lysed in 1 mL buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM NaF, 100 mM KCl, 0.2 mM EDTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 0.1 mM PMSF, 1 mM benzamidine, and 0.5 mM sodium vanadate) with a Polytron homogenizer and centrifuged at 12,000 *g* for 15 min at 4 °C. The supernatant fluid was aliquoted into micro-centrifuge tubes, to which 2× SDS sample buffer was added in a 1:1 ratio. The samples were boiled and cooled on ice before use for western blotting. Proteins were separated by electrophoresis on a 10 % polyacrylamide gel, and then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. Skim-milk powder in TBST buffer (1× Tris-buffered saline including 0.1 % Tween 20) was used to block membrane for 1 h at room temperature. Membranes were incubated overnight at 4 °C with following primary antibodies: mTOR (Cell signaling, 1:1,000), phosphorylated mTOR (Ser2448) (Cell signaling, 1:1,000), 4E-BP1 (Cell signaling, 1:1,000), phosphorylated 4E-BP1 (Thr70) (Cell signaling, 1:1,000), p70S6 kinase (Cell signaling, 1:1,000), phosphorylated p70S6 kinase (Thr389) (Cell signaling, 1:1,000), AMPKα (Cell signaling, 1:1,000), phosphorylated AMPKα (Thr172) (Cell signaling, 1:1,000), or β-actin (Sigma Chemicals, 1:5,000). Thereafter, the membranes were washed with TBST and incubated for 1 h at room temperature with anti-goat (mouse) immunoglobulin G horseradish peroxidase conjugated secondary antibody (Beijing ZhongShan Golden Bridge Biological Technology Co., Ltd, China; 1:5,000 dilution). After being washed with TBST, blots on the membrane were developed using an Enhanced Chemiluminescence Western blotting kit (ECL-plus, Amersham Biosciences, Sweden), visualized and quantified using an imaging system (Alpha Innotech FluorChem FC2, CA, USA). Abundances of all proteins of interest were normalized to those for β-actin.

### Statistical analysis

All values are expressed as mean ± SEM. Difference of means of two groups (e.g. mRNA levels of 0 and 2 mM Gln group) was determined by the Student's paired *t* test. Data for single factor experiments (e.g. different levels of Gln, Rap, and Cc) were performed by one-way ANOVA procedure of SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Data of 2 × 2 factorial experiments (e.g. Gln × Rap and Gln × Cc) were analyzed with a General Linear Model (Univariate) procedure of SPSS 13.0. Probability values ≤0.05 were taken to indicate statistical significance.

## Results

### Effect of Gln on IPEC-1 cells growth

The growth curves of IPEC-1 cells are shown in Fig. 1. On days 1 and 2, the number of IPEC-1 cells in 0.2, 0.5, 1, 2, and 5 mM Gln groups was higher ($P < 0.01$) than that in the control group (0 mM Gln), but there was no difference among the 1, 2, and 5 mM Gln groups. On day 3, the number of IPEC-1 cells reached the maximum in the presence of Gln and was dose-dependently increased by Gln from 0 to 2 mM ($P < 0.01$). Specifically, the number of IPEC-1 cells in 0.2, 0.5, 1, 2, and 5 mM Gln groups was 246, 288, 336, 388, and 343 % higher ($P < 0.01$) than that in the control group, respectively. On day 4, the number of IPEC-1 cells in the presence of Gln was also higher ($P < 0.01$) than that in the control group.

### Effects of Rap and Cc on IPEC-1 cells growth

IPEC-1 cell numbers on day 3 of the culture in the presence of Rap (an mTOR inhibitor) or Cc (an AMPK inhibitor) are given in Fig. 2. Compared with the 0 nM Rap, adding 10, 20, 40, 80, or 160 nM Rap to Gln-free culture medium for 72 h decreased ($P < 0.01$) the number of IPEC-1 cells, but there was no difference among the 10, 20, 40, 80, and 160 nM Rap groups (Fig. 2a). On the other hand, on day

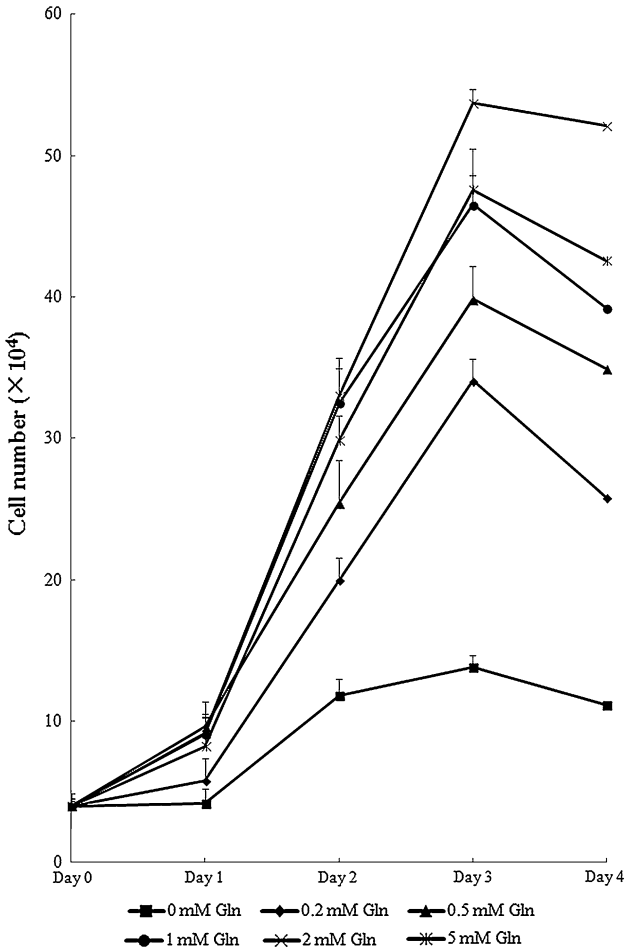


**Fig. 1** The number of IPEC-1 cells cultured in Gln-free DMEM supplemented with 0, 0.2, 0.5, 1, 2, or 5 mM Gln on days 0, 1, 2, 3, and 4. Data are mean ± SEM, $n = 8$

3 (72 h), cell number was gradually decreased ($P < 0.05$) with increasing Cc concentrations from 0 to 2 μM (Fig. 2b). Compared with the 0 μM Cc, adding 0.25, 0.5, 1, and 2 μM Cc decreased ($P < 0.05$) the number of IPEC-1 cells by 22, 31, 64, and 97 %, respectively.

## Effects of Gln, Rap, and Cc on IPEC-1 cells growth

Effects of Gln, Rap, and Cc on IPEC-1 cell number are illustrated in Fig. 3. Consistent with the previous results, cell number was increased ($P < 0.01$) when 2 mM Gln was added, but was decreased ($P < 0.01$) by administration of Rap (10 nM) or Cc (1 μM) for 72 h (Fig. 3). Moreover, there were significant ($P < 0.01$) interactions between Gln and Rap (Fig. 3a), as well as between Gln and Cc (Fig. 3b), on the number of IPEC-1 cells. The results indicated that adding 2 mM Gln to Gln-free culture medium in the presence of 10 nM Rap or 1 μM Cc significantly increased ($P < 0.01$) the IPEC-1 cell number.

## Effects of Gln on gene expression in IPEC-1 cells

Addition of 2 mM Gln to the Gln-free basal medium for 72 h affected expression of many genes in IPEC-1 cells (Fig. 4). Compared with 0 mM Gln, 2 mM Gln supplementation increased ($P < 0.05$) the mRNA levels for Bcl-2 (+26 %), mTOR (+36 %), p70S6 kinase (+29 %), 4E-BP1 (+117 %), COX7C (+32 %), ASCT2 (+67 %), ODC (+146 %), SGLT-1 (+117 %), CFTR (+71 %), $Na^+/K^+$-ATPase (+46 %), HSP70 (+65 %), and ZO-1 (+47 %), while decreasing ($P < 0.05$) the mRNA level for AMPKα2 (−27 %).

## Effects of Gln, Rap, and Cc on mTOR, p70S6 kinase, 4E-BP1, and AMPKα2 mRNA levels in IPEC-1 cells

Effects of Gln, Rap, and Cc on mTOR, p70S6 kinase, 4E-BP1, and AMPKα2 mRNA levels of IPCE-1 cells are shown in Tables 2, 3. Compared with the 0 Gln group, addition of 2 mM Gln or 10 nM Rap to Gln-free culture medium for 72 h increased ($P < 0.01$) the mRNA levels for mTOR, p70S6 kinase and 4E-BP1 (Table 2). However, Gln (2 mM) supplementation for 72 h decreased ($P < 0.01$) the AMPKα2 mRNA levels in IPEC-1 cells. Additionally, the 4E-BP1 and AMPKα2 mRNA levels were increased in IPEC-1 cells by administration of 1 μM Cc (72 h) (Table 3).

## Effects of Gln, Rap, and Cc on abundances of total and phosphorylated mTOR proteins in IPEC-1 cells

Abundances of total and phosphorylated mTOR proteins in IPEC-1 cells are shown in Fig. 5. In comparison with 0 Gln, no difference in abundances of total or phosphorylated mTOR proteins was detected when adding 2 mM Gln to the culture medium for 72 h. However, supplementation with 10 nM Rap reduced ($P < 0.01$) the amounts of total (−39 %) and phosphorylated (−54 %) mTOR (Fig. 5c, e). Interestingly, the abundance of phosphorylated mTOR was increased (+97 %, $P < 0.01$) by 1 μM Cc for 72 h (Fig. 5f).

## Effects of Gln, Rap, and Cc on abundances of total and phosphorylated 4E-BP1 proteins in IPEC-1 cells

Amounts of total and phosphorylated 4E-BP1 proteins in IPEC-1 cells are shown in Fig. 6. Compared with the 0 mM Gln group, supplementation with 2 mM Gln for 72 h increased ($P < 0.01$) both total (+92 %) and phosphorylated (+26 %) 4E-BP1 proteins (Fig. 6c, e). However, a lower (−37 %, $P < 0.01$) amount of phosphorylated 4E-BP1 was observed in the presence of 10 nM Rap (Fig. 6e). As compared with the 0 μM Cc group, 1 μM Cc supplementation

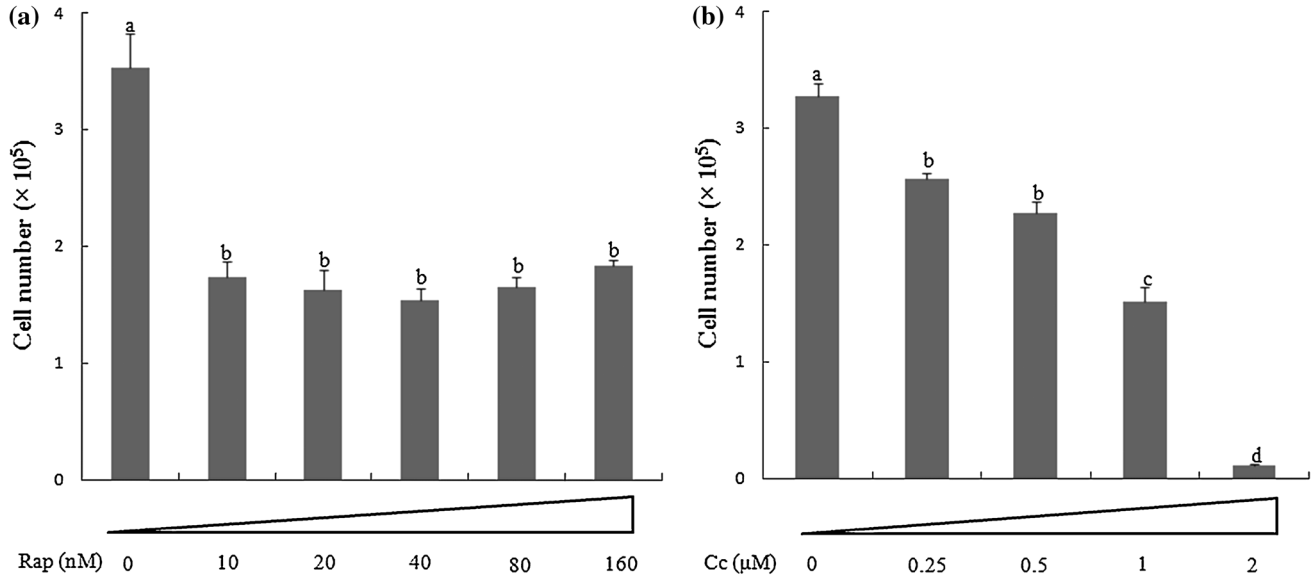


**Fig. 2** The number of IPEC-1 cells cultured in DMEM (0.5 mM Gln) containing 0, 10, 20, 40, 80, or 160 nM Rap (**a**), or 0, 0.25, 1, and 2 μM Cc (**b**) for 3 days. Data are mean ± SEM, $n = 8$. *a*, *b*, *c*, *d*, means sharing different letters differ ($P < 0.05$). *Rap* rapamycin, *Cc* compound C

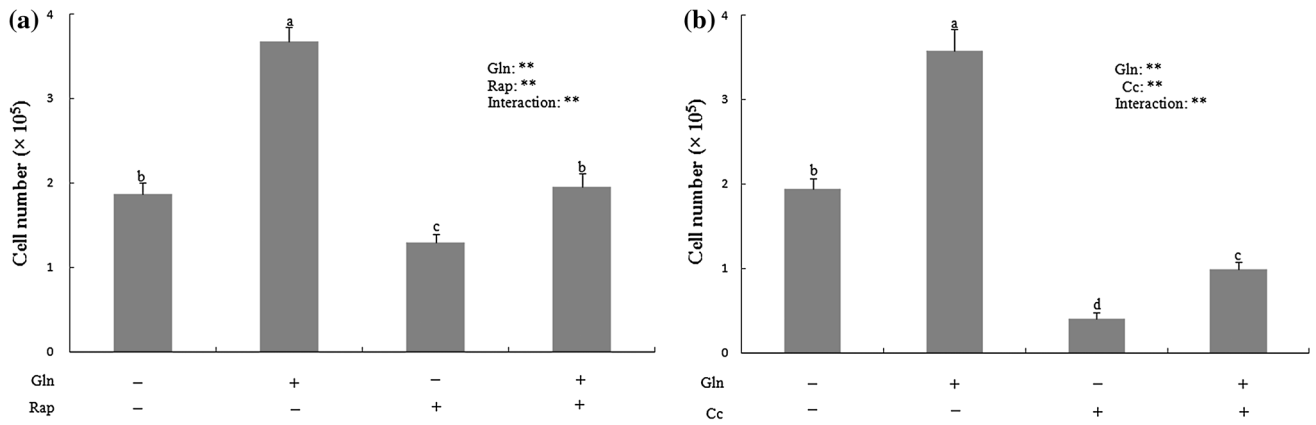


**Fig. 3** The number of IPEC-1 cells cultured in Gln-free DMEM supplemented with 0 or 2 mM Gln plus 0 or 10 nM Rap (**a**), or 0 and 1 μM Cc (**b**) for 3 days. Data are mean ± SEM, $n = 8$. *a*, *b*, *c*, *d*, means sharing different letters differ ($P < 0.05$). $^{**}P < 0.001$. *Rap* rapamycin, *Cc* compound C

increased (+27 %, $P < 0.05$) the abundance of phosphorylated 4E-BP1 in IPEC-1 cells (Fig. 6f).

### Effects of Gln, Rap, and Cc on abundances of total and phosphorylated p70S6 kinase proteins in IPEC-1 cells

Abundances of total and phosphorylated p70S6 kinase proteins in IPEC-1 cells are illustrated in Fig. 7. After 72 h incubation with 2 mM Gln, a higher (+95 %, $P < 0.01$) amount of phosphorylated p70S6 kinase was found in IPEC-1 cells, compared with the 0 mM Gln group (Fig. 7a, e, f). However, total and phosphorylated p70S6 kinase levels were decreased by 10 nM Rap (Fig. 7a, c, e).

### Effects of Gln, Rap, and Cc on abundances of total and phosphorylated AMPK proteins in IPEC-1 cells

Amounts of total and phosphorylated AMPK proteins in IPEC-1 cells are shown in Fig. 8. Supplementing 2 mM Gln to Gln-free culture medium for 72 h did not alter total or phosphorylated AMPK levels in IPEC-1 cells (Fig. 8c, d, e, f). However, supplementation with Cc, an AMPK inhibitor, significantly decreased ($P < 0.05$) the abundance of the total (−20 %) and phosphorylated (−24 %) AMPK (Fig. 8d, f). Meanwhile, the amount of phosphorylated AMPK in IPEC-1 cells was unexpectedly increased (+39 %, $P < 0.01$) by 10 nM Rap at 72 h (Fig. 8e).

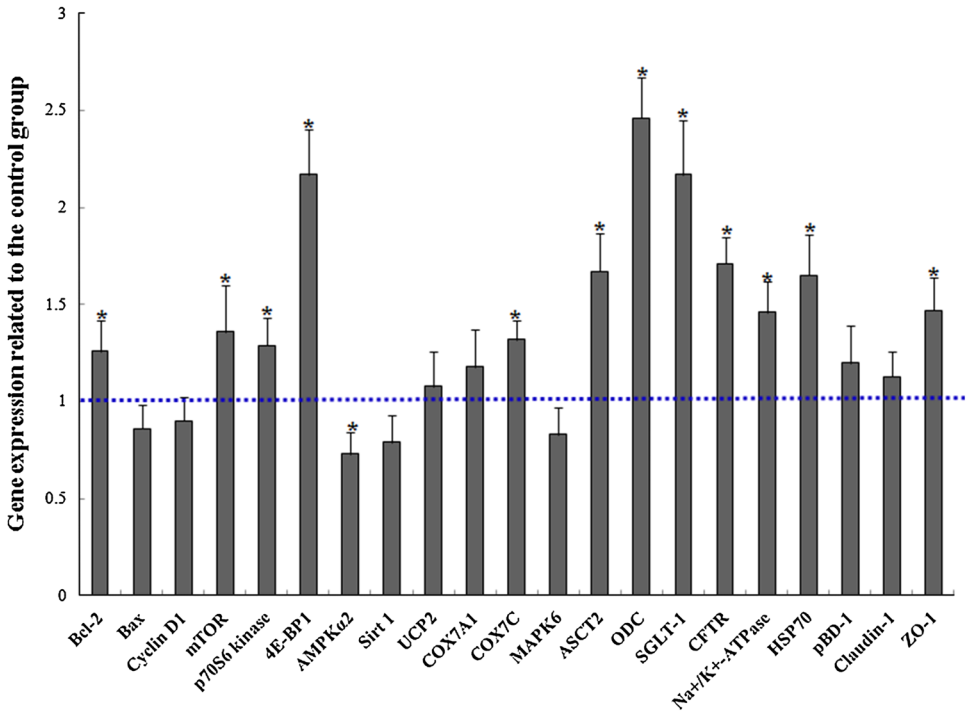


**Fig. 4** Gene expression of IPEC-1 cells. Cells were cultured in Gln-free DMEM supplemented with 0 or 2 mM Gln for 3 days. All mRNA levels in 0 mM Gln group were regarded as 1. Data are mean ± SEM, $n = 8$. *$P < 0.05$

**Table 2** Effects of Gln and Rap on mRNA levels of mTOR, p70S6 kinase, 4E-BP1, and AMPK in IPEC-1 cells

| Gene | 0 nM Rap | | 10 nM Rap | | SEM | *P* value | | |
|---|---|---|---|---|---|---|---|---|
| | 0 mM Gln | 2 mM Gln | 0 mM Gln | 2 mM Gln | | Gln | Rap | Gln × Rap |
| mTOR | 1.00$^{b}$ | 1.57$^{a}$ | 1.45$^{a}$ | 1.53$^{a}$ | 0.03 | <0.01 | <0.01 | <0.01 |
| p70S6 kinase | 1.00$^{c}$ | 1.54$^{b}$ | 0.96$^{c}$ | 2.01$^{a}$ | 0.04 | <0.01 | 0.001 | <0.01 |
| 4E-BP1 | 1.00$^{c}$ | 1.32$^{b}$ | 1.21$^{b}$ | 1.96$^{a}$ | 0.08 | <0.01 | <0.01 | <0.01 |
| AMPKα2 | 1.00$^{a}$ | 0.73$^{c}$ | 0.91$^{ab}$ | 0.82$^{bc}$ | 0.03 | <0.01 | 0.99 | 0.045 |

Data are mean ± SEM, $n = 8$

*Rap* rapamycin

$^{a, b, c}$ Means within rows with different superscripts differ ($P < 0.05$)

**Table 3** Effects of Gln and Cc on mRNA levels of mTOR, p70S6 kinase, 4E-BP1, and AMPK in IPEC-1 cells

| Gene | 0 μM Cc | | 1 μM Cc | | SEM | *P* value | | |
|---|---|---|---|---|---|---|---|---|
| | 0 mM Gln | 2 mM Gln | 0 mM Gln | 2 mM Gln | | Gln | Rap | Gln × Rap |
| mTOR | 1.00$^{b}$ | 1.45$^{a}$ | 0.98$^{b}$ | 1.36$^{a}$ | 0.02 | <0.01 | 0.27 | 0.48 |
| p70S6 kinase | 1.00$^{b}$ | 1.22$^{a}$ | 0.90$^{b}$ | 1.09$^{ab}$ | 0.03 | <0.01 | 0.24 | 0.55 |
| 4E-BP1 | 1.00$^{c}$ | 1.24$^{b}$ | 0.97$^{c}$ | 1.99$^{a}$ | 0.03 | <0.01 | <0.01 | <0.01 |
| AMPKα2 | 1.00$^{c}$ | 0.71$^{d}$ | 4.05$^{a}$ | 2.55$^{b}$ | 0.07 | <0.01 | <0.01 | <0.01 |

Data are mean ± SEM, $n = 8$

*Cc* compound C

$^{a, b, c, d}$ Means within rows with different superscripts differ ($P < 0.01$)

## Discussion

Over the past decades, Gln has attracted much attention from biomedical and nutritional scientists for its beneficial effects on human and animal intestinal health (Wu 2013). In the small intestine, large amounts of glutamine are hydrolyzed by the mitochondrial phosphate-activated glutaminase to from glutamate plus ammonia

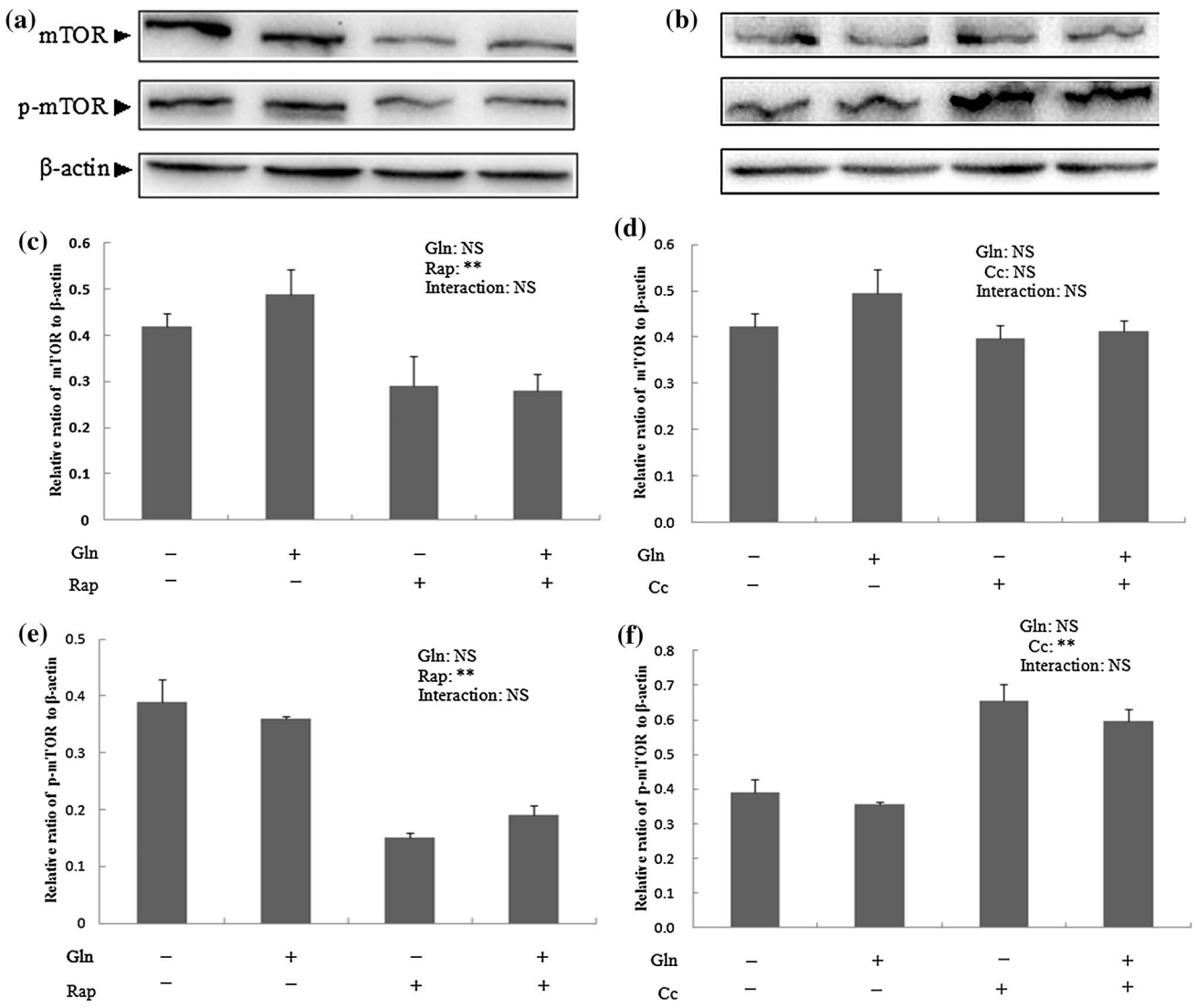


**Fig. 5** Amounts of total (**c** and **d**) and phosphorylated (**e** and **f**) mTOR proteins in IPEC-1 cells. Cells were cultured for 3 days (72 h) in Gln-free DMEM supplemented with 0 or 2 mM Gln plus 0 or 10 nM Rap (**a**), or 0 and 1 μM Cc (**b**). Data are mean ± SEM, $n = 6$. $^{**}P < 0.001$. *NS* no significance, *Rap* rapamycin, *Cc* compound C

(Wu et al. 1995). In contrast, synthesis of glutamine from glutamate is negligible in porcine enterocytes (Haynes et al. 2009; Li et al. 2009; Rezaei et al. 2013a). Besides serving a major metabolic fuel, Gln in both the small-intestinal lumen and the arterial blood is utilized for the synthesis of glutathione, a major antioxidant (Reeds and Burrin 2001; Wang et al. 2013, 2014b). Available evidence shows that glutamine plays a crucial role in maintaining gut architecture and integrity (Rhoads et al. 1997; Mates et al. 2002), enhancing the absorption of nutrients (Ban and Kozar 2010), stimulating mucosal cell proliferation (Rhoads et al. 1997), and improving intestinal energy metabolism (Hinshaw and Burger 1990a, b; Wu et al. 1995; Groening et al. 2011). Recently, elegant studies further elucidated that Gln stimulates protein synthesis and inhibits autophagy in enterocytes via mTOR and MAPK signaling pathways (Xi et al. 2012; Zhu et al. 2014). These findings aid in a new understanding of nutritional regulation of gut health by Gln. Additionally, while Gln was thought to improve cell energy metabolism, in part, via the AMPK (a energy sensor) signaling (Wu 2010), there is no sufficient data to support this notion. At present, it is largely unknown about the cross-talk between mTOR and AMPK signaling pathways or relationship between such a cross-talk with Gln.

In the current study, the optimum concentration and treatment time of Gln were 2 mM [similar to its

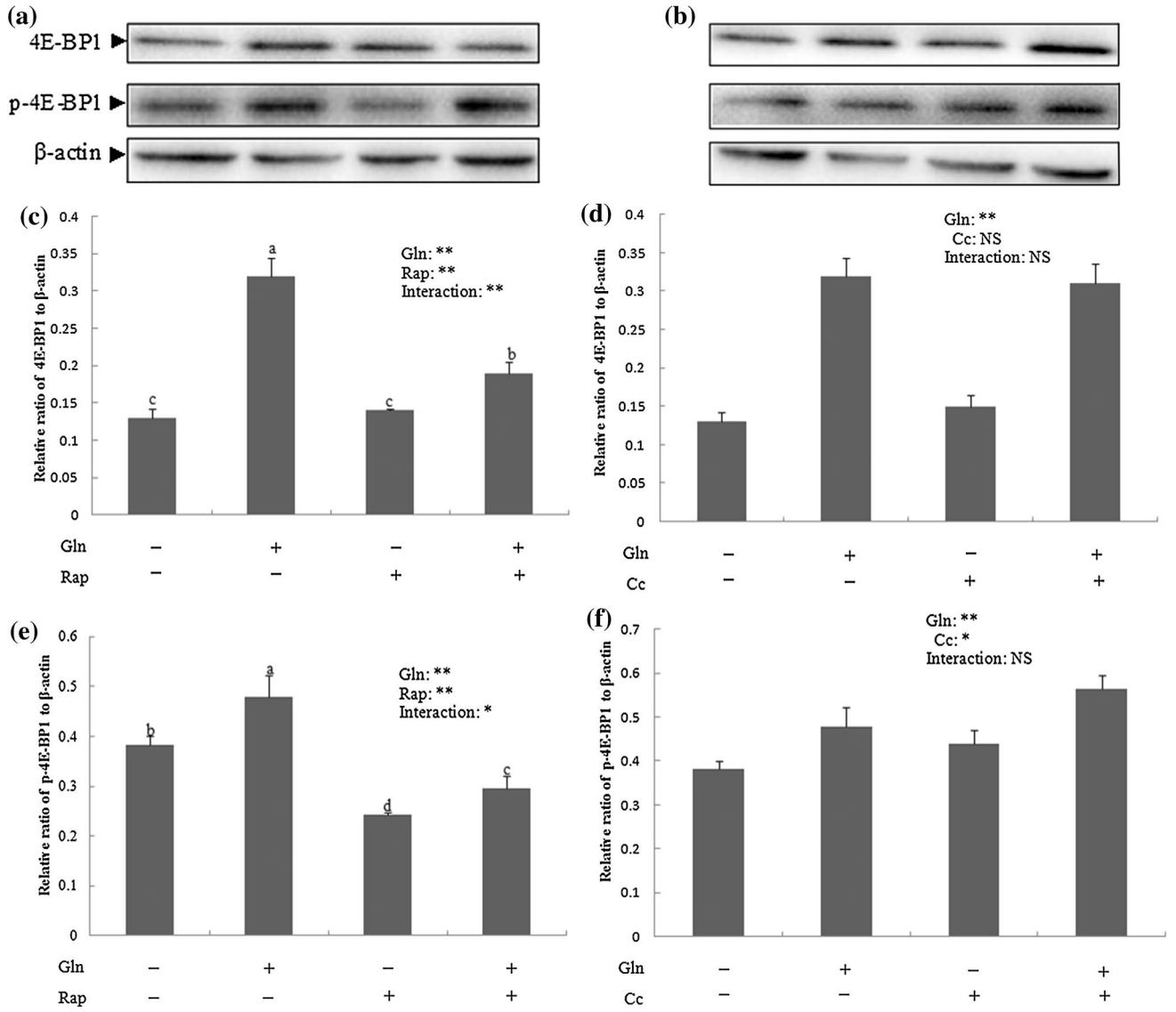


**Fig. 6** Amounts of total (**c** and **d**) and phosphorylated (**e** and **f**) 4E-BP1 proteins in IPEC-1 cells. Cells were cultured for 3 days (72 h) in Gln-free DMEM supplemented with 0 or 2 mM Gln plus 0 or 10 nM Rap (**a**), or 0 and 1 μM Cc (**b**). Data are mean ± SEM, $n = 6$. *a*, *b*, *c*, *d*, means sharing different letters differ ($P < 0.05$). *$P < 0.05$, **$P < 0.001$. *NS* no significance, *Rap* rapamycin, *Cc* compound C

concentration in the porcine small-intestinal lumen (Rezaei et al. 2013b)] and 3 days (72 h), respectively (Fig. 1). Moreover, mRNA levels for the genes associated with cell growth, energy metabolism, nutrient transporters, and barrier function were also altered by supplementation of 2 mM Gln for 72 h. For example, the mRNA levels of mTOR signaling molecules, particularly 4E-BP1 (+117 %), were dramatically increased, but the expression of AMPKα2 was decreased by Gln supplementation in IPEC-1 cells (Fig. 4). To our knowledge, virtually little is known about a regulatory effect of Gln on expression of mTOR and AMPK signaling molecules at the transcriptional level in porcine enterocytes. In skeletal muscle of rats, Gln supplementation had no effects on the mRNA levels of mTOR and 4E-BP1 (Lambertucci et al. 2012), but it remains unknown whether the dose of the supplemental Gln was sufficient to increase its intracellular concentrations. Bcl-2, a protein that inhibits cell autophagy and apoptosis (Xu et al. 2013), was upregulated by Gln supplementation, indicating that Gln improved cell growth possibly through regulating the expression of Bcl-2. This view is substantiated by the study of Zhu et al. (2014), who reported that Gln deprivation decreased the Bcl-2 protein abundance.

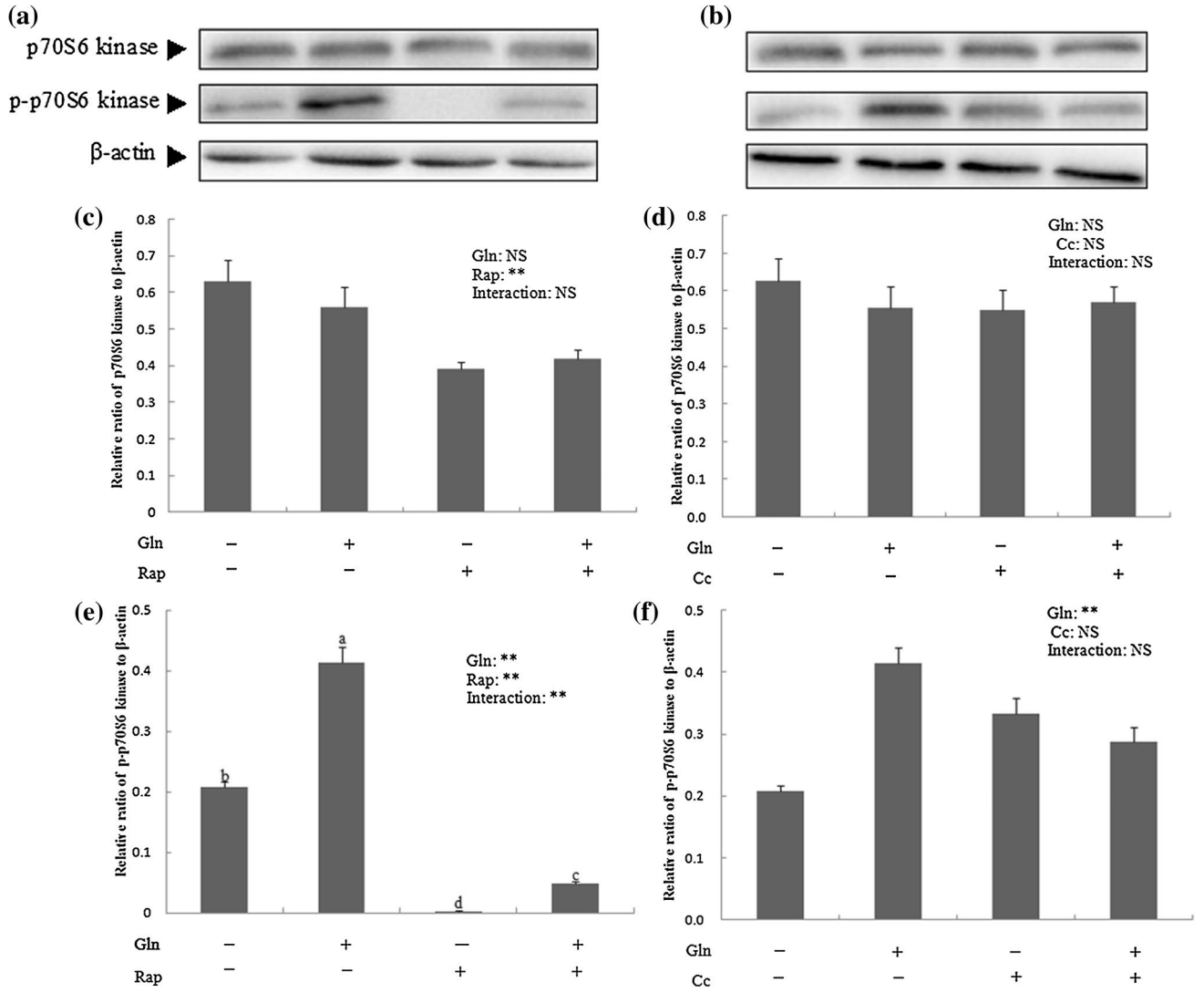


**Fig. 7** Amounts of total (**c** and **d**) and phosphorylated (**e** and **f**) p70S6 kinase proteins in IPEC-1 cells. Cells were cultured for 3 days (72 h) in Gln-free DMEM supplemented with 0 or 2 mM Gln plus 0 or 10 nM Rap (**a**), or 0 and 1 μM Cc (**b**). Data are mean ± SEM, $n = 6$. *a*, *b*, *c*, *d*, means sharing different letters differ ($P < 0.05$). $^{**}P < 0.001$. *NS* no significance, *Rap* rapamycin, *Cc* compound C

Concerning the effects of Gln on expression of genes associated with cell energy metabolism, no significant changes were observed in mRNA levels for Sirt1, UCP2, and COX7A1, with the exception of COX7C (+32 %), a component of the mitochondrial respiratory chain. Exogenous Gln was reported to increase Krebs cycle intermediates and restore COX activity (Groening et al. 2011). Interestingly, several genes encoding nutrient transporters, such as ASCT2, ODC, SGLT-1, CFTR, and $Na^+$/$K^+$-ATPase in IPEC-1 cells were up-regulated by Gln supplementation, implicating that Gln may improve the absorptive function of enterocytes via regulating transcription of genes for these nutrient transporters. Previous studies have also shown the Gln stimulated the ODC activity in IPEC-J2 cells (Kandil et al. 1995) and ASCT2 expression in HepG2 cells (Bungard and McGIVAN 2004). Additionally, Gln was reported to up-regulate expression of heat shock proteins (HSP) in many cell types, including intestinal cells (Rhoads and Wu 2009). Gln potentiation of HSP expression was associated with increased gut epithelial cell resistance to apoptotic injury (Ropeleski et al. 2005). Our results also indicate that 2 mM Gln supplementation increased HSP70 mRNA levels (+65 %) in IPEC-1 cells. Another important finding is that Gln supplementation enhanced the expression of zonula occluden (ZO)-1, a tight junction protein. Because ZO-1 is critical to tight junction assembly and maintenance (Turner 2009), elevated expression of ZO-1 in IPEC-1 cells by Gln may be of assistance in enhancing cell junction and barrier function.

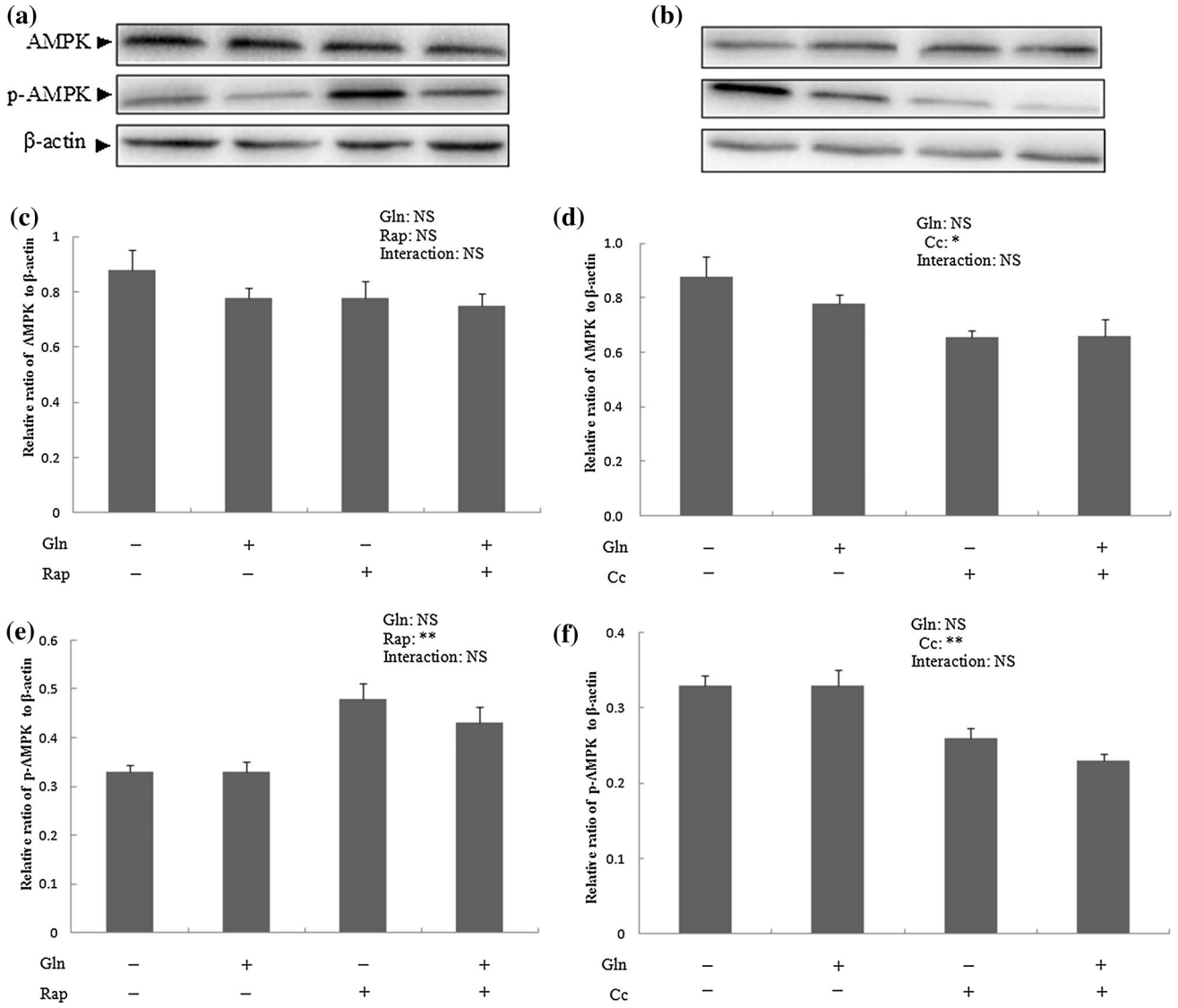


**Fig. 8** Amounts of total (**c** and **d**) and phosphorylated (**e** and **f**) AMPK proteins in IPEC-1 cells. Cells were cultured for 3 days (72 h) in Gln-free DMEM supplemented with 0 or 2 mM Gln plus 0 or 10 nM Rap (**a**), or 0 and 1 μM Cc (**b**). Data are mean ± SEM, $n = 6$. *$P < 0.05$, **$P < 0.001$. *NS* no significance, *Rap* rapamycin, *Cc* compound C

To determine the possible role of mTOR and AMPK signaling pathways in mediating the regulatory effect of Gln on IPEC-1 cell growth, we added Rap (a mTOR inhibitor) and Cc (a AMPK inhibitor) to the culture medium of IPEC-1 cells for 72 h in the presence or absence of Gln. Thereafter, the cell number, mRNA levels and protein expressions of mTOR, p70S6 kinase, 4E-BP1, and AMPK were measured. After administrated with 10 nM Rap or 1 μM Cc for 72 h, IPEC-1 cell numbers were decreased, but increased in the presence of 2 mM Gln (Figs. 2, 3). Moreover, there were significant Gln × Rap and Gln × Cc interactions for cell number (Fig. 3), indicating that mTOR and AMPK signaling pathways are involved in the stimulating effects of Gln on cell growth.

mTOR is an amino acid-responsive and highly conserved serine/threonine protein kinase that controls many aspects of cellular physiology, including protein synthesis (Bauchart-Thevret et al. 2010). Activated mTOR can phosphorylate 4E-BP1 and S6K1, resulting in the translation and polypeptide formation (Inoki et al. 2003; Fumarola et al. 2005). Our results showed that Gln had no effects on total or phosphorylated abundances of mTOR protein (Fig. 5a, c, e), while increasing the levels of phosphorylated p70S6 kinase (Fig. 7a, e) and 4E-BP1 proteins (Fig. 6a, c, e). Similar results were also observed in pig skeletal muscle (Yin et al. 2010) and enterocytes (Xi et al. 2012). When cells were treated with Rap, simultaneous supplementation of Gln increased the cell number, as well

as the abundances of phosphorylated p70S6 kinase and 4E-BP1 proteins (Figs. 6, 7), in comparison with no supplementation of Gln. Therefore, our results indicate that Gln may act directly to phosphorylate p70S6 kinase and 4E-BP1 independent of phosphorylated mTOR protein, further supporting the view of Xi et al. (2012) that Gln enhances intestinal protein synthesis through the activation of mTOR. Regarding the expression of genes in the mTOR signaling pathway, even though Gln could increase the mRNA levels for mTOR and p70S6 kinase (Tables 2, 3), the amounts of these two proteins were not affected by Gln supplementation, indicating different regulation of expression of both genes at transcriptional and translational levels. Similarly, Rap increased mTOR, p70S6 kinase, and 4E-BP1 mRNA levels, while decreasing their proteins abundance. These results suggest a compensatory increase in the transcription of these genes when mTOR activity is inhibited.

AMPK, a highly conserved heterotrimeric kinase, is activated under conditions of energy stress when intracellular ATP levels decline and AMP increases in response to nutrient deprivation (Liu et al. 2012). As an important fuel for enterocytes, Gln was reported to increase cellular ATP levels in normal condition and prevent the loss of ATP in Gln-deprived cells (Hinshaw and Burger 1990a, b; Ahmad et al. 2001). Thus, Gln may affect AMPK activation through elevating cellular ATP levels. In the present study, however, we found that Gln supplementation did not affect the abundance of either total or phosphorylated AMPK protein (Fig. 8), despite a decrease in mRNA levels for AMPKα2 in IPEC-1 cells (Table 2). Therefore, Gln may exert differential effects on the expression of AMPK at transcriptional and translational levels. Interestingly, when AMPK was inhibited by Cc, the abundances of phosphorylated mTOR and 4E-BP1 proteins were increased, but IPEC-1 cell number was reduced (Figs. 5, 6). A possible explanation for these results may be that the passive effects induced by AMPK inhibition override the positive effects of elevated phosphorylated mTOR and 4E-BP1 protein levels. On the other hand, the number of IPEC-1 cells was greater in the presence of both Gln and Cc than in the presence of Cc only (Fig. 3), substantiating our view that the positive effects of Gln on cell growth might not be associated with activation of AMPK. Collectively, these results suggest that Gln improves the growth of IPEC-1 cells independent of AMPK activation. Further studies are warranted to determine energy metabolism and downstream targets of AMPK in Gln-supplemented enterocytes. Nonetheless, these results support the concept that there are dietary requirements for Gln by animals to optimize intestinal health (Wu et al. 2013, 2014).

Furthermore, cellular abundance of the phosphorylated mTOR protein was increased when AMPK was inhibited by Cc in IPEC-1 cells (Fig. 5f), whereas the abundance of the phosphorylated AMPK protein was increased when mTOR was inhibited by Rap (Fig. 8e). A large body of evidence indicated that activated AMPK exerted an inhibitory effect on mTOR phosphorylation (Inoki et al. 2003; Gleason et al. 2007). Thus, it is reasonable to infer that AMPK inhibition could increase the amount of phosphorylated mTOR. Moreover, in animal models with impaired mTORC1 (mTOR complex 1) signaling, AMPK was highly activated (Long et al. 2011), suggesting a negative crosstalk between mTOR and AMPK signaling pathways in IPEC-1 cells.

In summary, Gln enhances the growth of IPEC-1 cells via activating the mTOR signaling pathway independent of AMPK. Gln regulates the expression of genes associated with nutrient transporters, energy metabolism, and cell growth. These results provide new molecular mechanisms for beneficial effects of Gln on intestinal health and growth. Our findings also have important implications for both animal and human nutrition.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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