Effect of glutamine on heat-shock protein beta 1 (HSPB1) expression during myogenic differentiation in bovine embryonic fibroblast cells

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Effect of glutamine on heat-shock protein beta 1 (*HSPB1*) expression during myogenic differentiation in bovine embryonic fibroblast cells

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Abstract The objective of this study was to examine the effects of glutamine on heat-shock protein beta 1 (*HSPB1*) expression in bovine embryonic fibroblast cells during myogenesis. First, to elucidate the role of glutamine on *HSPB1* expression during myogenesis, we treated with glutamine in myogenic lineage determinant (MyoD) over-expressed bovine embryonic fibroblast cells (BEFS-MyoD cells). Second, knockdown of *HSPB1* using small interference RNA was performed to evaluate whether muscle development by glutamine is dependent on *HSPB1* in BEFS-MyoD cells. As a result, glutamine promoted the mRNA level of *HSPB1*, *Myogenin*, *Desmin*, and mTOR as well as myotube formation, and protein synthesis ($p < 0.05$). The inhibition of *HSPB1* expression during myogenesis has shown to repress the expression of myogenic marker genes (MyoD, *Myogenin*, *Desmin*) ($p < 0.01$), formation of myotubes and protein synthesis ($p < 0.05$). According to the results, it is concluded that glutamine regulates *HSPB1* expression during myogenesis.

Keywords Glutamine · *HSPB1* · Bovine embryonic fibroblast cell · Myogenic differentiation · Protein synthesis

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Introduction

Efficient muscle growth and development is critical in beef cattle industry [1]. According to the previous studies, heat-shock protein beta 1 (*HSPB1*) is a candidate protein for muscle growth and development in beef cattle.

HSPB1, which is a 27 kDa protein expressed many tissues including muscle for protecting tissues from physiological stress, is known to enhance muscle development in bovine both in vitro and in vivo [2–4]. *HSPB1* expression is regulated by heat shock factor 1 (*HSF-1*) which is a transcription factor of *HSPB1* controlled by L-glutamine [5]. However, in bovine muscle, the effects of L-glutamine on the *HSPB1* expression is not clear. Thus, we hypothesize that glutamine activates *HSPB1* expression in bovine muscle cells leading to muscle development in myogenic differentiation. During myogenesis, cellular events such as cytoskeletal structure, protein synthesis, and mitochondrial metabolism are also dramatically changed [6]. Therefore, the objective of this study was to profile the gene expression of *HSPB1* as well as myogenic, protein synthesis, and mitochondrial biogenesis genes during myogenesis in myogenic lineage determinant (MyoD) over-expressed immortalized bovine embryonic fibroblast cells (BEFS-MyoD) after treatment with glutamine.

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Materials and methods

Cell culture

BEFS-MyoD cell line, myogenic lineage determinant over-expressed immortalized bovine embryonic fibroblast cells, was used [7]. The cells were grown in 100-mm tissue culture dish in growth medium containing Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences, Chicago, IL, USA) with high-glucose, 10% fetal bovine serum (FBS; GE Healthcare Life Sciences), 1% penicillin/streptomycin (GE Healthcare Life Sciences), 1% L-glutamine (ThermoFisher Scientific Inc., Waltham, MA, USA). Cultures were maintained in an incubator with 5% CO_2 and 37 °C and culture medium was replaced every 2 days. After reaching 100% confluence, cells were transferred to 6-well plates (Corning Inc., NY, USA). Then, the plate including cells were incubated until 100% confluence in growth medium for myogenic differentiation.

Induction of myogenesis

To induce myogenesis, cells were exposed to differentiation medium containing glutamine-free DMEM (GE Healthcare Life Sciences) with high glucose, 2% horse serum (GE Healthcare Life Sciences), 1% P/S (GE Healthcare Life Sciences), 10 μg/ml insulin (Sigma-Aldrich Co. LLC., St-Louis, MO, USA), 10 μg/ml doxycycline (Sigma-Aldrich Co. LLC.), and 0, 1, 2, 4 mM L-glutamine (ThermoFisher Scientific Inc.) respectively for 6 days. The medium was replaced every 2 days.

Small interference RNA treatment

HSPB1 small interference RNA (siRNA) transfection in BEFS-MyoD cells were conducted following the instruction of previous study [4]. Briefly, siRNA targeting *HSPB1* mRNA (sense, 5′-GUAGCCAUCACUGGACAUCUU-3′ and anti-sense, 5′-AAGAUGUCCAGUGAUGGCUAC-3′) was transfected in BEFS-MyoD cells using lipofectamine 2000 (ThermoFisher Scientific Inc.) at 100 nM during myogenic differentiation at day 2 and 4. Negative control siRNA was used as a control group (100 nM). Cells were maintained in differentiation medium supplemented with L-glutamine (4 mM) for 6 days. The medium was also changed every 2 days.

Gene expression analysis by quantitative real-time PCR

RNA was extracted from the differentiated cells at 0, 2, and 6 days respectively. Total RNA was extracted using Trizol (ThermoFisher Scientific Inc.) based on the manufacture protocol. RNA quantity test was conducted using Nanodrop 1000 (ThermoFisher Scientific Inc.). 1 μg RNA was reverse-transcribed with cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After reverse-transcription, a CFX-Connect Real-time system (Bio-Rad Laboratories, Inc.) was used to conduct qRT-PCR. A total reaction volume of real-time PCR mixture containing 50 μg of cDNA, 6 pmol of forward and reverse primers respectively, and 10 μl of SYBR-green master mixture (Bio-Rad Laboratories, Inc.) is 20 μl. Reaction mixture (Bio-Rad Laboratories, Inc.) was incubated at 95 °C for 3 min and then 50 cycles of 10 s at 95 °C, 10 s at 55–60 °C, and 30 s at 72 °C. The sequence information of primers is presented in Table 1. The mRNA expression of target genes normalized to the beta-actin is calculated by ΔΔCt method [8].

Cellular protein content determination

Cells were differentiated for 6 days. The total protein was extracted from cells using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific Inc.) containing 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, 2 mM EDTA, 6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 50 mM NaF, 200 μM Na_3VO_4, and 1x protease inhibitor (GE Healthcare Life Sciences). Protein concentrations were measured by BCA-assay kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions.

ATP concentration analysis

After myogenic differentiation for 6 days, ATP concentration in cells was measured with an ATP-assay kit (Abcam plc., Cambridge, MA, USA). Experiments were performed according to manufacturer's instruction.

Statistics

Student's *t* test was used to determine the significant difference between the two groups. More than three groups, data analysis was performed by one-way ANOVA with Duncan's multiple-range test. All data were presented as mean ± standard deviation (SD). Analyses were conducted using SPSS (SPSS Inc., Chicago, IL, USA). The statistical significance was set at $p < 0.05$.

Results and discussion

Firstly, we investigated the effects of L-glutamine on regulating *HSPB1* gene expression in bovine muscle cells during myogenesis using BEFS-MyoD cells treated with

Table 1 Primers used for quantitative real-time PCR

Gene	Primer	Sequence (5′–3′)	GeneBank accession no.
HSPB1	Forward	CCTGGACGTCAACCACTTC	NM_001025569
	Reverse	GCTTGCCAGTGATCTCCAC	
MyoD	Forward	CGTCTAGCAACCCAAACCAG	NM_001040478
	Reverse	GGCCTTCGATATAGCGGATG	
Myogenin	Forward	TGGGCGTGTAAGGTGTGTAA	NM_001111325.1
	Reverse	TGCAGGCGCTCTATGTACTG	
Desmin	Forward	GGACCTGCTCAATGTCAAGA	NM_001081575
	Reverse	GGAAGTTGAGGGCAGAGAAG	
mTOR	Forward	ATGCTGTCCCTGGTCCTTATG	XM_001788228.1
	Reverse	GGGTCAGAGAGTGGCCTTCAA	
PGC1-α	Forward	GTCCTTCCTCCATGCCTGAC	Q865B7
	Reverse	TAGCTGAGTGTTGGCTGGTG	
β-actin	Forward	GCGTGGCTACAGCTTCACC	NM_173979
	Reverse	TTGATGTCACGGACGATTTC	

HSPB1 heat shock protein beta-1, *MyoD* myogenic differentiation 1, *mTOR* mammalian target of rapamycin, *PGC1-α* peroxisome proliferator-activated receptor gamma coactivator 1-alpha

different L-glutamine concentrations (Fig. 1). As the concentration of L-glutamine increased, *HSPB1* gene expression tended to increase [Fig. 1(A)]. In the day 2 of differentiation, mRNA expression was higher ($p < 0.05$) in 2 mM and 4 mM treatment groups compared to non-treated group. In day 6, the expression of *HSPB1* gene was higher ($p < 0.01$) in all treatment groups of L-glutamine (1, 2, and 4 mM) than in non-treated group. Glutamine, one of the semi-essential amino acids in skeletal muscle [9], is known to induce *HSPB1* expression by regulating *HSF-1* which is the transcription factor of *HSP* genes [10]. As expected, the expression of *HSPB1* was higher in glutamine-treated groups than in non-treated group during myogenesis indicating that glutamine regulates *HSPB1* expression in myogenic differentiation [Fig. 1(A)]. Figure 1(B) shows cells cultured in myogenic differentiation conditions with glutamine (4 mM) formed a lot of fused myotubues, whereas cells without glutamine maintained their initial morphology. Consistent with these findings, as myogenic markers, the mRNA levels of *Myogenin* and *Desmin* were highly increased in 4 mM-treated group in the day 2 ($p < 0.05$), and day 6 ($p < 0.01$), respectively [Fig. 1(D, E)]. In contrast, *MyoD* mRNA level was decreased in glutamine treated group in day 2 compared with non-treated group [Fig. 1(C)]. Myogenesis is regulated by several myogenic genes such as *MyoD, Myogenin, MRF4,* and *Desmin* [11]. These myogenic marker genes are known to be upregulated during myogenic differentiation [12]. Similar to *HSPB1* expression*, Myogenin,* and *Desmin* expressions were higher in glutamine-treated group in our results [Fig. 1(D, E)]. However, it is still unclear how *HSPB1* regulates myogenesis. A recent study suggests that *HSPB1,* a major factor of actin polymerization in muscle, is known for maintaining muscle structure [13]. *HSPB1* also has chaperone function including preventing protein degradation, inhibiting muscle atrophy, and stabilizing muscle protein [14]. It is speculated that *HSPB1* enhances muscle development by protecting muscle proteins [15]. In contrast, *MyoD* expression was lower in glutamine-treated group [Fig. 1(C)]. *MyoD*, one of the initial myogenic marker genes, is known to regulate myogenic determination from myoblast to myotubes and early myogenesis comparing to other myogenic genes [16]. The mechanism about regulating *MyoD* expression is not clear. Therefore, we speculate that some of the feedback mechanism of downregulating *MyoD* expression was initiated due to *MyoD*-over expression cell characteristics [17].

A higher expression of *HSPB1* by L-glutamine promotes protein synthesis [Fig. 2(A, B)]. The expression of Mammalian Target of Rapamycin (mTOR) mRNA was higher ($p < 0.01$) in glutamine-treated group than in non-treated group in day 6. Consistently, cellular protein concentration was higher ($p < 0.01$) in glutamine-treated group than in non-treated group in day 6. mTOR known for master regulator of cellular protein metabolism, is regulated by leucine primarily and critical for protein synthesis in muscle [18, 19]. During myogenesis especially in late stage of differentiation, differentiated primary myotubes go on secondary myotube maturation stage, when cellular protein synthesis and fusions are occurred dramatically in muscle cells via mTOR-pathway [20]. We speculate that there is an upregulation of transcriptional levels of mTOR even if there are abundant activated mTOR proteins in cells. Moreover, *HSPB1* stabilizes protein structure and facilitates protein translation by inhibiting protein degradation in muscle cells during myogenesis [21]. We speculate that

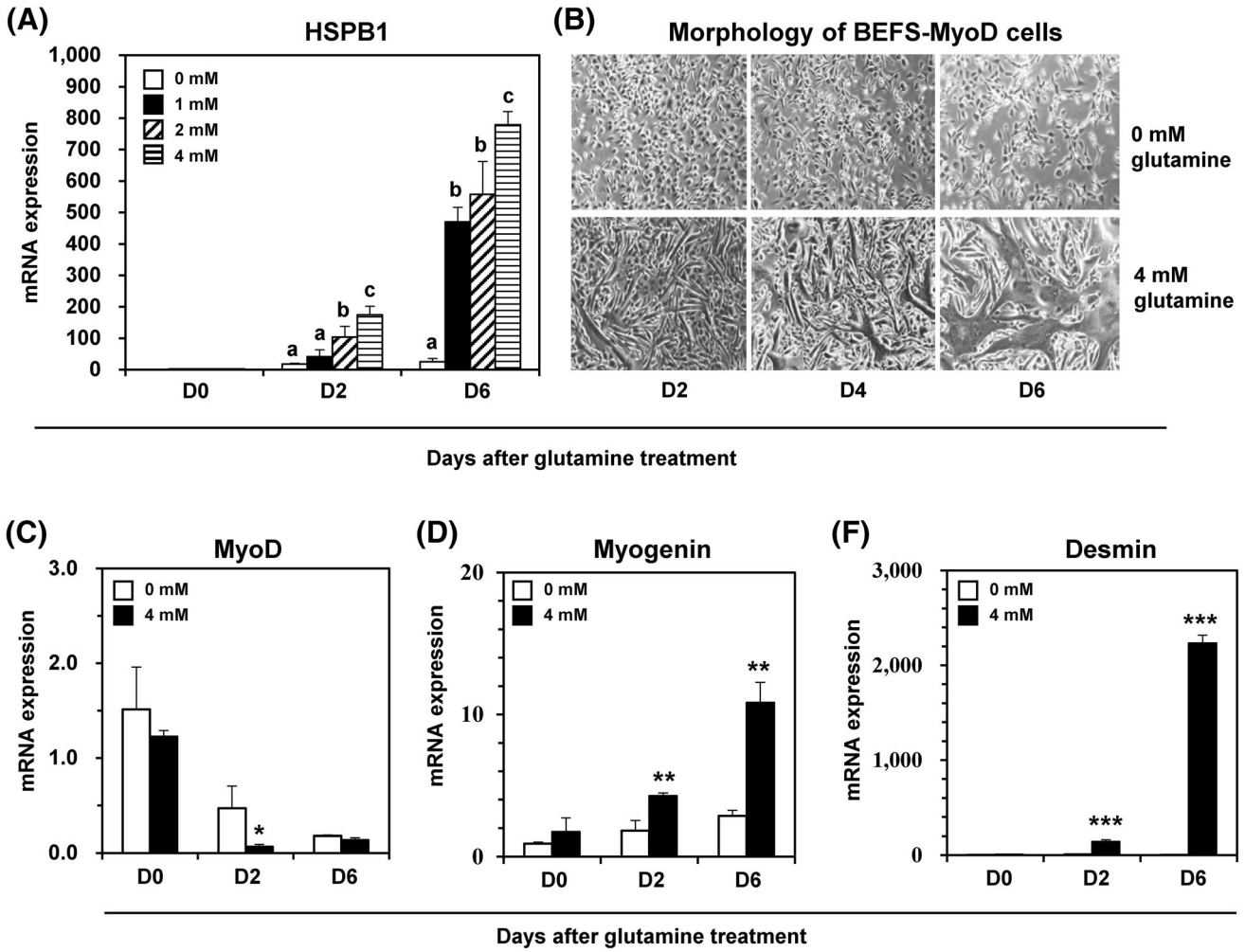

Fig. 1 The effect of L-glutamine on *HSPB1* expression, myogenesis in BEFS-MyoD cells during myogenic differentiation. Confluent cells were differentiated with different L-glutamine concentration (0, 1, 2, and 4 mM). (**A**) Relative mRNA levels of *HSPB1*, (**B**) representative images of BEFS-MyoD cells during myogenesis for 6 days (magnification × 10; Olympus Co., Shinjuku, Tokyo, Japan), (**C**, **D**, **E**) relative mRNA levels of *MyoD, myogenin, Desmin.* All data were represented as the mean ± SD (n = 3). Bars not labeled with same letter are statistically different ($p < 0.05$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

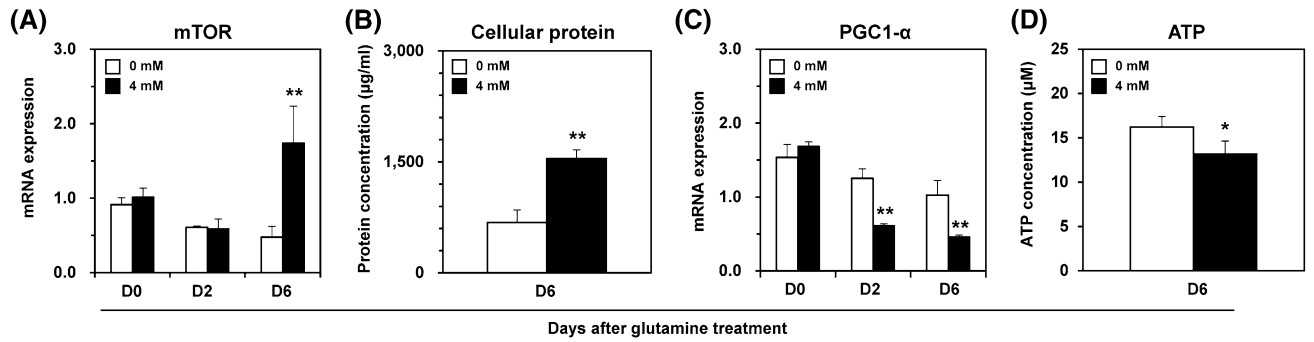

Fig. 2 The effect of L-glutamine on protein synthesis, and mitochondiral changes in BEFS-MyoD cells during myogenic differentiation. Confluent cells were differentiated with different L-glutamine concentration (0 and 4 mM). (**A**) Relative mRNA levels of *mTOR*, (**B**) cellular protein concentration, (**C**) relative mRNA levels of *PGC1-a*, (**D**) Cellular ATP concentration. All data were represented as the mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

due to molecular chaperone activities by *HSPB1*, muscle cells go on differentiation without any physiological stress resulting in upregulating mTOR mRNA expression. Thus, our data suggests that higher *HSPB1* expression by L-glutamine facilitates protein synthesis in muscle during myogenesis.

Mitochondrial biogenesis changes showed that the mRNA expression of *PGC1*-α, known for mitochondrial biogenesis gene, was higher ($p < 0.01$) in glutamine non-treated group than in treated-group in both day 2, and day 6 [Fig. 2(C, D)]. In the result of ATP concentration assay, cellular ATP amounts was lower ($p < 0.05$) in glutamine-treated group than in non-treated group in day 6. In general, mitochondria is a main metabolic organelle of muscle cells [22]. Mitochondrias are generated more in myotubes than in myoblasts during myogenesis due to high demand of ATPs [23]. Mitochondiral biogenesis is regulated by PGC-1α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha) which is a main determinant of mitochondrial numbers [24]. In the present study, PGC-1α mRNA expression and cellular ATP contents were lower in glutamine-treated group than in non-treated group [Fig. 2(C, D)]. According to the previous study, *HSPB1* prevents mitochondria fragmentation associated with apoptosis [25]. It is expected that PGC-1α expression is upregulated during myogenesis due to the reduced *HSPB1* expression.

We next examined whether L-glutamine enhances myogenesis in BEFS-MyoD cells only via *HSPB1* pathway (Fig. 3). Cells treated with L-glutamine (4 mM) were

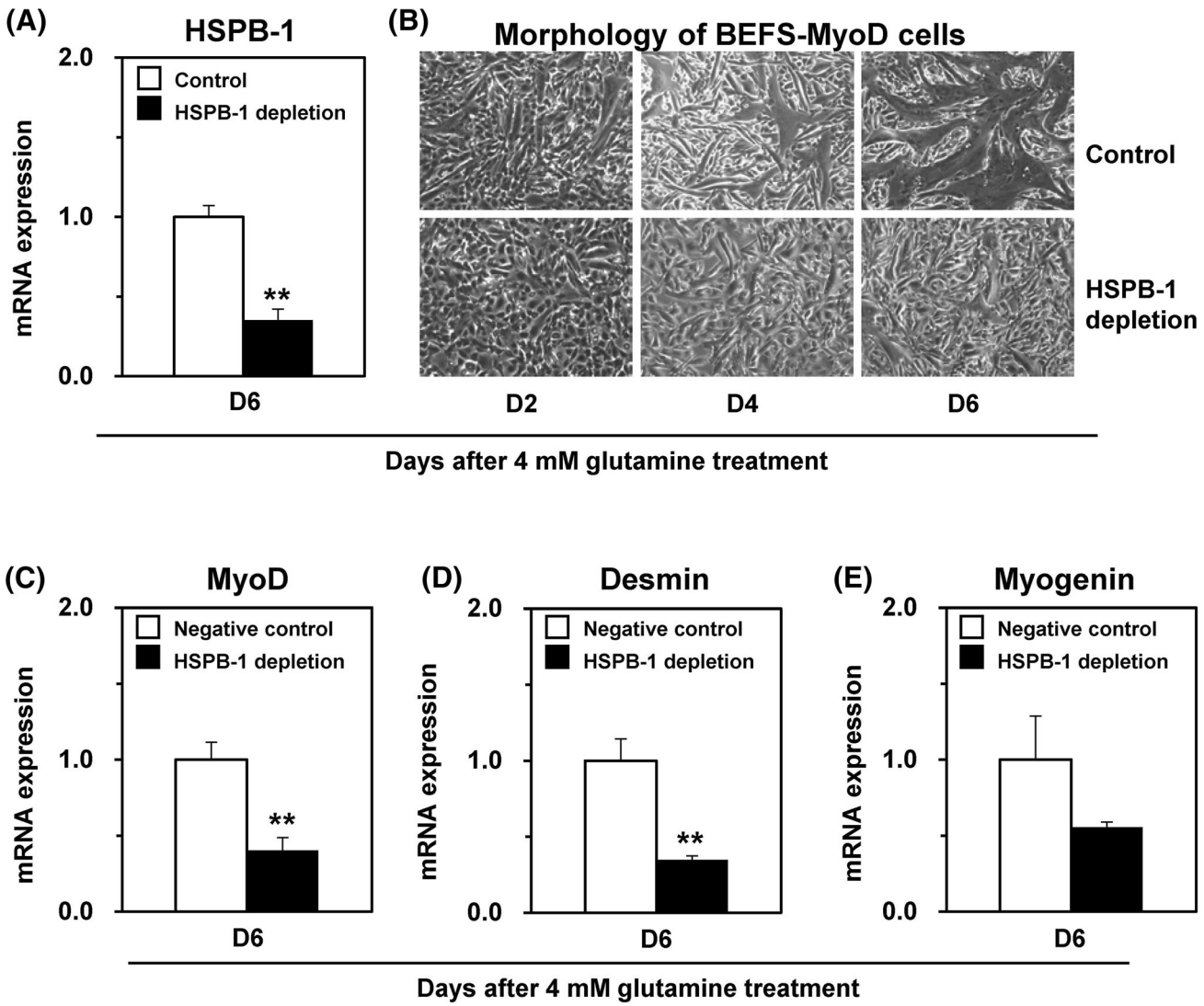

Fig. 3 The effect of *HSPB1* knockdown on glutamine-mediated myogenesis in BEFS-MyoD cells. (**A**) Relative mRNA levels of *HSPB1*, (**B**) representative images of BEFS-MyoD cells during myogenesis for 6 days (× 10), (**C**, **D**, **E**) relative mRNA levels of *MyoD, Desmin, myogenin*. All data were represented as the mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

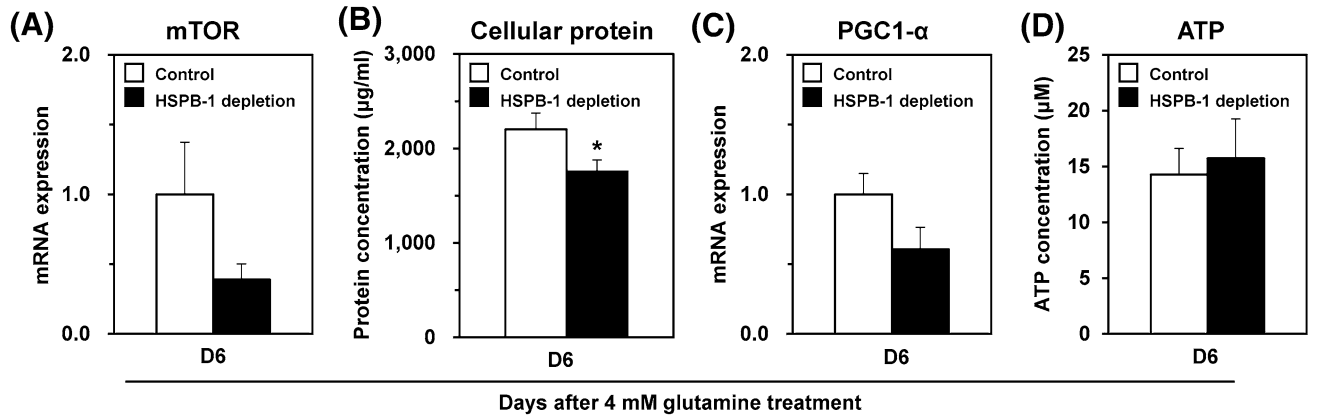

Fig. 4 The effect of *HSPB1* knockdown on protein synthesis, and mitochondiral changes during glutamine-mediated myogenesis in BEFS-MyoD cells. (**A**) Relative mRNA levels of *mTOR*, (**B**) cellular protein concentration, (**C**) relative mRNA levels of *PGC1-α,* (**D**) cellular ATP concentration. All data were represented as the mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

conducted *HSPB1* gene knockdown by siRNA transfection during myogenic differentiation day 2 and day 4, respectively. *HSPB1* knockdown caused a reduction in both *HSPB1* mRNA ($p < 0.01$) levels in day 6 [Fig. 3(A)]. Cell images shows that reduction of *HSPB1* mRNA inhibits myotubes formation [Fig. 3(B)], similarly in, *MyoD* and *Desmin* expression [Fig. 3(C, D)] ($p < 0.01$).

Reduction of *HSPB1* expression by siRNA transfection to BEFS-MyoD cells induces decreased cellular protein synthesis (Fig. 4). Cellular protein concentration was reduced ($p < 0.05$) in siRNA transfection group compared to control group [Fig. 4(B)]. mTOR mRNA expression was not significantly different between the two groups [Fig. 4(A)]. In the results of mitochondrial biogenesis changes, there were no significant differences between two groups in PGC-1α expression, and ATP concentration respectively [Fig. 4(C, D)].

HSPB1 knockdown inhibited myogenic differentiation during androgen-mediated myogenesis [4].

According to [Figs. 3(B)–(D), 4(B)] it is estimated that inhibition of myogenesis by *HSPB1* knockdown decreases cellular protein synthesis. However, comparing to results (Figs. 1, 2), the differences between the two groups in myogenic marker genes and protein synthesis were lower. We estimated that in the results (Figs. 3, 4), adequate supplements of L-glutamine (4 mM) recover myogenic differentiation. There were no significant differences in PGC-1α mRNA expression and cellular ATP contents [Fig. 4(C, D)]. Since the difference in the expression level of *HSPB1* is insignificant compared with result groups [0, 4 mM; Fig. 1(A)], it is expected that mitochondrial fragmentation was lower in *HSPB1* knockdown groups (Control, siRNA).

According to results, it is unclear that glutamine regulates myogenesis only via *HSPB1* pathway. However, as the expression level of *HSPB1* decreased, muscle development and protein synthesis also tended to decrease. Thus, it is estimated that glutamine enhances muscle development during myogenesis stage via targeting *HSPB1* expression considerably.

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