β-carotene attenuates muscle wasting in cancer cachexia by regulating myogenesis and muscle atrophy

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Abstract. Cancer cachexia is a metabolic disease involving multiple organs, which is accompanied by the depletion of muscle tissue and is associated with ~20% of cancer-related deaths. Muscle wasting is a critical factor in cancer cachexia. β-carotene (BC) has been shown to increase muscle mass and hypertrophy in healthy mice. However, its effects on muscle tissue dysregulation in cancer cachexia have yet to be studied. In the present study, 5-week-old male C57BL/6J mice were injected with $1x10^6$ Lewis lung carcinoma (LLC) cells to induce cancer cachexia; then the mice were administered BC (4 or 8 mg/kg) for 22 days to assess its effects on muscle atrophy in the gastrocnemius muscles. The effects of BC on inflammatory cytokines, myogenesis and muscle atrophy were evaluated using C2C12 myotubes treated with LLC-conditioned media. BC supplementation significantly suppressed tumor growth, inflammatory cytokines, and hepatic gluconeogenesis in the LLC-induced cancer cachexia mouse model, while also improving muscle weight and grip strength. These effects are considered to be mediated by the PI3K/Akt pathway and through regulation of muscle atrophy. Moreover, BC treatment was associated with the recovery of LLC-conditioned media-induced muscle differentiation deficits and muscle atrophy in C2C12 myotubes. These findings indicate BC as a potential novel therapeutic agent for cancer cachexia.

Introduction

Cancer cachexia is a devitalizing multifactorial syndrome that affects several metabolic processes in multiple organs. It is characterized by progressive weight loss due to the depletion of muscle with or without loss of adipose tissue mass, which cannot be recovered by nutritional therapy (1). Patients with cachexia exhibit decreased responses to chemotherapy and a diminished tolerance to anticancer treatments (1).

Skeletal muscle wasting is a hallmark of cachexia (2) and induces pathophysiological changes, such as a weak and fragile body (3). Moreover, patients with cachexia experience dysregulations in protein metabolism, decreased protein synthesis and upregulated whole-body protein turnover (4). The onset of muscle atrophy is a consequence of an imbalance between the synthesis and breakdown of skeletal muscle proteins (5). The muscle-specific E3 ubiquitin ligases, MAFbx/atrogin-1 (atrogin-1) and muscle RING-finger protein-1 (MuRF1) are the key regulators of muscle atrophy in cancer cachexia (6). Notably, they are upregulated in the skeletal muscles of patients with cancer cachexia, thereby indicating the presence of muscular atrophy (6). In addition, the knockdown of atrogin-1 or MuRF1 ameliorated muscle wasting in patients with cancer cachexia (6,7).

In cases where tumor and muscle tissues exist distantly from each other, proinflammatory cytokines, such as IL-6 and TNF-α, mediate signals to promote the breakdown of proteins, while also inhibiting protein synthesis (8). Systemic inflammation and the resulting catabolic stimuli cause cachexia by suppressing the synthesis of muscle protein and promoting muscle catabolism and atrophy (9). The liver is also involved in regulating metabolic processes in cancer cachexia (10). In patients with cancer cachexia, amino acids released by muscle wasting can be used for hepatic gluconeogenesis (11). Moreover, inflammatory cytokines, such as IL-6 produced by activated macrophages stimulate the liver to produce an acute phase response (12).

Muscle stemness can be damaged when cancer cachexia impedes muscle stem cell differentiation (13). In cancer cachexia, impaired muscle stem cell function can cause skeletal muscle atrophy by decreasing the regeneration of muscle myofibers (13). When quiescent muscle satellite cells are activated, they coexpress paired box 7 (Pax7) and myoblast determination protein 1 (MyoD), two major transcription factors of myogenic differentiation (14,15).

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The PI3K/Akt signaling pathway is reportedly involved in protein turnover in muscle tissues (16). Whereby the phosphorylation of PI3K causes the insulin-like growth factor (IGF)-1 to activate Akt and mTOR, which promotes protein synthesis and muscle proliferation (17). Thereby, cancer cachexia commonly exhibits an inhibited PI3K/Akt signaling pathway during muscle atrophy (18).

β-carotene (BC) is a provitamin A carotenoid and a strong antioxidant, which can scavenge free radicals (19,20). Oxidative stress can cause a reduction in the intracellular antioxidant pool, which results in the disturbance of the myogenic differentiation and muscle atrophy (21,22). Moreover, since BC acts as a strong antioxidant, it can defend against oxidative stress and DNA damage (23). In fact, BC exerted protective effects against oxidative stress (100 μM of H_2O_2)-induced muscle atrophy in C2C12 myotubes and soleus muscle atrophy in mice (24). In addition, combination treatments of the representative antioxidants including BC, astaxanthin, and resveratrol, increased protein synthesis in muscles during hypertrophy following atrophy in mice (25).

The concentration of serum carotenoid concentration has been revealed to be negatively associated with the risk of decreased muscle strength and walking pace (26,27). However, it has been reported that supplementation with BC decreased the soleus muscle mass loss in mice caused by denervation (24). It has been demonstrated that BC administration elevated muscle mass and functional hypertrophy of the soleus muscle in mice under physiological conditions (28). A combination of BC, astaxanthin and resveratrol increased protein synthesis and counteracted muscle hypertrophy in mice (25). BC supplementation also upregulated myoblast differentiation in chicken (29). In addition, the mean serum level of BC was significantly reduced in patients with cachexia (30). However, the effects of BC on the regulation of muscle atrophy and myogenic differentiation during cancer cachexia have yet to be investigated.

Thus, the aim of the present study was to investigate whether: i) BC can suppress aberrant muscle differentiation and atrophy induced by cancer cachexia; ii) the PI3K/Akt pathway can contribute to this effect; and iii) increased hepatic gluconeogenesis and systemic inflammation caused by cancer cachexia can be countered by BC supplementation.

Materials and methods

Cell culture and reagents. Lewis lung carcinoma (LLC) cells (CRL-1642, https://www.atcc.org/products/crl-1642) and C2C12 myoblasts (CRL-1772, https://www.atcc.org/products/crl-1772) were purchased from American Type Culture Collection and cultured in a growth medium containing Dulbecco's modified Eagle's medium (DMEM; Welgene, Inc.) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% streptomycin-penicillin (Invitrogen; Thermo Fisher Scientific, Inc.) in an incubator at 37˚C with a humidified atmosphere of 5% CO_2. BC was purchased from MilliporeSigma and dissolved in tetrahydrofuran (THF; MilliporeSigma). Mycoplasma testing was conducted for these cell lines and it was confirmed that they were mycoplasma-free. Fresh BC stock solution was prepared before each application and stored under dim light.

Cancer cachexia mouse model. Male C57BL/6J mice were purchased at 5 weeks of age from Central Lab Animal Inc. All mice were individually housed in a 12:12-h light-dark cycle and fed AIN-93G purified rodent pellet diets (RaonBio Inc.) *ad libitum*. After 1 week of acclimatization, the mice were randomized and sorted into four groups: i) Control mice (CTRL; n=12); ii) LLC cell-induced cancer cachexia mice (CC; n=12); iii) LLC cell-induced cancer cachexia mice supplemented with BC at 4 mg/kg body weight (BW) (BC 4; n=12); and iv) LLC cell-induced cancer cachexia mice supplemented with BC at 8 mg/kg BW (BC 8; n=12). To induce cancer cachexia, $1x10^6$ LLC cells were diluted in 100 μl of phosphate-buffered saline and subcutaneously inoculated into the right hindlimb of each mouse. The mice were orally administered BC 4 and BC 8 dissolved in 100 μl of corn oil twice a week throughout the experimental period.

BW and tumor volume were measured every other day. Tumor volume was calculated as width (mm) x length^2/2, with dimensions presented in millimeters (31). The diameter and volume of the largest tumor of mice on the day of sacrifice was 2.0 cm and 2.9 cm^3. Diameter measurements were decreased by 0.1 cm for skin and subcutaneous fat. Notably, 22 days after tumor cell inoculation, all mice were sacrificed by CO_2 inhalation, and the tissues and blood were collected. When sacrificing the mice, the CO_2 flow rate for euthanasia was calculated. CO_2 was delivered at three different flow rates: 30, 50 and 70% of the cage volume per minute, corresponding to rates of 5.9, 9.9 and 13.9 l/min, respectively. Serum samples were obtained by centrifugation at 25,553 x g for 15 min at 4˚C. Moreover, the weights of the organs (spleen, liver, etc.), muscles (gastrocnemius, pectoralis, etc.) and fats (subcutaneous, perirenal, etc.) were measured. Liver and gastrocnemius muscles were extracted for PCR, western blot and histological analyses.

The weights of the mouse carcasses and tumors were measured immediately after sacrifice. 'Carcass-tumor weight' was obtained by subtracting the tumor weight from the mouse carcass weight.

The present study was conducted according to the guidelines of the National Institutes of Health (NIH publication no. 8023, revised 1978), and approved (IACUC approval no. EWHA IACUC 21-003-1) by the Institutional Animal Care and Use Committee of Ewha Womans University (Seoul, Republic of Korea).

Grip strength assessment. Grip strength was determined using a grip strength meter (Jeung-Do Bio & Plant Co., Ltd.). After allowing the mouse to grip a mesh bar linked to a force transducer, the mouse tail was pulled gently until its grip was released, and the peak force (g) generated was recorded by the transducer. Reported values are the average of the measurements for each mouse, with a 1-min interval between sets.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells and gastrocnemius muscles using TRIzol reagent (Thermo Fisher Scientific, Inc.). Reverse transcription was then performed according to the manufacturer's protocol using a cDNA Reverse-Transcription kit (Thermo Fisher Scientific, Inc.). The resulting cDNA was used to perform quantitative polymerase

Table I. Primer sequences for reverse transcription-quantitative PCR.

Mouse species gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Murf1	TGACATCTACAAGCAGGAGTGC	TCGTCTTCGTGTTCCTTGC
Atrogin-1	AGTGAGGACCGGCTACTGTG	GATCAAACGCTTGCGAATCT
MyoD	CTACAGTGGCGACTCAGATG	TGTAGTAGGCGGTGTCGTAG
Pax7	CTGGATGAGGGCTCAGATGT	GGTTAGCTCCTGCCTGCTTA
G6pase	AGGAAGGATGGAGGAAGGAA	TGGAACCAGATGGGAAAGAG
Pepck	AGAGCAGAGAGACACAGTGC	AGGGCGAGTCTGTCAGTTCA
Gapdh	AACTTTGGCATTGTGGAAGG	TGTGAGGGAGATGCTCAGTG

chain reaction (PCR) amplification using a SYBR Green master mix (Qiagen GmbH). Briefly, cDNAs were mixed with 2X Rotor-Gene SYBR Green PCR Master Mix and the cycling program consisted of one cycle at 95˚C for 5 min, followed by 40 cycles at 95˚C for 5 sec, and 60˚C for 10 sec. The expression of all genes was normalized relative to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The sequences of the primers for RT-qPCR are listed in Table I. The relative quantification was performed by the common $2^{-\Delta\Delta Cq}$ method (32).

Western blot analysis. Protein samples from cells and gastrocnemius muscles were extracted using a radioimmunoprecipitation assay (RIPA) lysis buffer [150 mM NaCl, 50 mM Tris-hydrochloride (pH 7.5), 1% Nonidet, P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and 1 mM sodium fluoride]. Protein concentrations were determined by Bradford protein assay (Bio-Rad Laboratories, Inc.) and western blotting was then performed. The proteins (60 μg) were loaded and separated on a sodium dodecyl sulfate (SDS) denaturing polyacrylamide 12% gel and transferred to a PVDF membrane. The proteins were blocked with 5% skim milk for 1 h at room temperature and incubated with the respective primary antibodies overnight at 4˚C. Primary antibodies included anti-MuRF1 (1:200; cat. no. sc-398608; Santa Cruz Biotechnology, Inc.), anti-atrogin-1 (1:200; cat. no. sc-166806; Santa Cruz Biotechnology, Inc.), anti-phosphorylated (p)-Akt (Ser473) (1:1,000; cat. no. 9271; Cell Signaling Technology, Inc.), anti-Akt (1:1,000; cat. no. 9272; Cell Signaling Technology, Inc.), anti-p-PI3K p85 (Tyr458)/p55 (Tyr199) (1:1,000; cat no. 4228; Cell Signaling Technology, Inc.), anti-PI3K (1:1,000; cat no. 4292; Cell Signaling Technology, Inc.) and anti-α-tubulin (1:10,000; cat no. T5168; Sigma-Aldrich; Merck KGaA). Subsequently, the membranes were washed 3 times for 5 min with TBST containing 0.05% Tween-20, and then, incubated with the respective secondary anti-IgG (mouse and rabbit) antibodies [(1:2,000; cat no. 610-1302; Rockland Immunochemicals, Inc.) and (1:5,000; cat no. sc-2357; Santa Cruz Biotechnology, Inc.), respectively], which were purchased from Bio-Rad Laboratories, Inc. The protein of interest was visualized using an enhanced chemiluminescence reagent (cat no. SM801-0500; Gene DireX, Inc.). α-Tubulin was used as the internal control. Quantifications of blots were performed using an ImageJ software (v1.8.0; National Institutes of Health).

Enzyme-linked immunosorbent (ELISA) assay. The concentrations of serum IL-6 (cat. no. KMC0061) and TNF-α (cat. no. BMS607-3; Invitrogen; Thermo Fisher Scientific, Inc.) were measured by ELISA according to the manufacturer's protocols.

Hematoxylin and eosin (H&E) staining. The gastrocnemius muscle tissues were fixed in 4% formaldehyde for 48 h at room temperature, and cut into 5-micron-thick sections. Subsequently, the slices were stained with H&E at room temperature using an automated stainer (Thermo Fisher Scientific, Inc.). The duration for staining was 10 min for eosin Y and 5 min for Harris hematoxylin. Images were then captured using a light microscope before being analyzed by ImageJ software (v 1.8.0).

Immunohistochemistry (IHC). The gastrocnemius muscle tissues were fixed in 4% neutral buffered formaldehyde for 48 h at room temperature. The fixed muscle tissues were embedded in paraffin and sectioned into 4-μm thick sections. To perform immunohistocemical analysis, the sections were incubated at 37˚C with proteinase K (Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min for antigen retrieval and incubated at room temperature with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase/phosphatase activity. All sections were incubated with blocking reagent (2.5% normal horse serum; Vector Laboratories, Inc.) at room temperature for 1 h. The sections were incubated with anti-MyoD (1:500; cat. no. sc-377460; Santa Cruz Biotechnology, Inc.) and anti-atrogin-1 (1:80,00; cat. no. 67172-1-Ig; ProteinTech Group, Inc.) at 4˚C overnight. The sections were then incubated with the secondary antibody (biotinylated goat anti-mouse IgG; cat. no. PK-6102 ABC kit; Vector Laboratories, Inc.) at room temperature for 1 h, followed by incubation with avidin-biotin reagent (Vector Laboratories, Inc.) at room temperature for 30 min. After incubation, all sections were incubated with 1X 3,3-diaminobenzidine tetrahydrochloride (DAB; Thermo Fisher Scientific, Inc.). The sections were counterstained with Mayer's hematoxylin for 30 sec at room temperature. At least three randomly selected image fields per sample were examined under a light microscope (Olympus Corporation) and imaged.

Cell culture and conditioned medium collection. LLC cells were seeded at $5x10^6$ cells per dish of 75 cm^2 and cultured to >90% confluence, after which, their growth medium was

removed and replenished with serum-free DMEM. After 48 h of incubation, the conditioned medium (CM) was collected into a centrifuge tube and centrifuged at 3,062 x g for 15 min at 4˚C. The collected CM was passed through a syringe filter with 0.2-μm pores (Sartorius AG) and aliquoted for storage at -20˚C until required for further use. Cell cultures were maintained in a 5% CO_2/95% air atmosphere at 37˚C.

Differentiation and LLC CM treatment of C2C12 cells. C2C12 cells seeded at a density of 50,000 cells/well in a 6-well plate and were incubated in a growth medium (DMEM with 10% FBS and 1% streptomycin-penicillin) until reaching 90-100% confluence. The growth medium was then replaced by a differentiation medium [high-glucose DMEM containing 2% horse serum (Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin] to induce myogenic differentiation for 2 days. Subsequently, the differentiation medium was replaced by a 1:1 mixture of DMEM supplemented with 2% horse serum and LLC CM for 3 days in the presence of BC or THF. The medium was changed every day during the experiment. All the cellular experiments were performed under 5% CO_2/95% air atmosphere at 37˚C.

Myotube length assessment. Cellular differentiation was analyzed by measuring the myotube length using images captured by a microscope at magnifications of x10 or x20 (Leica, DMI 6000 B; Leica Microsystems, Inc.). Myotube length (μm) was assessed using ImageJ (v1.8.0) software.

Cell viability assay. Cell viability was estimated using MTT (MilliporeSigma) assay. C2C12 myotubes were seeded at a concentration of 10,000 cells/well and differentiated in 96-well plates and exposed to LLC CM for 4 days with BC or THF at 37˚C. The media were removed, and 100 μl MTT solution was added to each well and allowed to incubate for 3 h at 37˚C. The plate was analyzed using a plate reader at 560 nm (Molecular Devices, LLC).

Statistical analysis. All data are expressed as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc test was conducted using GraphPad PRISM software (version 3.02; GraphPad Software; Dotmatics). A P-value threshold of <0.05 was considered to indicate a statistically significant difference. Each experiment involved at least three replicates.

Results

Effects of BC on cachectic progression in an LLC-induced cancer cachexia mouse model. On days 10, 16, 18, 20 and 22 after cancer cell inoculation, tumor volumes had decreased significantly in the BC-supplemented groups compared with the tumor volumes in the CC group (Fig. 1A). An MTT assay was then conducted to evaluate the direct effects of BC on the number of viable LLC cells. After 3 days of treatment, BC induced significant reductions in the viability of LLC cells, in a dose-dependent manner. Thus, it can be inferred that BC has direct antitumor effects on LLC cancer cells (Fig. S1). Carcass weight after tumor removal (carcass-tumor weight) was significantly decreased in the CC group compared with the CTRL group (P<0.05). The body weight changes during the experiment period were measured (Fig. S2A). While the body weights of the BC 4 and BC 8 groups were significantly higher than the CC group on day 18, there were no statistically significant differences among groups on the last day of the experiment. Carcass-tumor weight tended to be increased by BC supplementations at 4 and 8 mg/kg BW compared with the CC group, although the levels of statistical significance were both P>0.05 (Fig. 1B). The weights of the mice spleens, hearts, livers, kidneys and lungs from the mice were also measured. BC supplementation did not exert significant effects on the organ weight recovery compared with the CC group weights (Table SI). As anorexia is normally accompanied by cachexia, food intake was measured every two days. However, there were no significant differences throughout the experimental period among the groups for food intake (Fig. S2B).

Subsequently, the effects of BC on cancer cachexia-induced muscle and adipose tissue weight losses were observed. As illustrated in Table II, LLC tumor inoculation caused significant reductions in the weights of the gastrocnemius (P<0.001), pectoralis (P<0.01), triceps (P<0.05) and quadriceps (P<0.001) compared with the CTRL group. However, the weights in the BC 8 group were significantly increased for the gastrocnemius muscle (P<0.01), triceps (P<0.05) and quadriceps (P<0.05) compared with the CC group. Moreover, the weight of the gastrocnemius muscle in the BC 4 group was also significantly increased (P<0.001) compared with the CC group. In addition, the weights of the adipose tissues, including subcutaneous (P<0.001), perirenal (P<0.001), mesenteric (P<0.01) and epididymal fats (P<0.01) of the CC group were decreased compared with the CTRL group. By contrast, BC supplementation at 8 mg/kg BW significantly restored the mesenteric fat weight loss (P<0.05) compared with the CC group. These results indicated that BC effectively attenuated cancer cachexia-related muscle wasting.

To identify whether the increased muscle mass in the mice fed the BC supplement was related to functional improvement, the grip strength was analyzed at days 0, 7, 14 and 21 after inoculating the LLC cells (Fig. 1C). The grip strength in all mice decreased after day 7, an effect that was considered to be due to the stress caused by repeated practices. Moreover, compared with the CTRL group, there was a greater reduction in grip strength in the CC group 7 days after the LLC cell inoculation (P<0.01 on day 7; P<0.001 on days 14 and 21). Furthermore, the mice in the BC 8 group significantly recovered their grip strength on days 14 and 21 after LLC cell inoculation by 21 and 23%, respectively (P<0.05 for both).

Serum IL-6 and TNF-α levels were measured using ELISA (Fig. 1D). The levels of these proinflammatory cytokines were significantly increased in the CC group compared with the CTRL group, whereby IL-6 increased by 14.1% (P<0.001) and TNF-α increased by 11.5% (P<0.01). Nonetheless, supplementation with BC at 4 and 8 mg/kg BW significantly decreased the IL-6 serum levels by 7.0 and 6.1%, respectively, compared with those observed in the CC group (P<0.05 for both). In addition, a BC supplementation of 8 mg/kg BW effectively decreased the TNF-α serum levels by 7.5% compared with the CC group (P<0.05).

Hepatic gluconeogenesis regulation by BC in LLC cancer cachexia mice is depicted in Fig. 1E. The mRNA expression levels of both *G6pase* and *Pepck* were significantly

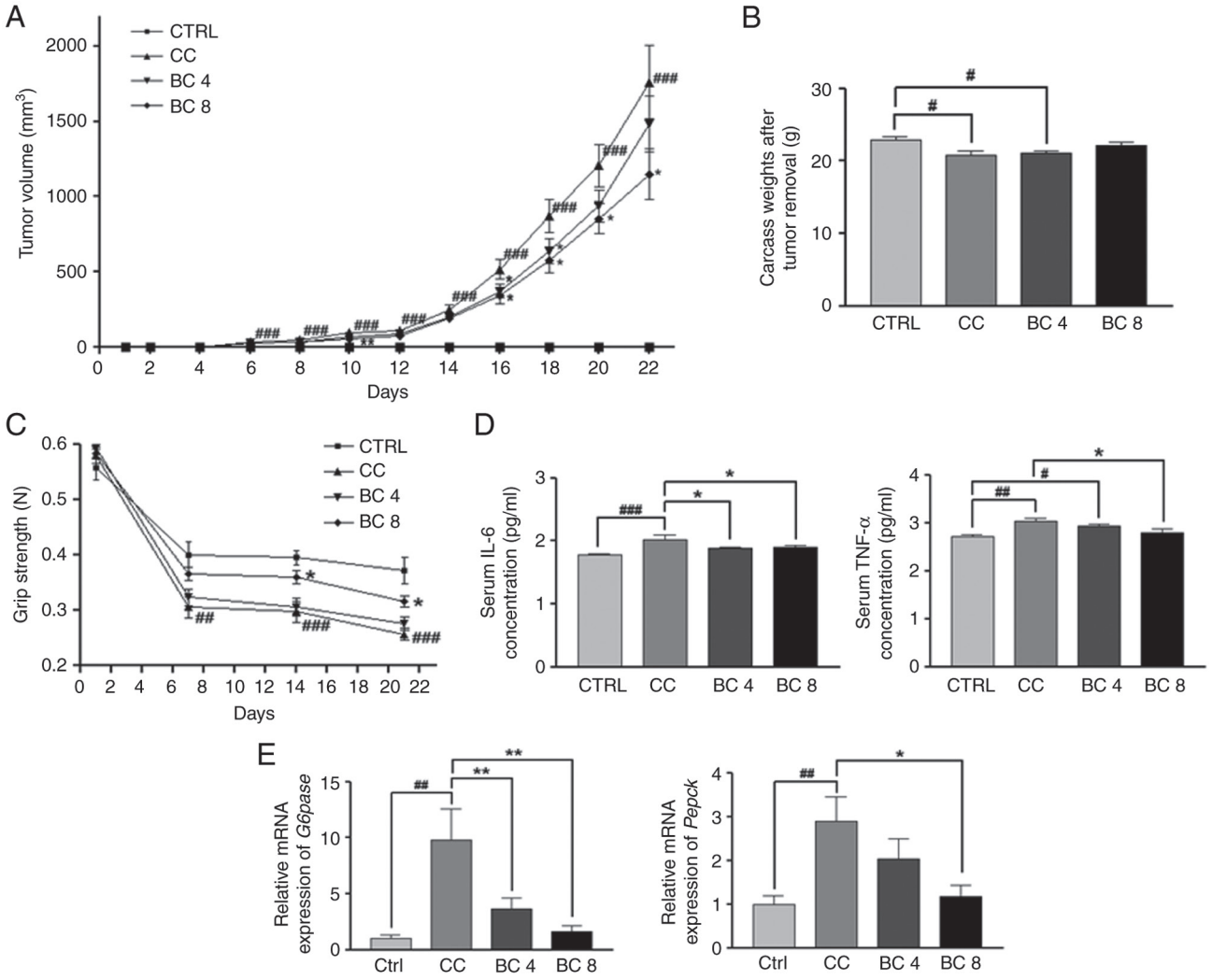

Figure 1. Effects of BC on cachectic progression in an LLC cancer cachexia mouse model. (A) Tumor volumes, (B) carcass weights after tumor removal (carcass-tumor weight) and (C) grip strength were analyzed. (D) Serum IL-6 and TNF-α levels were assessed using ELISA. (E) mRNA expression of gluconeogenesis-related genes, *Pepck* and *G6pase* in the liver was determined by reverse trascription-quantitative PCR. *Gapdh* was used as a loading control. All data are shown as the mean ± standard error of the mean and were analyzed using one-way ANOVA with a Newman-Keuls post hoc test. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the CTRL; *P<0.05 and **P<0.01 vs. CC. BC, β-carotene; LLC, Lewis lung carcinoma; CTRL, control; CC, LLC-induced cancer cachexia; BC 4, LLC-induced cancer cachexia + 4 mg/kg BW of β-carotene; BC 8, LLC-induced cancer cachexia + 8 mg/kg BW of β-carotene; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; *G6pase*, glucose 6-phosphatase; *Pepck*, phosphoenol-pyruvate carboxykinase.

increased in the CC group compared with the CTRL group (P<0.01 for both, respectively). In addition, compared with the CC group, the expression of both genes was significantly downregulated with BC supplementation at 8 mg/kg BW, by 83.3 and 59.2%, compared with the CC group (P<0.01 and P<0.05, respectively). The *G6pase* mRNA expression was also significantly decreased by 63.1% in the BC supplementation group of 4 mg/kg BW compared with the CC group (P<0.01).

Effects of BC on gastrocnemius muscle atrophy in an LLC-induced cancer cachexia mouse model. As revealed in Fig. 2A, H&E staining of the gastrocnemius muscles in the CC group revealed a decrease in the size of the muscle fibers, while this was restored by BC supplementation. The myofiber size distribution tended to shift rightward in the BC 8 group (the most frequent value range was 1,500-2,000 μm^2) compared with the CC group (the most frequent value range was 1,000-1,500 μm^2), indicating the myofibers in the BC group were longer than those in the CC group (data not shown).

In Fig. 2B, the atrogin-1 protein expression in gastrocnemius muscles was upregulated in the CC group compared with the CTRL group (P<0.01), which indicated an increase in muscle atrophy. However, this expression was significantly suppressed by 52.2 and 48.6% in the BC supplementation groups of 4 and 8 mg/kg BW, respectively, compared with the CC group (both P<0.05).

As demonstrated in Fig. 2C, the *atrogin-1* and *Murf-1* mRNA levels were significantly increased in the CC group compared with the CTRL group (P<0.01 and P<0.05, respectively), while BC supplementation of 4 or 8 mg/kg BW significantly suppressed these increases (P<0.05 for both). As revealed in Fig. 2D, mRNA expression of muscle stem cell markers, *MyoD* and *Pax7* in gastrocnemius muscles was significantly higher in the CC group compared with the CTRL group (P<0.05 for both). Conversely, the *MyoD* mRNA level

Table II. Effects of BC on various types of fat and muscle weights in an LLC cancer-cachexia mouse model.

Muscle or fat tissue	CTRL	CC	BC 4	BC 8
Gastrocnemius muscle (g)	0.222±0.011	0.152±0.010[c]	0.205±0.012[f]	0.208±0.006[e]
Pectoralis muscle (g)	0.055±0.005	0.035±0.004[b]	0.045±0.003	0.043±0.004
Triceps (g)	0.113±0.010	0.085±0.005[a]	0.093±0.006	0.113±0.008[d]
Quadriceps (g)	0.153±0.006	0.110±0.007[c]	0.118±0.007[b]	0.135±0.008[d]
Tibialis anterior muscle (g)	0.066±0.005	0.050±0.005	0.058±0.005	0.061±0.005
Subcutaneous fat (g)	0.245±0.025	0.099±0.009[c]	0.098±0.011[c]	0.105±0.014[c]
Perirenal fat (g)	0.107±0.007	0.067±0.006[c]	0.067±0.005[c]	0.085±0.007[a]
Mesenteric fat (g)	0.213±0.019	0.136±0.011[b]	0.158±0.009[a]	0.193±0.018[d]
Epididymal fat (g)	0.297±0.023	0.211±0.017[b]	0.222±0.013[a]	0.265±0.019
Brown fat (g)	0.094±0.007	0.075±0.004	0.076±0.005	0.082±0.009

All data are shown as mean ± standard error of the mean and were analyzed by one-way ANOVA with a Newman-Keuls post hoc test. [a]P<0.05, [b]P<0.01 and [c]P<0.001 vs. the CTRL; [d]P<0.05, [e]P<0.01, and [f]P<0.001 vs. the CC group. BC, β-carotene; LLC, Lewis lung carcinoma; CTRL, Control; CC, LLC-induced cancer cachexia; BC 4, LLC-induced cancer cachexia + 4 mg/kg body weight of BC; BC 8, LLC-induced cancer cachexia + 8 mg/kg body weight of BC.

was significantly reduced compared with the CTRL level following BC supplementation of 4 and 8 mg/kg BW (P<0.05 and P<0.01, respectively), while the *Pax7* mRNA level was significantly downregulated by BC supplementation of 8 mg/kg BW (P<0.05).

In Fig. 2E, the IHC staining of gastrocnemius muscles revealed that both MyoD and atrogin-1 were highly overexpressed in the nuclei of the CC group compared with those of the CTRL group. The expression of MyoD and atrogin-1, however, was decreased and weakened in the BC 4 and BC 8 groups when compared with the CC group (magnification, x100).

Effects of BC on the PI3K/Akt pathway in gastrocnemius muscles of LLC-induced cancer cachexia mice. To examine the effects of BC on the PI3K/Akt pathway in the gastrocnemius muscle in cancer cachexia mice, p-PI3K and p-Akt protein expression was analyzed. As shown in Fig. 3, the ratio of p-PI3K/PI3K and p-Akt/Akt in the CC group was lower than that of the CTRL group (both P<0.05). However, BC supplementation at 4 mg/kg suppressed the downregulation of phosphorylation of PI3K by 71.9%, whereas PI3K and Akt phosphorylation was increased in the BC 8 group by 80.4 and 282.7%, respectively (both P<0.05). These results demonstrated that BC stimulated the PI3K/Akt signaling pathway to inhibit muscle wasting in the gastrocnemius muscle induced by cancer cachexia.

Effects of BC on myogenesis and muscle atrophy in C2C12 myoblasts treated with LLC CM. As demonstrated in Fig. 4A-a, the addition of LLC CM inhibited the formation of normal myotubes. Specifically, LLC CM incubation shortened myotubes (P<0.05), suggesting that LLC CM caused myotube atrophy. However, treatment with 10 and 20 μM of BC significantly suppressed myotube length shortening by 38.2 and 46.7%, respectively, compared with the CC group (P<0.05 for both) (Fig. 4A-b). In Fig. 4B, the absorbance values from the MTT assay indicated that BC (10 or 20 μM) did not affect the cellular viability of the myotubes within 3 days of treatment. Moreover, no cytotoxic effects were observed on the C2C12 myotubes at any of the BC concentrations up to 20 μM.

The culture medium was isolated to measure the IL-6 and TNF-α levels. As revealed in Fig. 4C, the secretion of IL-6 and TNF-α was significantly increased by 20.7 (P<0.01) and 41.4%, respectively in the CC group (P<0.001) compared with the CTRL group. Interestingly, while the IL-6 level tended to be restored by BC treatments, albeit without statistical significance, the elevated TNF-α level caused by LLC CM incubation was significantly suppressed by 20 μM BC (P<0.05).

Next, the regulatory roles of BC on the levels of muscle atrophy-related markers in C2C12 myotubes, while incubated with LLC CM, were analyzed. As revealed in Fig. 4D, C2C12 myotubes treated with LLC CM exhibited an upregulation of protein expression levels of the muscle atrophy-related markers atrogin-1 and Murf1 compared with the CTRL group (P<0.05 for both). However, these expression levels were suppressed by treatment with BC at 20 μM (P<0.05). In Fig. 4E, LLC CM treatment also significantly upregulated *atrogin-1* and *Murf1* mRNA expression levels compared with the CTRL group (P<0.05 for both). However, these expression levels were significantly downregulated by treatment with BC at 10 and 20 μM (P<0.05 for *atrogin-1* and P<0.01 for *Murf1*). These data indicated that BC could restore the LLC CM-induced dysregulations of myogenesis, muscle atrophy and proinflammatory cytokines in C2C12 myotubes.

In addition, the protein expression levels of atrogin-1 and Murf1 in C2C12 myotubes treated with BC at 20 μM were evaluated in the presence or absence of LLC CM. As a result, treatment with BC at 20 μM in the absence of LLC CM tended to suppress the expression levels of Murf1 and atrogin-1 compared with the CTRL group, but they were not statistically significant (P>0.05 for both). Nevertheless, as already described in the present study, LLC CM-induced upregulation of atrogin-1 and Murf1 compared with the CTRL group were significantly suppressed by 20 μM BC treatment (P<0.01 for

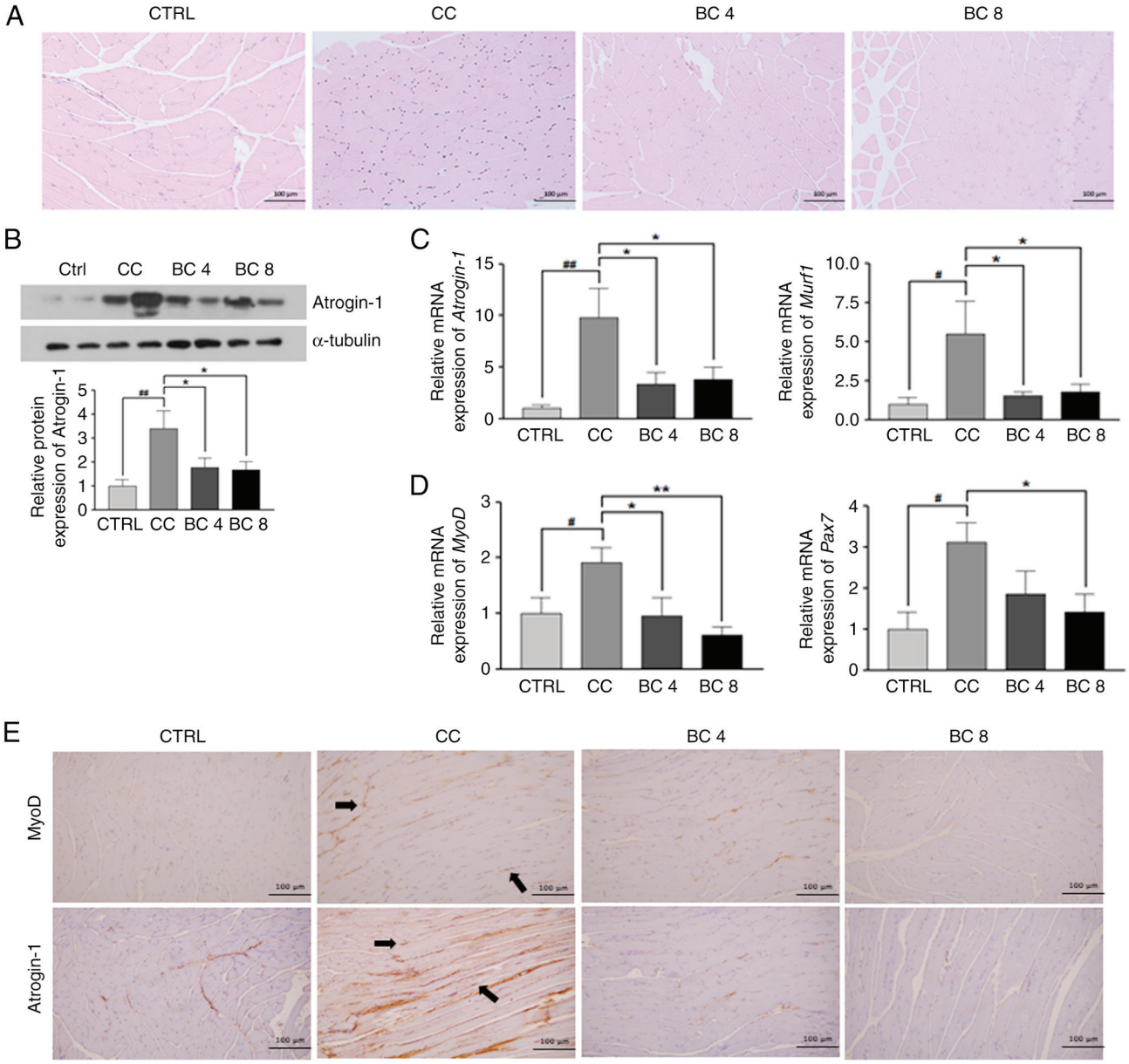

Figure 2. Effects of BC on gastrocnemius muscle atrophy in an LLC cancer cachexia mouse model. (A) Representative images of hematoxylin-eosin stained sections of the gastrocnemius muscle (magnification, x100; scale bar, 100 μm) (B) Protein expression level of atrogin-1 was analyzed by western blotting. (C) mRNA expression levels of muscle atrophy-related genes, *atrogin-1* and *Murf1*, and (D) muscle stem cell-related genes, *MyoD* and *Pax7* in gastrocnemius muscle were determined by reverse trascription-quantitative PCR. *Gapdh* was used as a loading control. (E) Representative images of IHC staining of MyoD and atrogin-1 in gastrocnemius muscle (magnification, x100). All data are shown as the mean ± standard error of the mean and were analyzed using one-way ANOVA with a Newman-Keuls post hoc test. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the CTRL; *P<0.05 and **P<0.01 vs. CC. BC, β-carotene; LLC, Lewis lung carcinoma; CTRL, control; CC, LLC-induced cancer cachexia; BC 4, LLC-induced cancer cachexia + 4 mg/kg BW of β-carotene; BC 8, LLC-induced cancer cachexia + 8 mg/kg BW of β-carotene; Murf1, muscle RING-finger protein-1; MyoD, myoblast determination protein 1; Pax7, paired box 7.

atrogin-1 and P<0.05 for Murf1). These results indicated that BC is only effective in inhibiting the muscle atrophy in C2C12 myotubes with LLC CM incubation (Fig. S3).

Discussion

The present study identified the protective effects of BC on cancer cachexia-induced muscle wasting both *in vitro* and *in vivo*. BC supplementation prevented the loss of muscle mass and grip strength in the LLC cell-bearing cancer cachexia mouse model. Moreover, BC administration also suppressed muscle atrophy by regulating the PI3K/Akt pathway and muscle stemness. *In vitro* analysis confirmed that LLC CM caused reductions in myotube length and myogenesis and increased muscle atrophy and inflammatory cytokine secretions compared with the CTRL group. However, these alterations were effectively restored by treatments with BC.

LLC cells were used to induce cancer cachexia both *in vitro* and *in vivo* (33-35). Consistent with the model of the present study, a previous study revealed that LLC cell injection effectively induced cancer cachexia with muscle wasting in mice (36). Moreover, LLC CM caused a reduction in C2C12 myotube size and the upregulation of muscle atrophy markers, atrogin-1 and MuRF1 (37,38).

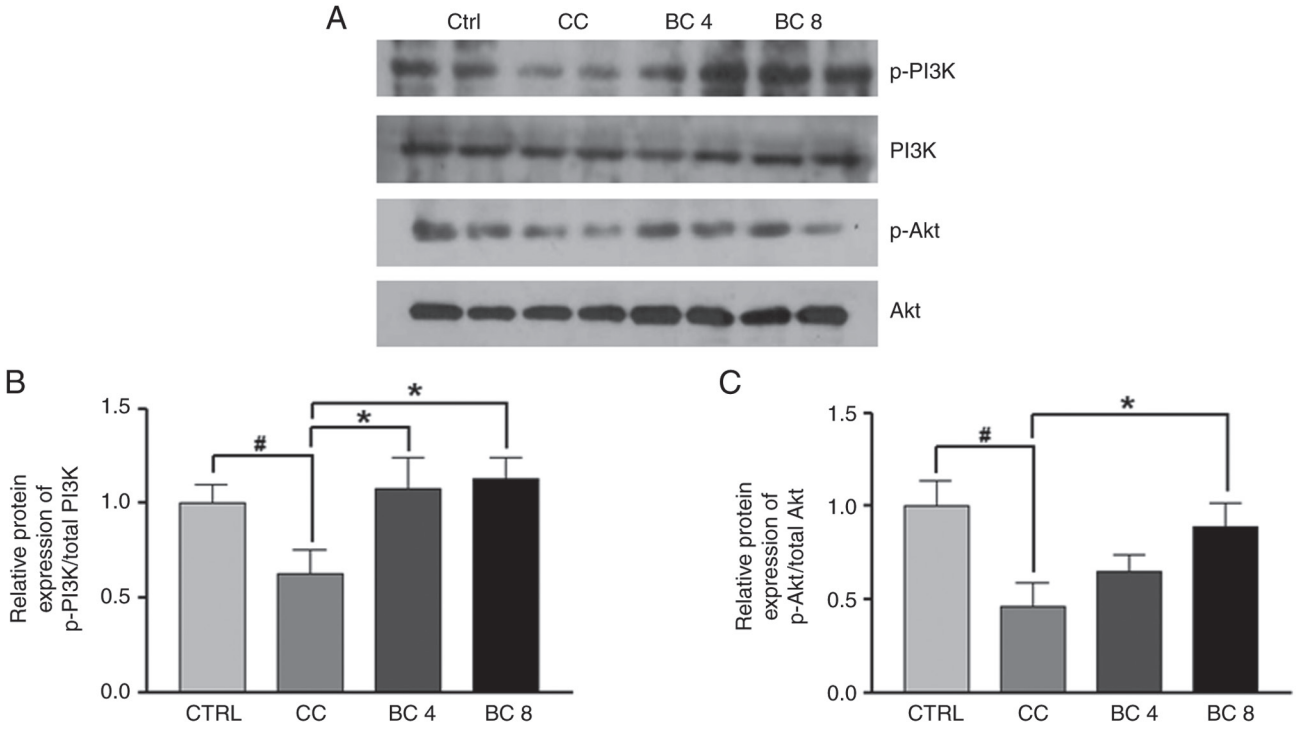

Figure 3. Effects of BC on protein expression of the PI3K/Akt pathway in gastrocnemius muscles of an LLC cancer cachexia mouse model. (A-C) Protein expression of the PI3K/Akt pathway in gastrocnemius muscle was determined by western blotting. All data are shown as mean ± standard error of the mean and were analyzed using one-way ANOVA with a Newman-Keuls post hoc test. $^{\#}$P<0.05 vs. the CTRL; *P<0.05 vs. CC. BC, β-carotene; LLC, Lewis lung carcinoma; CTRL, control; CC, LLC-induced cancer cachexia; BC 4, LLC-induced cancer cachexia + 4 mg/kg BW of β-carotene; BC 8, LLC-induced cancer cachexia + 8 mg/kg BW of β-carotene; PI3K, phosphoinositide 3-kinase.

Since overgrown and large tumors can cause great pain in mice, the mice were sacrificed before the average length of their tumor reached 2 cm in accordance with IACUC regulations. Moreover, based on the preliminary study results, 22 days of $1x10^6$ LLC cell inoculation could successfully induce cancer cachexia phenotypes, such as muscle atrophy and adipose depletion. Therefore, mice were sacrificed 22 days after cancer cell inoculation in the present study.

While a previous study investigating the effects of BC on muscle metabolism mainly focused on the soleus muscle (39), the gastrocnemius muscle was the primary focus in the present study because it represents the most common skeletal muscle associated with cachexia. Additionally, it is more involved in cancer cachexia pathogenesis, such as myogenesis and muscle atrophy, than the soleus muscle. Myogenic transcription and muscle-specific E3 ubiquitin ligases, which are important in regulating cancer cachexia, were more prominently expressed in the gastrocnemius muscle than in soleus muscles after nerve injury (40). Muscle atrophy, which was accompanied by a reduction in muscle fiber size and upregulation of MuRF1 and atrogin-1 expression levels, was also more predominant in the gastrocnemius muscle than in the soleus muscle after injury (40).

Similar to the finding that supplementation with BC attenuated the LLC-induced cancer cachexia by downregulating the expression levels of atrophy markers (atrogin-1 and MuRF1), it was previously reported that BC attenuated soleus muscle loss by suppressing the expression levels of *atrogin-1* and *Murf1* against denervation-induced muscle atrophy (24). BC also reportedly promoted protein synthesis in the soleus muscles and suppressed ubiquitin conjugates under normal conditions, however, it did not regulate mRNA expression of the atrogenes, *atrogin-1* and *Murf1* (28). Since the muscle-specific E3 ubiquitin ligases, atrogin-1 and MuRF1, are regulated by BC, these findings suggest that BC exerts protective effects on muscle wasting in cancer cachexia through the ubiquitin-proteasome pathway.

However, in contrast to the findings in the present study, the previous studies showed that BC administration was only effective in increasing the mass of the soleus muscle against the denervation (24) and under physiological conditions (28), whereas no effect was observed in the gastrocnemius muscle. In addition, BC treatment inhibited the denervation-induced upregulation of ubiquitin conjugates in the soleus muscle, yet not in the gastrocnemius muscle (24). The gastrocnemius and soleus have distinct anatomical and physiological features, whereby the gastrocnemius is mainly composed of fast twitch muscle fibers, whereas the soleus muscle mostly comprises slow twitch muscle fibers (41). In contrast to the gastrocnemius, there is also less risk of injury in the soleus muscle, while should injury occur, soleus injuries have a tendency of being less severe in clinical presentation and less acute compared with gastrocnemius injuries (42). Thus, it is assumed that the gastrocnemius was more affected by cancer cachexia, which is a devastating muscle-wasting disease than the soleus. Similar to the findings of the present study, previous studies have reported that fast twitch muscle fibers, such as the gastrocnemius muscles, were selectively targeted in the cancer cachexia rodent model (43,44).

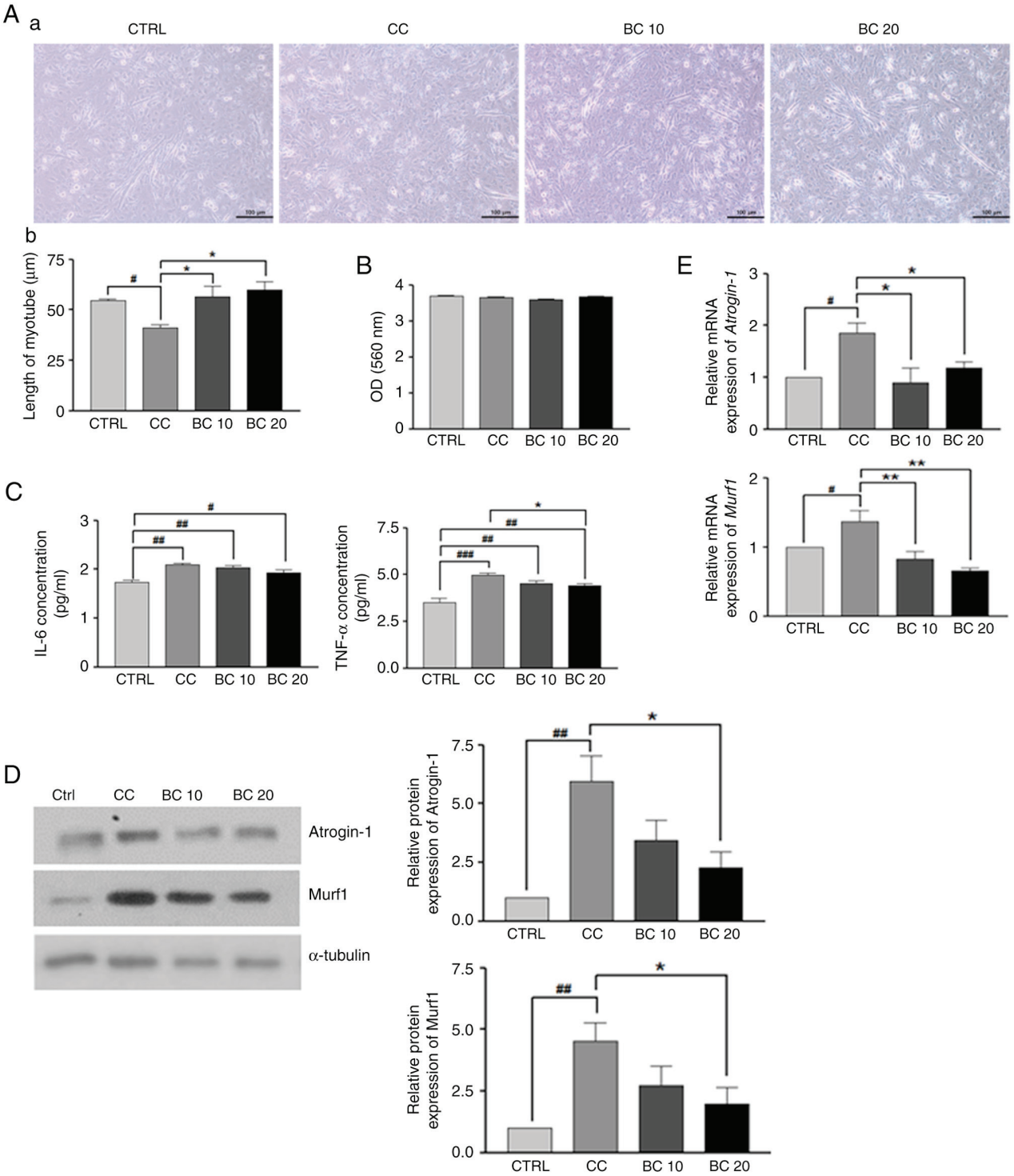

Figure 4. Effects of BC on myogenesis and muscle atrophy in C2C12 myotubes treated with LLC CM. (A-a) Cells were observed under a microscope. (A-b) Length of myotube as formed by differentiated C2C12 cells. (B) Cell viability in C2C12 myotubes treated with LLC CM was evaluated with 10 and 20 μM BC for 3 days using an MTT assay. (C) Secreted IL-6 and TNF-α levels in the media were determined. (D) Protein levels of atrogin-1 and MuRF1 were measured by western blotting and the relative band intensities were calculated after normalization to a-tubulin expression. (E) The mRNA levels of *atrogin-1* and *Murf1* were measured by reverse trascription-quantitative PCR. *Gapdh* was used as a loading control. All data are shown as the mean ± standard error of the mean and were analyzed using one-way ANOVA with a Newman-Keuls post hoc test. $^{\#}$P<0.05, $^{\#\#}$P<0.01, and $^{\#\#\#}$P<0.001 vs. the CTRL; *P<0.05 and **P<0.01 vs. CC. BC, β-carotene; LLC, Lewis lung carcinoma; CM, conditioned medium; CTRL, Control; CC, Cancer-cachexia; BC 10, 10 μM of BC; BC 20, 20 μM of BC; Murf1, muscle RING-finger protein-1; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.

TNF-α, IL-1 and IL-6 are major proinflammatory cytokines that are released from immune or cancer cells and promote muscle loss in patients with cachexia (45). The present study also demonstrated that BC effectively suppresses serum cytokines (TNF-α and IL-6) in cancer cachexia, implying that BC may have effects on immune or cancer cells, and their interactions with muscle tissues. The ability of BC to modulate the immune system and attenuate inflammatory diseases has been well

studied (46). Previously, it was reported that treatments with BC significantly reduced the production of the proinflammatory cytokines, TNF-α and IL-6, following induction by lipopolysaccharides in macrophages (47). Pretreatment with BC significantly decreased cytokines levels of TNF-α and IL-6 in the kidneys of bromobenzene-induced rats (48). Additionally, another study reported that subjects with lower plasma BC levels exhibited increased IL-6 levels compared with healthy children (49).

As a result of muscle depletion in cachexia or muscle wasting, amino acids are released from skeletal muscles and function as precursors for hepatic gluconeogenesis (10,50). In addition, the upregulation of gluconeogenesis also results in a low concentration of skeletal muscle amino acids, leading to muscle wasting. Thus, this aggressive feedback loop worsens the disease (51). During muscle atrophy, the activity of the glycolytic enzymes is increased in the muscle, while gluconeogenesis is increased in the liver (52). Hepatic gluconeogenesis is also affected by inflammatory cytokines, such as TNF-α, IL-6 and IL-1. Production of inflammatory proteins, such as acute-phase proteins, in the liver also requires muscle deterioration as a source of amino acids in cancer cachexia (11,53). In the present study, treatment with BC suppressed IL-6 and TNF-α serum levels, which may inhibit hepatic gluconeogenesis. Thus, further investigation into the hepatic levels of inflammatory cytokines and other related mechanisms are required in future studies.

In the present study, the PI3K/Akt signaling pathway was suppressed by cancer cachexia. It was previously reported that myotubes become hypertrophic via the PI3K/Akt pathway, which increases protein synthesis and decreases the expression of the muscle atrophy-related markers, MAFbx and MuRF1 (54,55). BC supplementation was observed to upregulate the PI3K/Akt signaling pathway in cancer cachexia-induced mouse gastrocnemius muscles. Oral BC administration enhanced skeletal muscle mass by upregulating IGF-1 (28), which is known to activate the PI3K/Akt signaling pathway (54).

The mRNA levels of the muscle stem cell markers, *Pax7* and *MyoD*, were upregulated following the induction of cancer cachexia; however, these levels were recovered after treatment with BC. Further stem cell-based regenerative treatment was used to restore the muscle homeostasis following injury (56). The muscle satellite cells were activated to participate in regenerative procedures following cancer cachexia-associated muscle damage (13). This implies that the upregulation of the stem cell markers caused by cancer cachexia was a consequence of the feedback system to counteract muscle wasting. A previous study reported that BC inhibited proliferation and promoted the differentiation of Pax7-enriched chicken myoblasts via b-carotene oxygenase 1 (29), thereby supporting the findings of the present study. Additionally, BC supplementation suppressed cancer stemness in colon cancer (57) and neuroblastoma (58,59).

In the present study, BC effectively restored LLC CM-induced myogenesis inhibition, while it has also been reported in a previous study regarding neuroblastoma, that BC induced neuronal cell differentiation in SK-N-BE(2)C neuroblastoma cells (59). Retinoic acid (RA), produced by cleavage of BC, also reportedly upregulates the differentiation of embryonic and cancer stem cells (60). The present study revealed that treatments with BC downregulated *atrogin-1* and *Murf1* levels against LLC CM-induced wasting in C2C12 myotubes. Similar to the results of the present study, treatments with BC decreased hydrogen peroxide-induced increases in *atrogin-1* and *Murf1* levels in C2C12 myotubes (24). BC was also converted into RA, which suppressed proliferation and increased the differentiation of chicken myoblasts (29). Treatments with BC have been shown to alleviate H_2O_2-induced ubiquitin ligases of *atrogin-1* and *Murf1*, dose-dependently in C2C12 myotube cells (24).

For *in vivo* analysis, mice were treated with BC supplements of 4 and 8 mg/kg BW, administered twice weekly, for 22 days. These doses corresponded to 1.12 and 2.24 mg/kg BW per day, respectively, and can be converted to 5.44 and 10.88 mg/day for a 60-kg person (61). As orange-colored carrots (*Daucus carota* L.) contain BC, in quantities of 7 to 17 mg/100 g (62), the current doses used in the present study were physiological. At 10 and 20 μM, BC treatments did not result in significant toxic effects in differentiated C2C12 cells.

Recently, it was reported that relatively low doses of BC (0.5 and 1 μM for *in vitro* experiments and 0.5 and 2 mg/kg BW for *in vivo* experiments) were effective in restoring adipose wasting in cancer cachexia induced by CT26 cancer cell inoculation (63). However, in the present study, relatively high doses of BC (10 and 20 μM for *in vitro* experiments and 4 and 8 mg/kg BW for *in vivo* experiments) restored muscle wasting-associated dysregulations in cancer cachexia. Likewise, it was observed that BC plays a preventive role against cancer cachexia at different concentration ranges depending on the model. This suggests that relatively low BC concentrations were effective in improving the recovery of a patient from early cancer cachexia-induced impairments. CT26 and LLC cancer cells are known to induce different molecular mechanisms causing cancer cachexia (64). A combination treatment of exercise training and erythropoietin appeared to have different anticancer cachexia effects in CT26- and LLC-induced mouse models, respectively (65). The precise underlying mechanism by which BC provides protective effects in each of the CT26- and LLC-induced cancer cachexia models and with their respective concentration ranges should be studied further.

A limitation of the present study was that the protein expression of Murf1 was only measured in *in vitro* but not *in vivo* experiments. Examination of the protein expression of Murf1 in the cancer cachexia mouse model was attempted. In spite of numerous attempts and troubleshooting, the uniformly distributed high background in the western blot films continued to appear. It was hypothesized that this was due to the non-specific bindings of the proteins with the primary or secondary antibodies. Thus, since it was difficult to draw reliable conclusions from the blurred and unclear western Murf1 bands from the mouse samples, the data could not be presented.

In conclusion, the present study determined that BC supplementation can alleviate muscle wasting in cancer cachexia by regulating myogenesis and muscle atrophy dysregulations in gastrocnemius muscles. These effects may be mediated by the regulation of PI3K/Akt phosphorylation. BC supplementation also effectively downregulated the increased hepatic gluconeogenesis and systemic inflammation caused by cancer cachexia. Cachexia also occurs in several diseases, including heart failure, kidney disease and chronic obstructive pulmonary disease (66). Thus, the present study provides insights into the possible roles of BC as a novel therapeutic agent for other diseases associated with muscle wasting.

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Availability of data and materials

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

Authors' contributions

YuK contributed to the conception, design of the study, and acquisition of funding. YeK, YO, YSK, JHS, YSL and YuK analyzed and interpreted the data, and wrote and reviewed the manuscript. YeK, YO and YSL performed the experiments. YSK, JHS, and YuK provided technical support to perform the experiments. YeK and YuK confirm the authenticity of all the raw data. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

The present study was conducted according to the guidelines of the National Institutes of Health (NIH publication no. 8023, revised 1978), and approved (IACUC approval no. EWHA IACUC 21-003-1) by the Institutional Animal Care and Use Committee of Ewha Womans University (Seoul, Republic of Korea).

Patients consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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